New treatment strategies in myelodysplastic syndromes and acute myeloid leukemia

Hypomethylating agents and proteasome inhibitors

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Hypomethylating agents and proteasome inhibitors

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Chapter 1

General introduction and scope of this thesis

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

Prognostic variable	0	0.5	1	1.5	2	3	4
Cytogenetics *(Table 1b)*	Very good	—	Good	—	Inter-mediate	Poor	Very poor
BM blast, %	≤ 2	—	> 2%- < 5%	—	5%-10%	> 10%	—
Hemoglobin, g/dL	≥ 10	—	8- < 10	< 8	—	—	—
Platelets, x 10^9/L	≥ 100	50-< 100	< 50	—	—	—	—
ANC, x 10^9/L	≥ 0.8	< 0.8	—	—	—	—	—

IPSS-R risk	Very low	Low	Intermediate	High	Very high
Risk score	**≤1.5**	**>1.5–3.0**	**>3.0–4.5**	**>4.5–6.0**	**>6.0**
Median survival (years)	8.8	5.3	3.0	1.6	0.8
Time to 25% evolution to AML (years)	NR	10.8	3.2	1.4	0.73

BM, bone marrow; ANC, absolute neutrophil count; —, not applicable. IPSS, international prognostic scoring system; AML, acute myeloid leukemia; NR, not reached.

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

Prognostic subgroups	Cytogenetic abnormalities
Very good	−Y, del(11q)
Good	Normal, del(5q), del(12p), del(20q), double anomalies including del(5q)
Intermediate	del(7q), +8, +19, i(17q), any other single or double independent clones
Poor	−7, inv(3)/t(3q)/del(3q), double abnormalities including −7/del(7q), complex: 3 abnormalities
Very poor	Complex: > 3 abnormalities

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).[3] 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

AML with recurrent genetic abnormalities
AML with t(8;21)(q22;q22); RUNX1-RUNX1T1
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11
APL with t(15;17)(q22;q12); PML-RARA
AML with t(9;11)(p22;q23); MLLT3-MLL
AML with t(6;9)(p23;q34); DEK-NUP214
AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1
AML (megakaryoblastic) with t(1;22)(p13;q13); RBM15-MKL1
Provisional entity*: AML with mutated NPM1
Provisional entity*: AML with mutated CEBPA
AML with myelodysplasia-related changes
Therapy-related myeloid neoplasms
Acute myeloid leukemia, not otherwise specified
AML with minimal differentiation
AML without maturation
AML with maturation
Acute myelomonocytic leukemia
Acute monoblastic/monocytic leukemia
Acute erythroid leukemia
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis

** 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).[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.[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.[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.[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).[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.[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.[24]

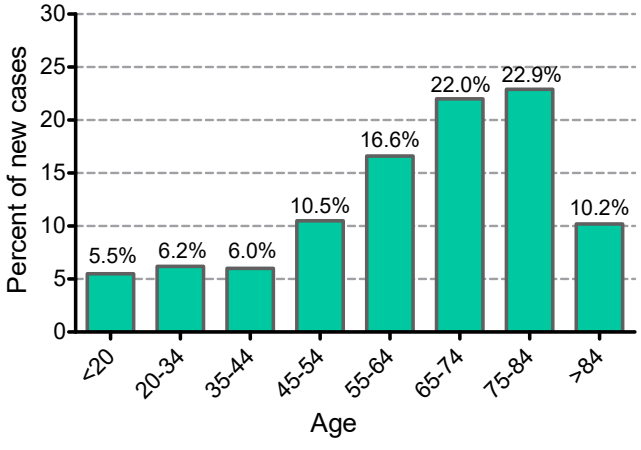

Figure 1. Incidence of acute myeloid leukemia by age group

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

Risk Profile	Subsets
Favorable	t(8;21)(q22;q22); RUNX1-RUNX1T1 inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11 Mutated NPM1 without FLT3-ITD (normal karyotype) Biallelic mutated CEBPA (normal karyotype)
Intermediate-I†	Mutated NPM1 and FLT3-ITD (normal karyotype) Wild-type NPM1 and FLT3-ITD (normal karyotype) Wild-type NPM1 without FLT3-ITD (normal karyotype)
Intermediate-II	t(9;11)(p22;q23); MLLT3-KMT2A Cytogenetic abnormalities not classified as favorable or adverse
Adverse	inv(3)(q21q26.2) or t(3;3)(q21;q26.2); GATA2–MECOM (EVI1) t(6;9)(p23;q34); DEK-NUP214 t(v;11)(v;q23); KMT2A rearranged −5 or del(5q); −7; abnl(17p); complex karyotype§

† 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) or 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.[25] 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.[26-28] 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.[27,29-31] 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.[15] 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.[32] 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.[33,34] 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.[33,34] 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).*[32] 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.[29,35] 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.[36]

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.[37] 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

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.[38] Theoretically, epigenetic changes are reversible and are therefore an attractive target for therapeutic intervention.[39] 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.[40] 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.[40,41] Hypermethylation of a promoter region results in transcriptional silencing and is associated with aging and cancer.[42-44] 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.[12,45] In MDS, promoter hypermethylation of specific cancer-related genes such as *CDKN2A* and genes of the WNT signaling pathway has often been described.[46-49] A recent genome-wide 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.[45] Although mutations in genes involved in DNA methylation are detected in about 50% of MDS patients[33], aberrant methylation was observed in all tested MDS samples in the studies of Jiang et al. and Figueroa et al.[12,45] 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.[50]

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.[51] 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.[51] 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.[52,53] 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.[54] 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.[55] 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.[56] 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.[57,58]

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.[15] 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.[59] 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.[60] 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.[31,61,62] 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.[63] 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.[62]

Secondary AML may arise from different mutation patterns compared to *de novo* AML. A recent mutational analysis indicated that mutations in *SRSF2*, *SF3B1*, *U2AF1*, *ZRSR2*, *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.[64] 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.[64]

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%).[65] 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.[65] 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 origin 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.[77] 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.[79] 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.[23,80-87] 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.[88] 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 myelo-ablative conditioning in patients aged 35-60 years.[89-92] 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 myelo-ablative conditioning have been reported.[89,93] 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.[94]

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).[95] 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.[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'-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 methyltranspherases (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.[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.[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.[97,98] Moreover, various studies have reported hypermethylation of tumor suppressor genes in MDS, such as *CDKN2A, CDKN2B*, Wnt inhibitors, *CDH1*, and *SOCS1*, and showed that this hypermethylation could be reversed by azacitidine or decitabine.[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.[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.[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.[101]

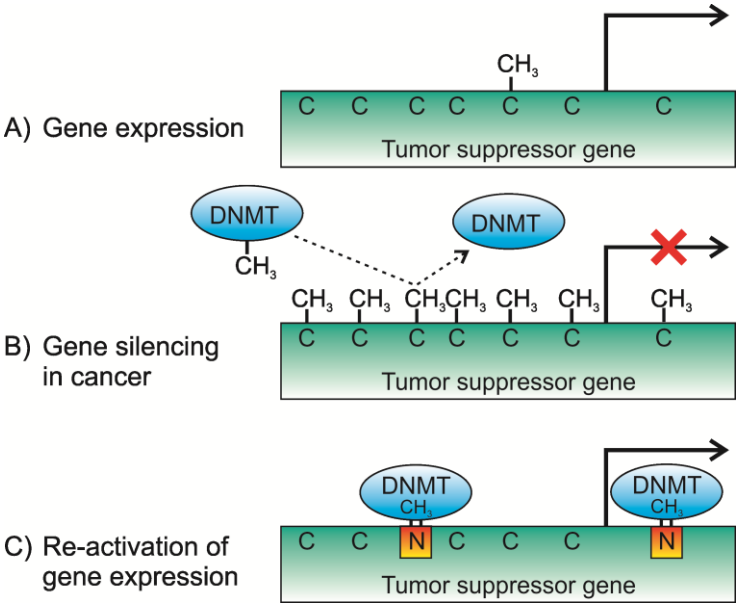

Figure 2. Conceptual working mechanisms of hypomethylating agents.

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 transpherase; CH_3, methylgroup; C, carbon atom in cytosine; N, nitrogen atom in cytosine analog.*

Azacitidine in higher-risk MDS and AML

After discovery of the hypomethylating effect, azacitidine has been tested in a dose of 75 mg/m^2 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.[102] 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.[103] 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

analysis of the AZA-001 study confirmed that azacitidine treatment induced a survival benefit in AML with 20-30% blasts.[104] 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 [105,106], whereas outcome of conventional therapy in these patients is notoriously poor.[24]

Decitabine in MDS and AML

Decitabine initially has been studied in two randomized trials that compared decitabine (9 doses of 15 mg/m^2 intravenously in 3 days every 6 weeks) with best supportive care in MDS patients aged ≥ 60 years who were not eligible for intensive chemotherapy.[107,108] 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/m^2 intravenously for 5 days per 4 weeks, which resulted in complete remissions in 39% of patients (compared to 21% and 24% with other schedules).[109] 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/m^2 in 1 hour for 5 days/month) was compared with conventional care, which consisted of best supportive care or low-dose cytarabine.[110] 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/m^2 for 10 days per month and reported complete remission rates as high as 47%, which is comparable to intensive chemotherapy in older AML patients.[111]

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.[112] 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.[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.[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 $CD34^+$ cells.[117-119] Inhibition of NF-κB with the *in vitro* proteasome inhibitor MG-132 induced apoptosis in AML $CD34^+$ cells, but not in normal $CD34^+$ cells, suggesting a therapeutical window.[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 *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.[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.[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 *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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Chapter 2

Platelet doubling after the first azacitidine cycle is a promising predictor for response in MDS, CMML and AML patients in the Dutch azacitidine compassionate patient named program

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ABSTRACT

The efficacy of azacitidine in the treatment of high-risk myelodysplastic syndromes (MDS), chronic myelomonocytic leukemia (CMML) and acute myeloid leukemia (AML) (20-30% blasts) has been demonstrated. To investigate the efficacy of azacitidine in daily clinical practice and to identify predictors for response, we analyzed a cohort of 90 MDS, CMML and AML patients who have been treated in a Dutch compassionate patient named program.

Patients received azacitidine for a median of 5 cycles (range 1-19). The overall response rate (complete/partial/hematological improvement) was 57% in low-risk MDS, 53% in high-risk MDS, 50% in CMML, and 39% in AML patients. Median overall survival (OS) was 13.0 (9.8-16.2) months. Multivariate analysis confirmed circulating blasts (Hazard Ratio (HR) 0.48, 95% CI 0.24-0.99; p = 0.05) and poor risk cytogenetics (HR 0.45, 95% CI 0.22-0.91; p = 0.03) as independent predictors for OS. Interestingly, this analysis also identified platelet doubling after the first cycle of azacitidine as a simple and independent positive predictor for OS (HR 5.4, 95% CI 0.73-39.9; p = 0.10). In conclusion, routine administration of azacitidine to patients with variable risk groups of MDS, CMML and AML is feasible, and subgroups with distinct efficacy of azacitidine treatment can be identified.

INTRODUCTION

Myelodysplastic syndromes (MDS) are hematopoietic stem cell disorders, characterized by dysplasia leading to cytopenias and a high probability of progression to acute myeloid leukemia (AML). Morphologically, MDS are categorized by the French-American-British (FAB) classification and more recently by the world health organization (WHO) classification.[1-3] In the WHO classification, MDS with 20-30% bone marrow blasts was reclassified as AML and chronic myelomonocytic leukemia (CMML) was reclassified as a separate myelodysplastic/myeloproliferative disorder.

In 2004 the US Food and Drug Administration (FDA) approved the hypomethylating drug azacitidine for the treatment of myelodysplastic syndromes, mainly based on the significant delay in time to transformation to acute myeloid leukemia and death, compared with best supportive care.[4] A recent phase 3 study demonstrated that azacitidine significantly improved overall survival (OS) in higher-risk MDS patients when compared with conventional care regimens.[5] In this study, azacitidine induced responses in about 50% of treated high-risk MDS patients, including 17% complete remissions (CR) and 12% partial remissions (PR). A *post hoc* analysis of this study showed improved OS in the subgroup of AML patients with 20-30% blasts treated with azacitidine compared to best supportive care.[6] Although these studies have convincingly shown that azacitidine has beneficial effects, it remains difficult to predict which particular subgroup of patients will benefit from azacitidine treatment. Also in the perspective of the high cost of azacitidine treatment, identification of patients who optimally benefit from treatment is an important issue. Recently, an azacitidine prognostic scoring system for OS based on performance status, circulating blasts, red blood cell transfusions and th International Prognostic Scoring System (IPSS) cytogenetic risk was proposed.[7]

In the present study we analyzed a cohort of 90 patients treated with azacitidine in a compassionate named patient program in the Netherlands. We assessed the efficacy of azacitidine in routine daily clinical practice, validated the proposed azacitidine prognostic scoring system and identified platelet doubling after the first cycle of azacitidine as a promising predictor for response in MDS, CMML and AML patients.

METHODS

Patients and data collection

After FDA approval of azacitidine in the US and before European Medicines Agency (EMA) approval in the European Union, a compassionate patient named program was initiated in the Netherlands. All patients with intermediate-2 and high-risk MDS, CMML and AML (20-30% blasts) could be included. Exceptionally, patients with low-risk MDS (n = 7) or AML with more than 30% blasts (n = 11) could be included as well. Applications were reviewed by an expert panel before

acceptance, and patients were included after informed consent in accordance with the Declaration of Helsinki. After critical review of applications, seven patients with low-risk MDS were accepted for azacitidine treatment since they had a strong indication for treatment but had no other suitable treatment options.

Between December 2008 and August 2010, 90 patients, treated in 18 centers, were included in this compassionate named patient program. Diagnoses were made using World Health Organization (WHO)-2008 criteria.[2] Cytogenetic abnormalities were classified according to the International System for human Cytogenetic Nomenclature (ISCN) criteria.[8] Risks were assessed by the IPSS.[9] Data were collected between August 2010 and November 2010 by studying case records of all individual patients to complete case report forms. Peripheral blood counts, performance status and transfusions were evaluated at start of every treatment cycle.

Treatment

Azacitidine was administered subcutaneously at the approved schedule of 75 mg/m²/day for 7 days every 28 days. Physicians intended to give at least six cycles of treatment. Patients who responded well were to continue treatment until progression. Red blood cell (RBC) transfusions were given in agreement with general recommendations: Hb < 8 g/dL. RBC transfusion dependency was defined as having ≥1 RBC transfusion every 8 weeks over at least 2 months.

Response criteria and study endpoints

Response was evaluated after every cycle by blood counts and by bone marrow aspirate if available. Eighty-nine patients had received a bone marrow aspirate at diagnosis. Complete remission (CR), partial remission (PR), CR with incomplete blood count recovery (CRi), stable disease (SD), hematologic improvement (HI), and progression were defined according to IWG 2006 criteria.[10] Response was analyzed on an intention-to-treat basis. Besides two patients who had normal pre-treatment blood values, all patients were considered eligible for assessment of HI of the erythroid, neutrophil, and/or platelet lineages (HI-E, HI-N, and HI-P respectively). Response duration was measured from the cycle in which marrow evaluation took place in patients achieving CR, CRi, or PR, or from the cycle in which blood counts first met HI criteria, until the date of progression. Overall survival (OS) was measured from the onset of azacitidine. Patients who remained alive were censored at the time of the last visit to the hospital. MDS, CMML and AML were defined according to WHO-2008 criteria.[2]

Statistical analysis

Survival curves were estimated with the Kaplan-Meier method and log rank tests were used for evaluating differences in OS. To assess the impact of between-patient differences in the onset of response after the initiation of treatment, the Mantel-Byar method was also applied. Predictive factors for overall survival were analyzed by Wald tests for univariate and multivariate

comparisons. Cox proportional hazards regression models were used to estimate hazard ratios (HRs) and associated 95% confidence intervals (CIs). A P value < 0.05 was considered significant in all analysis. The Statistical Package for the Social Sciences (SPSS) version 16 was used for analysis.

RESULTS

Table I. Baseline patient characteristics

	All patients (*N* = 90)	Low-risk MDS (*N* = 7)	High-risk MDS (*N* = 40)	CMML (*N* = 12)	AML (*N* = 31)
Age (years)	71 (39-84)	69 (60-79)	73 (39-83)	65 (51-74)	71 (40-84)
Sex					
Male	60 (67%)	5 (71%)	26 (65%)	6 (50%)	23 (74%)
Female	30 (33%)	2 (29%)	14 (35%)	6 (50%)	8 (26%)
Interval from diagnosis (months)	4 (0-89)	25 (0-87)	3 (0-42)	2 (0-40)	4 (0-89)
Prior treatment					
No	51 (57%)	3 (43%)	27 (68%)	7 (58%)	14 (45%)
Erythropoietin/G-CSF	10 (11%)	3 (43%)	6 (15%)	0 (0%)	1 (3%)
Chemotherapy	19 (21%)	0 (0%)	6 (15%)	0 (0%)	13 (42%)
Cyclosporine	5 (6%)	1 (14%)	1 (3%)	2 (17%)	1 (3%)
Hydroxycarbamide	5 (6%)	0 (0%)	0 (0%)	3 (25%)	2 (7%)
RBC transfusion dependency[1]	63 (73%)	7 (100%)	26 (70%)	7 (58%)	23 (77%)
Cytogenetic risk					
Good	24 (28%)	4 (57%)	13 (34%)	7 (58%)	0 (0%)
Intermediate	28 (33%)	1 (14%)	3 (8%)	5 (42%)	19 (68%)
Poor	33 (39%)	2 (29%)	22 (58%)	0 (0%)	9 (32%)
Not done	5 (6%)	0 (0%)	2 (5%)	0 (0%)	3 (10%)

Abbreviations: MDS, myelodysplastic syndromes; CMML, chronic myelomonocytic leukemia; AML, acute myeloid leukemia; G-CSF, granulocyte colony-stimulating factor. Results are reported as N (%) or median (range).

[1] Defined as having ≥1 red blood cell transfusion every 8 weeks over at least 2 months.

Baseline characteristics of the study population

The study population included 90 patients from 18 centers (Table I). Median age was 71 years (range 39-84) and 33% of the patients were women. WHO diagnoses included 10 (11%) MDS-refractory anemia with ringed sideroblasts (RARS) or MDS-refractory cytopenia with multilineage dysplasia (RCMD), 9 (10%) MDS-refractory anemia with excess blasts (RAEB)-1, 28 (31%) MDS-RAEB-2, 12 (13%) CMML and 31 (34%) AML (according to WHO definition (i.e. >20% blasts)). Of the AML patients, 19 were *de novo* AML en 12 had relapsed AML (two after previous allogeneic hematopoietic cell transplantation). The IPSS was determined for the MDS patients and AML patients with ≤ 30% blasts, and was intermediate-low in 9 (14%), intermediate-high in 31 (46%) and high in 27 (40%) patients. Median interval from diagnosis until azacitidine treatment was 4 months (range 0-89) in the total cohort, but was longer in low-risk MDS patients (with IPSS <1.5; 25 months (range 0-87)). Subgroups of patients were previously treated with erythropoietin and/or granulocyte colony-stimulating factor (G-CSF) (11%), cyclosporine (6%), hydroxycarbamide (6%) or cytarabine-containing chemotherapy (21%). At inclusion, 63 (73%) patients were RBC transfusion dependent (Table I).

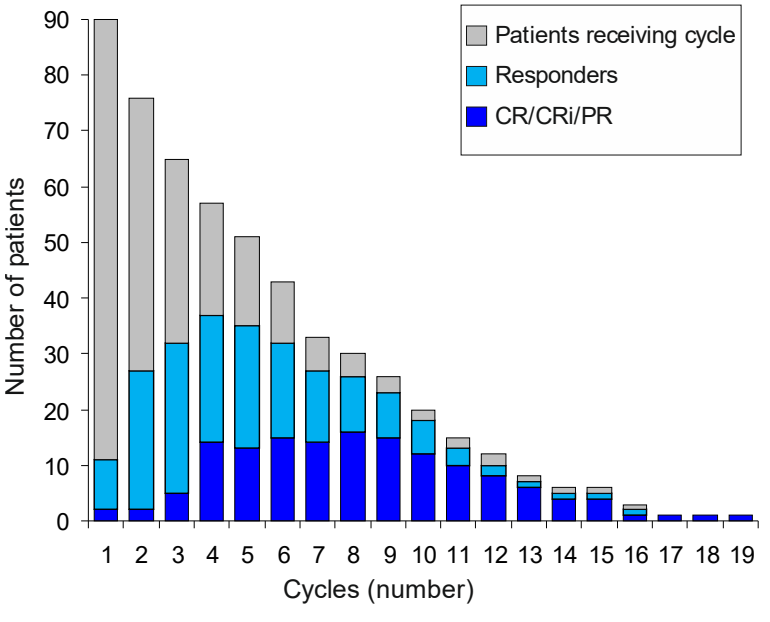


Figure 1. The numbers of patients receiving azacitidine and responses per cycle

The numbers of patients receiving the corresponding cycle of azacitidine treatment and responders at the end of this cycle are shown. Of the patients who are receiving treatment, overall response is 49% after 3 cycles, 74% after 6 cycles, and 88% after 9 cycles. After 6 cycles, all patients who do not show a response have stable disease. Abbreviations: CR, complete remission; mCR, marrow CR/CRi; PR, partial remission.

Treatment and response

The median number of treatment cycles was 5 (range 1-19) (Fig 1, Table II). After a median follow up of 8 months (range 1-21), overall response was achieved in 43 (48%) patients, including 13 (14%) CR, 1 (1%) PR, 10 (11%) CRi, and 19 (21%) HI alone (Table II). Of the patients with HI, 10 had HI-E, 16 had HI-P and 2 had HI-N. Eight patients met criteria for combined HI of two or three lineages. Overall response rates were the highest in low-risk MDS (57%) and the lowest in AML (39%). Median time to response (CR, PR, CRi, HI) was two months (range 1-6).

Table II. Treatment outcome in the various disease groups according to WHO-2008 criteria

	All patients ***(N = 90)***	**Low-risk MDS** ***(N = 7)***	**High-risk MDS** ***(N = 40)***	**CMML** ***(N = 12)***	**AML** ***(N = 31)***
Number of cycles	5 (1-19)	7 (5-10)	5 (1-19)	8 (1-15)	4 (1-15)
Overall response (CR, PR, CRi, HI)	**43 (48%)**	**4 (57%)**	**21 (53%)**	**6 (50%)**	**12 (39%)**
CR	13 (14%)	0 (0%)	5 (13%)	3 (25%)	5 (16%)
PR	1 (1%)	0 (0%)	0 (0%)	0 (0%)	1 (3%)
CRi	10 (11%)	1 (14%)	6 (15%)	1 (8%)	2 (7%)
HI	19 (21%)	3 (43%)	10 (25%)	2 (17%)	4 (13%)
SD	17 (19%)	3 (43%)	6 (15%)	4 (33%)	4 (13%)
Disease progression/ death	26 (29%)	0 (0%)	6 (15%)	2 (16%)	14 (46%)
Not evaluable	4 (4%)	0 (0%)	3 (8%)	0 (0%)	1 (3%)
Time to response (months)	2 (1-6)	2 (1-4)	2 (1-4)	2 (1-6)	2 (1-6)
Time to CR, PR, CRi (months)	4 (1-8)	6 (6-6)	4 (1-8)	4 (2-7)	4 (2-6)
Failure to achieve 3 cycles	25 (28%)	0 (0%)	9 (23%)	3 (25%)	13 (42%)
Failure to achieve 6 cycles	47 (52%)	2 (29%)	23 (58%)	5 (42%)	17 (55%)
Deaths (at end of study)	45 (50%)	1 (%)	20 (50%)	2 (17%)	22 (71%)
before 2nd cycle	5 (6%)	0 (0%)	0 (0%)	0 (0%)	5 (16%)
before 4th cycle	18 (20%)	0 (0%)	4 (20%)	2 (17%)	12 (39%)
Allogeneic transplantation after azacitidine	4 (4%)	0 (0%)	3 (8%)	1 (8%)	0 (0%)

Abbreviations: MDS, myelodysplastic syndromes; CMML, chronic myelomonocytic leukemia; AML, acute myeloid leukemia; CR, complete remission; PR, partial remission; CRi, CR with incomplete blood count recovery; SD, stable disease; HI, hematologic improvement.
Results are reported as N (%) or median (range).

Failure to receive at least three cycles of azacitidine was reported in 25 (28%) patients. The cause of interruption was early death in 11, fast progression of disease in five, severe side-effects (gastro-intestinal bleeding, nausea, malaise) in five, stroke in one, consent withdrawal in one and

allogeneic hematopoietic cell transplantation after the first cycle in two patients. Failure to achieve at least 6 cycles of azacitidine was reported in 47 (52%) patients with the highest rates in patients with high-risk MDS (58%) and AML (55%).

Dose adjustments or schedule changes were made in 21 (23%) patients. Schedule changes from 7 to 5 days were made in 15 patients, dose reductions of 50% were made in four patients; one patient had both 50% dose reduction and a 5-day schedule, and one patient had a schedule change to 3 days. In 15 (17%) patients, these dose adjustments were made before the sixth treatment cycle.

The number of patients receiving azacitidine cycles and the number of responses associated with each cycle are depicted in Fig 1. After six cycles, 74% of patients that were still being treated showed a response (CR/CRi/PR/HI).

Median OS was 13.0 months (range 9.8-16.2). Responders had a better OS than non-responders (16.0 months (range 13.8-18.2) versus 6.0 months (range 3.3-8.7), $p < 0.001$; Fig 2). To correct for the necessity to survive long enough to achieve a response, an analysis according to the Mantel-Byar method was performed. This analysis confirmed the survival benefit in the responders group ($p = 0.001$) (data not shown). In addition, we also performed a time-dependent Cox regression analysis, in which the onset of response was included as a time-dependen variable to assess the association between response and OS ($p = 0.0002$) (data not shown). This analysis confirmed findings from the survival analysis using the Mantel-Byar method. Duration of CR and PR was 1-17 months after a median follow up of 8 months (range 1-21), but was ongoing in 20 of the 24 patients at the end of study.

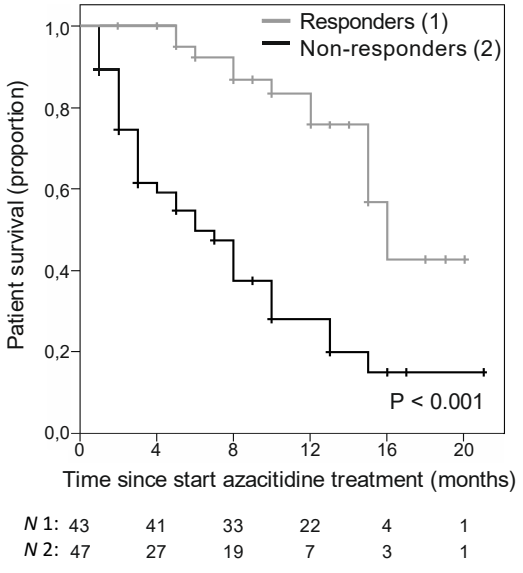


Figure 2. Overall Survival in patients with and without any response on azacitidine.

The median Overall Survival was significantly better in responders (16.0 months, range 13.8-18.2) compared to non-responders (6.0 months, range 3.3-8.7).

Table III. Univariate and multivariate analysis of predictors for overall survival

	Median OS (months)	HR (95% CI)	*P* value
(a) Univariate analysis			
All patients	13.0 (9.8-16.2)		
Circulating blasts		0.44 (0.24-0.79)	0.006
Present	8.0 (4.3-11.6)		
Absent	15.0 (11.3-18.7)		
Bone marrow blasts		0.46 (0.25-0.85)	0.01
<20%	15.0 (11.2-18.8)		
≥20%	7.0 (0.7-13.3)		
Cytogenetic risk		0.53 (0.29-0.97)	0.04
Good/intermediate	15.0 (9.1-20.9)		
Poor	8.0 (5.0-11.0)		
RBC transfusion dependency at inclusion		0.69 (0.34-1.4)	0.32
Yes	10.0 (6.2-13.8)		
No	15.0 (12.4-17.6)		
WHO performance score		0.49 (0.24-0.99)	0.047
0-1	13.0 (10.9-15.1)		
≥ 2	8.0 (2.4-13.6)		
Platelet ratio second and first cycle*		7.8 (1.1-57.4)	0.04
≥ 2 fold increase	Not reached		
< 2 fold increase or decrease	13.0 (10.0-16.0)		
(b) Multivariate analysis			
Cirulating blasts		0.48 (0.24-0.99)	0.05
Present	8.0 (4.3-11.6)		
Absent	15.0 (11.3-18.7)		
Cytogenetic risk		0.45 (0.22-0.91)	0.03
Good/intermediate	15.0 (9.1-20.9)		
Poor	8.0 (5.0-11.0)		
Platelet ratio second and first cycle*		5.4 (0.73-39.9)	0.10
≥ 2 fold increase	Not reached		
< 2 fold increase or decrease	13.0 (10.0-16.0)		

Table IIIa depicts the results of the univariate analysis of factors assumed to be associated with OS. Subsequently, all these factors were analysed in a multivariate Cox proportional hazards regression model which identified circulating blasts, cytogenetic risk group and platelet doubling after the first cycle as independent predictors for OS (Table IIIb).
Abbreviations: OS, overall survival; HR, hazard ratio; CI, confidence interval.
** Ratio of the number of platelets at the start of the second azacitidine cycle divided by the number of platelets at the start of the first cycle.*

Predictors for response and survival

We selected potential predictors for OS that were suggested to be important in previous studies. Based on our clinical experience, we also were interested in the predictive value of platelet doubling after the first cycle of azacitidine. In univariate analysis, potential predictors for OS were the presence of circulating blasts (HR 0.44, 95% CI 0.24-0.79; p=.006), bone marrow blasts ≥20% (HR 0.46, 95% CI 0.25-0.85; p = 0.01), poor risk cytogenetics at inclusion (HR 0.53, 95% CI 0.29-0.97; p = 0.04), RBC transfusion dependency at inclusion (HR 0.69, 95% CI 0.34-1.4; p = 0.32) and an (at least) two-fold increase in platelet counts at the start of the second azacitidine cycle compared to the start of the first cycle (HR 7.8, 95% CI 1.1-57.4; p = 0.04) (Table IIIa). In contrast to Itzykson et al, we used the bone marrow blast percentage of below or above 20% as variable in our analysis (instead of 15%), since this criterion is also used in the WHO-2008 criteria[2] to separate MDS from AML. Multivariate analysis confirmed the presence of circulating blasts (HR 0.48, 95% CI 0.24-0.99; p=.05), poor risk cytogenetics (HR 0.45, 95% CI 0.22-0.91; p=.03) and an at least two-fold platelet increase (HR 5.4, 95% CI 0.73-39.9; p=.10) as independent predictors for OS (Table IIIb).

Of the 90 treated patients, 14 (16%) had an at least two-fold increase in platelet counts after the first cycle of azacitidine, which was associated with significant better OS (p = 0.01, logrank test) (Fig 3). Median baseline platelet count of these patients was 35 $x10^9$/L (range 2-290 $x10^9$/L). Figure 4 depicts the absolute increase in platelet counts of the 14 patients who had at least doubling of platelet counts after the first azacitidine cycle. The characteristics of this subgroup of patients were not significantly different from the patients without platelet doubling. This subgroup consisted of seven patients with MDS, four with CMML and three with AML. Interestingly, platelet doubling was observed in all cytogenetic risk groups, in patients with and without circulating blasts, and in patients who were transfusion dependent and independent.

In order to validate the prognostic scoring system that was recently proposed by Itzykson *et al.*[7], we determined this score for each patient, assigning one point to: performance score ≥ 2, presence of circulating blasts, RBC transfusion dependency of ≥ 4 units/ 8 weeks and intermediate-risk cytogenetics, and assigning two points to poor-risk cytogenetics. The score could be determined in 83 patients who could subsequently be separated into three risk groups: low (score 0), 13 patients; intermediate (score 1-3), 61; high (score 4-5), nine patients. Kaplan-Meier survival curves revealed that the median OS was not reached in the low-risk group, it was 12.0 months (7.8-16.2) in the intermediate-risk group, and 8.0 months (0.0-22.6) in the high-risk group (p=0.004) (Fig 5A). The group of 13 patients with a low risk score contained six patients with an at least two-fold increase in platelet counts after the first cycle of azacitidine. Interestingly, within the 61 patients with an intermediate risk score, seven patients had an at least two-fold increase in platelet counts and had a superior survival. So, when considering platelet doubling, a favourable subgroup can be identified within the intermediate-risk group (Fig 5B). None of the nine patients with a high risk score had an at least two-fold increase in platelet counts.

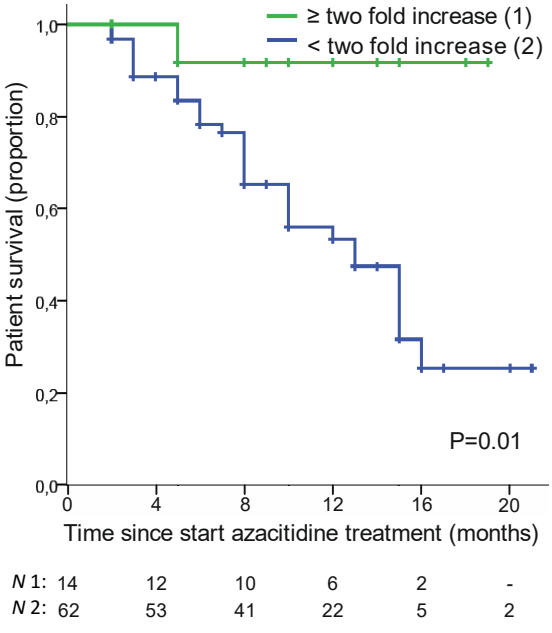


Figure 3. Overall Survival in patients with and without a two-fold platelet increase

An at least two fold increase in platelet counts at the start of the second azacitidine cycle compared to the start of the first cycle was observed in 14 of 76 patients. The platelet ratio could not be calculated in 14 patients because they did not start a second cycle of treatment. Patients with doubling of their platelets after the first azacitidine cycle have longer median Overall Survival (median not reached versus13.0 months (range 10.0-16.0)), p=.01, according to logrank test).

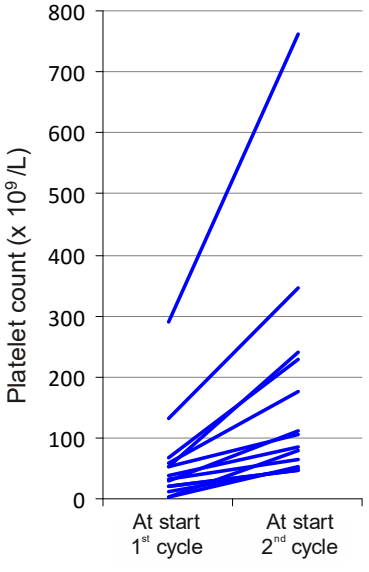
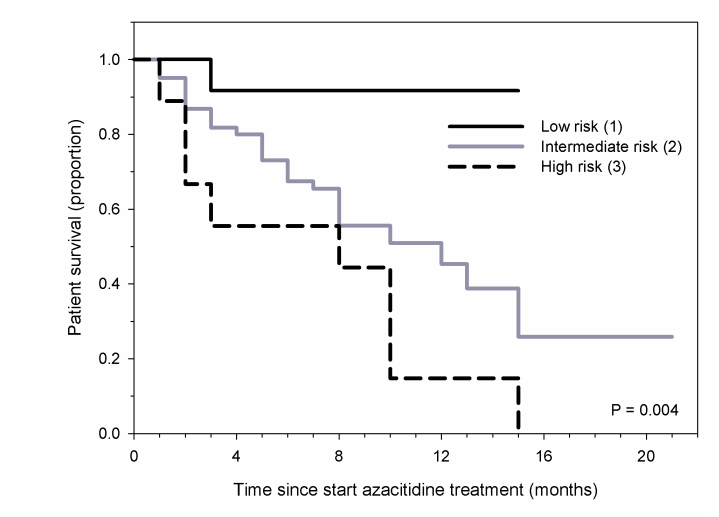


Figure 4. Absolute platelet increase in patients who had at least a doubling of platelet counts after the first azacitidine cycle

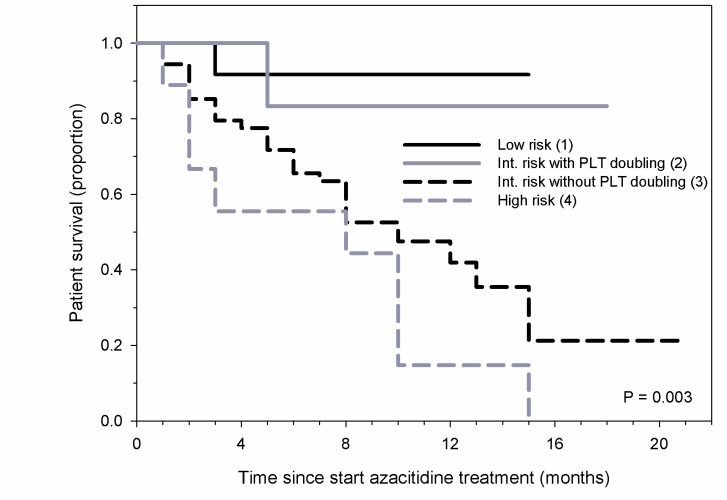
Fourteen patients had at least a doubling of their platelet counts at start of the second cycle of azacitidine treatment compared to the start of the first cycle. Twelve patients started with platelet counts below 100 x 10^9/L and after one azacitidine cycle 7 of 14 patients had platelet counts above 100 x 10^9/L.

DISCUSSION

In this multicenter retrospective analysis, 90 patients with variable risk groups of MDS, CMML and AML received azacitidine for a median of five cycles. About half of the patients achieved a response and 26% achieved CR/PR. These response rates are comparable to the results of the AZA-001 trial and to a large French named patient program study, in which overall response rates were 49% and 43%, respectively, and CR/PR rates were 29% and 28%, respectively.[5,7] Further, in our study the OS was 13.0 months, which was comparable to 13.5 months in the French named patient program.[7] Altogether, these data show that the data of the AZA-001 study were confirmed by the Dutch named patient program.



N group 1:	13	11	9	5	-	-
N group 2:	61	47	33	18	4	2
N group 3:	9	5	5	1	-	-



N group 1:	13	11	9	5	-	-
N group 2:	54	41	29	17	3	2
N group 3:	7	6	4	1	1	-
N group 4:	9	5	5	1	-	-

Figure 5A and 5B. Validation of Itzykson's prognostic score for overall survival

A) The score was computed for each patient, assigning one point to: performance score ≥2, presence of circulating blasts, RBC transfusion dependency of ≥ 4 units/ 8 weeks and intermediate-risk cytogenetics; assigning two points to poor risk cytogenetics. Patients were segregated into three risk groups with low (0), intermediate (1-3), and high (4-5) risk scores. According to this risk score, 13 patients had low-risk, 61 patients had intermediate-risk and 9 patients had high-risk scores. B) The intermediate-risk group was subdivided into patients with and without an at least two-fold increase in platelet counts after the first cycle of azacitidine.

Unlike conventional chemotherapy, it often takes several cycles of azacitidine before its effectiveness becomes apparent. Therefore, the ability to predict response at an earlier time point would be of help. So far, four routine factors have been identified in a cohort of 282 higher-risk MDS patients that are predictive for response and OS.[7] The prognostic relevance of these factors (performance status, circulating blasts, red blood cell transfusions, IPSS cytogenetic risk) was confirmed in our study cohort. Identification of patients who are likely or unlikely to benefit from azacitidine treatment is, besides from a scientific perspective, also an important issue in the perspective of the cost of the healthcare system.

In addition, univariate analysis identified doubling of platelet counts after the first cycle of azacitidine treatment as a strong predictor for response in our study. It appeared to be an independent predictor for OS after adjustment for known predictors (circulating blasts and poor risk cytogenetics) and was marginally significant in this relatively small group of patients. These results are in line with results reported from 162 patients treated with 5-aza-2'-deoxycytidine (decitabine) in which a rise in platelet counts preceded a good trilineage response and predicted for a superior OS.[11] However, in this study absolute platelet numbers were used instead of platelet ratios. Moreover, time to platelet recovery following decitabine-primed induction chemotherapy in patients with AML was shorter than generally observed with cytarabine-containing induction chemotherapy.[12]

The plateau in the OS curve, although hampered by a relatively short follow-up, suggests that patients with platelet doubling respond extremely well to azacitidine treatment. The value of platelet doubling should be validated in other cohorts of patients treated with azacitidine. Intriguingly, a doubling of the number of platelets after the first cycle of azacitidine was seen in all cytogenetic risk groups, in patients with and without circulating blasts, and in patients who were transfusion dependent and -independent. For example, five of the 16 patients with platelet doubling had unfavorable cytogenetics. This suggests the existence of a subgroup of patients who are extremely sensitive to azacitidine. Unfortunately, the molecular abnormalities of the patients in the studied cohort are unknown, but it could be hypothesized that this subgroup is characterized by certain molecular abnormalities that modify the epigenome, like *TET2* or *DNMT3A* mutations. Recently, a French study revealed that *TET2* status may be an independent genetic predictor for response to azacitidine, independently of karyotype, in high-risk MDS and AML with low blast counts.[13] In addition, it has been shown that also variable risk groups can be identified using flowcytometry.[14] It would be of interest to determine whether doubling of platelets is associated with a certain immunophenotype of myeloid progenitor cells.

In conclusion, treatment with azacitidine is effective in routine daily clinical practice in patients with variable risk groups of MDS, CMML and AML. An at least two fold increase in platelet counts after the first cycle of azacitidine treatment predicted longer OS and may be a useful early indicator for a favourable outcome of azacitidine treatment.

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Chapter 3

Azacitidine results in comparable outcome in newly diagnosed AML patients with more or less than 30% bone marrow blasts

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ABSTRACT

The efficacy of azacitidine has been demonstrated in acute myeloid leukemia (AML) patients with 20-30% bone marrow (BM) blasts, but limited data is available on patients with ≥30% blasts. We analyzed 55 newly diagnosed AML patients, treated with azacitidine. The overall response rate was 42%. Median overall survival (OS) was 12.3 months. We confirmed poor-risk cytogenetics, therapy-related AML, performance score ≥2, and white blood cell count ≥15x10^9/l as independent adverse predictors for OS. The BM blast percentage, however, had no impact on OS (*P*=0.55).

In conclusion, administration of azacitidine is effective in AML patients with 20-30% and >30% BM blasts.

INTRODUCTION

Acute myeloid leukemia (AML) is a heterogeneous clonal disorder of hematopoietic progenitor cells with different molecular genetic abnormalities, clinical characteristics, and variable outcomes with currently available treatment.[1,2] AML is most common in the elderly with a median age at presentation of approximately 70 years.[2] Older AML patients generally have a limited benefit with currently available treatment due to a combination of poor chemotherapeutic tolerance and inherent disease resistance.[3-10] Nevertheless, several studies on intensive and non-intensive treatment types suggest that older AML patients benefit from treatment.[11,12]

Recently, azacitidine has become available for (older) AML patients with 20-30% bone marrow (BM) blasts. A phase 3 study demonstrated that azacitidine significantly improved OS in higher-risk MDS patients compared to best supportive care and low-dose cytarabine.[13] A post-hoc analysis of this study showed improved OS in the subgroup of AML patients with 20-30% blasts treated with azacitidine compared with best supportive care.[14] The efficacy of azacitidine in previously untreated AML patients has been confirmed in a retrospective analysis of an Italian compassionate program and in a small German prospective multicenter study.[15,16] However, until now no comparison of treatment outcome has been made between AML patients with 20-30% BM blasts, for whom azacitidine is generally available, and patients with 30% or more BM blasts, who can only be treated off-label. Therefore, we analyzed the treatment results of 55 newly diagnosed AML patients who have been treated with azacitidine.

PATIENTS AND METHODS

Patients and data collection

After FDA approval of azacitidine in the US and before EMA approval in the EU, a compassionate named patient program (NPP) was initiated in the Netherlands for MDS, CMML, and AML patients with 20-30% blasts. The results of this Dutch NPP have been reported.[17] In the present retrospective study, the NPP was extended and an analysis was made of 55 consecutive newly diagnosed AML patients (with 20-30% and >30% BM blasts) who have been treated upfront with azacitidine. Data of this extended compassionate NPP has been collected between August 2010 and March 2012 after informed consent in accordance with the Declaration of Helsinki by studying health records. Diagnoses were made using World Health Organization (WHO)-2008 criteria.[18] Cytogenetic risk could be determined in 52 of 55 patients according to the refined cytogenetic classification of the Medical Research Council.[19] The BM blast count refers to myeloblasts or monoblasts.

Treatment

Azacitidine was administered subcutaneously at the approved schedule of 75 mg/m²/day during 7 days every 28 days. Physicians intended to give at least 6 cycles of treatment. Patients who responded well were to continue treatment until progression. Red blood cell (RBC) transfusions were given in agreement with general recommendations: Hb <8 g/dl. RBC transfusion dependency was defined as receiving ≥2 RBC transfusions every 8 weeks.

Response criteria and study endpoints

Response was evaluated after every cycle by blood count and by bone marrow aspirate if available. All patients had received a bone marrow aspirate at diagnosis. Morphologic complete remission (CR), CR with incomplete blood count recovery (CRi), and partial remission (PR) were defined according to IWG-2003 criteria for AML.[20] Hematological improvement of the erythroid, neutrophil, and platelet lineages was defined by IWG-2006 criteria.[21] Overall survival (OS) was defined as the time from onset of azacitidine treatment to death. Patients who remained alive were censored at the last visit to the hospital.

Statistical analysis

Analyses were made on an intention-to-treat basis. Differences between groups in patient characteristics and response rates were compared using 2-sided Fisher's exact tests or chi-square tests for categorical variables and Student's t-tests or Mann-Whitney U tests for quantitative variables. Survival curves were estimated with the Kaplan-Meier method. Predictive factors for OS were analyzed by Wald tests for univariate and multivariate comparisons. Cox proportional hazards regression models were used to estimate hazard ratios (HR) and associated 95% confidence intervals (CI). A *P*-value <0.05 was considered significant. SPSS-19 was used for analysis.

RESULTS

Baseline characteristics of the study population

The study population included 55 newly diagnosed and previously untreated AML patients from 12 different hospitals. Baseline characteristics are depicted in table 1. Median age was 73 years (range 59-84). The median BM blast count was 25% (14-85%); 38 (69%) patients had <30% BM blasts and 17 (31%) patients had ≥30% BM blasts. There were no significant differences in baseline characteristics between patients with <30% and ≥30% BM blasts (Table 1).

Table 1. Baseline patient characteristics by BM blast count.

	All patients (*N*=55)	<30% BM blasts (*N*=38)	≥30% BM blasts (N=17)	*P*
Age (years)				0.15
Median (range)	73 (59-84)	72 (60-84)	75 (59-82)	
Sex, female	14 (25%)	10 (26%)	4 (24%)	1.00
AML, type				0.50
De novo	34 (62%)	25 (66%)	9 (53%)	
Previous MDS	11 (20%)	6 (16%)	5 (29%)	
Therapy related	10 (18%)	7 (18%)	3 (18%)	
Cytogenetic risk				0.53
Favorable	4 (4%)	2 (6%)	0 (0%)	
Intermediate	33 (64%)	21 (60%)	12 (71%)	
Poor	17 (33%)	12 (34%)	5 (29%)	
BM blasts (%)				<0.001
Median (range)	25 (14-85)	22 (14-29)	48 (31-85)	
Circulating blasts				0.36
Absent	19 (35%)	15 (39%)	4 (24%)	
Present	36 (65%)	23 (61%)	13 (76%)	
WBC (x10^9/l)				
Median (range)	3 (0-146[§])	3 (0-146[§])	2 (0-61)	0.44
≥ 15 x10^9/l	12 (22%)	8 (21%)	4 (24%)	1.00
LDH increased	26 (51%)	18 (51%)	8 (50%)	1.00
Transfusion dependency	33 (61%)	24 (63%)	15 (53%)	0.56
WHO performance score				0.72
0-1	41 (79%)	29 (81%)	12 (75%)	
2-4	11 (21%)	7 (19%)	4 (25%)	

Results are reported as N (%) or median (range). Abbreviations: BM, bone marrow; AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; WBC, white blood cell count; LDH, lacate dehydrogenase; WHO, world health organisation. [§] One patient with 27% BM blasts had myelofibrosis with a high leukocyte count of 146 x10^9/l including 70 x10^9/l peripheral blasts at baseline.

Response to treatment and overall survival

The median number of azacitidine cycles was 6 (range 1-27) (Table 2). Response was achieved in 23 (42%) patients, including 13 (24%) CR, 4 (7%) CRi, and 6 (11%) PR. Hematological improvement was achieved in 23 (42%) patients, of whom 15 patients had improvement of the erythroid lineage, 19 of the platelet lineage, and 7 of the neutrophil lineage, with combined responses of

Table 2. Treatment outcome by BM blast count.

	All patients (*N*=55)	<30% BM blasts (*N*=38)	≥30% BM blasts (N=17)	*P*
Number of azacitidine cycles	6 (1-27)	6 (1-27)	5 (1-24)	0.96
< 3 cycles	14 (26%)	10 (26%)	4 (24%)	0.83
Response, overall	23 (42%)	16 (42%)	7 (41%)	1.00
CR	13 (24%)	10 (26%)	3 (18%)	0.81
CRi	4 (7%)	2 (5%)	2 (12%)	
PR	6 (11%)	4 (11%)	2 (12%)	
Hematological improvement (HI)	23 (42%)	15 (39%)	8 (47%)	1.00
Time to response (months)	4 (1-10)	4 (2-10)	4 (1-6)	0.28
Time to HI (months)	2 (1-7)	2 (1-4)	2 (1-7)	0.63
Overall survival (months)	12.3	14.3	11.7	0.55
95% CI	(7.8-18.0)	(7.8-20.6)	(1.5-NR)	

Results are reported as N (%) or median (range). Abbreviations: BM, bone marrow; CR, complete remission; CRi, CR with incomplete blood count recovery; PR, partial remission; HI, hematological improvement; CI, conficence interval; NR, not reached.

two or three lineages in 16 patients. Three patients had peripheral blood counts that were too high at baseline to evaluate the hematological improvement. Four patients had hematological improvement, but achieved no CR or PR. Median time to response was 4 months (range 1-10) and median time to hematological improvement was 2 months (range 1-7). Duration of response ranged from 9 to at least 27 months. Median duration of response was not reached; response was ongoing in 14 patients at the end of study. Transfusion dependency was present in 33 (61%) patients at baseline and 14 patients became transfusion dependent during the first two cycles. After 1 to 6 cycles, 14/47 (30%) patients became transfusion independent.

Failure to complete at least 3 cycles of azacitidine was reported in 16 (29%) patients. The reasons for discontinuation were disease progression and/or early death (*N*=12), side-effects (pancytopenia and fever; *N*=2), and consent withdrawal (*N*=2). Dose adjustments or schedule changes were made in 15 (27%) patients, of which 11 before the sixth treatment cycle. Schedule change from 7 to 5 days was applied in 9 patients; dose reductions of 50% were applied in 4 patients, and 2 patients had dose reductions of 33% and 25%.

No differences in response rates (CR, CRi, PR) or hematologic improvement rates were found in patients with <30% versus ≥30% BM blasts (Table 2). Median OS of all patients was 12.3 months (95% CI 7.8-18.0 months). Patients with ≥30% BM blasts did not have a survival disadvantage (Table 2). Responders had a longer OS than non-responders (median OS 24.3 versus 4.5 months, *P*<0.001; Fig 1A). Since it generally takes several cycles to achieve a response, we compared also

the patients who completed at least three cycles of azacitidine. This analysis also demonstrated a longer median OS in responders compared to non-responders (24.3 versus 9.7 months, *P*<0.001; Supplementary fig 1). The OS in responders and non-responders was independent of the BM blast percentage (*P*=0.33 and *P*=0.47, respectively; Fig 1B).

Interestingly, of the 17 patients with poor-risk cytogenetics, four achieved a response (3 CR, 1 PR), which was associated with an improved OS compared to non-responding poor-risk patients (14.3 versus 3.7 months; *P*=0.01), illustrating the efficacy of azacitidine also in patients with poor risk cytogenetics.

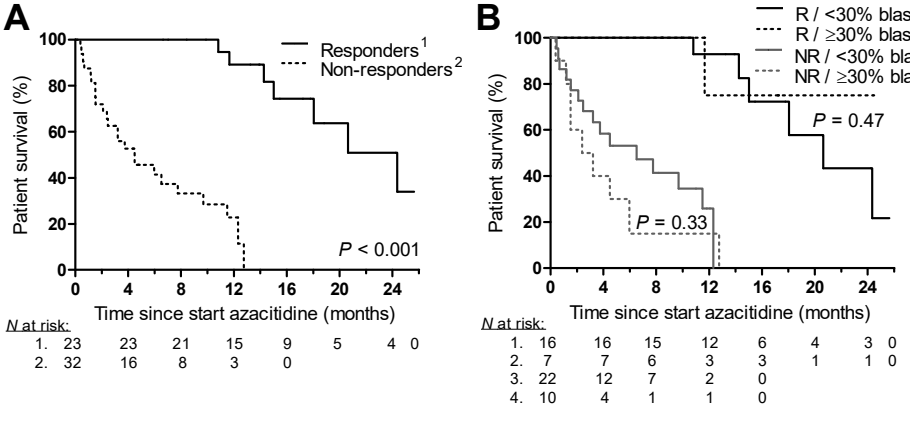

Figure 1. Patient survival by response to azacitidine.
(A) Median OS was significantly better in responders compared to non-responders (24.3 versus 4.5 months; *P*<0.001). (B) The BM blasts percentage had no impact on OS in responders (R) or non-responders (NR).

Predictors for overall survival

We analyzed potential predictors for OS that were selected based on previous studies.[2,22] Univariate analysis revealed no difference in OS in patients with <30% versus ≥30% BM blasts (Table 3a; Fig 2A). In contrast, in univariate analysis, poor OS was associated with poor-risk cytogenetics, therapy-related AML, and WHO performance score ≥2 (Table 3a; Figs 3B,C,D). Based on our previous experience, we were also interested in the predictive value of platelet doubling after the first cycle of azacitidine.[17] However, only 4 patients showed a platelet doubling after the first cycle of azacitidine. Therefore, platelet doubling was not included in further analyses.

For multivariate analysis, we selected predictors for OS with *P*<0.15 in univariate analysis. Multivariate analysis confirmed poor-risk cytogenetics, therapy-related AML, baseline WHO performance score ≥2, and baseline WBC ≥15x10^9/l as independent adverse predictors for OS (Table 3b, Fig 3E).

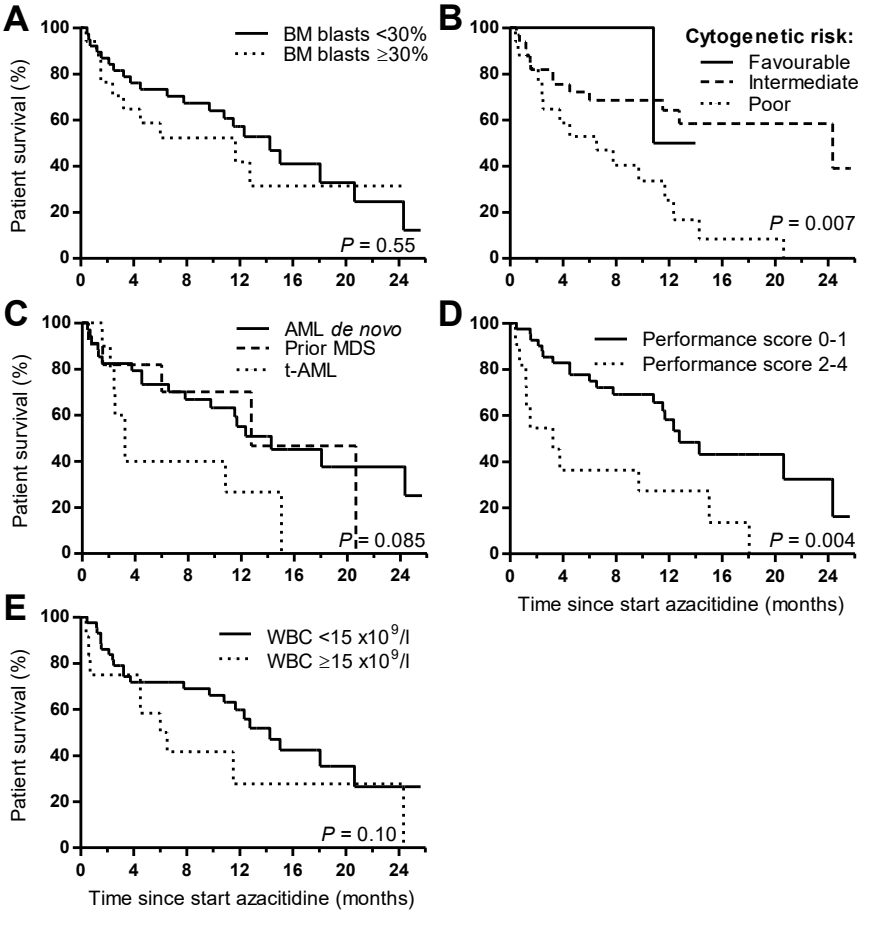

Figure 2. Impact of risk factors on survival of all patients.

(A) BM blasts <30% versus ≥30%. (B) Cytogenetic risk score. (C) AML *de novo* versus prior MDS versus therapy-related AML (t-AML). (D) WHO performance score 0-1 versus 2-4. (E) WBC<15 versus ≥15x10^9/l.

In addition, we assessed in our cohort of AML patients the azacitidine-specific prognostic scoring system that was designed and validated for higher-risk MDS patients and AML patients with 20-30% BM blasts.[17,22] The azacitidine-specific prognostic score was determined as follows: one point was assigned to performance score ≥2, presence of circulating blasts, RBC transfusion dependency of ≥4 units/8 weeks, and intermediate-risk cytogenetics; and two points were assigned to poor-risk cytogenetics. The score could be determined in 47 patients who could subsequently be divided into low- (score 0; *N*=0), intermediate- (score 1-3; *N*=37), and high (score 4-5; *N*=10) risk groups. Median OS was 12.7 months in the intermediate-risk group versus 4.1 months in the high-risk group (*P*=0.013) (Supplementary fig 2). In our cohort, the risk score predicted OS in patients with 20-30% BM blasts, as was shown before[17,22], and also significantly predicted OS in the subgroup with ≥30% BM blasts (*P*=0.006).

Table 3. Predictors for overall survival: univariate and multivariate analysis.

	Median OS (months)	HR (95% CI)	*P*
a. Univariate analysis			
Bone marrow blasts			0.55
< 30%	14.3	ref.	
≥ 30%	11.7	1.3 (0.59-2.7)	
Cytogenetic risk			<0.008*
Favorable	NR	1.1 (0.1-8.8)	0.91
Intermediate	10.2	ref.	
Poor	2.6	3.1 (1.5-6.8)	0.003*
AML, type			0.086
De novo	14.3	ref.	
Previous MDS	12.7	1.0 (0.38-2.8)	0.96
Therapy related	3.2	2.5 (1.1-5.9)	0.038*
WHO performance score			0.006*
0-1	12.7	ref.	
≥ 2	3.2	3.0 (1.4-6.5)	
WBC			0.11
< 15 x10^9/l	14.3	ref.	
≥ 15 x10^9/l	6.0	1.9 (0.86-4.2)	
LDH			0.34
Normal	12.3	ref.	
Increased	11.5	1.4 (0.69-2.9)	
Circulating blasts			0.44
Absent	15.0	ref.	
Present	11.5	1.4 (0.6-2.9)	
Transfusion dependency			0.17
No	18.0	ref.	
Yes	11.5	1.7 (0.80-3.8)	
b. Multivariate analysis			
Cytogenetic risk			0.001*
Favorable	NR	0.12 (0.01-1.2)	0.073
Intermediate	10.2	ref.	
Poor	2.6	3.9 (1.6-9.3)	0.002*
AML, type			0.002*
De novo	14.3	ref.	
Previous MDS	12.7	2.1 (0.68-6.8)	0.19
Therapy related	3.2	8.9 (2.7-29.3)	<0.001*
WHO performance score			<0.001*
0-1	12.7	ref.	
≥ 2	3.2	7.3 (2.6-20.0)	
White blood cells			0.003*
< 15 x10^9/l	14.3	ref.	
≥ 15 x10^9/l	6.0	5.3 (1.7-15.8)	

*Abbreviations: OS, overall survival; HR, hazard ratio; CI, confidence interval; AML, acute myeloid leukemia; WBC, white blood cell count; LDH, lacate dehydrogenase; ref., reference group; NR, not reached. *Statistically significant difference.*

DISCUSSION

In this multicenter retrospective analysis, 55 patients with newly diagnosed and untreated AML received azacitidine for a median of 6 cycles. In 42% of the patients a response was achieved, including 24% CR. These response rates are comparable with the results of the AML patients in the AZA-001 trial (18% CR), with the previously untreated AML patients enrolled in an Italian compassionate program (overall response 50%), and with the previously untreated AML patients included in a German prospective multicenter trial (overall response 48%).[14-16] Further, in our study the median OS was 12.3 months, which was less than the 24.5 months in the AZA-001 trial, but superior compared to the 9 months observed in the untreated patients in the Italian NPP and 7.7 months in the German trial.[14-16] Altogether, these data show that the efficacy of azacitidine in newly diagnosed AML patients can also be confirmed by the extended Dutch NPP. Interestingly, also patients with poor-risk cytogenetics may benefit from azacitidine treatment. Indeed, in our study, 4 of 17 patients with poor-risk cytogenetics showed a response to azacitidine which was associated with an improved OS.

Although azacitidine is currently only registered for the treatment of AML with 20-30% BM blasts, no differences in survival and response rates were observed in patients with <30% and ≥30% BM blasts. The ongoing AML-001 trial, which compares azacitidine with intensive chemotherapy or best supportive care in AML patients with ≥30% blasts, should be awaited to confirm our observations in a prospective randomized clinical trial. The earlier mentioned German and Italian studies did not compare the efficacy of azacitidine based on the percentage of BM blasts.[15,16] Factors that, in contrast to the percentage of BM blasts, significantly and independently predicted for OS were cytogenetic risk status, AML type (therapy-related), baseline WBC, and WHO performance score.

Identification of patients who are likely or unlikely to benefit from azacitidine treatment is, besides from a scientific- and patient perspective, also an important issue in managing the costs of healthcare. Therefore, it would be of help to have response predictors. In the French NPP, in a cohort of 282 higher-risk MDS patients, four routine factors have been identified that were predictive for OS. The prognostic relevance of these factors (WHO performance score, circulating blasts, RBC transfusion dependency, and cytogenetic risk) could be confirmed in our cohort of 55 untreated AML patients and might be of benefit for the design of future trials.

Finally, the treatment of older AML patients is clinically challenging. Our data on 55 newly diagnosed and previously untreated AML patients suggest that administration of azacitidine to older AML patients is feasible and also has a favorable impact on outcome in patients with more than 30% BM blasts.

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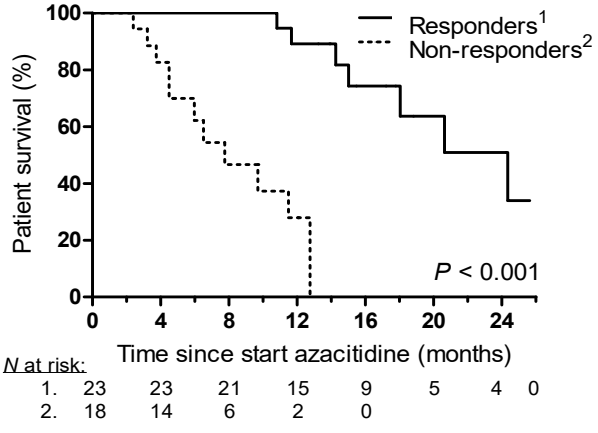
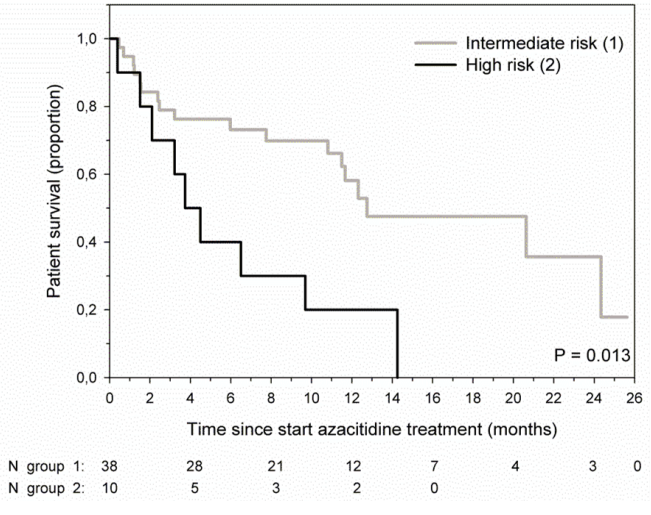
SUPPLEMENTARY DATA



Supplementary figure 1. Survival of patients who received at least three azacitidine cycles by response.
Median OS was significantly better in responders compared to non-responders who achieved three cycles or more (24.3 versus 9.7 months; *P*<0.001).

Supplementary figure 2. Validation of the azacitidine-specific prognostic scoring system for overall survival in AML patients.

The score was computed for each patient, assigning one point to: performance score ≥2, presence of circulating blasts, RBC transfusion dependency of ≥ 4 units/ 8 weeks and intermediate-risk cytogenetics; assigning two points to poor-risk cytogenetics. Patients were segregated into three risk groups: low (score 0; 0 patients), intermediate (score 1-3; 38 patients), and high (score 4-5; 10 patients) risk groups. Median OS was significantly longer in intermediate-risk compared to high-risk patients (12.7 versus 4.1 months; *P*=0.013).



Chapter 4

Azacitidine might be beneficial in a subgroup of older AML patients compared to intensive chemotherapy: a single center retrospective study of 227 consecutive patients

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ABSTRACT

Background: Treatment options in older acute myeloid leukemia (AML) patients include intensive chemotherapy, best supportive care (BSC), and hypomethylating agents. Currently, limited data is available on hypomethylating agents in older AML patients in unselected patient populations.

Methods: To compare the effectiveness of azacitidine with conventional therapy, we collected data of 227 consecutive AML patients (≥60 years) who were treated with azacitidine (*N*=26), intensive chemotherapy (*N*=90), and BSC (*N*=97).

Results: Azacitidine-treated patients were older and had more comorbidities, but lower white blood cell- and bone marrow blast counts compared with intensive chemotherapy patients. Complete or partial response was achieved in 42% of azacitidine-treated patients and in 73% of intensive chemotherapy patients (*P*=0.005). However, the overall survival (OS) was similar (1-year-OS 57% versus 56%, *P*=0.93; 2-year-OS 35% versus 35%, *P*=0.92), and remained similar after correction for risk factors in a multivariate analysis. Patients treated with BSC had an inferior OS (1-year- and 2-year-OS 16% and 2%, *P*<0.001). Compared to intensive chemotherapy, azacitidine-treated patients spent less days in the hospital (0.5 versus 56, *P*<0.001), and needed less red blood cell and platelet transfusions (2.7 versus 7, *P*<0.001 and 0.3 versus 5, *P*<0.001) in the first three months.

Conclusions: Azacitidine treatment is associated with a comparable OS but higher tolerability in a subgroup of older AML patients compared with intensive chemotherapy. Patients receiving BSC had a poor prognosis.

INTRODUCTION

Acute myeloid leukemia (AML) is characterised by a differentiation defect of hematopoietic stem- and progenitor cells, leading to the accumulation of blast cells and cytopenias. The incidence of AML increases with age, with a median age at diagnosis of approximately 70 years.[1] Older AML patients generally have a poor prognosis compared to younger patients due to a higher incidence of comorbidities, higher rates of treatment related mortality, and adverse disease characteristics, associated with resistant disease and relapses.[2-5] Median overall survival (OS) of patients over 60 years of age treated with intensive chemotherapy is less than 1 year, with complete remission (CR) rates of about 50% and a treatment related mortality of at least 15%, indicating an unfavourable risk-benefit ratio of intensive chemotherapy.[6,7]

Nevertheless, several studies suggest that older AML patients benefit from treatment. A small randomized clinical trial by the HOVON study group and an analysis of the Swedish Acute Leukemia Registry showed that standard intensive treatment improves early death rates and long-term survival compared to best supportive care only (BSC) in older patients.[8-10] In an additional prospective randomized trial it was demonstrated that low-dose cytarabine treatment was superior to BSC and hydroxyurea in patients with favourable- or intermediate-risk cytogenetics.[11]

The optimal treatment of older AML patients in daily clinical practice remains challenging. A choice should be made between intensive chemotherapy, less intensive treatment, and palliation, considering individual risks and benefits.[7,12] To guide physicians in their decisions, several prognostic factors have been identified and risk scores have been developed based on age, performance status, comorbidities, cytogenetics, molecular markers, clinical variables, and laboratory measurements.[13-17]

Recently, the DNA methyltransferase inhibitor azacitidine has become available for MDS and AML patients with up to 30% bone marrow blast. A superior OS has been demonstrated in AML patients with 20-30% bone marrow blasts treated with azacitidine compared to conventional treatment.[18,19] Two recent studies showed a beneficial outcome in previously untreated AML patients, including patients with more than 30% bone marrow blasts, who were treated with azacitidine.[20,21] However, limited data is available on the treatment of older unselected AML patients with azacitidine compared to conventional treatment options. To study the impact of azacitidine and conventional care options in routine clinical practice, we analysed the treatment results of 227 consecutive newly diagnosed AML patients of 60 years and older in our center.

METHODS

Patients and data collection

For this retrospective study, data has been collected from 227 consecutive AML patients of 60 years and older who were diagnosed and treated between January 2002 and May 2012 at the University Medical Center Groningen. The minimal follow-up time was six months. Data has been collected by studying health records of individual patients between October 2011 and October 2012 in accordance with the Dutch code of conduct for medical research and the Declaration of Helsinki. Diagnoses were made using French-American-British criteria and World Health Organization (WHO)-2008 criteria.[27,28] Cytogenetic risk was defined according to the National Comprehensive Cancer Network (NCCN) guidelines.[29] Baseline comorbidity was quantified by the hematopoietic cell transplantation (HCT) comorbidity index, which was previously demonstrated to be a predictive score in AML patients over 60 years of age treated with intensive chemotherapy.[14,30,31]

Treatment

Azacitidine was available in The Netherlands from December 2008 onwards in a compassionate named patient program. Azacitidine was administered subcutaneously at the approved schedule of 75 mg/m²/day during 7 days every 28 days. It was intended to give at least 6 cycles of azacitidine and to continue treatment until progression in patients who responded well. Dose reductions and delays of treatment cycles could be made.

Intensive chemotherapy was administered according to one of the HOVON studies[9,32,33], which all contain standard dose cytarabine and an anthracycline (www.hovon.nl). As part of the subsequent HOVON studies, patients were randomised to receive or not G-CSF, intermediate dose cytarabine, bevacizumab, clofarabine, or lenalidomide in addition to the chemotherapy. Of the patients treated according to HOVON studies, 57% was officially included in a HOVON study. Allogeneic hematopoietic stem cell transplantation could be applied following induction therapy. Patients with acute promyelocytic leukemia (APL) were treated with ATRA-containing chemotherapy, according to the HOVON 79 study.[34]

Best supportive care (BSC) consisted of transfusions, antibiotics, and hospital admissions as needed. 6-Mercaptopurine and hydroxycarbamide could be added to the treatment. Red blood cell- or platelet transfusions were given in agreement with general recommendations: Hb <8 g/dl, or higher in case of comorbidity, and platelets <20 $x10^9$/l or higher in case of bleeding or anticoagulant therapy.

Response criteria and study endpoints

Response was evaluated after every treatment cycle of intensive chemotherapy and azacitidine by blood count and by bone marrow aspirate if available. Morphologic CR and partial remission (PR) were defined according to IWG-2003 criteria for AML.[145] Response duration was measured from the date at which marrow evaluation took place in patients achieving CR or PR, until relapse or

death or censoring. OS was measured from the date of diagnosis. Patients who remained alive were censored at the time of the last visit to the hospital.

Statistical analysis

Response was evaluated after every treatment cycle of intensive chemotherapy and azacitidine by blood count and by bone marrow aspirate if available. Morphologic CR and partial remission (PR) were defined according to IWG-2003 criteria for AML.[35] Response duration was measured from the date at which marrow evaluation took place in patients achieving CR or PR, until relapse or death or censoring. OS was measured from the date of diagnosis. Patients who remained alive were censored at the time of the last visit to the hospital.

RESULTS

Baseline characteristics of the study population

The study population included 227 consecutive newly diagnosed AML patients of 60 years and older. Patients were treated according to one of the following main strategies: 26 (11%) patients were treated with azacitidine, 90 (40%) patients were treated with intensive chemotherapy, 97 (43%) patients were treated with best supportive care (BSC), and 14 (6%) patients were diagnosed with acute promyelocytic leukemia (APL) and treated with all-trans retinoic acid (ATRA)-containing intensive chemotherapy (Additional figure 1). These fourteen APL patients were excluded from comparisons of azacitidine with intensive chemotherapy, because of the alternative treatment strategy and superior OS. Patients who were treated with azacitidine completed a median of 6 (1-30) cycles. All azacitidine patients started with the standard dose (7 days 75 mg/m²/day), except for one patient with pancytopenia and malaise at diagnosis who received 50 mg/m²/day. A dose reduction of 30% was made in one patient after 10 cycles and a schedule change from 7 to 5 days was applied in one patient after 13 cycles. Failure to receive at least three cycles of azacitidine was reported in six (23%) patients; the reason of interruption was early death (*N*=4), or pancytopenia (*N*=2).

Baseline patient- and disease characteristics of the different treatment groups are shown in Table 1. Considering patient related factors: patients who were treated with azacitidine and BSC were significantly older than patients treated with intensive chemotherapy (*P*<0.001). Patients receiving azacitidine had a superior performance (*P*=0.002), and patients receiving BSC had a worse performance (*P*=0.003) compared to patients receiving intensive chemotherapy. The HCT-comorbidity score was worse in patients treated with azacitidine (*P*=0.029) and BSC (*P*<0.001) compared to intensive chemotherapy. Considering disease related factors: the percentage of secondary AML (including therapy related AML, prior MDS, and prior myeloproliferative neoplasms) was not significantly different between the treatment groups (*P*=0.089). Patients who

Table 1. Baseline patient- and disease characteristics by treatment strategy

	All patients (*N* =213)	Azacitidine (*N* =26)	Intensive chemo-therapy (*N* =90)	BSC (*N* =97)	*P*-value	
					overall	Aza vs IC
Age						
Median (range)	68 (60-96)	70 (60-81)	66 (60-74)	71 (60-96)	<0.001	<0.001
≥ 70 years	80 (38%)	14 (54%)	10 (11%)	56 (58%)	<0.001	<0.001
Sex, male	119 (56%)	17 (65%)	47 (52%)	55 (57%)	0.48	0.27
Performance score ≥ 2	121 (59%)	5 (19%)	47 (54%)	69 (75%)	<0.001	0.002
HCT-comorbidity index						
Low (0)	98 (46%)	9 (35%)	57 (63%)	32 (33%)	<0.001	0.029
Intermediate (1-2)	66 (31%)	8 (31%)	18 (20%)	40 (41%)		
High (> 2)	49 (23%)	9 (35%)	15 (17%)	25 (26%)		
AML FAB classification						
M0/M1	41 (20%)	4 (17%)	21 (21%)	16 (19%)	0.27	0.28
M2	87 (42%)	8 (33%)	40 (40%)	39 (47%)		
M4/M5	51 (25%)	11 (46%)	22 (22%)	18 (22%)		
M6/M7	13 (6%)	1 (4%)	4 (4%)	8 (10%)		
AML type						
De novo	139 (65%)	13 (50%)	65 (72%)	61 (63%)	0.089	0.056
Secondary	74 (35%)	13 (50%)	25 (28%)	36 (37%)		
Bone marrow blasts						
Median (range)	45 (16-100)	27 (20-88)	52 (20-100)	47 (16-93)	<0.001	<0.001
≥ 30%	135 (70%)	11 (42%)	67 (77%)	57 (72%)	0.003	0.001
WBC						
Median (range)	5 (0-360)	3 (0-15)	5 (1-236)	7 (1-360)	0.13	<0.001
≥ 15 x10^9/l	65 (31%)	0 (0%)	31 (34%)	34 (35%)	0.002	<0.001
LDH						
Median (range)	324 (116-4835)	259 (136-1133)	340 (134-2664)	332 (116-4835)	0.15	0.092
> 600 U/l	49 (23%)	2 (8%)	21 (23%)	26 (27%)	0.13	0.15
Cytogenetic risk					0.003	0.48
Favourable	8 (4%)	0 (0%)	4 (4%)	4 (4%)		
Intermediate	135 (63%)	18 (69%)	62 (69%)	55 (57%)		
Unfavourable	47 (22%)	8 (31%)	21 (23%)	18 (19%)		
Not available	23 (11%)	0 (0%)	3 (3%)	20 (21%)		
Molecular markers					0.27	0.45
NPMc+/ITD-	13 (7%)	1 (4%)	9 (11%)	3 (4%)		
Others	163 (93%)	23 (96%)	75 (89%)	65 (96%)		

Patients with promyelocytic leukemia (N=14) were excluded from this analysis. Abbreviations: BSC, best supportive care only; Aza vs IC, azacitidine versus intensive chemotherapy; HCT, hematopoietic cell transplantation; AML, acute myeloid leukemia; BM, bone marrow; WBC, white blood cell count; LDH, lactate dehydrogenase; NPMc+/ITD-, cytoplasmic NPM1 without FLT3 internal tandem duplication. Results are reported as N (%) unless otherwise indicated.

were treated with azacitidine had lower bone marrow (BM) blast counts (*P*<0.001), and white blood cell (WBC) counts (*P*<0.001) compared to patients treated with intensive chemotherapy. Indeed, none of the patients treated with azacitidine had WBC ≥ 15 x10^9/l. The cytogenetic risk score was not significantly different in patients treated with azacitidine compared with intensive chemotherapy (*P*=0.48), but cytogenetic risk was worse in patients treated with BSC compared to intensive chemotherapy (*P*=0.005). In 23 patients, the karyotype was not evaluated at baseline.

Allogeneic hematopoietic cell transplantation (allo-HCT) was applied in 14 patients following induction chemotherapy and also in one patient after azacitidine treatment. Baseline characteristics of these allo-HCT patients are shown in additional table 1. All allo-HCT patients received reduced intensity conditioning with fludarabine (30 mg/m^2 for 3 subsequent days) and 2 Gray total body irradiation (TBI) before transplantation. All patients received mobilised peripheral blood stem cells, that were obtained from HLA matched siblings in 11 patients and from matched unrelated donors in 4 patients. Since transplant strategies have evolved during the study period, the last years, patients in CR after two cycles of chemotherapy with a 10/10 matched donor available under the age of 70 received an allo-HCT. Indeed, of the patients younger than 70 years in CR, 12 (24%) received an allo-HCT, which is 10 (48%) considering this patient group since 2008.

Response

Response (CR, PR) was achieved in 11 (42%) patients who were treated with azacitidine, and in 66 (73%) patients who were treated with intensive chemotherapy, which was significantly different (*P*<0.001; Table 2). Of the 55 patients treated with 6-mercaptopurine, two patients met criteria for PR. In the azacitidine group, median time to response from the start of therapy was 4 months (range 3-7 months) and median duration of response was 16 months. Of the 15 azacitidine-treated patients who did not meet criteria for response, 5 patients had a stable disease for 5-15 months. In the intensive chemotherapy group, CR was achieved in 46 (51%) patients after the first induction cycle and, cumulatively, in 58 (64%) patients after the second induction cycle. Median duration of response in the intensive chemotherapy group was 11 months.

Early mortality and supportive care

The 4- and 8-week mortality rates and the relapse rates were not significantly different in the azacitidine group compared with the intensive chemotherapy group. However, the number of days in the hospital was significantly lower in patients treated with azacitidine compared to intensive chemotherapy during the first three months (0.5 versus 56 days, *P*<0.001) and the following 3 months, i.e. months 4-6 (0 (range 0-8) versus 0 (range 0-81) days, *P*=0.036) after diagnosis (Table 2; Figure 1A). Patients treated with azacitidine needed less red blood cell transfusions (2.7 versus 7, *P*<0.001) and less platelet transfusions (0.3 versus 5, *P*<0.001) during the first three months after diagnosis compared to patients treated with intensive chemotherapy, but the number of red blood cell transfusions during months 4-6 was similar in both treatment

groups (0 versus 0.7, *P*=0.97) (Table 2; Figure 1BC). Similar results were obtained when excluding patients who underwent allo-HCT (Table 2).

Table 2. Treatment outcome of patients treated with azacitidine or intensive chemotherapy

	Azacitidine *(N =26)*	Intensive chemotherapy *(N =90)*		P-value	
		All *(N =90)*	Excl. allo-HCT *(N=76)*	Aza vs all IC	Aza vs IC excl. allo-HCT
Overall survival					
1-year	57%	56%	50%	0.93[1]	0.80[1]
2-year	35%	35%	31%	0.92[1]	0.50[1]
Response, overall	**11 (42%)**	**68 (76%)**	**54 (71%)**	<0.001	0.005
CR	9 (35%)	63 (70%)	49 (65%)		
PR	2 (8%)	5 (6%)	5 (7%)		
No CR or PR	15 (58%)	22 (24%)	22 (29%)		
Early death					
within 4 weeks	1 (4%)	4 (4%)	4 (4%)	0.88[1]	0.79[1]
within 8 weeks	2 (8%)	11 (12%)	11 (12%)	0.51[1]	0.40[1]
Relapse/death after response					
within 1 year	4 (36%)	39 (57%)	34 (63%)	0.21[1]	0.18[1]
within 2 years	5 (45%)	42 (62%)	37 (69%)	0.30[1]	0.14[1]
Days in hospital, median per month (range)					
month 1-3	0.5 (0-30)	56 (2-85)	54 (2-85)	<0.001	0.029
month 4-6	0 (0-8)	0 (0-81)	0 (0-81)	0.036	0.006
RBC transfusions, median per month (range)					
month 1-3	2.7 (0-10)	7 (0-32)	7 (0-32)	<0.001	<0.001
month 4-6	0 (0-13)	1 (0-8)	0 (0-8)	0.97	0.65
PLT transfusions, median per month (range)					
month 1-3	0.3 (0-7)	5 (0-19)	5 (0-19)	<0.001	<0.001
month 4-6	0 (0-1)	0 (0-8)	0 (0-8)	0.016	0.047

[1]*Log rank test. Patients with promyelocytic leukemia (N=14) were excluded from this analysis. Abbreviations: excl. allo-HCT, excluding patients undergoing allogeneic hematopoietic stem cell transplantation; IC, intensive chemotherapy; Aza, azacitidine; vs, versus; CI, confidence interval; CR, complete remission; PR, partial remission; RBC, red blood cell; PLT, platelet. Results are reported as N (%) unless otherwise indicated.*

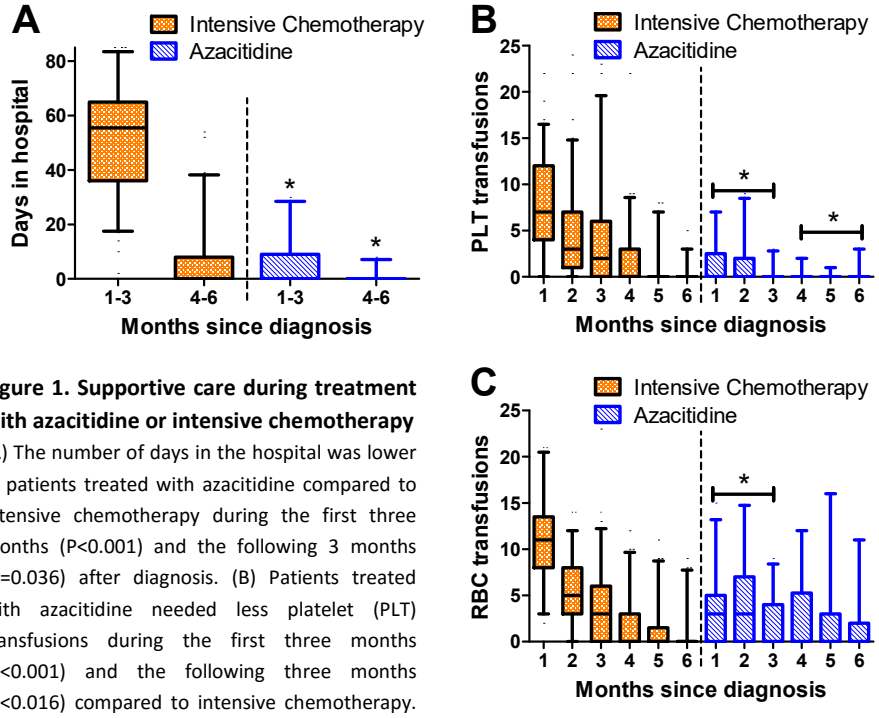

Figure 1. Supportive care during treatment with azacitidine or intensive chemotherapy
(A) The number of days in the hospital was lower in patients treated with azacitidine compared to intensive chemotherapy during the first three months (P<0.001) and the following 3 months (P=0.036) after diagnosis. (B) Patients treated with azacitidine needed less platelet (PLT) transfusions during the first three months (P<0.001) and the following three months (P<0.016) compared to intensive chemotherapy. (C) Patients treated with azacitidine needed less red blood cell (RBC) transfusions (P<0.001) during the first three months compared to intensive chemotherapy. The median, 5th, 25th, 75th, and 95th percentile are depicted.

4

Complications and causes of death

To compare the number of complications and the causes of death, we selected patients who were treated in the time period that azacitidine was applied (2009-2012). Grade 3/4 infections occurred in 9 (35%) azacitidine-treated patients, in 9 (32%) BSC-treated patients, and in all 46 (98%, 1 missing) patients who received intensive chemotherapy. Of the patients treated with azacitidine, 17 (65%) had grade 3/4 anemia or thrombocytopenia at some time during the treatment. Causes of death in patients treated with azacitidine, BSC, or intensive chemotherapy were disease progression in 8 (62%), 19 (79%), and 12 (50%) patients, respectively; infection in combination with progressive disease in 4 (31%), 5 (21%), and 4 (17%) patients, respectively; and infection without disease progression in 0 (0%) azacitdine and BSC patients, but in 7 (30%) intensive chemotherapy patients. One patient treated with intensive chemotherapy died because of ischemic heart disease. The patient who was treated with azacitidine and allo-HCT died because of graft-versus-host disease.

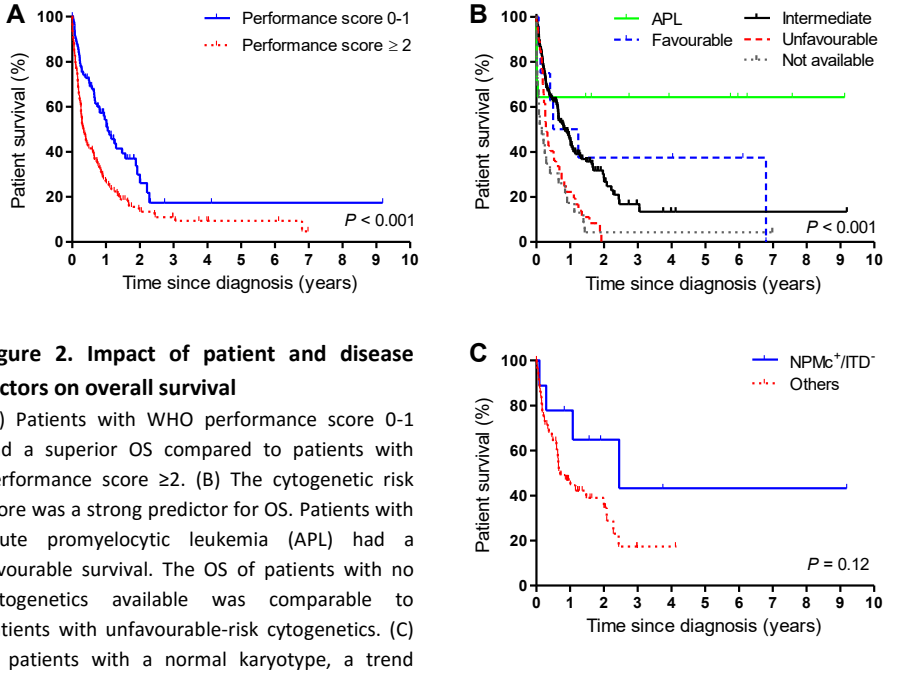

Figure 2. Impact of patient and disease factors on overall survival

(A) Patients with WHO performance score 0-1 had a superior OS compared to patients with performance score ≥2. (B) The cytogenetic risk score was a strong predictor for OS. Patients with acute promyelocytic leukemia (APL) had a favourable survival. The OS of patients with no cytogenetics available was comparable to patients with unfavourable-risk cytogenetics. (C) In patients with a normal karyotype, a trend towards better OS was observed in the presence of cytoplasmic *NPM1* without *FLT3-ITD* (*NPMc+/ITD-*) compared to other patients.

Impact of patient- and disease related factors on overall survival

Median OS of all 227 patients was 7.8 months. Patients with a good performance score (0-1) at baseline had a better OS than patients with an adverse score (≥2) (12.6 versus 4.0 months, respectively; *P*<0.001) (Figure 2A and additional table 2). Cytogenetic risk significantly predicted the survival with a median OS of 5.9 months in patients with favourable-risk cytogenetics, excluding APL patients, 9.7 months in patients with intermediate risk cytogenetics, and 3.6 months in patients with unfavourable-risk cytogenetics (*P*<0.001). Patients with a translocation t(15;17) (APL) had a superior OS (median not reached) compared to other AML patients. The median OS of patients with no karyotype available was 1.9 months, which was similar to the OS of patients with unfavourable-risk cytogenetics (*P*=0.33) (Figure 2B and additional table 2). The nucleophosmin 1 (*NPM1)* mutation status and presence of *FLT3*-internal tandem duplication (ITD) were determined in 67 of the 80 patients with a normal karyotype. Nine patients had cytoplasmic *NPM1* without *FLT3-ITD* (*NPMc+/ITD-*). Although numbers are small, a trend towards better OS was observed in these patients compared to patients without *NPMc+/ITD-* (median OS 29.5 versus 8.5 months; *P*=0.12; Figure 2C).

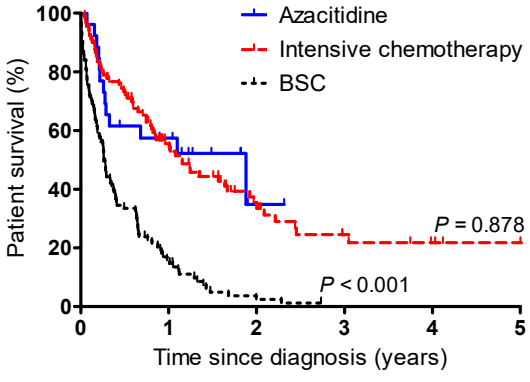

Figure 3. Overall survival by treatment strategy

The OS is similar in patients who were treated with azacitidine (N=26) and intensive chemotherapy (IC; N=90), and is worse in patients who received BSC (N=97).

Impact of treatment on overall survival

The OS in the different treatment groups is depicted in figure 3. The OS was similar in patients receiving azacitidine and patients receiving intensive chemotherapy (table 2; 1-year OS 57% versus 56%, P=0.93; 2-year OS 35% versus 35%, P=0.92). Also when we compared the OS of patients treated since 2009, when azacitidine became available, we observed a similar OS in patients receiving azacitidine and patients receiving intensive chemotherapy (additional figure 2; 1-year OS 57% versus 51%, P=0.80; 2-year OS 35% versus 38%, P=1.00). Since baseline differences were present among patients treated with azacitidine versus intensive chemotherapy, we assessed the OS in the subgroups of patients aged ≥70 years, patients with HCT comorbidity score >0, patients with performance scores <2, patients with <30% BM blasts, and patients with <15x10^9/l WBC's. Also in these subgroups, no significant differences in OS were observed between azacitidine and intensive chemotherapy (*P*=0.74*; P*=0.71*; P*=0.25*; P*=0.71*; P*=0.95, respectively).

Patients who received BSC had a significantly worse OS (1-year and 2-year OS 16% and 2%) compared to azacitidine and intensive chemotherapy (*P*<0.001 and *P*<0.001). When we selected for patients with a good performance score (<2), which was the lesser part of the BSC group, we still observed a significantly worse OS in the BSC group compared to azacitidine (*P*=0.025) and intensive chemotherapy (*P*=0.004).

The median OS of fifteen patients who underwent allo-HCT was 22.5 months from the date of diagnosis, while other patients treated with intensive chemotherapy or azacitidine had a median OS of 12.9 months (p=0.05). After allo-HCT, five patients died due to a relapse (*N*=4) or graft versus host disease (*N*=1).

Of the 90 patients treated with intensive chemotherapy, 51 (57%) were included in a clinical trial and 39 (43%) were treated off-study. Patients treated off-study had more comorbidities (p<0.001) than patients included in a trial. Between these patient groups, no differences in overall response rates (75% versus 72%; *P*=0.81), and no differences in median OS (14.9 versus 12.9 months, respectively; *P*=0.77) were observed (data not shown).

Predictors for overall survival

To assess whether the OS was similar in patients who were treated with azacitidine and intensive chemotherapy after correction for patient- and disease related factors, we performed a multivariate regression analysis. First, we determined which factors were associated with OS. In univariate analysis, unfavourable OS was associated with BSC (versus azacitidine), unfavourable cytogenetic risk or cytogenetic risk not evaluated (versus intermediate risk), age ≥70 years, performance score ≥2, and LDH >600 U/l (Additional table 2). Next, we selected from univariate analysis predictors for OS with *P*<0.10. Multivariate analysis confirmed BSC, unfavourable cytogenetic risk, and LDH >600 U/l as independent adverse predictors for OS. The survival of patients treated with azacitidine versus intensive chemotherapy was not significantly different after correction for these factors (P=0.84).

DISCUSSION

In this single center retrospective study, treatment results of 227 newly diagnosed consecutive AML patients aged ≥60 years who have been treated with BSC, azacitidine, or intensive chemotherapy, were analysed. This study confirms the dismal prognosis of older AML patients who receive only BSC, which was either related to adverse characteristics at baseline or to the treatment type. To optimise treatment in older patients who are unfit for chemotherapy, new therapies are developed, including azacitidine. A treatment benefit for azacitidine compared to BSC was observed in a post-hoc analysis of the AML patients in the AZA-001 randomized trial.[19] In the same trial, also a limited number of patients treated with intensive chemotherapy was included, but no significant differences in OS were observed between patients treated with azacitidine versus intensive chemotherapy.

In our retrospective study, despite the limitations of the relatively small number of patients and disparities between the treatment groups, we observed no significant differences in OS in patients treated with azacitidine compared to intensive chemotherapy. Also a time-dependent effect could be excluded. When corrected for baseline differences in a multivariate analysis, a HR of 1.07 was found with a 95% CI of 0.58-2.0 when azacitidine and intensive chemotherapy were compared. Despite relatively small numbers resulting in a wide CI, our point estimate (HR=1.07) does suggest a comparable treatment effect of azacitidine treatment versus intensive chemotherapy in older AML patients with good performance scores and low WBC counts. Comparable results have been reported recently by the MD Andersen Cancer Center in a cohort study of 671 patients, including 114 patients treated with hypomethylation-based (either azacitidine or decitabine) therapy.[22] In this study they also reported a significant difference in CR rates but similar OS in patients treated with epigenetic therapy versus intensive chemotherapy. These observations, in a larger cohort, are in line with our observations and might suggest that the currently used response criteria are not sufficient for evaluating some (less intensive) treatment strategies. Further, in the perspective of comparing intensive treatment with less intensive treatment, it is also interesting to note that a

small prospective randomised trial between chemotherapy and low-dose cytarabine did not result in a survival benefit for intensive treatment.[23]

An important issue, though difficult to analyse, is the reason why some patients received only BSC, others azacitidine and others intensive chemotherapy. The patients receiving azacitidine differed from the intensive chemotherapy patients in terms of older age, and more comorbidities, but also better performance, lower WBC counts, and lower BM blast counts, while the BSC group consisted of older patients with a high cytogenetic risk score, a poor performance score, and a high HCT-comorbidity index. Apparently, although no defined guidelines were used, the treating physicians seem to have integrated these baseline characteristics in their clinical decisions. Azacitidine is currently only registered for the treatment of AML with bone marrow blasts between 20% and 30%. However, we have recently analysed a cohort of 55 AML patients treated in different hospitals with azacitidine, which included 31% patients with ≥30% bone marrow blasts. A comparable OS and response rates were demonstrated in patients with <30% and ≥30% bone marrow blasts (van der Helm, 2012, in publication). These findings are in line with the results of the Italian named patient program and a German trial.[20,21] An additional advantage of azacitidine is the tolerability[19,24,25], which is reflected in our study by a lower number of days in the hospital and a lower number of red blood cell- and platelet transfusions compared to intensive chemotherapy. In addition, only two of 26 azacitidine-treated patients discontinued treatment because of drug toxicity.

The ongoing phase III trial of azacitidine versus BSC versus intensive chemotherapy (AZA-AML-001 trial) is expected to finally provide the decisive answers for the optimal treatment schedule for elderly AML patients. Recently, the results have been reported of a large phase III trial, comparing the efficacy and safety of decitabine (20 mg/m^2, days 1-5) (*N*=242) with treatment choice (supportive care (*N*=28) and low dose cytarabine (*N*=215) of older patients with newly diagnosed AML and poor- or intermediate-risk cytogenetics.[26] The authors concluded that there was a significant improvement in median OS with decitabine versus treatment choice.

CONCLUSIONS

Azacitidine treatment is associated with a comparable OS but higher tolerability in a subgroup of older AML patients compared with intensive chemotherapy. Patients receiving BSC had a poor prognosis. Therefore, our data suggest that azacitidine treatment might be a valuable alternative to intensive chemotherapy and should be considered instead of BSC in older AML patients.

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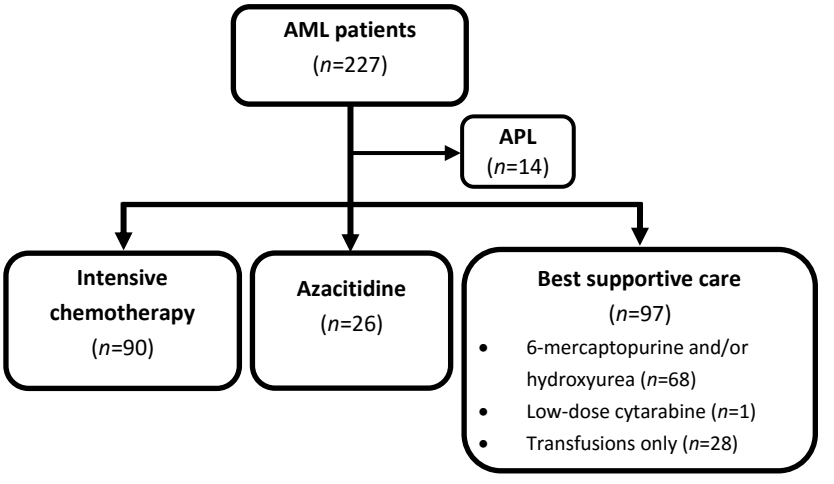
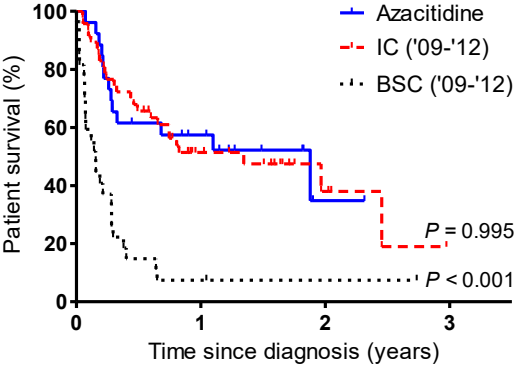
SUPPLEMENTARY FIGURES AND TABLES



Additional figure 1. Flow diagram of the study population

Between January 2002 and May 2012, 227 consecutive AML patients aged ≥60 years were diagnosed and treated in our hospital. Abbreviations: AML, acute myeloid leukemia; APL, acute promyelocytic leukemia.



Additional figure 2. Overall survival by treatment strategy in the time period that azacitidine was available.

The OS is similar in patients who were treated with azacitidine (*N*=26) and intensive chemotherapy (IC; *N*=47), and is worse in patients who received BSC (*N*=28).

Additional table 1. Baseline characteristics of patients who underwent allogeneic hematopoietic cell transplantation

	Allo-HCT patients (*N* =14)
Age, median (range)	64 (60-68)
Sex, male	9 (60%)
Performance score, ≥ 2	5 (33%)
HCT-comorbidity index	
Low (0)	11 (73%)
Intermediate (1-2)	4 (27%)
High (> 2)	0 (0%)
AML FAB classification	
M0/M1	2 (13%)
M2	8 (53%)
M4/M5	4 (27%)
M6/M7	1 (7%)
AML type	
De novo	9 (60%)
Therapy related	2 (13%)
Prior MDS/myeloproliferative disease	4 (27%)
Bone marrow blasts, median (range)	47 (21-69)
≥ 30%	10 (67%)
WBC, median (range)	4 (1-31)
≥ 15 x10^9/l	4 (27%)
LDH, median (range)	253 (134-1224)
> 600 U/l	4 (27%)
Cytogenetic risk	
Favourable	0 (0%)
Intermediate	11 (73%)
Unfavourable	4 (27%)
Molecular markers	
NPMc+/ITD-	0 (0%)
Other	15 (100%)

Abbreviations: HCT, hematopoietic cell transplantation; AML, acute myeloid leukemia; FAB, French-American-British; MDS, myelodysplastic syndrome; WBC, white blood cell count; LDH, lactate dehydrogenase; NPMc+/ITD-, cytoplasmic NPM1 without FLT3 internal tandem duplication

Additional table 2. Predictors for overall survival: univariate and multivariate analysis

	Median OS (months)	HR (95% CI)	P-value
a. Univariate analysis			
Treatment strategy			<0.001
Intensive chemotherapy	13.8	Ref.	
Azacitidine	22.5	0.95 (0.52-1.7)	0.87
BSC	3.1	Ref.	
Azacitidine	22.5	0.30 (0.17-0.53)	<0.001
Intensive chemotherapy	13.8	Ref.	
BSC	3.1	3.2 (2.3-4.5)	<0.001
Cytogenetic risk			<0.001
Favourable	5.9	0.78 (0.34-1.8)	0.56
Intermediate	9.7	Ref.	
Unfavourable	3.6	2.0 (1.4-2.9)	<0.001
Not available	1.9	2.4 (1.5-3.8)	<0.001
AML FAB classification			0.77
M0/M1	10.3	Ref.	
M2	18.8	1.0 (0.68-1.6)	0.85
M4/M5	13.8	0.94 (0.57-1.5)	0.80
M6/M7	20.0	1.4 (0.68-2.7)	0.38
AML type			
De novo	7.9	Ref.	
Secondary	4.8	1.3 (0.91-1.7)	0.16
Age			
< 70 years	9.5	Ref.	
≥ 70 years	3.9	1.5 (1.1-2.1)	0.007
Performance score			
0-1	12.6	Ref.	
≥ 2	4.0	1.8 (1.3-2.5)	<0.001
WBC			
< 15 x10^9/l	8.0	Ref.	
≥ 15 x10^9/l	4.8	1.4 (0.98-1.9)	0.068
Bone marrow blasts			
< 30%	13.4	Ref.	
≥ 30%	7.5	1.4 (0.94-1.9)	0.10
LDH			
≤600 U/l	8.1	Ref.	
>600 U/l	3.4	1.6 (1.1-2.3)	0.012
b. Multivariate analysis			
Treatment strategy			<0.001
Intensive chemotherapy	13.8	Ref.	
Azacitidine	22.5	1.07 (0.58-2.0)	0.84
BSC	3.1	Ref.	
Azacitidine	22.5	0.32 (0.18-0.59)	<0.001

Intensive chemotherapy	13.8	Ref.	
BSC	3.1	3.3 (2.3-4.7)	<0.001
Cytogenetic risk			<0.001
Favourable	5.9	0.85 (0.36-2.0)	0.71
Intermediate	9.7	Ref.	
Unfavourable	3.6	2.4 (1.6-3.5)	<0.001
Not available	1.9	1.5 (0.89-2.4)	0.14
LDH			
≤600 U/l	8.1	Ref.	
>600 U/l	3.4	1.8 (1.2-2.6)	0.002

Patients with promyelocytic leukemia (N=14) are excluded from this analysis. Abbreviations: OS, overall survival; HR, hazard ratio; CI, confidence interval; Ref., reference group; BSC, best supportive care only; AML, acute myeloid leukemia; WBC, white blood cell count; LDH, lactate dehydrogenase.

Chapter 5A

Long-term outcome of older AML patients treated with hypomethylating agents compared to conventional chemotherapy

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Treatment of older AML patients is clinically challenging due to a combination of more unfavorable disease characteristics and comorbidities. The hypomethylating agents (HMAs) azacitidine and decitabine comprise a new treatment strategy in addition to high- and low-intensity conventional treatment modalities including best supportive care (BSC), cytarabine-based intensive chemotherapy (IC), and allogeneic hematopoietic cell transplantation (allo-HCT). Previously, longer overall survival (OS) after treatment with HMAs versus BSC and comparable OS with HMAs versus IC has been observed by us and others in older AML patients in a daily clinical practice setting.[1,2] Recently, the prospective randomized AZA-AML-001 trial revealed similar results in AML patients aged ≥65 years.[3] However, the long-term outcome of HMAs in comparison with conventional care in unselected AML patients, is still unclear. A substantial number of older AML patients is treated with IC followed by allo-HCT whereas allo-HCT after HMAs is still rare, which might influence long-term survival.

To evaluate the long-term outcome of treatment with HMAs versus conventional therapy, taking into account allo-HCT, we re-assessed our cohort of AML patients aged 60 years and older after inclusion of 326 consecutive patients. Patients were diagnosed between January 2002 and August 2015 at the University Medical Center Groningen. Baseline characteristics are depicted in Table 1. Patients treated with HMAs were older, had better WHO performance scores, and had less frequently *FLT3-ITD* or *NPM1* mutations compared to patients treated with IC. In addition, peripheral blood- and bone marrow analyses demonstrated lower blast counts, white blood cell (WBC) counts and lactate dehydrogenase (LDH) in patients treated with HMAs. BSC was associated with poor WHO performance scores, high WBC counts and high LDH compared to HMAs. After a median follow-up (as calculated with the reverse Kaplan-Meier method[4]) of 4.1 years for the entire cohort and 2.4 years for patients treated with hypomethylating agents, 255 (78%) deaths had occurred, including 40 (69%) deaths in the HMA group. Seventeen patients had acute promyelocytic leukemia (APL) and were excluded from comparisons of treatment outcome.

HMA therapy was associated with improved survival compared to BSC as previously observed (median OS 13.3 versus 3.0 months, hazard ratio (HR) 0.33 (95% confidence interval (CI) 0.23-0.48), $p < 0.001$)). To compare the OS of HMA patients with IC patients we used a time-dependent survival analysis that allowed patients to switch to the allo-HCT group at the time of allo-HCT. This method allowed us to compare HMAs with IC while correcting for the effect of allo-HCT, and to separately depict the survival of allo-HCT patients (Figure 1). Our time-dependent survival estimation showed that the median OS was similar in patients treated with HMAs (13.3 months) compared to IC (13.8 months; HR 1.13 (95% CI 0.76-1.66), $p = 0.55$). Of note, none of the patients survived beyond 40 months with HMAs in our cohort in contrast to IC. However, 14 HMA patients (including four with favorable genetic risk) were censored at the end of study and may have lived longer. Due to small numbers at risk after 36 months, no statistical or clinical significance could be assigned to the plateau of the IC curve. One- and 2-year OS rates were 55% and 43% with HMAs, 54% and 33% with IC, and 14% and 3% with BSC. Within the patient group treated with HMAs, median OS with azacitidine was 13.2 months (95% CI 4.3-22.1). Due to the small number (N = 6)

Table 1. Baseline characteristics

	AZA/DAC (N = 48/6)	BSC (N = 114)	IC (N = 101)	Allo-HCT (N = 40)
Age (years), median (min-max)	72 (60-83)	72 (60-95)	67 (60-74)*	64 (60-72)*
≥ 70	38 (70%)	71 (62%)	17 (17%)*	3 (8%)*
Male gender	33 (61%)	65 (57%)	55 (55%)	27 (68%)
WHO performance score ≥ 2	15 (28%)	80 (71%)*	51 (51%)*	11 (28%)
HCT-comorbidity index				
0	14 (26%)	31 (27%)	43 (43%)	25 (63%)*
1-2	21 (39%)	36 (32%)	36 (36%)	3 (8%)*
>2	19 (35%)	47 (41%)	22 (22%)	12 (30%)
WHO diagnosis				
Recurrent genetic abnormalities	1 (2%)	0 (0%)	4 (4%)	0 (0%)
Myelodysplasia-related changes	24 (44%)	45 (40%)	32 (32%)	13 (33%)
Therapy-related changes	11 (20%)	19 (17%)	9 (9%)	6 (15%)
Not otherwise specified	18 (33%)	48 (43%)	56 (55%)*	21 (53%)
Diagnosis before 2009	0 (0%)	69 (61%)*	41 (41%)*	2 (5%)
Prior MDS	9 (17%)	18 (16%)	17 (17%)	4 (10%)
BM blasts (%), median (min-max)	32 (20-96)	44 (16-96)	55 (20-100)*	37 (21-99)
>30%	31 (59%)	77 (72%)	77 (78%)*	25 (64%)
PB blasts (%), median (min-max)	2 (0-92)	15 (0-96)	17 (0-95)*	10 (0-99)
ELN genetic risk group				
Favorable	5 (10%)	10 (11%)	16 (17%)	1 (3%)
Intermediate I or II	24 (46%)	56 (61%)	55 (57%)	24 (60%)
Adverse	23 (44%)	26 (28%)	26 (27%)	15 (38%)
Monosomal karyotype	11 (21%)	17 (19%)	13 (14%)	4 (10%)
NPM1 mutation	6 (11%)	7 (6%)	22 (22%)*	3 (8%)*
NPM1 wild-type	41 (76%)	50 (44%)	56 (55%)	33 (83%)
Missing	7 (13%)	57 (50%)	23 (23%)	4 (10%)
FLT3-ITD	1 (2%)	11 (10%)	24 (24%)*	6 (15%)*
FLT3 wild-type	43 (80%)	66 (58%)	68 (67%)	31 (78%)
Missing	10 (19%)	37 (33%)	9 (9%)	3 (8%)
WBC (>20 x10^9/L)	7 (13%)	33 (29%)*	32 (32%)*	8 (20%)
Hb (mmol/L), median (min-max)	6.1 (3.0-9.6)	5.8 (3.9-8.9)	5.6 (2.5-9.0)	6.0 (4.4-9.3)
Platelets (x10^9/L), median (min-max)	49 (6-304)	46 (2-353)	53 (7-404)	60 (13-477)
LDH > ULN	24 (45%)	79 (71%)*	71 (70%)*	22 (56%)

**p<0.05 compared to azacitidine/decitabine. AZA, azacitidine; DAC, decitabine; BSC, best supportive care only; IC, intensive chemotherapy; allo-HCT, allogeneic hematopoietic cell transplantation; HCT, hematopoietic cell transplantation; MDS, myelodysplastic syndromes; BM, bone marrow; PB, peripheral blood; ELN, European LeukemiaNet; WBC, white blood cell*

count; LDH, lactate dehydrogenase; ULN, upper limit of normal.

and short follow-up time of patients receiving decitabine, no separate analysis for decitabine could be conducted. Median OS in patients diagnosed with APL was 8.2 years.

Fourty patients underwent allo-HCT after IC (N = 36), azacitidine (N = 2), or decitabine (N = 2). Four patients received allo-HCT in second remission. Allo-HCT was associated with younger age (p = 0.003) and lower HCT-comorbidity scores (p = 0.001) compared to IC or HMA without allo-HCT (Table 1). Cytogenetic risk was intermediate in 60% and adverse in 38% of the allo-HCT patients. After a median follow-up of 2.3 years, median OS of patients who underwent allo-HCT was 33.8 months from transplantation and 39.6 months from diagnosis. Allo-HCT was associated with longer OS than HMAs (HR 0.63 (95% CI 0.35-1.15), p = 0.14) or IC without allo-HCT (HR 0.71 (95% CI 0.41-1.24), p = 0.23), although in our limited and retrospective cohort not statistically significant in time-dependent analysis. Favorable OS after allo-HCT in older AML patients was also observed in an observational study, in a large post-hoc analysis of four prospective HOVON/SAKK trials, and in a phase II prospective trial.[5-7] In our cohort, causes of death in patients who underwent allo-HCT were relapse (N = 10), graft-versus-host disease (N = 2), infection (N = 2), graft failure (N = 1), and unknown (N = 1).

Peripheral blood- and bone marrow responses were more frequently observed with IC compared to HMAs. Overall response rates (complete remission (CR), CR with incomplete blood count recovery (CRi), partial remission (PR) and stable disease with hematologic improvement (HI)) were 52% in HMA patients versus 78% in IC patients (p < 0.001). Specifically, CR, CRi, PR, or HI was observed in 29%, 3%, 9%, and 10% of HMA patients, respectively; and in 50%, 18%, 7%, and 3% of IC patients. A disparity between response and survival has been observed more often with HMAs, suggesting that new response criteria for HMAs may have to be considered.[8] Median response duration was 24.3 months in HMA patients versus 20.1 months in IC patients (p = 0.66). Therapy-associated mortality, as reflected by 4-week- and 8-week mortality rates, was not signifficantly different between HMAs (N = 2 (3%) and 7 (12%), respectively) and IC (N = 11 (8%) and 22 (16%), respectively; p = 0.26 and 0.49).

Subgroup analyses revealed no significant differences in OS between patients treated with HMAs versus IC for patients with favorable, intermediate or adverse genetic risk scores; monosomal karyotype; WBC counts above or below 20 $x10^9$/L; bone marrow blast counts above or below 30%; age above or below 70 years; low, intermediate, or high HCT-comorbidity index; therapy-related AML; or AML with myelodysplasia-related changes. Also responses to HMAs were observed across all subgroups, suggesting that HMAs might be considered in all subgroups of patients.

Since allo-HCT is regarded as the only curative option in (older) AML patients[9] and since upfront treatment with HMAs or IC may induce similar OS in older subgroups[10-12], it would be interesting to explore the possibilities of azacitidine or decitabine as cytoreductive therapy before allo-HCT. A recent small prospective study reported favorable results of the addition of decitabine to a fludarabine/total body irradiation-conditioning regimen.[13] In addition, induction of CD8+ T-cell

responses against epigenetically silenced tumor-associated antigens has been observed upon addition of decitabine, which may contribute to disease control post-transplant.[13-15] Further, HMAs are associated with relatively mild side-effect profiles possibly allowing more older AML patients to stay in good physical condition to proceed with allo-HCT.[16] In summary, our data suggests that HMAs and IC without allo-HCT induce similar OS in AML patients aged 60 years and older. To select the best treatment strategy for subgroups of older AML patients, our observations warrant further evaluation in prospective randomized trials.

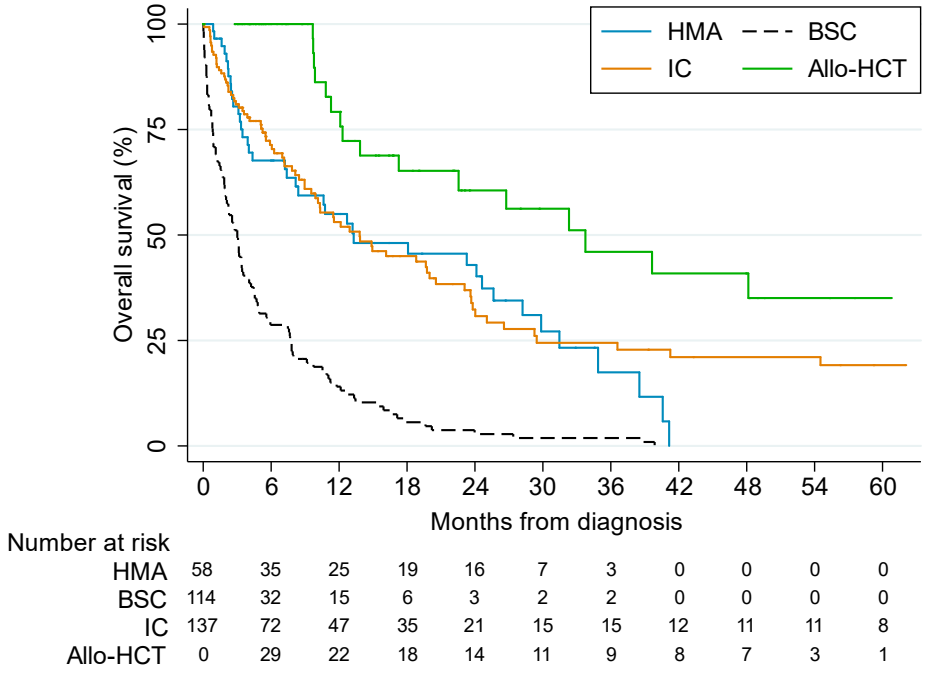

Figure 1. Overall survival by treatment strategy

Survival curves were estimated by a time-dependent Cox regression analysis that allowed patients to switch from the HMA group (N = 4) or IC group (N = 36) to the allo-HCT group at the time of transplantation. Patients were at risk in the HMA or IC group before allo-HCT and at risk in the allo-HCT group from the day of allo-HCT. Therefore, the numbers at risk differ from Table 1. Median time to allo-HCT was 5.0 months (range 2.8-23.5 months). Statistical analysis was performed in STATA 13.0 by splitting the allo-HCT cases in a time-before-transplantation with censoring at allo-HCT and a time from allo-HCT until death or end of follow-up. The first patient received allo-HCT after 2.8 months; therefore the OS curve of the allo-HCT group starts at 2.8 months. *BSC, best supportive care; HMA, hypomethylating agent; IC, intensive chemotherapy; allo-HCT, allogeneic hematopoietic cell transplantation.*

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Chapter 5B

Overexpression of TP53 is associated with poor survival, but not with reduced response to hypomethylating agents in older patients with acute myeloid leukemia.

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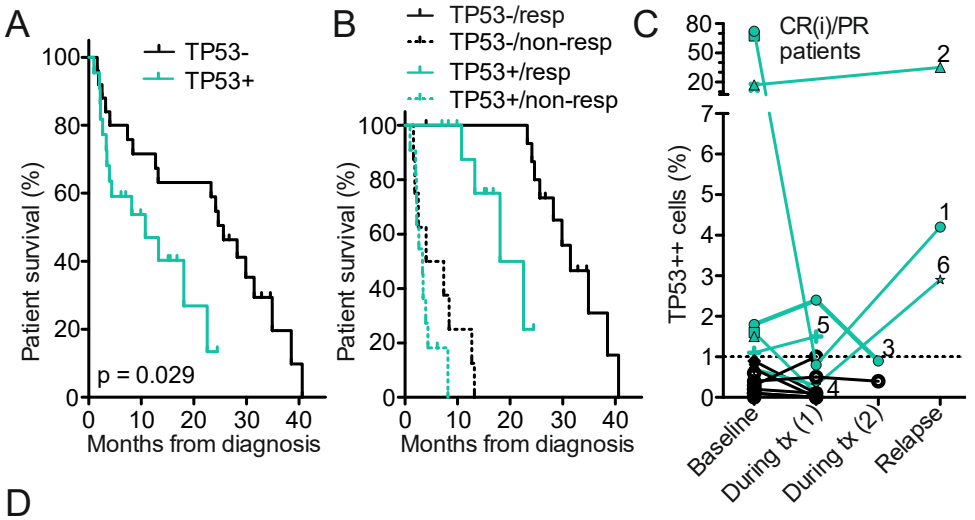
Over the past few decades, it has become clear that some gene mutations are important for risk stratification and predicting response to therapy in AML patients. Mutations in *TET2* and *DNMT3A* genes are associated with unfavourable outcome in AML patients treated with high-dose chemotherapy, while improved outcome has been reported in the setting of hypomethylating agents (HMAs).[1,2] Additional mutations with unfavourable prognosis are *TP53* mutations, frequently coinciding with complex karyotypes.[3] Outcome following high-dose chemotherapy is poor in AML patients with a *TP53* mutation, with a median overall survival (OS) of about ten months.[3] However, treatment outcome following HMAs is less well defined. Studies in myelodysplastic syndromes (MDS) and secondary- or low-blast-count AML suggest that patients with *TP53* mutations may benefit from HMAs.[4,5] To investigate the impact of HMA treatment in *TP53*-mutated AML patients, the prevalence of TP53-overexpression at baseline was assessed in a cohort of 47 AML patients treated with HMAs by using a validated immunohistochemistry method.[6,7] In addition, bone marrow biopsies were studied at different time points during treatment in 19 patients. Bone marrow biopsies were stained with the anti-TP53 mouse monoclonal antibody (clone Bp53-11, Ventana Medical Systems, Tucson, USA). At least 800 nucleated cells in two microscopic fields were evaluated at 40x magnification and scored as no staining, weak (TP53+), or strong (TP53++) nuclear staining. Biopsies showing strong TP53 staining in >1% of nucleated cells ('TP53-positive') were considered as *TP53*-mutated[6], which was confirmed by whole exome sequencing in seven separate patients.

Baseline characteristics were comparable among TP53-positive (*N*=22) and TP53-negative (*N*=25) patients; however, TP53-positive patients had more often adverse-risk cytogenetics, monosomal karyotypes, and higher peripheral blast counts (Supplementary table 1). Patients were treated with a median of eight (1-38) azacitidine cycles (75 mg/m^2 7/28 days), or two (1-24) decitabine cycles (20 mg/m^2 10/28 days; after bone marrow response 5/28 days).[8] Two patients received azacitidine after one to three cycles of decitabine. Three patients, all TP53-positive, had an allogeneic hematopoietic cell transplantation with reduced-intensity conditioning following HMA treatment. Overall response rates (complete remission (CR), CR with incomplete blood count recovery (CRi), partial remission (PR), and hematologic improvement (HI)) were not significantly different between TP53-positive and TP53-negative AML patients (p=0.23); eleven (50%) TP53-positive patients achieved a response (36% CR, 5% PR, and 9% HI), compared to sixteen (70%) TP53-negative patients (44% CR, 4% CRi, 17% PR, and 4% HI).

Figure 1. Survival of TP53-positive and TP53-negative patients and percentages of TP53++ cells during HMA treatment

A) Overall survival in TP53-negative (*N* = 25) and TP53-positive (*N* = 22) patients treated with HMAs. B) Overall survival in TP53-positive responders (CR(i), PR or HI) versus non-responders (p<0.001) and in TP53-negative responders versus non-responders (p<0.001). C) Percentage of TP53++ cells in patients who achieved CR(i) or PR. Blue lines indicate patients with a TP53-positive bone marrow biopsy (>1% TP53++ cells) at some time during the course of AML. Numbers correspond with patients listed in the table below. D) Details of patients with more than one evaluable bone marrow biopsy who are TP53-positive at some time during the course of AML. E) Example of TP53 staining of the bone marrow of patient 1.



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Patient	Baseline		During treatment (1)				During treatment (2)				Relapse		
	Blasts (%)	TP53++ (%)	Months of HMA tx	Response	Blasts (%)	TP53++ (%)	Months of HMA tx	Response	Blasts (%)	TP53++ (%)	Months of HMA tx	Blasts (%)	TP53++ (%)
1	44	72.0	3	CR	3	0.8					9	2†	4.2
2	27	16.8		CR							11	40	35
3	26	1.8	1	CRi	4	2.4	6*	CR	3	0.9			
4	21	1.6	3	SD/HI	10	0.1	7	CR	1	n.e.			
5	82	1.1	5	PR	29	1.5							
6	48	0.7	19	CR	2	0.3					31	50	2.9
7	46	4.4	4	SD/HI	33	3.5	7	SD/HI	26	10.3			
8	21	0.0	4	SD	13	1.3							
9	33	n.e.	4	SD/HI	14	4.5	8	SD/HI	15	6.6			

**Allo-HCT in 3rd month. †Relapse 3 months after biopsy. HMA, hypomethylating agent; tx, therapy; CR, complete remission, CRi, CR with incomplete blood count recovery; PR, partial remission; SD/HI, stable disease with hematological improvement; TP53++, cells with TP53 overexpression; n.e., not evaluable.*

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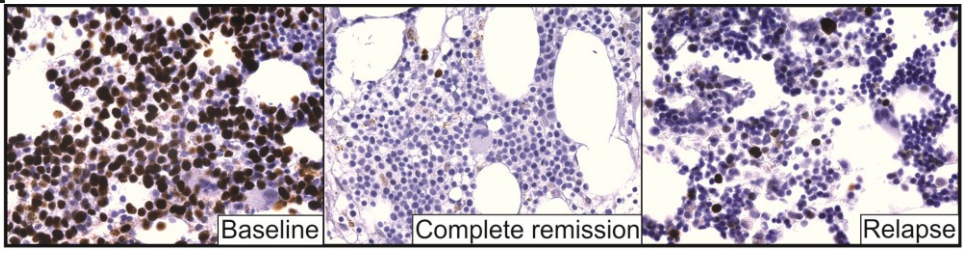

Median OS was 25.6 months in TP53-negative patients versus 10.8 months in patients with TP53 overexpression at baseline (HR 2.5 (95% CI 1.1-5.7), p=0.029; Figure 1A). TP53-positive responders (CR/CRi/PR/HI) had a longer median OS than TP53-positive non-responders (18.1 vs. 3.3 months, respectively; p<0.001; Figure 1B). Median OS was also improved in TP53-negative responders (31.5 months vs. 7.4 months in non-responders; p<0.001). Time to response was not significantly different (4.1 months in TP53-positive vs. 2.8 months in TP53-negative patients; p=0.46). No difference was observed in response duration (21.1 months in TP53-positive vs. 23.2 months in TP53-negative patients; p=0.22), in contrast to a recent study that reported shorter response duration in *TP53*-mutated MDS patients.[9] In our cohort, the shorter median OS of TP53-positive patients was related to a trend towards shorter survival following relapse (1.9 months in TP53-positive vs. 11.2 months in TP53-negative patients; p=0.069) and a shorter median OS of TP53-positive non-responders (3.3 vs. 7.4 months in TP53-negative patients; p=0.10).

Univariate analysis revealed that response to HMAs was associated with WHO performance scores <2 (p=0.004), non-increased LDH (p=0.013), and favourable or intermediate cytogenetic risk scores (p=0.052). These factors remained significant in multivariate analysis. In contrast, no significant impact on response was observed for TP53 overexpression (p=0.18), WBC count >20 $x10^9$/L (p=0.72), age above 70 years (p=0.36), or bone marrow blast count (p=0.55). Univariate analysis of factors influencing survival revealed that poor OS was associated with TP53 overexpression (p=0.034), WHO performance scores ≥2 (p=0.001), increased LDH (p=0.025), poor cytogenetic risk scores (p=0.005), and monosomal karyotypes (p=0.011). In multivariate analysis, WHO performance scores ≥2 remained independently significant, but TP53 overexpression and poor-risk cytogenetics did not predict OS independently of each other, as could be expected. These results indicate that TP53 overexpression is associated with poor survival, however, not with reduced response to HMAs.

Subsequent bone marrow samples were available in 19 patients, which allowed us to evaluate percentages of TP53++ cells during HMA treatment and/or relapse. Percentages of TP53++ cells in TP53-positive patients ranged from 1.1% to 72% (Figure 1C-E). TP53++ cell percentages were often lower than the blast percentage in the bone marrow biopsy, suggesting that TP53++ cells often represented AML subclones. In six patients (including patient 1), TP53++ percentages were higher than the blast count, which could be related to TP53++ lineage-committed precursors and morphologic characteristics of MDS. Nine out of nineteen patients with more than one bone marrow sample were TP53-positive somewhere during the course of treatment (Figure 1C-E). Achievement of CR was associated with a decrease of TP53++ cells below 1% (patient 1 and 3). Relapse was associated with an increase of TP53++ cells (patient 1 and 2). Patient 6 became TP53-positive at relapse after 31 months of azacitidine treatment. This attainment of TP53-positivity might be due to expansion (associated with clonal selection) of a previously undetectable dormant clone, since a recent report showed that *TP53*-mutated cells can be present at low frequencies (0.003-0.7%) years before development of therapy-related AML or MDS.[10] CR(i), PR, or HI was in our cohort sometimes accompanied by a reduction of TP53++ cells (patient 4 and 7), suggesting repression of the *TP53*-mutated AML clone by HMAs; and was sometimes associated

with persistence of TP53++ cells (patient 3, 5, 7, 9), suggesting insensitivity of the *TP53*-mutated clone to HMAs. Together, these data indicate that *TP53*-mutated cells can be targeted by HMAs in a subset of AML patients.

In summary, our data indicate that AML patients with *TP53* mutations generally have a poor OS but can respond to and benefit from treatment with HMAs, with temporary suppression of the *TP53*-mutated clone in a subset of patients. However, to determine whether HMAs improve outcome of *TP53*-mutated patients, prospective trials are needed.

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Supplementary table 1. Baseline characteristics

	TP53-negative (N=25)	TP53-positive (N=22)	p-value
Age (years), median (min-max)	72 (67-83)	72 (60-79)	0.24
≥ 70	19 (76%)	14 (64%)	0.52
Male gender	18 (72%)	12 (55%)	0.24
WHO performance score ≥ 2	5 (2%)	6 (27%)	0.73
HCT-comorbidity index			0.33
0	7 (28%)	5 (23%)	
1-2	11 (44%)	6 (27%)	
>2	7 (28%)	11 (50%)	
WHO diagnosis			0.58
Recurrent genetic abnormalities	0 (0%)	1 (5%)	
Myelodysplasia-related changes	12 (48%)	7 (32%)	
Therapy-related changes	5 (20%)	6 (27%)	
Not otherwise specified	8 (32%)	8 (36%)	
Prior MDS	5 (20%)	2 (9%)	0.42
BM blast %			
Median (min-max)	31 (20-71)	38 (20-95)	0.17
>30%	14 (56%)	13 (62%)	0.77
ELN genetic risk group			0.004*
Favorable	3 (13%)	2 (10%)	
Intermediate I or II	17 (71%)	6 (29%)	
Poor	4 (17%)	13 (62%)	
Monosomal karyotype	0 (0%)	8 (38%)	0.001*
Complex karyotype	3 (13%)	8 (38%)	0.081
Other mutations			
NPM1	0 (0%)	1 (5%)	0.48
FLT3-ITD	2 (9%)	4 (19%)	0.40
WBC >20 x10^9/L	2 (8%)	4 (18%)	0.40
Hb (mmol/L), median (min-max)	6.8 (3.0-8.6)	5.6 (4.1-9.6)	0.17
Platelets (x10^9/L), median (min-max)	52 (9-230)	49 (12-304)	0.38
LDH > ULN	11 (46%)	14 (64%)	0.25
Treatment			0.040*
Azacitidine	24 (96%)	16 (73%)	
Decitabine	1 (4%)	6 (27%)	

HCT, hematopoietic cell transplantation; WHO, World Health Organization; MDS, myelodysplastic syndromes; BM, bone marrow; ELN, European LeukemiaNet; WBC, white blood cell count; Hb, hemoglobin; LDH, lactate dehydrogenase; ULN, upper limit of normal.

Chapter 6

Effective targeting of primitive AML CD34+ cells by the second-generation proteasome inhibitor carfilzomib

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ABSTRACT

In the present study the sensitivity of AML cells to the second-generation proteasome inhibitors carfilzomib and oprozomib was investigated in comparison with the first-generation proteasome inhibitor bortezomib. In patient-derived AML $CD34^+$ cells and AML cell lines, bortezomib and carfilzomib induced a similar reduction in survival and proteasome activity in short-term cultures, whereas the effect of oprozomib was less pronounced. Importantly, carfilzomib was more effective than bortezomib in targeting the more primitive leukemic cells, as reflected by a significant reduction in quiescent $CD34^+CD38^-$ cells and stem cell frequency by carfilzomib. In contrast, normal bone marrow (NBM) $CD34^+$ cells were only mildly affected. In parallel with the increased sensitivity, proteasome activity tended to be higher in AML cells compared to NBM and transcriptome analysis showed an increased expression of several proteasome subunits in AML $CD34^+$ cells. Anti-apoptotic MCL-1 was upregulated upon proteasome inhibition, and the effect of the proteasome inhibitors on the survival of primary AML $CD34^+$ cells could be further enhanced by inhibition of MCL-1. Our results indicate that the proteasome inhibitor carfilzomib is more effective in reducing the long-term survival of AML cells as compared to bortezomib and oprozomib, and might be a promising agent for the treatment of AML.

INTRODUCTION

According to the cancer stem cell model, acute myeloid leukemia (AML) is maintained by rare populations of (preleukemic and) leukemic stem cells that are relatively quiescent, resistant to therapy, and causing frequent relapses after intensive chemotherapy.[1,2] These relapses contribute to 5-year survival rates of only 5-55% in adults, dependent on the cytogenetic risk group.[3] Moreover, with a median age of about 70 years, a large group of AML patients is not eligible for intensive chemotherapy.[4] Therefore, new treatment strategies with lower toxicities which target leukemic stem cells are warranted.

The ubiquitin-proteasome system plays an essential role in protein homeostasis of eukaryotic cells through selective degradation of abnormal and regulatory proteins. The proteasome is a barrel-like complex composed of two outer α-rings and two central β-rings, each containing seven subunits, and two regulatory caps that recognize ubiquitinated proteins. Proteins are cleaved at the proteolytic sites on the β5, β1, and β2 subunit, encoded by the *PSMB5*, *PSMB6*, and *PSMB7* gene, respectively. The proteasome is involved in the regulation of various critical cellular processes including cell proliferation, apoptosis, and DNA repair, and is important for the activation of the pro-survival transcription factor NF-κB by degradation of p-IκBα.[5,6] In hematopoietic cells, a proteasome variant known as the immunoproteasome containing distinct catalytic subunits ($\beta5_i$ (*PSMB8*), $\beta1_i$ (*PSMB9*), and $\beta2_i$ (*PSMB10*)) is present besides the constitutive proteasome.[7,8] The immunoproteasome is associated with processes of antigen presentation on top of constitutive proteasomal functions.

Several abnormalities of the ubiquitin-proteasome system have been described in leukemic cells including a higher expression of the proteasome in leukemic cells compared to normal peripheral blood cells as determined by immunohistochemistry and elevated proteasome activity in the plasma of AML patients. Moreover, NF-κB activity is shown to be increased in stem cell-enriched AML subpopulations as compared to normal bone marrow (NBM) $CD34^+$ cells[9,10], which might be related to the increased activity of various components upstream of NF-κB, such as IRAK1 and TAK1.[11-13] Inhibition of NF-κB with the proteasome inhibitor MG-132 (which is only applicable *in vitro*) induced apoptosis in AML $CD34^+$ cells but not in normal $CD34^+$ cells.[14] Therefore, proteasome inhibition may be a promising treatment strategy in AML.

The first-in-class proteasome inhibitor bortezomib shows clinical effectiveness in multiple myeloma and mantle cell lymphoma.[15] In AML, bortezomib reduces NF-κB activity in particular in the more mature $CD34^-$ AML cell fraction *in vitro* whereas the AML $CD34^+$ cells are less sensitive to bortezomib, which can be ascribed to upregulation of the anti-apoptotic protein MCL-1 and improper inhibition of NF-κB.[16-18] The second-generation proteasome inhibitor carfilzomib and its orally bio-available derivate oprozomib may be more effective since these inhibitors bind irreversibly and more specifically to the proteasome and the immunoproteasome.[19-21] In clinical trials, carfilzomib was active in bortezomib-refractory and -relapsed multiple myeloma patients

and induced fewer side effects than bortezomib.[22-24] Although limited data is currently available on the effect of second-generation proteasome inhibitors in AML, they suggest that AML cells are sensitive to carfilzomib.[25,26] However, the effect on primitive AML $CD34^+$ cells is unclear. In the present study we demonstrate that carfilzomib was more effective in reducing the long-term survival of AML $CD34^+$ cells as compared to bortezomib and oprozomib, whereas normal $CD34^+$ cells were less affected by carfilzomib. Moreover, addition of an MCL-1 inhibitor increased the cytotoxic effects on AML $CD34^+$ cells.

MATERIALS AND METHODS

Reagents

Carfilzomib (lot #6012-85) and oprozomib (lot #1262-071) were kindly provided by Onyx pharmaceuticals. Bortezomib (lot #9EZT500) was obtained from Janssen-Cilag. Obatoclax mesylate (Cat. #S1057) was obtained from Selleckbio.

Cell lines, patient material and healthy controls

The human leukemia cell lines HL-60, OCI-AML3, MOLM13, and THP-1 were cultured in RPMI 1640 supplemented with 10% FCS. Bone marrow and peripheral blood samples from AML patients (See Table SI for patient characteristics) and healthy controls were obtained after informed consent in accordance with institutional guidelines and the Declaration of Helsinki. Normal bone marrow (NBM) was obtained from potential donors for allogeneic bone marrow transplantation and patients who underwent elective total hip replacement. Mobilized peripheral blood cells were obtained from healthy donors who underwent apheresis for allogeneic bone marrow transplantation. Neonatal cord blood was obtained from healthy full-term pregnancies from the obstetrics departments of the UMCG and the Martini Hospital Groningen, the Netherlands. Mononuclear cells were isolated by density gradient centrifugation (Lymphoprep, Stem Cell Technologies), and $CD34^+$ cells were selected by MicroBeads on the AutoMACS Pro Separator (Miltenyi Biotec).

Short-term culture of primary cells

AML, NBM, and normal peripheral blood $CD34^+$ cells were expanded for 2-3 days prior to analysis on mouse stromal (MS5) cells in LTC medium (α-minimum essential medium supplemented with heat-inactivated 12.5% fetal calf serum, heat-inactivated 12.5% horse serum, penicillin and streptomycin, 2 mM glutamine, 57.2 μM β-mercaptoethanol and 1 μM hydrocortisone (all from Sigma, Zwijndrecht, The Netherlands) supplemented with interleukin 3 (IL-3; Gist-Brocades, Delft, the Netherlands), granulocyte colony-stimulating factor (G-CSF; Rhone-Poulenc Rorer, Amstelveen, the Netherlands), and thrombopoietin (TPO; Kirin, Tokyo, Japan) (20 ng/mL each).[27-

[30] Cord blood $CD34^+$ cells were expanded for 2-3 days in hematopoietic progenitor cell growth medium (HPGM; Lonza, Leusden, The Netherlands) supplemented with c-Kit ligand, Flt-3 ligand (both from Amgen, Thousand Oaks, CA), and TPO (100 ng/mL each) prior to analysis. Cultures were kept at 37^oC and 5% CO2.

Cell viability measurements

Cell viability was assessed using MTS assays (Promega, Madison, USA). Cells were seeded in 96 wells plates and incubated with bortezomib, carfilzomib, or oprozomib for 24 hrs at 37°C after which MTS reagent was added. Cell viability was determined after 2-4 hrs by measuring the absorption at 490 nm using an iMark microplate reader (Bio-Rad Veenendaal, the Netherlands).

Flow cytometry analysis and cell sorting

Fluorescence activated cell sorting (FACS) analyses were performed on an LSR II flow cytometer, FACScalibur (Becton Dickinson (BD), Alpen a/d Rijn, The Netherlands), or MacsQuant (Miltenyi Biotec). Cell sorting was performed by MoFLo (Dako Cytomation, Carpinteria, CA, USA) after staining with CD34-APC (Cat. #555824, Lot #3191690, BD). For analysis of apoptotic cells, cells were stained with AnnexinV-FITC (IQP-120F, Miltenyi Biotec) and PI (IQP-121, IQ Products, Groningen, the Netherlands). For analysis of quiescent cells, cells were re-suspended in hematopoietic progenitor cell growth medium (HPGM, Lonza) and stained with 5 µg/mL Hoechst 33342 (Lot #458868, Invitrogen, Bleiswijk, the Netherlands) at 37°C for 45 min, followed by addition of 1 µg/mL Pyronin Y (Sigma) for 30 min. Cells were washed in the solution containing Hoechst and Pyronin Y, followed by staining with CD34-APC and CD38-Alexa700 (Cat. #303524, Lot #B169984, Biolegend, San Diego, CA) at 4°C for 20 min and FACS analysis. Samples with a maximum of 50% cell death in untreated controls after 24 hrs incubation were analyzed using FlowJo V10 software.

Chymotrypsin-like proteasome activity measurements

After 4 hrs incubation at 37°C with proteasome inhibitors, cells were lysed in lysis buffer (50 mM HEPES [pH 7.5], 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, and 2mM ATP).[31] Lysis was performed on ice for 30 minutes with vortexing every 10 minutes. After 15 minutes centrifugation at maximum speed, the supernatant was collected and transferred in 96 wells plates containing assay buffer (115 mM NaCl, 1 mM KH_2PO_4, 5 mM KCl, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM sodium HEPES buffer [pH 7.4].[32] Suc-LLVY-aminomethylcoumarin (AMC; Enzo Life Sciences) was added to each well in a final concentration of 50 mM at the start of the assay. This substrate is cleaved by chymotrypsin-like (proteasome) activity, releasing fluorescent AMC. The rate of fluorescence was measured for 60 minutes at 5 minute-intervals by a Synergy 2 plate reader (Miltenyi Biotek) using 360 nm excitation- and 460 nm emission filters. Non-proteasome background activity was

measured by total inhibition of the proteasome using 1 uM bortezomib and was distracted from all measurements.

Long-term culture initiating cell (LTC-IC) assays

$CD34^+$ cells from AML, NBM, or CB samples were sorted and plated in limiting dilution in 96-well plates pre-coated with MS5 stromal cells and cultured for five weeks in LTC medium. For AML samples, wells containing cobblestone-area forming cells (CFCs) were scored as positive. For NBM and CB samples, wells containing CFCs two weeks after addition of methylcellulose (MethoCult H4230; StemCell Technologies) were scored as positive. The stem cell frequency was calculated using L-Calc Limiting Dilution Software (StemCell Technologies).

Colony forming cell assays

NBM $CD34^+$ cells were incubated in RPMI with the different proteasome inhibitors. After 24 hrs, methylcellulose supplemented with 20 ng/mL IL-3, 20 ng/mL IL-6, 20 ng/mL G-CSF, 20 ng/mL c-kit ligand, and 6 U/mL erythropoietin (Cilag Eprex) was added. After two weeks, CFCs were counted.

Lentiviral transduction

The pRRL-CMVd2EGFP-SFFV-tdTomato vector was made by inserting the XhoI-XbaI fragment (containing the CMV-d2EGFP cassette) from pCMV-d2EGFP[33] (kindly provided by Hiroshi Harada, Radiation and Tumor Biology, Kyoto University, Kyoto, Japan), into the XhoI-NheI cut pRRL-SFFV-tdTomato vector. Lentiviral particles were produced by transient transfection of 293T cells. HL-60 cells were transduced in one round with lentiviral supernatant supplemented with polybrene (4 µg/mL; Sigma, Zwijndrecht, The Netherlands). Tomato positive cells were sorted and used for measurement of proteasome activity.

Micro-array analysis

Gene expression profiling of 66 AML $CD34^+$ samples, 51 AML $CD34^-$ samples, and 22 NBM $CD34^+$ samples was performed previously using the Illumina HumanHT-12 Expression BeadChips as published.[34,35] We selected from this dataset the seventeen proteasome subunit-coding genes and compared the expression rates by the Mann–Whitney U test. To assess the degree of multiple testing, we performed this analysis within a multivariate permutation test with 1000 permutations, a false discovery rate of 5% and a confidence level of 80%. This resulted in a list of significantly upregulated genes, which, based on permutations, contains no more than 5% false positive associations.

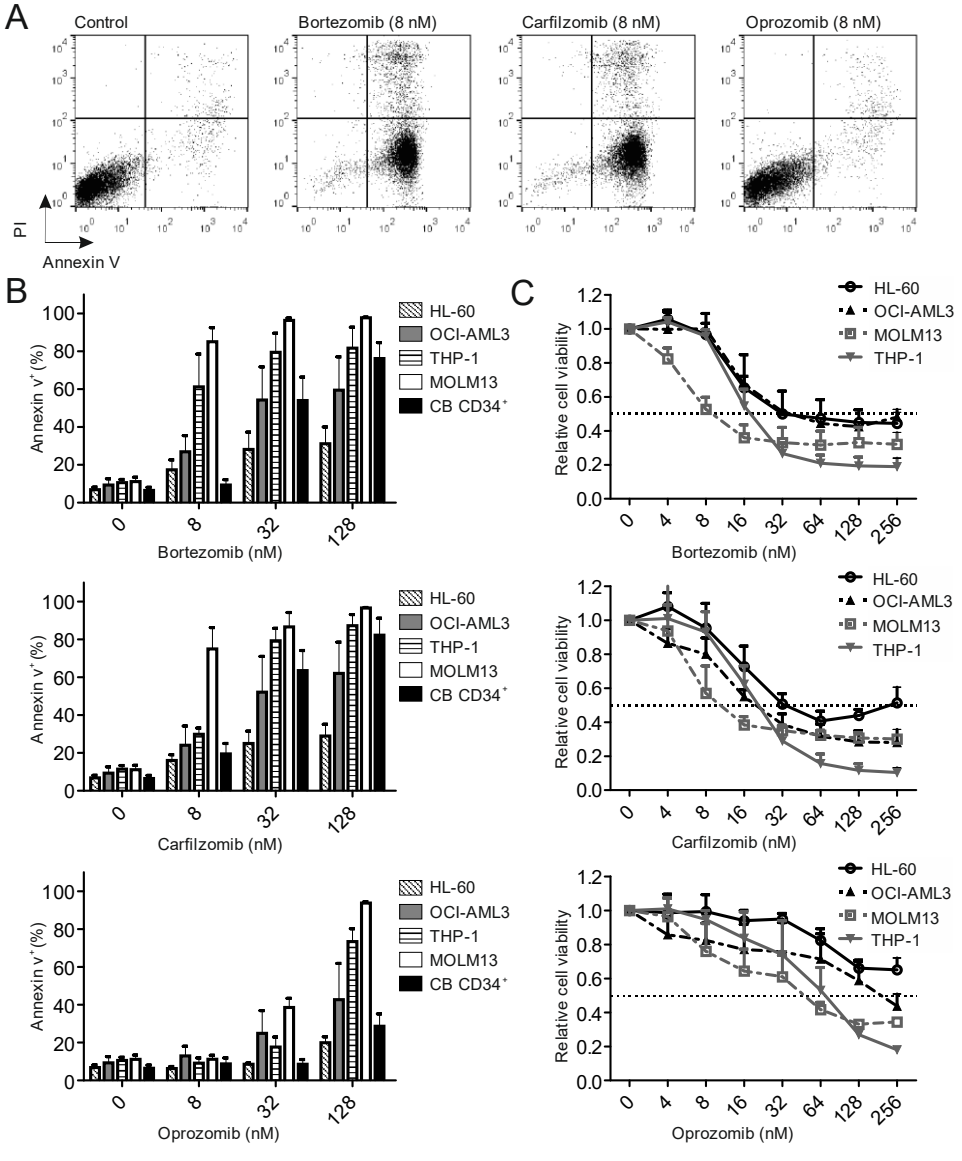

Figure 1. Survival of AML cells was equally reduced by bortezomib and carfilzomib and was less reduced by oprozomib after short-term incubation.

(A) Representative example of apoptosis measurements. MOLM13 cells were incubated with 8 nM bortezomib, carfilzomib, or oprozomib for 24 hrs.

(B) Apoptosis in AML cell lines and CB CD34$^+$ cells after 24 hrs of incubation (*n* = 3 each).

(C) Cell viability in AML cell lines was measured after 24 hrs of incubation by MTS assays (*n* = 3 each).

Immunoblotting

Antibodies against MCL-1 (Calbiochem, Darmstadt, Germany, AM50 (RC13),) and β-Actin (C4) (Santa Cruz Biotechnology, CA, USA) were used in dilutions of 1:300 and 1:1000, respectively. Secondary fluorescent antibodies were obtained from Invitrogen (1:10000). Binding of antibodies was detected by an Odyssey infrared imager (Li-Cor Biosciences, Lincoln, NE).

Statistical analysis

All values are expressed as means ± SE. The Student *t* test and Wilcoxon signed-rank test were used for comparisons. P-values below 0.05 were considered to be significant.

RESULTS

After short-term incubation, carfilzomib and bortezomib induce an equal reduction of survival in AML cell lines whereas oprozomib induces less cell death.

To investigate whether AML cells and normal $CD34^+$ cells are sensitive to the second-generation proteasome inhibitors, the AML cell lines MOLM13, THP1, OCI-AML3, HL-60, and cord blood $CD34^+$ cells were incubated with carfilzomib and oprozomib and effects on apoptosis and survival were compared with effects of the first-generation proteasome inhibitor bortezomib. After 24 hrs, we observed a concentration-dependent reduction in viability and induction of apoptosis in all four cell lines (Fig 1A-C). The most sensitive were the MLL-AF9 translocation-bearing cell lines MOLM13 and THP-1, as was in accordance with previous reports on MLL-fusion leukemia cells.[36,37] The effects of carfilzomib were comparable to bortezomib, e.g. the IC50 in MOLM13 cells was 7.1 nM for carfilzomib versus 6.2 nM for bortezomib, as measured by MTS viability assays (Fig 1C). Annexin V staining showed comparable results (Fig 1A-B). Cord blood $CD34^+$ cells were also affected upon treatment with bortezomib and carfilzomib in this short-term culture setting, but they were less sensitive than MOLM13 and THP1 cells (e.g. 75% ± 23% apoptotic cells in MOLM13 versus 19% ± 14% in cord blood $CD34^+$ cells upon 24 hrs incubation with 8 nM carfilzomib; $p < 0.001$; Fig 1B). At equimolar concentrations, oprozomib induced less cytotoxicity than carfilzomib and bortezomib in all four cell lines. Nevertheless, cord blood $CD34^+$ cells were less sensitive to oprozomib than MOLM13 and THP-1 cells (e.g. 94% ± 1.2% apoptotic cells in MOLM13 vs. 29% ± 10% in cord blood $CD34^+$ cells upon incubation with 128 nM carfilzomib; $p < 0.001$).

Proteasome activity is reduced in AML cell lines after incubation with carfilzomib, oprozomib and bortezomib.

To verify whether the reduction in cell survival after 24 hrs of exposure was preceded by a reduction in proteasome activity, we used the fluorogenic substrate SSLVY-AMC to measure the chymotrypsin-like activity as an indicator of the effect on the proteasome enzymatic activities.

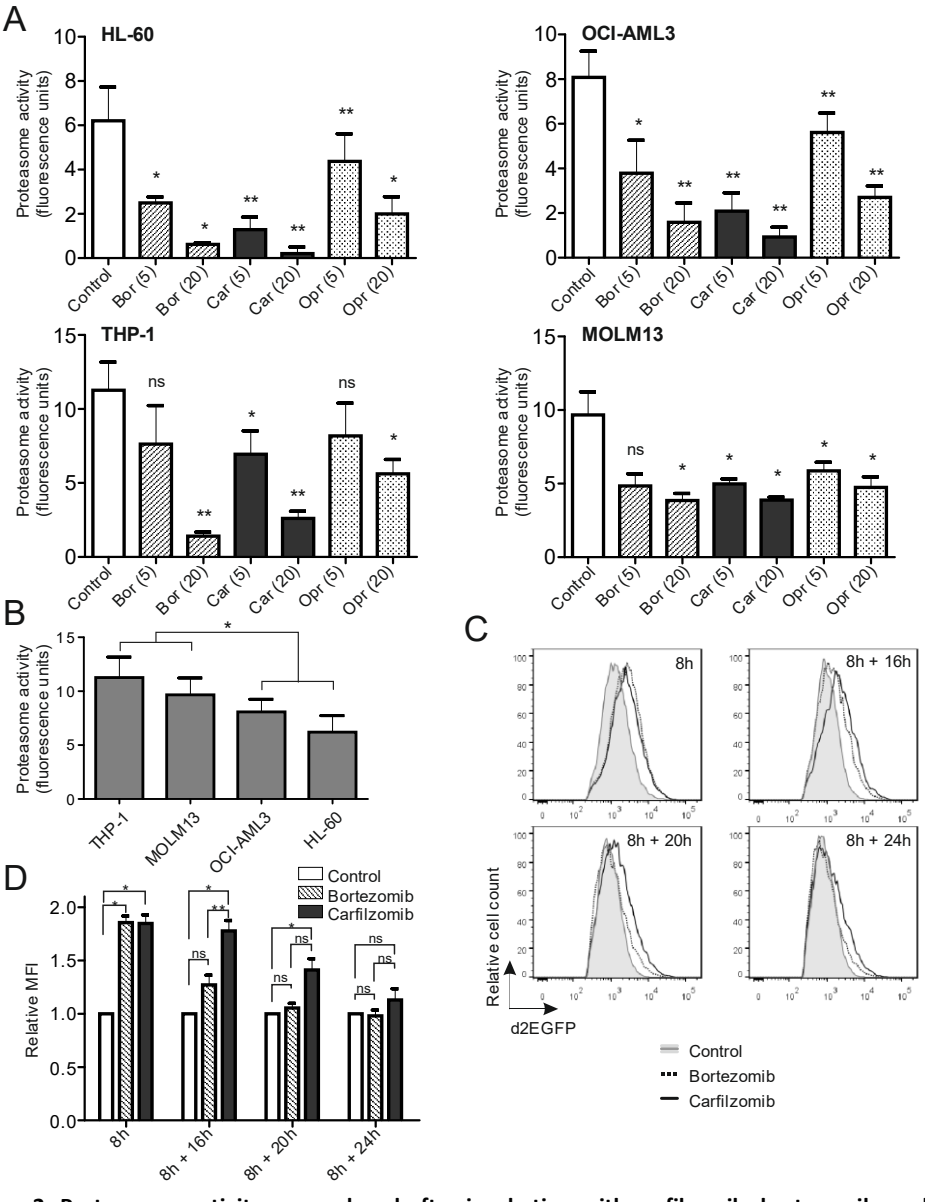

Figure 2. Proteasome activity was reduced after incubation with carfilzomib, bortezomib, and oprozomib with prolonged effectivity of carfilzomib.

(A) Chymotrypsin-like proteasome activity was measured in cell lines after 4 hrs of incubation with 5 nM or 20 nM bortezomib, carfilzomib, or oprozomib, and was quantified with fluorescence produced upon cleavage of the proteasome substrate SLLVY-AMC (*n* = 4 each). (B) Basal chymotrypsin-like proteasome activity in untreated cell lines. (C - D) HL-60 cells were transduced with d2EGFP, incubated for 8 hrs with 20 nM bortezomib or carfilzomib and then washed thoroughly three times with PBS. The mean fluorescence intensity (MFI) of d2EGFP was measured after incubation and 16, 20, and 24 hrs after washing. A representative example (C) and the MFI relative to the untreated control (D) are shown (*n* = 3).

Indeed, we observed a reduction of chymotrypsin-like proteasome activity in all AML cell lines after 4 hrs of exposure to the three proteasome inhibitors (Fig 2A). Again, carfilzomib and bortezomib showed similar effects (e.g. 90% and 97% reduction, respectively, in HL60 after 4 hrs 20nM; p = 0.019 and p = 0.009), whereas the effect of oprozomib on chymotrypsin-like proteasome activity was smaller (e.g. 68% reduction in HL60; p = 0.013). Basal chymotrypsin-like proteasome activity was higher in the more sensitive MOLM13 and THP-1 cells compared to the less sensitive HL-60 and OCI-AML3 cells (Fig 2B). Because carfilzomib binds irreversibly to the proteasome in contrast to bortezomib[19], we assessed whether carfilzomib was able to reduce the proteasome activity for a longer period compared to bortezomib. For this purpose HL-60 cells were transduced with short-lived d2EGFP which is rapidly degraded by the proteasome in time, but will accumulate upon proteasome inhibition. Cells were incubated for 8 hrs with bortezomib or carfilzomib, after which the accumulation of d2EGFP was measured at different time points. Directly after incubation, carfilzomib and bortezomib induced similar accumulation of d2EGFP (MFI relative to the untreated control was 1.85 vs. 1.86, respectively; p = 0.79), suggesting that both compounds equally inhibited the proteasome activity. However, 16 hrs after removal of the inhibitors, the MFI declined in bortezomib-treated cells but not in carfilzomib-treated cells (relative MFI 1.27 vs. 1.78, respectively; p = 0.007), suggesting that carfilzomib indeed had a longer-lasting inhibitory effect on proteasome activity (Fig 2C-D), in line with the described irreversible binding of carfilzomib to the proteasome.

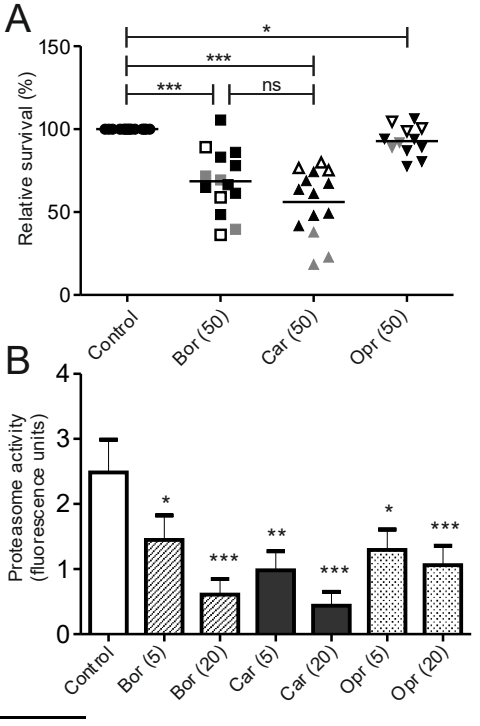

Figure 3. Survival and chymotrypsin-like proteasome activity were reduced in primary AML CD34$^+$ cells upon proteasome inhibition.

(A) Primary AML CD34$^+$ cells (n = 14) were incubated for 24 hrs with 50 nM bortezomib, carfilzomib, or oprozomib, after which survival was measured by flow cytometry. The three most sensitive samples towards carfilzomib are shown in grey and the three least sensitive samples are shown in white.

(B) Chymotrypsin-like proteasome activity was measured in primary AML CD34$^+$ cells after 4 hrs of incubation with 5 or 20 nM bortezomib, carfilzomib, or oprozomib (n = 12).

Survival and proteasome activity of primary AML CD34$^+$ cells are also equally affected by carfilzomib and bortezomib after short-term incubation.

Next, we investigated the sensitivity of the patient-derived AML CD34$^+$ cell subfraction to the proteasome inhibitors (*n* = 20, Table SI). After 24 hrs of incubation, we observed a cytotoxic effect of bortezomib and carfilzomib in most of the samples (69% ± 19% survival, $p < 0.0001$; and 56% ± 20% survival, $p < 0.0001$, respectively), whereas AML cells were relatively resistant to oprozomib (93% ± 9% survival, $p = 0.020$) (Fig 3A). Although the variation between the AML samples was distinct, we observed again a similar reduction of cell survival after short-term incubation with carfilzomib and bortezomib. The survival reduction was again associated with a decrease in chymotrypsin-like proteasome activity in primary AML CD34$^+$ cells (Fig 3B).

Carfilzomib slightly reduced the percentage of quiescent AML CD34$^+$CD38$^-$ cells and reduced the stem cell frequency of primary AML cells.

We have previously shown that bortezomib primarily affects the more mature AML CD34$^-$ cell fraction and has limited impact on AML CD34$^+$ cells.[18] Therefore, we assessed the effects of carfilzomib and oprozomib on the AML CD34$^-$ and CD34$^+$ cell fractions, and focused in particular on the quiescent cell population. Following 24 hrs of incubation with carfilzomib and oprozomib, we noticed that the CD34$^-$ and CD34$^+$CD38$^+$ cells were also in this case more affected than the relatively immature CD34$^+$CD38$^-$ cells in this short-term assay. For example, upon carfilzomib treatment there was an 81% reduction in CD34$^-$ cells (p = 0.04), a 47% reduction in CD34$^+$CD38$^+$ cells (p = 0.003), and a 10% reduction in CD34$^+$CD38$^-$ cells (p = 0.19; Fig 4A-B). However, we observed with carfilzomib, but not with bortezomib and oprozomib, a slight but significant reduction of quiescent AML CD34$^+$CD38$^-$ cells (41% quiescent CD34$^+$CD38$^-$ cells vs. 52% in untreated control; p = 0.03; Fig 4C-D). To examine whether the AML stem cell-enriched cell fractions are also functionally affected upon addition of carfilzomib, AML CD34$^+$ cells (n = 10) were cultured on an MS5 stromal layer in limiting dilution for five weeks to determine the long-term-culture-initiating cell (LTC-IC) frequency. Importantly, upon a single treatment for 24 hrs with carfilzomib, the LTC-IC frequency was reduced to 47% of the untreated control (± 24%; p = 0.003), while bortezomib and oprozomib did not affect the LTC-IC frequency (97% ± 36%, p = 0.44; and 89% ± 39%, p = 0.21, respectively) (Fig 4E). To address the variable sensitivity of AML CD34$^+$ cells to carfilzomib, we assessed the proteasome activity in three sensitive and two less-sensitive AMLs. We did not detect a difference in basal proteasome activity between these different AMLs. However, following 4 hrs of incubation with carfilzomib, we did not demonstrate any proteasome activity in the sensitive AMLs, while in the insensitive AMLs remaining proteasome activity could still be demonstrated, i.e. 37% and 60% of the starting value (data not shown).

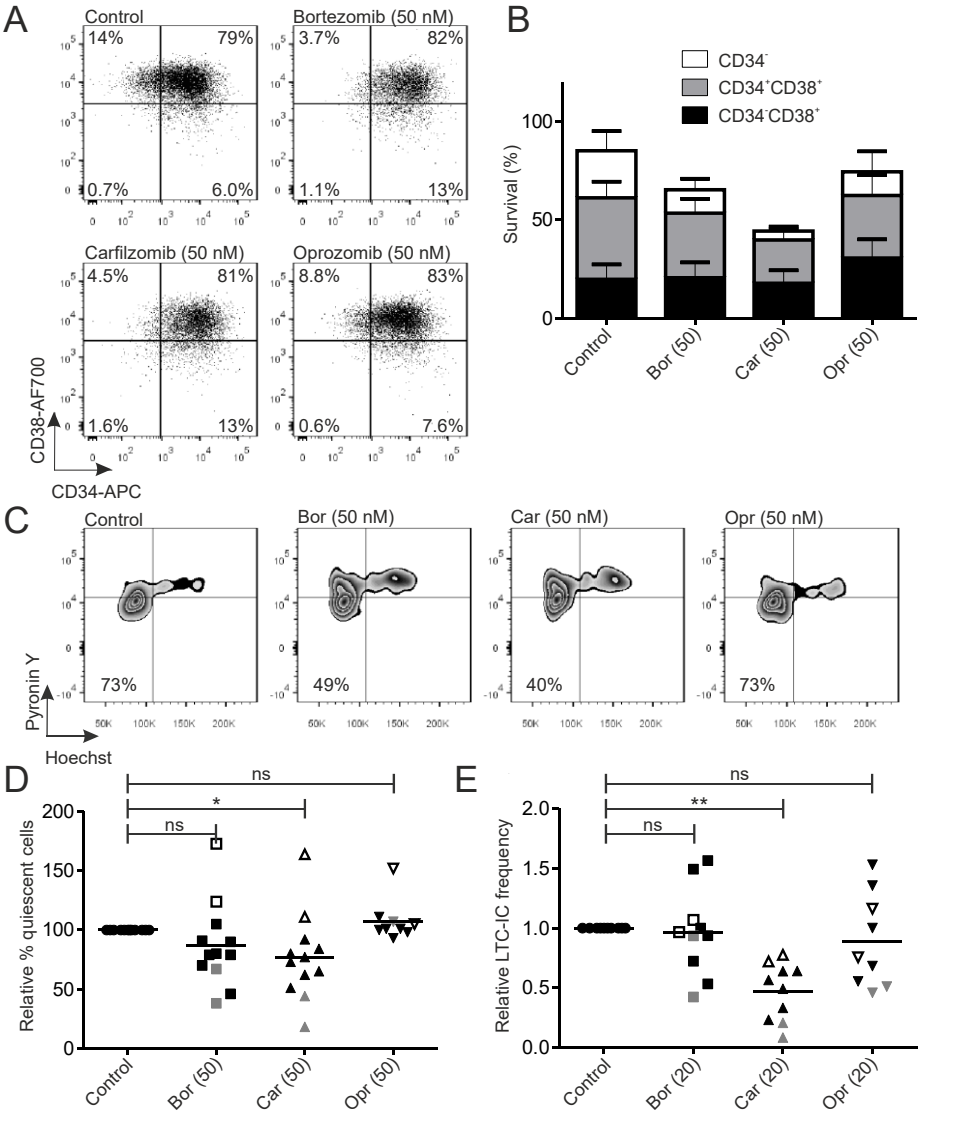

Figure 4. Carfilzomib reduced the percentage of quiescent cells and the stem cell frequency of primary AML cells.

(A-D) AML CD34$^+$ cells were expanded on MS5 stromal cells for two days and were incubated in liquid with proteasome inhibitors (50 nM) for 24 hrs. Survival of the different cell populations was determined by flow cytometry. (A) Representative example of CD34/CD38 stainings. (B) Survival percentages of CD34$^+$CD38$^-$, CD34$^+$CD38$^-$, and CD34$^-$ cell populations (*n* = 9). (C) Representative example of the analysis of quiescent CD34$^+$CD38$^-$ cells. (D) Percentage of AML CD34$^+$CD38$^-$ cells in the *G0* phase of the cell cycle (*n* = 10). The average quiescent cell percentage of the untreated control cells was 52% ± 17%. (E) AML CD34$^+$ cells were incubated for 24 hrs on MS5 stromal cells with proteasome inhibitors (20 nM) and then demi-populated weekly in LTC-IC assays (*n* = 10). The average LTC-IC frequency of the untreated controls was 1/43 ± 1/29. The two most sensitive samples towards carfizomib are shown in grey and the two least sensitive samples are shown in white.

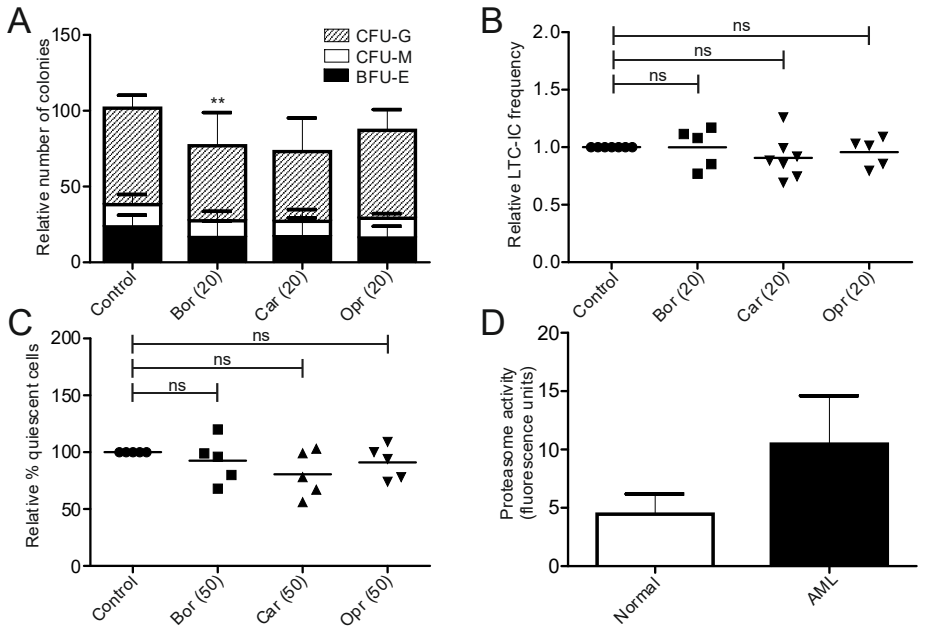

Figure 5. Normal control cells were only mildly affected by proteasome inhibition.

(A) Normal bone marrow (n = 9) and normal peripheral blood (n = 2) $CD34^+$ cells were treated with the proteasome inhibitors (20 nM) for 24 hrs and then put in methylcellulose. CFCs were scored after two weeks. The average colony count of the untreated controls was 225 ± 112 per 1000 cells. (B) NBM (n = 3) and cord blood (n = 4) $CD34^+$ cells were incubated for 24 hrs on MS5 stromal cells with proteasome inhibitors (20 nM) and then demi-populated weekly in LTC-IC assays. The average LTC-IC frequency of the untreated controls was 1/145 ± 1/177 (C) Percentage of NBM $CD34^+CD38^-$ cells in the *G0* phase of the cell cycle after 24 hrs incubation with the proteasome inhibitors (50 nM; n = 10). The average quiescent cell percentage of the untreated control cells was 64% ± 25%. (D) Chymotrypsin-like proteasome activity in primary AML $CD34^+$ cells (n = 6) and NBM or peripheral blood $CD34^+$ cells (n = 8).

6

Carfilzomib does not affect NBM $CD34^+$ cells.

To evaluate the effect of carfilzomib on NBM $CD34^+$ cells, we assessed the colony forming potential and LTC-IC frequency of NBM cells upon treatment with carfilzomib. Both the colony forming potential and the LTC-IC frequency were not significantly affected by carfilzomib (i.e. 68% ± 51% in the carfilzomib group compared to controls normalized to 100% (p = 0.053); LTC-IC frequency 91% ± 19% in the carfilzomib group compared to the untreated control (p = 0.11); Fig 5A-B). Furthermore, the frequency of quiescent NBM $CD34^+CD38^-$ cells was not significantly altered upon exposure to carfilzomib (55% ± 30% vs. 64% ± 25% in controls; Fig 5C). Together, these data suggest that NBM $CD34^+$ cells are less sensitive to carfilzomib than AML $CD34^+$ cells. A difference in sensitivity of AML $CD34^+$ and normal $CD34^+$ cells might be explained by a difference in proteasome activity in AML cells compared to normal cells. By measuring the chymotrypsin-like activity of AML $CD34^+$ (n=6) and NBM $CD34^+$ cells (n=8), we indeed observed a trend towards

increased proteasome activity in AML CD34$^+$ cells (Fig 5D). Higher proteasome activity in AML cells might be the result of higher levels of proteasome complexes due to increased expression of proteasomal subunits. To study this, we compared the expression levels of proteasome subunit coding genes in primary AML CD34$^+$ cells of 66 patients and NBM CD34$^+$ cells of 22 donors from a recently performed microarray.[34,35] We observed significantly increased expression levels of nine out of 17 subunit-coding genes and a trend towards increased expression of all subunits in AML CD34$^+$ versus NBM CD34$^+$ cells (Fig S1A). We also observed increased expression of seven proteasome subunit-coding genes in AML CD34$^+$ compared to AML CD34$^-$ cells, suggesting that the more primitive AML cells might have increased proteasome activity compared to the more mature cell population (Fig S1B).

MCL-1 is upregulated in AML CD34$^+$ cells after incubation with bortezomib, carfilzomib, and oprozomib and MCL-1 inhibition sensitizes AML CD34$^+$ cells to proteasome inhibitor-induced cell death.

The cytotoxic effects of proteasome inhibitors may be limited by the induction of anti-apoptotic signaling. We and others previously observed that treatment of AML CD34$^+$ cells with bortezomib is hampered by upregulation of the anti-apoptotic protein MCL-1.[18,38,39] Here, we observed that MCL-1 is also upregulated in HL-60 cells upon treatment with carfilzomib and oprozomib (Fig 6A). Whereas MCL-1 is required for the survival and maintenance of leukemic cells, and inhibition of MCL-1 abrogates leukemic outgrowth, we wondered whether inhibition of MCL-1 further sensitizes primary leukemic cells to carfilzomib and oprozomib. AML CD34$^+$ cells were treated for

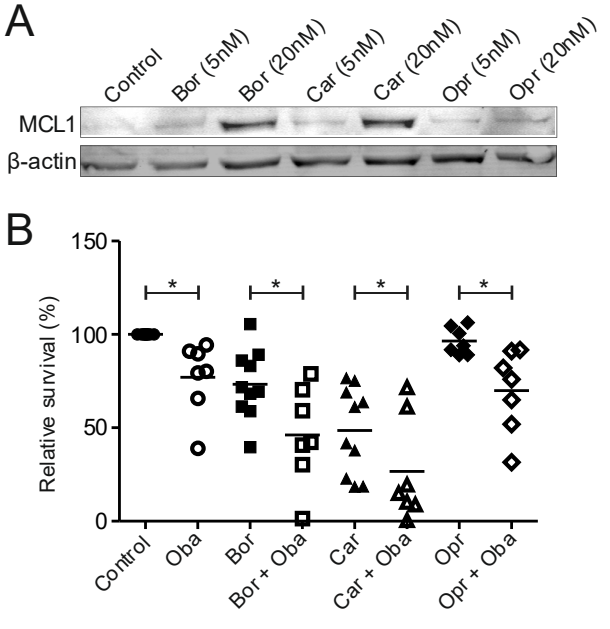

Figure 6. MCL-1 was upregulated in AML cells after incubation with bortezomib, carfilzomib, and oprozomib and MCL-1 inhibition sensitized AML CD34$^+$ cells to proteasome inhibitor-induced cell death.

(A) HL-60 cells were incubated with proteasome inhibitors for 24 hrs. The anti-apoptotic MCL-1 isoform 1 was detected by western blot.

(B) Primary AML CD34$^+$ cells (n = 10) were incubated with proteasome inhibitors (50 nM), the MCL-1 inhibitor obatoclax (5 uM), or both for 24 hrs. Survival was measured by flow cytometry.

24 hrs with carfilzomib or oprozomib in combination with obatoclax, a pan-BCL-2 family member inhibitor which is currently under clinical investigation. Co-treatment with obatoclax increased the sensitivity of primary AML cells to the proteasome inhibitors. Survival of AML $CD34^+$ cells after 24 hrs of incubation with 50 nM bortezomib, carfilzomib, or oprozomib was 73%, 49%, and 96%, respectively, in the absence of obatoclax, versus 46% (p = 0.03), 27% (p = 0.03), and 65% (p = 0.04), respectively, when combined with obatoclax (Fig 6B).

DISCUSSION

The results of the present study demonstrate that carfilzomib and bortezomib are equally effective in targeting AML cells in short-term assays, but, more importantly, that carfilzomib was more effective in targeting the stem cell-enriched $CD34^+$ AML cell fraction. The higher sensitivity of primitive AML cells to carfilzomib could be related to the irreversible binding of carfilzomib to the proteasome, which does not result in an advantage in short-term readouts, but results in a decreased viability in long-term assays due to prolonged proteasome inhibition.

NBM $CD34^+$ cells were only mildly affected upon carfilzomib treatment. The variation in sensitivity between AML $CD34^+$ cells and NBM $CD34^+$ cells, and also within different AML cells, is likely related to differences in proteasome- and NF-κB activity. We and others indeed observed an increased chymotrypsin-like proteasome activity and increased proteasome subunit expression in AML $CD34^+$ cells compared to NBM cells (Fig 5D, Fig S1A, and.[40,41] Furthermore, previous studies have shown that AML $CD34^+$ cells frequently gain constitutive NF-κB activity, in contrast to NBM $CD34^+$ cells[9,13,14], which might be related to the increased activity of various components upstream of NF-κB, such as IRAK1 and TAK1.[11,12] As a consequence of the increased proteasome- and NF-κB activity, the stem cell-enriched AML $CD34^+$ cells might be more dependent on these pathways for their survival, which provides a therapeutical window. In addition, the higher sensitivity of cell lines bearing the MLL-AF9 fusion (MOLM13 and THP-1) might be due to an accumulation of the MLL fusion protein upon proteasome inhibition, which is at higher levels detrimental to leukemia cell survival and triggers latent tumor suppression programs.[36]

In the present study, we measured the chymotrypsin-like proteasome activity as an indicator of the effect on proteasome activity. Chymotrypsin-like activity is shown to be a biomarker for clinical response on standard therapies in AML and multiple myeloma patients in contrast to trypsin-like and caspase-like activities.[41] The measurement of chymotrypsin-like proteasome activity covers both the constitutive proteasome- and the immunoproteasome activity which are both inhibited by bortezomib, carfilzomib and oprozomib.[7,21] It has recently been suggested that higher ratios of the immunoproteasome correlate with sensitivity of AML cells to proteasome inhibition.[8] However, the effects were only determined in AML blasts in short-term read-outs.

Our data suggest that AML cells were less sensitive to oprozomib compared to carfilzomib and bortezomib *in vitro* at equimolar concentrations. Lower efficacy of oprozomib was previously also observed in head and neck cancer cells and various cell lines. Nevertheless, oprozomib largely

inhibited tumor growth *in vivo*.[42] In addition, the limited toxicity to cord blood and normal bone marrow $CD34^+$ cells might provide the opportunity to apply higher doses of oprozomib.

To optimally eradicate AML cells, a combination of anti-cancer agents targeting different oncogenic pathways is presumably the most successful. An interesting target in combination with proteasome inhibitors is the anti-apoptotic protein MCL-1. MCL-1 is required for the maintenance of early hematopoietic progenitors and is highly expressed in AML $CD34^+CD38^-$ cells as compared to AML progenitors and normal $CD34^+$ cells.[43,44] In this study, we showed that MCL-1 is upregulated upon incubation with carfilzomib and oprozomib and that simultaneous inhibition of MCL-1 by obatoclax has an additive cytotoxic effect on AML $CD34^+$ cells.

In summary, our data indicate that the second-generation proteasome inhibitor carfilzomib might be more effective in reducing the long-term survival of AML $CD34^+$ cells as compared to bortezomib and oprozomib. Addition of an MCL-1 inhibitor increased the cytotoxic effects on AML $CD34^+$ cells. Therefore, carfilzomib in combination with MCL-1 inhibition is a promising therapeutic option for the treatment of AML patients.

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SUPPLEMENTARY TABLES AND FIGURES

Table SI. Clinical characteristics of studied patients

AML	Diagnosis (WHO)	% CD34$^+$	NPM	FLT3	Cytogenetics
1	AML with cytogenetic aberrations	29%	n.a.	wt	-Y, t(8;21)
2	AML without maturation	30%	wt	ITD	NK
3	Acute biphenotypic leukemia	57%	wt	ITD	NK
4	Acute monocytic leukemia	10%	mut	ITD	NK
5	Acute monocytic leukemia	85%	n.a.	wt	n.a.
6	AML with dysplasia	31%	wt	wt	NK
7	AML without maturation	86%	n.a.	n.a.	n.a.
8	AML with minimal differentiation	90%	n.a.	n.a.	del 5q
9	AML with maturation	57%	n.a.	n.a.	n.a.
10	Acute myelomonocytic leukemia	16%	wt	ITD	NK
11	Acute monocytic leukemia	67%	wt	ITD	NK
12	AML with dysplasia	25%	n.a.	wt	n.a.
13	AML without maturation	70%	wt	wt	NK
14	AML with genetic aberrations	87%	n.a.	n.a.	inv(3), -7, -10
15	Acute basophilic leukemia	34%	wt	wt	t(9;22), inv(16)
16	AML with maturation	29%	wt	ITD	NK
17	AML with dysplasia	58%	wt	ITD	NK
18	AML with maturation	39%	mut	ITD	t(3;5), +8
19	AML with genetic aberrations	76%	wt	wt	inv(16)
20	AML with dysplasia	18%	wt	wt	t(9;22), t(4;11)

AML was classified according to WHO classification. AML, acute myeloid leukemia; NPM, nucleophosmin; n.a., not available; wt, wildtype; mut, mutated; FLT3-ITD, Fms-like tyrosine kinase 3-internal tandem duplication; NK, normal karyotype.

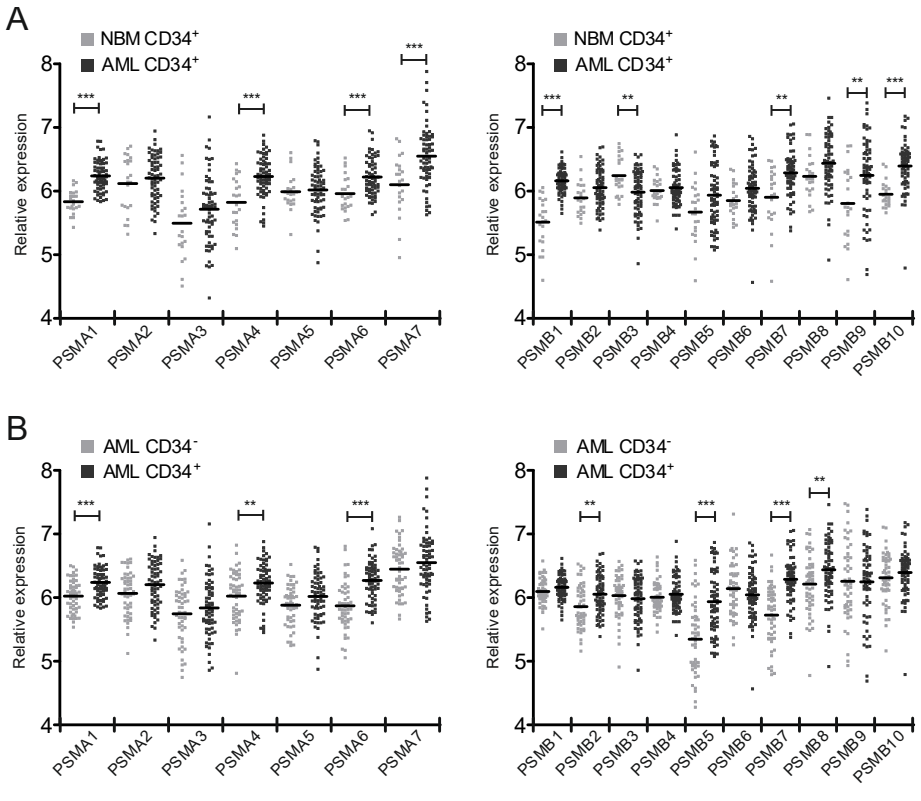

Figure S1. Proteasome subunit-coding genes were higher expressed in AML CD34$^+$ cells (*n* = 66) versus NBM CD34$^+$ cells (*n* = 22) or AML CD34$^-$ cells (*n* = 51).

Seventeen subunit-coding genes from a micro-array were compared by the Mann–Whitney U test corrected by a multivariate permutations test. (A) Expression levels of proteasome subunit-coding genes in AML CD34$^+$ cells versus NBM CD34$^+$ cells. (B) Expression levels of proteasome subunit-coding genes in AML CD34$^+$ cells versus AML CD34$^-$ cells.

Chapter 7

Summary, discussion and future perspectives

SUMMARY

The “3+7 regimen” that is based on a combination of an antracyclin and cytarabine is since more than 40 years the backbone for the treatment of AML. This regimen has cured a considerable number of patients. However, the results in older patients are disappointing, also when post-remission maintenance therapy was added to the regimen.[1,2] The unfavorable results at older age are partially related to increased comorbidity. However, more dominant are unfavorable disease characteristics, such as adverse cytogenetic abnormalities, high rates of MDS-related AML and therapy-related AML, high incidence of multidrug resistance and distinct gene expression profiles in older patients.[3-5] Therefore, new therapies that more efficiently target malignant cells and are also applicable in less fit patients, are desired. In this thesis, two new treatment strategies were studied in a clinically relevant setting and *in vitro*, respectively.

With the emergence of the hypomethylating agents (HMAs) azacitidine and decitabine, a new less intensive treatment strategy became available for higher-risk MDS and AML patients. A survival benefit of azacitidine compared to conventional care (including intensive chemotherapy, best supportive care (BSC), or low-dose cytarabine) was demonstrated in 2009 in a phase III randomized controlled trial in higher-risk MDS en AML patients with less than 30% bone marrow blasts.[6] However, little was known about the efficacy of azacitidine in daily clinical practice, without strict inclusion and exclusion criteria, and in AML patients with more than 30% bone marrow blasts. In **Chapter 2**, we analyzed the treatment results of a cohort of 90 MDS, CMML, and AML patients who participated in the Dutch compassionate use named patient program. In this program, patients could receive azacitidine before registration in the Netherlands, i.e. before December 2008. Results revealed overall response rates (complete- or partial remission (CR, PR) and hematologic improvement (HI)) of about 50% with CR/PR rates of 26%, which was comparable to the AZA-001 trial. Median overall survival (OS) tended to be shorter with 13.0 months (range 9.8-16.2), but was comparable to the OS in the French named patient program of 13.5 months. In our cohort, several previously described predictors for poor outcome could be confirmed, including poor-risk cytogenetics, circulating blasts, and poor WHO performance score. Interestingly, a significantly longer OS was observed in the small group of patients (16%) who had an early platelet response, defined as platelet count doubling after the first cycle (four weeks) of azacitidine.

In the extended named patient program, also patients with a bone marrow blast count over 30% could be treated with azacitidine. In **Chapter 3**, the treatment results of these patients were analyzed and compared with AML patients with 20%-30% blasts. Results revealed no differences in response rates and OS in patients with blast counts higher or lower than 30%. More predictive for disadvantageous outcome than the percentage of bone marrow blasts, were lack of response to azacitidine, poor cytogenetic risk, poor WHO performance score, therapy-related AML, and white blood cell (WBC) count over 15 x 10^9/L. The efficacy of azacitidine in AML patients with more than 30% blasts has recently been confirmed in a large phase III trial.[7]

In our center, a relatively large population of older AML patients has been treated with azacitidine. In **Chapter 4**, we analyzed the characteristics and outcome of all consecutive AML patients of 60 years and older diagnosed and treated in the University Medical Center Groningen between 2003 and 2010. We compared patients receiving azacitidine with patients receiving intensive chemotherapy or BSC. A significantly improved OS was observed in the azacitidine-treated patients compared to the BSC group, which was partly related to the therapy and partly to more favorable patient- and disease characteristics in the azacitidine group. Interestingly, azacitidine and intensive chemotherapy resulted in similar OS. Also after correction for baseline characteristics and known risk factors, OS did not differ between both groups. Because the follow-up time and number of azacitidine-treated patients in this study were relatively limited, we reassessed our cohort in **Chapter 5** after a median follow-up time of four years and with extended inclusion of older AML patients until August 2015. Compared to patients receiving BSC only, azacitidine-treated patients showed a significant survival advantage. The survival of azacitidine-treated patients after the longer follow-up time was still comparable with patients receiving intensive chemotherapy, unless the latter had acute promyelocytic leukemia or subsequently underwent allogeneic hematopoietic cell transplantation (allo-HCT).

Mutations in the tumor suppressor gene *TP53* are frequently associated with complex karyotypes, poor response to intensive chemotherapy (about 28% CR in *TP53*-mutated patients versus 50% CR in other adults with complex karyotype AML), and shorter OS and relapse-free survival after CR.[8,9] An important question is whether these patients can benefit from an alternative type of therapy, i.e. HMAs. In **Chapter 5**, we assessed bone marrow samples of 47 older AML patients treated with HMAs by using immunohistochemistry to identify TP53 overexpression in AML cells, which is indicative for *TP53* mutation. OS of the 22 patients with TP53 overexpression was shorter compared to patients with normal TP53 expression. Nonetheless, response rates did not significantly differ and both patient groups had improved survival when achieving a response to azacitidine. Further, achieving CR in two patients with baseline TP53 overexpression was associated with disappearance of TP53++ cells, indicating that *TP53*-mutated AML cells can be targeted by HMAs. These data suggest that HMAs may be beneficial in older AML patients with a *TP53* mutation.

Understanding the tumor biology of AML and differences between AML stem cells and normal hematopoietic stem cells can lead to new therapeutic strategies. One of the observed differences between AML (stem) cells and normal hematopoietic stem cells concerns the ubiquitin-proteasome system. Increased proteasome expression, proteasome activity, and NF-κB activity have been described in AML stem cell-enriched cell populations, suggesting that the proteasome is a potential druggable target. The first-generation proteasome inhibitor bortezomib reduced NF-κB activity and induced apoptosis, especially in more mature $CD34^-$ AML cell populations, whereas the more primitive AML $CD34^+$ cells were less sensitive. In **Chapter 6,** we investigated whether the second-generation proteasome inhibitors carfilzomib and oprozomib had improved efficacy to target patient-derived primitive AML cells compared to bortezomib. We observed a larger reduction of AML stem cell frequencies in long-term cultures after incubation with carfilzomib as

compared to bortezomib. Also quiescent AML $CD34^+CD38^-$ cell percentages were more reduced after incubation with carfilzomib. A higher efficacy of carfilzomib could be related to its irreversible binding to the proteasome, resulting in prolonged proteasome inhibition, whereas shorter-lived effects can be expected of bortezomib that binds reversibly. Indeed, we observed reduction of proteasome activity for a longer period after incubation with carfilzomib compared to bortezomib. Importantly, normal $CD34^+$ cells were less affected, which could be due to lower normal proteasome abundance and proteasome activity, associated with lower dependency on the proteasome. In AML $CD34^+$ cells, proteasome activity indeed tended to be increased. Further, 9 out of 17 proteasome subunit-coding genes were significantly higher expressed in AML $CD34^+$ cells compared to normal $CD34^+$ cells. Previous studies have indicated that upregulation of anti-apoptotic MCL-1 inhibited the apoptotic effect of bortezomib in AML $CD34^+$ cells. Likewise, we observed upregulation of MCL-1 upon incubation with carfilzomib and oprozomib. Co-treatment with the pan-Bcl-2 inhibitor obatoclax, which also inhibits MCL-1, enhanced the apoptotic effects of the proteasome inhibitors on primitive AML $CD34^+$ cells, suggesting that carfilzomib in conjunction with an apoptotic drug might be a potential new treatment strategy in AML patients.

DISCUSSION AND FUTURE PERSPECTIVES

Treatment of older patients with higher-risk MDS or AML

Treatment of older AML- or higher-risk MDS patients is clinically challenging. With a life expectancy of approximately 20 years at 65 years of age[10], there is much to gain by effective therapy. Different studies have demonstrated that treatment with either intensive or non-intensive therapy results in better survival and quality of life than BSC.[11,12] Results of the Swedish Acute Leukemia registry indicate that most older AML patients still benefit from intensive chemotherapy, since OS was better in regions where more patients received intensive therapy.[12] Besides, a phase II clinical trial and post-hoc analyses of phase III trials indicate that allo-HCT following reduced-intensity conditioning is feasible and associated with better overall survival and relapse-free survival in AML patients between 60 and 75 years of age, especially in those with intermediate and adverse risk AML.[13-15]

However, many MDS and AML patients are not fit enough for standard intensive induction therapy and for many older patients with adverse disease characteristics it is questionable whether the expected treatment benefit outweighs the treatment-related side effects and mortality risk. Especially in these patients, less-intensive treatment strategies are essential. Less-intensive therapy with the hypomethylating agent azacitidine has become available for higher-risk MDS and AML patients after a survival benefit has been reported in randomized phase III studies in 2009 and 2015 (see indications in Table 1).[6,7,16] As AML and MDS patients included in randomized clinical trials tend to be younger, have better performance scores, less comorbidities, and less adverse disease characteristics compared to patients not included in trials[17], caution should be taken to extrapolate trial data to the general population of MDS and AML patients. In this thesis, we studied the use of azacitidine in MDS, CMML, and AML patients in daily clinical practice. Our data from the Dutch named patient program confirm the effectivity of azacitidine in clinical practice with responses in about half of the patients and a median OS of 13.0 months. Also AML patients with more than 30% bone marrow blasts showed favorable responses to azacitidine in our extended named patient program; they had similar response and survival rates as patients with less than 30% blasts. Recently, the AZA-AML-001 trial confirmed the efficacy of azacitidine in AML patients (age ≥65 years) with more than 30% bone marrow blasts. In this trial, median OS of AML patients treated with azacitidine was 10.4 months versus 6.5 months with conventional care (i.e. preselected low-dose cytarabine, intensive chemotherapy, or BSC). This difference did not reach statistical significance in the primary analysis (p=0.1009), but a pre-specified analysis censoring patients from the moment they received subsequent therapy after study drug discontinuation indicated a significant survival benefit of 5.2 months for azacitidine. Further, analyses of the preselected subgroups revealed a significant survival benefit for azacitidine compared to BSC (median OS 5.8 versus 3.7 months; HR 0.60 (95% CI 0.38-0.95), p = 0.029), a trend towards better OS compared to low-dose cytarabine (median OS 11.2 versus 6.4 months;

HR 0.90 (95% CI 0.70-1.16), p = 0.43), and a similar median OS of azacitidine and intensive chemotherapy (13.3 versus 12.2 months; HR 0.85 (95% CI 0.52-1.38), p = 0.50). Based on these results, azacitidine was approved by the European Medicine Agency in October 2015 for the treatment of AML patients of 65 years and older with more than 30% blasts and ineligible for allo-HCT (Table 1). Likewise, a marginally significant survival benefit was observed in older AML patients treated with decitabine (20 mg/m^2 in 1 hour for 5 days/month) compared to low-dose cytarabine or BSC (7.7 versus 5.0 months, HR 0.82 (95% CI 0.68-0.99), p = 0.037 at secondary analysis), which has led to approval of decitabine in 2012 for AML patients of 65 years and older unfit for intensive chemotherapy (Table 1).[18]

Table 1. Approved indications for HMAs by the European Medicine Agency

	Indications
Azacitidine 75 mg/m^2 s.c. 7/28d	Adults who are not eligible for hematopoietic stem cell transplantation with: • MDS with IPSS intermediate-2 or high risk • CMML with 10–29% bone marrow blasts without myeloproliferative disorder • AML with 20–30% blasts and multi-lineage dysplasia, according to WHO classification • AML with > 30% bone marrow blasts and age ≥ 65 years
Decitabine 20 mg/m^2 i.v. 5/28d	Adults aged ≥ 65 years who are not eligible for standard induction therapy with: • AML, newly-diagnosed *de novo* or secondary

IPSS, International Prognostic Scoring System; MDS, myelodysplastic syndromes; CMML, chronic myelomonocytic leukemia; AML, acute myeloid leukemia; WHO, World Health Organization.

Side-effects of azacitidine and decitabine are relatively mild in MDS as well as in older AML patients.[19,20] They include hematologic toxicity (including grade 3-4 cytopenias), most often resolving after the first two cycles, gastro-intestinal side effects (especially for azacitidine) that are usually controllable with simple measures, and injection site reactions in case of azacitidine.[21] Tolerability of HMAs is generally much better in older patients compared to intensive chemotherapy, which was reflected in our AML cohort by a lower number of days in the hospital, lower transfusion requirements, and a limited drop-out due to drug toxicity. An additional advantage of HMAs is the administration in an outpatient setting. Development of oral azacitidine (CC-486), at present assessed in phase I studies in low-risk MDS and AML patients, may further enhance applicability in older and frail patients.[22,23] In summary, the favorable toxicity profile of HMAs has made it feasible to treat a larger group of AML patients. However, it should be noted that the prospect of these patients is still very unfavorable with an OS of only 6-13 months.

Position of azacitidine and decitabine among conventional treatment types

Now that azacitidine has been approved for higher-risk MDS and both azacitidine and decitabine have been approved for older AML patients, the question is how to define their position among other treatment types and how further improvements can be made on this backbone. In higher-risk MDS, azacitidine is currently considered as standard of care in the large group of patients ineligible for allo-HCT, given the reported survival benefit over conventional care. Azacitidine largely replaced BSC and low-dose cytarabine in MDS patients, as improved survival, improved response rates, and lower toxicity have been observed compared to BSC and low-dose cytarabine.[6,24] Further, unlike low-dose cytarabine, azacitidine has been shown to improve outcome of patients with adverse genetic risk, including the very-poor risk monosomal karyotypes.[25]

For AML patients, results from this thesis and previous studies suggest that treatment with HMAs (decitabine and azacitidine) must be strongly considered instead of BSC in all patients who are able and willing to undergo treatment.[7,16,18] Of the 114 BSC patients in our cohort, 54% received 6-mercaptopurine (6-MP; 200-250 mg 2 times a week). Median OS was improved with 6-MP but still unfavorable compared to azacitidine (4.8 months with 6-MP versus 1.9 months without 6-MP versus 13.3 months with azacitidine, $p < 0.001$; Figure 1).

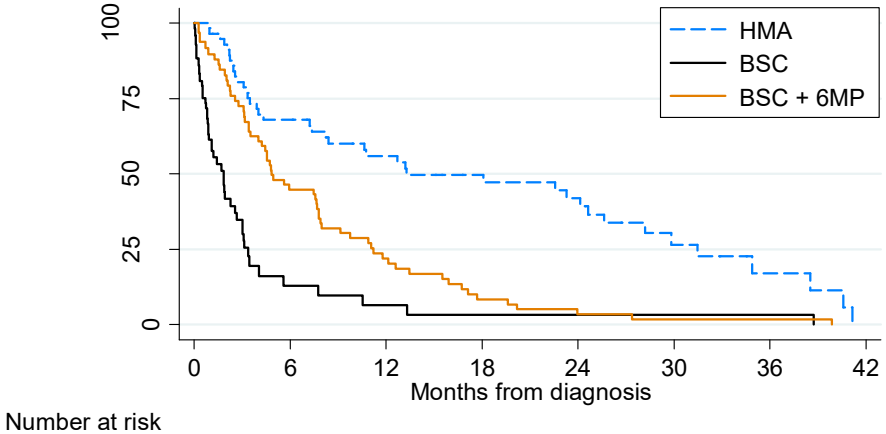

Figure 1. Overall survival in patients treated with best supportive care (BSC) only, BSC plus 6-mercaptopurine (BSC + 6MP), or hypomethylating agents (HMA).

To correct for the time for starting 6-MP, which could bias a favorable outcome in the 6-MP group, a time-dependent survival analysis was conducted in STATA 13.0. All BSC (N = 53) and BSC + 6-MP (N = 61) patients started in the BSC group. At the time they started 6-MP treatment, patients were censored in the BSC group and were counted at risk in the BSC + 6-MP group.

The results of our cohort of consecutive older AML patients indicate that azacitidine and intensive chemotherapy might induce a similar survival benefit in a subgroup of patients, even though response rates were higher with intensive chemotherapy (75% remissions versus 42% with azacitidine (Chapter 5)). The comparable OS despite lower response rates is in line with the general clinical experience that achievement of CR or PR is not a prerequisite for prolonged OS by azacitidine.[26,27] To address this matter, new response criteria have recently been proposed for AML patients treated with HMAs, which include HI in the absence of bone marrow blast clearance as valid response.[28] Similar OS with HMAs and intensive chemotherapy was also observed in a large Spanish cohort of 671 older AML patients, in a subgroup analysis of the AZA-AML-001 trial, and in the AZA-001 trial.[6,7,29] Superior OS has been reported with HMAs compared to intensive chemotherapy in patients over 70 years of age.[30] However, also the opposite has been reported, with significantly superior survival after intensive chemotherapy compared to azacitidine in a retrospective study of 334 AML patients aged 60 years or older.[31] Anyhow, older AML patients treated with intensive chemotherapy generally have inferior outcome compared to the young[3,32-35], which provides opportunities for less toxic therapies such as HMAs. Future research is needed to more precisely determine which subgroups (e.g. patients >70 years old, patients with monosomal karyotypes, etc.) benefit more from HMAs and which subgroups benefit more from intensive chemotherapy. Data from our extended patient named program and from others indicates that the outcome with azacitidine is poorer in AML patients with proliferative disease, reflected by higher WBC counts (>15 x 10^9/L), suggesting that the hypomethylating effects are slow-acting and not dependent on the rate of cell proliferation.[36,37] With decitabine, however, a trend towards improved outcome has been observed regardless of baseline WBC count in a post-hoc analysis of a large phase III trial.[38]

Since allo-HCT is currently the only potential curative therapy for MDS and AML, an interesting treatment approach to assess in older patients would be to combine cytoreductive therapy with an HMA and allo-HCT. It should be noted that most treatment schedules with HMAs (except for the 10-day decitabine schedule) are associated with lower response rates compared to IC[39] and that especially remission without minimal residual disease is associated with favorable outcome after allo-HCT[40]. Nevertheless, retrospective studies in MDS patients indicate that azacitidine followed by allo-HCT is equally effective as intensive chemotherapy followed by allo-HCT in indolent disease.[41-43] HMAs may trigger an enhanced graft-versus-leukemia effect via increasing regulatory T-cell numbers, activating immune cells and upregulation of tumor antigens through hypomethylation.[44-46] First results of the addition of decitabine to a fludarabine/total body irradiation-conditioning schedule followed by allo-HCT are encouraging.[46] Currently, a prospective trial (EORTC-1301) randomizing older AML patients for induction therapy with decitabine versus conventional chemotherapy before allo-HCT is ongoing.

Furthermore, different schedules of HMAs may be feasible and effective. For example, a combination of intensive chemotherapy and an HMA followed by allo-HCT might be an effective treatment strategy in fit older patients, as different cell death pathways in the heterogeneous leukemia cell population will be targeted. First results indicate that combining azacitidine or

decitabine with intensive chemotherapy is feasible in older AML patients and induces favorable response rates.[47-50] Further, azacitidine may be used as consolidation therapy after allo-HCT, to prevent relapse and to augment the graft-versus-leukemia effect.[51] Encouraging results indicate that low-dose azacitidine (35-50 mg/m^2 for 5 days/4weeks) is feasible after allo-HCT, is associated with low incidence of graft-versus-host disease, and can be applied at a dose of 75 mg/m^2 for 7 days in case of minimal residual disease.[45,52,53] If allo-HCT is not feasible due to unavailability of a matched donor or frailty, the ideal consolidation therapy in older AML patients is undetermined. Post-remission maintenance therapy with azacitidine after intensive chemotherapy seems safe and feasible in older AML patients.[52,54] A randomized trial (HOVON 97) to evaluate azacitidine maintenance versus no maintenance after intensive chemotherapy is ongoing.

As both azacitidine and decitabine are currently registered for treating older (≥65 years) AML patients ineligible for intensive chemotherapy and/or allo-HCT, the question arises which drug is preferred. In this perspective it should be noted that 80% of azacitidine is incorporated into RNA and 20% in DNA, while 100% of decitabine is incorporated into DNA. Different gene expression profiles have been observed in cell lines after treatment with azacitidine and decitabine, suggesting different effects of these drugs on cell biology.[55] In our single-center cohort, the number of AML patients treated with decitabine was too small to compare its efficacy with azacitidine. A recent meta-analysis and a retrospective analysis comparing azacitidine with decitabine in MDS patients demonstrated better results of azacitidine treatment, especially in patients older than 75 years and patients with IPSS risk ≥3.[19,56] However, the decitabine dosing schedules probably were suboptimal. Inversely, a retrospective study comparing outcome after intensive chemotherapy with outcome after HMAs (both azacitidine and decitabine) revealed superiority of decitabine.[29] Therefore, comparative studies of azacitidine and decitabine (including the 10-day intravenous 20 mg/m^2 dosing schedule) in MDS and AML patients will be of interest.

Prognostic and predictive factors in patients treated with hypomethylating agents

Response to HMAs requires time and is usually observed after several treatment cycles. Therefore it is recommended to administer at least six cycles of azacitidine or four cycles of decitabine (5 days schedule) before discontinuation. In view of the long time frame and costs (azacitidine costs about € 5.300 per 7-day cycle and decitabine costs about €7.000 per 5-day cycle in the Netherlands), it would be of value to have predictive markers for response. Globally used prognostic scores, including the IPSS and the IPSS-R in MDS and the cytogenetic/molecular risk stratification in AML, provide information on the likely outcome of the disease, but are not designed to predict treatment benefit. To more precisely estimate the progression-free survival and OS, specific prognostic scores in patients treated with HMAs have been designed. The prognostic score of Itzykson *et al.* includes WHO performance score, circulating blasts, red blood cell transfusion dependency and cytogenetics (good versus intermediate versus poor), and assigns patients up to 30% bone marrow blasts in three risk groups with significantly different OS.[26] In the Dutch named patient program, we were able to confirm the prognostic value of this scoring system in azacitidine-treated patients. For unfit AML patients treated with azacitidine, the

European ALMA score has been developed, which uses the WHO performance score, WBC count and cytogenetics (normal versus abnormal) to discriminate between three risk groups with different OS.[57] These prognostic scores are often used to guide decisions in favor or against azacitidine treatment, although they formally do not predict treatment benefit.[58] Ideally, prospective or retrospective data from a randomized trial should be used to identify response predictors.[58] In absence of these data, prognostic factors for response to treatment (instead of survival) or early markers for response may indicate treatment benefit.

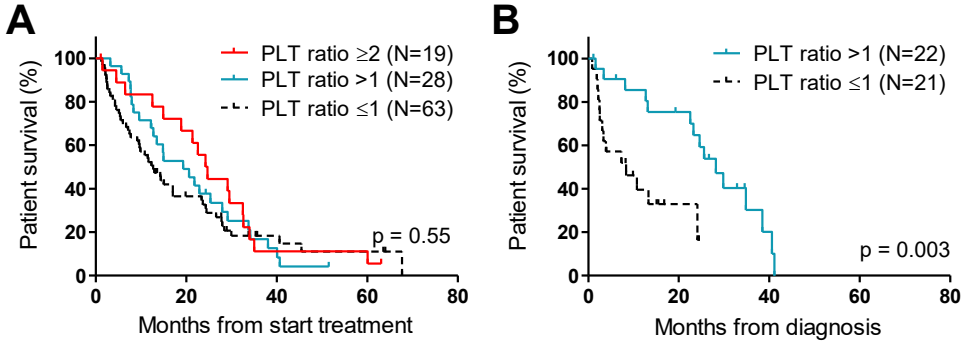

Figure 2. Overall survival by platelet increase after the first month of HMA treatment

A) OS by platelet (PLT) count ratio of MDS, CMML and AML patients treated within the extended Dutch azacitidine named patient program. Patients without available information on platelet counts at start of the second cycle (N = 15) were excluded. **B)** OS by PLT count ratio of AML patients from our single-center cohort treated with azacitidine (N = 36) or decitabine (N = 7). Patients without available information on platelet counts at start of the second cycle (N = 15) have been excluded. Platelet ratios were determined by dividing platelet counts at start of the second azacitidine cycle by platelet counts at start of the first cycle. Patients who received platelet transfusions within 10 days before the second cycle were considered as PLT ratio ≤1.

In the Dutch azacitidine named patient program we identified a possible early marker for treatment benefit. Platelet doubling after the first cycle of azacitidine was observed in a small group of patients (about 15%) and was an independent prognostic factor for favorable OS, although statistically not significant in multivariate analysis. Importantly, also in the majority of patients who did not show early platelet increase, responses were observed. Therefore, platelet doubling could be regarded as encouragement to continue treatment, while lack of platelet doubling should not be used to withhold further azacitidine treatment. We re-assessed the extended named patient program in January 2015 (3.5 years later) to evaluate the prognostic value of early platelet increase on long-term survival. After this longer follow-up time, median OS of patients with platelet doubling after the first azacitidine cycle was 24.3 months compared to 19.3 months in patients with a less than 2 fold platelet increase, and 12.7 months in patients without platelet increase, which did not reach statistical significance (p = 0.55; Figure 2A). We also assessed early platelet increases in our cohort of older AML patients treated with azacitidine or decitabine. Only six of 43 evaluable patients showed platelet doubling after the first cycle.

Therefore, we combined this group of patients with patients who had a platelet increase less than 2 fold. AML patients with a more than 1 fold platelet increase after the first cycle (N = 21 azacitidine/1 decitabine) had a significantly longer median OS (28.2 months) compared to patients without any platelet increase (N = 15 azacitidine/6 decitabine; median OS 8.4 months; p = 0.003; Figure 2B).

A prognostic value of early platelet increase during HMA treatment has also been observed by other groups. Zeidan et al. validated platelet doubling after the first azacitidine cycle in a cohort of 102 patients with MDS or AML with less than 30% bone marrow blasts and confirmed a longer median OS in patients with platelet count doubling (21.0 months) compared to those without (16.7 months, HR 1.88 (95% CI 1.03-3.40), p = 0.04).[59] Raffoux et al. reported that early platelet responses were associated with higher remission rates in AML and MDS patients treated with azacitidine, valproic acid, and all-trans retinoic acid.[60] In the Australian azacitidine named patient program, a non-significant trend towards longer OS was observed in AML patients with platelet count doubling after one cycle (N=23) compared to others (N = 220; median OS 574 versus 330 days, p = 0.141).[61] Van den Bosch et al. and Jung et al. observed superior OS in MDS patients treated with decitabine who had an early platelet response.[62,63] Apparently HMAs affect megakaryocyte development. A mouse study suggests that decitabine enhances megakaryocyte maturation and platelet release.[64] It is unclear whether the aberrant MDS or AML cells or the residual normal cells are responsible for the increased production. Several studies suggest that normal hematopoietic cells can be stimulated to megakaryocyte differentiation. Experimental treatment with decitabine in eight patients with sickle cell anemia led to an increase in platelet counts and bone marrow megakaryocytes in all patients.[65] Also in five patients with β-thalassemia and fifteen patients with metastatic lung cancer, experimental treatment with decitabine resulted in 1.3 to 3 fold increases in platelet counts.[66] These data are in line with the observation that cytogenetically normal $CD34^+$ cells in MDS patients expand in response to treatment with erythropoietin and granulocyte-colony stimulating factor.[67] On the other hand, increases of dysplastic megakaryocytes have been observed in at least one study in MDS patients who had a platelet response upon treatment with decitabine, suggesting that also dysplastic cells can be stimulated to production of platelets.[62]

Gene mutations and response to HMAs

In recent years, various gene mutations have been identified in MDS and AML by next-generation sequencing. Increasing evidence demonstrates the association of these mutations with clinical outcome (Table 2). Some mutations, such as *TET2* and *DNMT3A* mutations, are generally associated with adverse outcome, but are predictive for improved response to HMAs.[68-70] One of the most unfavorable mutations is mutation of *TP53*. Very poor survival has been reported with conventional therapy.[71,72] Although we also observed poor survival in our cohort of AML patients, our results indicate that patients with *TP53* mutations may benefit from hypomethylating agents with similar response rates compared to *TP53*-wild type patients and temporary suppression of

the *TP53*-mutated AML clone. However, prospective studies are needed to determine whether *TP53*-mutated patients indeed benefit from HMAs.

Table 2. Mutations and their clinical significance in MDS and AML

Category	Mutant gene	Frequency in MDS (%)	Frequency in AML (%)	Clinical findings and prognosis
DNA methylation	DNMT3A	10-15	20-50	Adverse; but favorable response to HMAs
DNA de-methylation	TET2	20-30	10-20	Poorer in int-risk AML; but favorable response to HMAs
	IDH1	3	7	Poorer in FLT3-ITD-negative AML
	IDH2-R140	5	7	Favorable
	IDH2-R172		2	Adverse
	WT1	<1	9	Poorer in NK-AML
Activated signalling	FLT3-ITD	<1	27	Poorer in int-risk AML
	FLT3-TKD	<1	11	Variable according to study
	KIT	<1	4	Poorer outcome in CBF AML
Myeloid transcription factors	RUNX1	10	5	Adverse
	bi-CEBPA	<1	4	Favorable
Tumor suppressor/ multifactorial	TP53	5-18	8-16	Adverse
	NPM1	<1	33	Favorable in absence of FLT3-ITD and mutant DNMT3a
Chromatin regulation	ASXL1	15-25	5	Poorer in int-risk AML
	MLL-PTD	<1	5	Adverse
Splicosome	SF3B1	25-30	3	Favorable in MDS
	SRSF2	15	2	Adverse in MDS

ITD, internal tandem duplication; TKD, tyrosine kinase domain; int, intermediate; HMAs, hypomethylating agents; bi-CEBPA, bi-allelic CEBPA mutations. Table based on Grimwalde et al.[252] and [33,34,65,132,173,251,253-256].

Proteasome inhibition in AML

To improve cure rates in AML patients, investigation of new therapeutic strategies based on differences between AML (stem) cells and normal hematopoietic stem- and progenitor cells is essential. One of the potential treatment targets is the transcription factor NF-κB, which is constitutively activated in the leukemic cells of a majority of AML patients and appears to be important for the survival of AML blasts.[81,82] Various pathways have been proposed to be involved in constitutive NF-κB activity, including genetic aberrations leading to increased NF-κB activation (e.g. *AML1-ETO* translocation, *C/EBPα* mutation, deletion of 5q), autocrine/paracrine signaling loops, upregulation of upstream components of the NF-κB pathway such as IRAK1, TAK1, and BTK; and increased proteasome activity leading to increased degradation of the NF-κB-inhibitory protein IκBα.[81] Based on these pathways, various treatment options have been proposed to inhibit NF-κB. Inhibition of BTK by ibrutinib for example resulted in cell death and decreased NF-κB

activity in primary AML cells, and showed cytotoxic effects in chronic lymphocytic leukemia in phase I and II clinical studies.[83,84] IRAK1 is recently shown to be inhibited by the FLT3/JAK inhibitor pacritinib in AML cells.[85]

In this thesis, we evaluated the effects of the proteasome inhibitors carfilzomib, oprozomib and bortezomib on patient-derived AML cells *in vitro*. We observed a cytotoxic effect of carfilzomib on both primitive AML $CD34^+$ cell fractions and more mature AML $CD34^-$ cell fractions, whereas the first-generation proteasome inhibitor bortezomib mainly targeted more mature AML $CD34^-$ cell populations. To evaluate whether carfilzomib induces apoptosis in AML $CD34^+$ cells with acceptable effects on normal tissue, *in vivo* studies are required. Oprozomib, when tested at equimolar concentrations, showed limited effectiveness. However, also the effects on normal $CD34^+$ cells were smaller, suggesting that oprozomib could be safely added at higher concentrations that may result in higher effectiveness. Future experiments are needed to define the optimal dose schedule for oprozomib to target primitive AML cells. Furthermore, we observed that the anti-apoptotic protein MCL-1 was upregulated in AML cells incubated with carfilzomib, oprozomib, or bortezomib. Inhibition of MCL-1 by obatoclax enhanced the apoptotic effects of the proteasome inhibitors, suggesting that combinations of proteasome inhibitors with drugs that target anti-apoptosis pathways may be worthwhile to further investigate. Currently, several drugs that target MCL-1 or other anti- or pro-apoptotic BCL-2 family members are under investigation, including the pan-BCL-2 inhibitors obatoclax and gossypol, the MCL-1-specific inhibitors maritoclax and MIM1, and the Bcl-X_L/ Bcl-2/Bcl-w inhibitors ABT-737 and ABT-263.[86] All of these compounds induced apoptosis in various cancer cells *in vitro* and *in vivo*, especially in hematologic malignancies.[86] However, phase I and II clinical studies with some of these drugs as single agents have reported only modest effects so far. Still, given the preclinical results, studies with drug combinations with BCL-2 family inhibitors may be of interest.

In light of predicting response to treatment, we assessed baseline chymotrypsin-like activity in several sensitive and less-sensitive AML samples. In this limited amount of samples, we did not observe an association of chymotrypsin-like activity with sensitivity to carfilzomib. This might be related to variable affinity of carfilzomib to chymotrypsin-like activity sites of the constitutive proteasome versus the immunoproteasome present in hematopoietic cells.[87] A recent study suggests that higher ratios of immunoproteasome versus constitutive proteasome subunit expression are associated with sensitivity of AML cells to carfilzomib.[88] Future studies to assess possible associations between response and chymotrypsin-like activity, as well as other related proteins such as NF-κB and Nrf2, are of interest and could shed more light on the mechanism of action of carfilzomib in AML cells.

In AML patients, carfilzomib has not been tested yet, but bortezomib treatment has been associated with some favorable first results. In a phase II clinical trial in AML patients aged 60 to 75 years, efficacy and safety of adding bortezomib to cytarabine/daunorubicine induction and intermediate-dose cytarabine consolidation therapy was assessed.[89] Results revealed favorable CR rates of 65% with a median disease-free survival of 8 months and median OS of 12 months. Further, it was concluded that 1.3 mg/m^2 bortezomib combined with intensive chemotherapy had

an acceptable toxicity profile, although 11 out of 95 patients developed grade 3 sensory neuropathy. Since carfilzomib has not been associated with neurotoxicity or other severe side-effects, carfilzomib is a promising alternative to bortezomib. Several subgroups of patients may be more sensitive to proteasome inhibition. For example, AML cell samples bearing a *FLT3-ITD* mutation are shown to be more sensitive to bortezomib compared to wild type cells, which was related to bortezomib-induced degradation of FLT3 and FLT3-ITD by autophagy.[90] In the above mentioned phase II study, six out of eight (75%) patients with *FLT3-ITD* achieved CR.[89]

Interestingly, a DNA hypomethylating effect of bortezomib has been observed in AML cells, which was related to downregulation of *DNMT1* via interfering with its Sp1/NF-κB transcription complex.[91] Therefore, addition of a proteasome inhibitor may enhance the efficacy of HMAs, which inhibit DNMTs by direct interaction. A phase I clinical trial combining bortezomib with decitabine in poor-risk AML patients reported CR in 9/17 patients with good initial tolerability but occurrence of neurotoxicity after two cycles requiring discontinuation of bortezomib in three patients.[92] Unfortunately, a subsequent randomized study of decitabine versus decitabine plus bortezomib was closed prematurely because an interim analysis indicated that the combination was unlikely to be superior to decitabine alone.[93] Another phase I trial combined bortezomib with azacitidine in patients with relapsed or refractory AML.[93] Of these 23 poor-risk patients, five achieved a remission after a median of (only) 2 cycles (range 1-12+). However, again significant neurotoxicity was reported, urging the need of replacing bortezomib in future studies by proteasome inhibitors with lower toxicity profiles, such as carfilzomib.

Other new treatment strategies in higher-risk MDS and AML

Currently, various other promising therapeutic approaches that may improve future outcome in higher-risk MDS and AML patients are being investigated. The novel hypomethylating agent guadecitabine is a dinucleotide of decitabine and deoxyguanosine that is resistant to degradation by cytidine deaminase, resulting in a prolonged half-life compared to azacitidine and decitabine, which have a half-life of less than 30 minutes. The prolonged half-life could potentially improve response rates especially in lower proliferative disease, since azanucleosides must be incorporated into DNA during the S-phase of cell division to exert a hypomethylating effect.[94] Phase I and II studies in AML patients ineligible for intensive chemotherapy demonstrated that subcutaneous guadecitabine at 60 mg/m^2 for 5 days is well tolerated and is clinically active with CR rates of 57% and median OS of 10.5 months.[94,95] A phase III study is currently being conducted.

Another strategy to improve treatment results with HMAs is addition of histone deacetylase (HDAC) inhibitors, since *in vitro* studies revealed a synergistic effect of histone deacetylation and DNA hypomethylation on re-expression of genes silenced by malignant transformation.[96] However, clinical studies investigating combinations of an HMA with the HDAC inhibitor valproic acid or entinostat not only showed lack of improved response and survival, but also found lower degrees of hypomethylation when both drug types were combined, suggesting pharmacodynamic antagonism.[97,98] Nevertheless, combination of the HDAC inhibitor pracinostat with azacitidine

induced promising remission rates of 54% with an estimated 1-year survival rate of 60% in AML patients aged ≥65 years ineligible for intensive therapy.[99]

Further, several low-intensity drugs and drug combinations have recently reached phase II and III clinical trials, however with at the moment only modest efficacy in older AML and higher-risk MDS patients. Gemtuzumab ozogamicin is an anti-CD33 antibody conjugate that in combination with low-dose cytarabine induced higher CR rates compared to single agent low-dose cytarabine (30% versus 17%, p = 0.006), but did not improve OS.[100] Likewise, combination of gemtuzumab ozogamicin with azacitidine or decitabine led to increased OS rates but not to improved OS rates in phase II studies compared to historical data, except for poor risk AML patients who seemed to benefit from azacitidine plus gemtuzumab ozogamicin.[101,102] Volasertib is a selective cell cycle kinase inhibitor that targets Polo-like kinase 1 inducing cell cycle arrest and apoptosis. Combination with low-dose cytarabine resulted in improved remission rates (31.0% versus 13.3%, p =0.052) and median OS rates (8.0 versus 5.2 months, p = 0.047), but also increased neutropenic fever/infections and gastro-intestinal effects compared to low-dose cytarabine alone in AML patients ineligible for intensive therapy.[103] Interestingly, responses were observed across all genetic risk groups, including 5/14 patients with adverse risk. A phase III study of volasertib plus low-dose cytarabine and studies combining volasertib with decitabine or intensive chemotherapy are currently being conducted. Tipifarnib, a farnesyltransferase inhibitor affecting Ras-signaling, might improve survival as maintenance therapy in AML patients at high risk for relapse.[104] The Aurora B kinase inhibitor barasertib and the nucleoside analogue prodrug sapacitabine did not appear to improve outcome in AML.[105]

An interesting upcoming development is the emergence of personalized therapy or 'precision medicine' in AML, which takes into account inter-patient differences in disease- and patient characteristics and possible intra-patient differences due to clonal evolution over time.[106,107] Besides currently available therapies, emerging targeted therapies such as inhibitors of FLT3, isocitrate dehydrogenase 1 (IDH1), IDH2, NF-κB, or bromodomain and extra terminal protein (BET), will in particular fit well in this concept.

Age-related changes in hematopoiesis and AML

To improve outcome in older AML patients, it is important to enlarge our insight in the causes of their poor outcome. It has become increasingly clear that differences exist between young and old individuals in normal hematopoiesis. Ageing has been associated with an increased prevalence of clonal hematopoiesis, considered as a pre-leukemic state.[108] Further, whereas normal hematopoiesis under physiological conditions is largely maintained by short-term hematopoietic stem cells (HSCs) and progenitors, increased numbers and increased activity of HSCs is observed with ageing.[109,110] These aged HSCs seem to be skewed to the myeloid compartment and have a diminished function as reflected by a reduced self-renewal capacity and reduced engraftment following stem cell transplantation.[109,111,112] Besides contributing to a higher risk of developing myeloid malignancies, age-related changes might also limit peripheral blood recovery after

treatment, for example due to possibly increased vulnerability of normal HSCs to anti-leukemic therapy.

In AML, older age is associated with increased numbers of gene mutations in intermediate- and adverse-risk AML.[113,114] Also the type of mutations is different in older patients, as mutations more frequently occur in *ASXL1*, *MLL*, *RUNX1*, and *TET2*, while *NRAS* mutations appear less often in older AML patients.[114] These data suggest that older AML patients often have a disease with different properties than younger AML patients, which might confer different sensitivity towards treatment. More research is needed to further characterize AML cells of older patients and to adapt treatment to their specific alterations.

Further, age-related changes may contribute to the higher relapse rates in older AML patients after treatment. As preleukemic cells are shown to be able to survive therapy and to generate new clones or activate dormant clones that initiate relapse[115,116], the higher prevalence of (preleukemic) clonal hematopoiesis in older individuals contains an increased risk of relapse. The appearance of TP53-overexpressing cells upon relapse that we observed in one of our azacitidine-treated AML patients in Chapter 5 might have been due to the outgrowth of a small (preleukemic) clone. In addition, due to impaired sensitivity of older AML cells, low numbers of AML cells might persist during remission, causing relapse. In Chapter 5, two patients showed re-appearance or expansion of TP53-overexpressing cells at time of relapse, suggesting persistence of small amounts of *TP53*-mutated AML cells during HMA treatment in remission.

CONCLUSIONS

In conclusion, in this thesis we have shown that azacitidine is effective and feasible in daily clinical practice in higher-risk MDS patients and older AML patients, including AML patients with more than 30% bone marrow blasts or with *TP53* mutations. We demonstrated that azacitidine in older AML patients is associated with better outcome compared to BSC, and with similar outcome compared to intensive chemotherapy not followed by allo-HCT. Further, we showed that the second-generation proteasome inhibitor carfilzomib reduces survival of primitive AML cell fractions *in vitro*. More research is required to improve current treatment with HMAs, to guide treatment decisions in higher-risk MDS and older AML patients, and to assess *in vivo* and clinical effectivity of new possible treatment strategies including carfilzomib.

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Chapter 8

Nederlandse samenvatting

De normale vorming van bloedcellen, het myelodysplastisch syndroom en leukemie

Dagelijks worden er in het beenmerg vele miljarden nieuwe bloedcellen gevormd vanuit stamcellen volgens een strikt gereguleerd en complex proces. Bloedcellen bestaan grofweg uit drie groepen: rode bloedcellen voor het transport van zuurstof en koolstofdioxide, bloedplaatjes voor de bloedstolling en verschillende soorten witte bloedcellen die belangrijk zijn voor de afweer. Door veranderingen in de stamcellen of vroege voorlopercellen kan de uitrijping naar functionele bloedcellen verstoord worden, zoals bij het myelodysplastisch syndroom (MDS). Patiënten hebben hierbij last van een mild tot levensbedreigend tekort aan één of meer soorten bloedcellen, wat zich uit in vermoeidheid (bij een tekort aan rode bloedcellen), bloedingen (bij een tekort aan bloedplaatjes), en/of infecties (bij een tekort aan witte bloedcellen). Ook kunnen afwijkende cellen zich ongeremd gaan delen en zo de normale bloedaanmaak verdringen, zoals bij ernstigere vormen van MDS en bij leukemie. Er zijn verschillende typen leukemieën, afhankelijk van het type voorlopercel waaruit ze ontstaan. Dit proefschrift gaat over MDS en een agressief type leukemie dat net als MDS uit voorlopercellen van het myeloïde soort ontstaat: acute myeloïde leukemie (AML). AML kan zich ontwikkelen vanuit MDS, maar kan ook op zichzelf staan. In sommige gevallen kan radioactieve straling of chemotherapie voor een andere aandoening als oorzaak worden aangewezen, maar in de meeste gevallen is de oorzaak van de celveranderingen die leiden tot MDS en/of AML onbekend. Een belangrijke risicofactor voor het ontstaan van MDS en AML is veroudering. Meer dan de helft van de MDS- en AML-patiënten is dan ook ouder dan 70 jaar.

Behandeling van het myelodysplastisch syndroom en acute myeloide leukemie

MDS wordt ingedeeld in verschillende risicogroepen met een verschillende kans op de ontwikkeling van AML of op overlijden aan de gevolgen van MDS. Laag-risico MDS wordt vooral ondersteunend behandeld met zo nodig medicijnen die de aanmaak van bloedcellen stimuleren of met bloedtransfusies. Hoog-risico MDS wordt vaak net als AML behandeld met intensieve chemotherapie, zo mogelijk gevolgd door een transplantatie van gezonde bloedvormende cellen van een gematchte donor, die – als alles goed gaat – het bloedsysteem van de patiënt vervangen en een goede kans bieden op genezing. In jongere patiënten worden hiermee goede resultaten behaald, maar de resultaten in de grote groep oudere patiënten vallen tegen. Dit is voor een deel gerelateerd aan bijkomende gezondheidsproblemen en voor een nog groter deel aan ongunstige eigenschappen van de zieke cellen zelf, die zich in ouderen vaak anders lijken te gedragen. Bovendien is deze behandeling voor veel oudere patiënten te zwaar. Daarom worden er vaak behandelschema's met lagere doseringen chemotherapie toegepast, vaak gericht op het in toom houden van de ziekte in plaats van op genezing. Deze minder zware chemotherapie geeft doorgaans matige resultaten en is voor sommige ouderen alsnog te zwaar. Daarom zijn er – vooral voor oudere patiënten – nieuwe behandelstrategieën nodig gericht tegen MDS en AML.

Nieuwe behandelstrategieën

Om uiteindelijk de behandeling van voornamelijk deze oudere, moeilijk te behandelen groep MDS- en AML-patiënten te verbeteren, hebben we ons in dit proefschrift gericht op twee soorten nieuwe behandelingen. Als eerste hebben we de effectiviteit van het nieuwe middel azacitidine en het vergelijkbare decitabine in de klinische praktijk onderzocht. Deze medicijnen grijpen volgens de huidige inzichten aan op de regulatie van genexpressie, oftewel de regulatie van het 'aan- en uitzetten' van genen. Vaak is dit verstoord in MDS- en AML-cellen, waardoor genen die de kankergroei onderdrukken 'uit' staan. Door de inbouw van azacitidine of decitabine in het DNA van de cellen kunnen deze genen weer geactiveerd worden. Deze middelen hebben relatief milde bijwerkingen en kunnen poliklinisch of in dagbehandeling gegeven worden in maandelijkse kuren.

In **hoofdstuk 2** hebben we gegevens verzameld van de eerste 90 MDS- en AML-patiënten die in Nederland werden behandeld met azacitidine, nadat de superioriteit van azacitidine ten opzichte van conventionele behandelingen (intensieve chemotherapie, lage dosis chemotherapie, of ondersteunende behandeling) aangetoond was in een internationaal onderzoek. Onze gegevens laten zien dat in de klinische praktijk ongeveer de helft van de patiënten een respons heeft op azacitidine, wat vergelijkbaar is met de internationale studie, maar met een kortere mediane overleving van 13 maanden. Vervolgens hebben we onderzocht of bepaalde kenmerken van de patiënten of hun ziekte voorspellend waren voor een goede of slechte behandeluitkomst. Interessant was dat een kleine groep (16%) patiënten al na de eerste azacitidinekuur een verdubbeling had van het aantal bloedplaatjes. Deze patiënten hadden een opvallend lange overleving. Een effect van azacitidine op de vorming van bloedplaatjes is al eens eerder beschreven en het lijkt erop dat een stijging van bloedplaatjes een vroeg teken kan zijn van een goede respons op de behandeling.

Vóór oktober 2015 was azacitidine alleen geregistreerd voor MDS, AML met slechts 20-30% blasten (voorlopercellen) in het beenmerg (want deze AML-patiënten vielen eerder onder de definitie 'MDS') en chronische myelomonocytaire leukemie (CMML). In **hoofdstuk 3** hebben we onderzocht of er een verschil was in behandeluitkomst tussen AML-patiënten met 20-30% blasten en AML-patiënten met meer dan 30% blasten. In een groep van 55 patiënten zagen we vergelijkbare responspercentages en overleving in beide categorieën AML-patiënten. Hieruit concludeerden we dat het blastenpercentage niet voorspellend was voor de behandeluitkomst. Andere eerder beschreven factoren waren in deze studie wel voorspellend, waaronder een hoog cytogenetisch risico (bepaalde patronen van afwijkingen aan het erfelijk materiaal van cellen), een verhoogd aantal witte bloedcellen, een slechte klinische conditie bij aanvang van de behandeling en AML als gevolg van eerdere chemo- of radiotherapie. Inmiddels heeft een groter onderzoek onze resultaten bevestigd en is azacitidine ook geregistreerd voor AML-patiënten met meer dan 30% blasten.

In het Universitair Medisch Centrum Groningen is een relatief grote groep oudere AML-patiënten behandeld met azacitidine. In **hoofdstuk 4** hebben we de karakteristieken en behandeluitkomsten bekeken van de AML-patiënten van 60 jaar en ouder die hier tussen 2002 en 2012

gediagnosticeerd en behandeld werden. De drie hoofdgroepen van behandeling waren azacitidine, intensieve chemotherapie, en alleen ondersteunende behandeling met waar nodig bloedtransfusies en antibiotica. AML-patiënten die behandeld waren met azacitidine hadden een betere overleving dan patiënten die alleen ondersteunende behandeling hadden gekregen. Dit was deels gerelateerd aan de behandeling zelf, maar ook deels aan slechtere patiënt- en ziektekenmerken bij diagnose, waardoor waarschijnlijk voor ondersteunende therapie gekozen was. Opvallend was dat de azacitidine-patiënten en de intensieve chemotherapie-patiënten een vergelijkbare overleving hadden, ook na correctie voor verschillen in kenmerken tussen de groepen en bekende risicofactoren. Azacitidine-patiënten verbleven echter korter in het ziekenhuis en hadden minder bloedtransfusies nodig. Dit suggereert dat bepaalde groepen oudere AML-patiënten meer baat hebben bij een behandeling met azacitidine dan met intensieve chemotherapie of met enkel ondersteunende behandeling.

Omdat de studieduur voor de azacitidinegroep relatief kort was, hebben we in **hoofdstuk 5A** de groep oude AML-patiënten drie jaar later nog een keer geanalyseerd met inclusie van nieuwe patiënten inclusief decitabine-patiënten tot in 2015. Azacitidine en decitabine waren geassocieerd met betere uitkomsten dan alleen ondersteunende behandeling en gaven resultaten vergelijkbaar met intensieve chemotherapie. Alleen wanneer patiënten vervolgens, meestal na intensieve chemotherapie, een transplantatie hadden gehad van bloedvormende donorcellen (volgens een gereduceerd behandelschema met minder bijwerkingen), hadden ze een betere overleving. Interessant in dit kader zijn nieuwe ontwikkelingen waarbij behandelschema's met decitabine of azacitidine gevolgd door een transplantatie onderzocht worden voor oudere patiënten, met als doel het bereiken van een zo groot mogelijke effectiviteit met beperkte bijwerkingen.

DNA-mutaties in het gen *TP53* in AML-patiënten zijn geassocieerd met een slechte overleving en een slechte respons op intensieve chemotherapie, maar over de respons op azacitidine en decitabine is weinig bekend. In **hoofdstuk 5B** hebben we beenmergsamples van 47 oudere AML-patiënten onderzocht op de aanwezigheid van het eiwit TP53 in de celkernen, wat boven een bepaalde grenswaarde duidt op een *TP53*-mutatie. De 22 AML-patiënten met een *TP53*-mutatie die we op deze manier identificeerden hadden weliswaar een kortere overlevingsduur, maar hadden vergelijkbare responspercentages en even lange responsduur onder behandeling met azacitidine of decitabine in vergelijking met patiënten zonder deze mutatie. Bij een complete respons verdwenen de meeste *TP53*-gemuteerde cellen uit het beenmerg, wat aangeeft dat deze cellen ondanks de mutatie gevoelig kunnen zijn voor azacitidine en decitabine. De resultaten uit dit hoofdstuk laten zien dat azacitidine en decitabine een goede behandeloptie vormen in oudere AML-patiënten met een *TP53*-mutatie.

Om nieuwe medicijnbehandelingen te ontwikkelen is het van groot belang om onderzoek te doen naar de eigenschappen van AML-cellen en de verschillen tussen gezonde stamcellen en AML-stamcellen. Eén van de verschillen tussen normale- en AML-stamcellen werd gevonden in de proteasomen (complexen in de cel die verantwoordelijk zijn voor de afbraak van ongebruikte of schadelijke eiwitten) en de daaraan gerelateerde activiteit van het eiwit NF-κB, wat onder andere apoptose (gecontroleerde celdood) tegengaat. AML-stamcellen blijken vaak een verhoogde

proteasoom- en NF-κB-activiteit te hebben, wat inhoudt dat ze mogelijk gevoeliger zijn voor proteasoomremmers dan normale cellen. Eerder onderzoek met de proteasoomremmer bortezomib liet zien dat vooral de verder ontwikkelde AML-cellen gevoelig waren voor dit medicijn, maar dat de primitieve AML-celpopulaties waarin de stamcellen zich bevinden, de behandeling overleefden. In **hoofdstuk 6** onderzochten we of de tweede generatie proteasoomremmers carfilzomib en oprozomib een grotere effectiviteit hebben ten opzichte van bortezomib in AML-cellen afkomstig van patiënten. Behandeling van primitieve AML-cellen in lange-termijnkweken liet zien dat carfilzomib meer effect had dan bortezomib en oprozomib in vergelijkbare doseringen. Carfilzomib gaf een daling van de AML-stamcelfrequenties en van het aantal niet-delende cellen. Deze grotere effectiviteit is mogelijk gerelateerd aan een langere werkingsduur door een sterkere binding van carfilzomib aan het proteasoom. Na behandeling met carfilzomib vonden we inderdaad een langdurigere remming van proteasoomactiviteit dan met bortezomib. Gezonde primitieve celpopulaties bleken minder gevoelig voor carfilzomib dan AML-cellen, wat verklaard zou kunnen worden door een lagere proteasoomactiviteit en een kleiner aantal proteasomen, zoals we observeerden in normale cellen. Uit eerder onderzoek was gebleken dat het anti-apoptose-eiwit MCL-1 het effect van bortezomib in primitieve AML-cellen tegengaat. Ditzelfde effect van MCL-1 zagen we na behandeling met carfilzomib en oprozomib. Gelijktijdige behandeling met obatoclax, een medicijn dat onder andere MCL-1 remt, vergrootte de effectiviteit van de proteasoomremmers op de AML-cellen. De resultaten uit dit hoofdstuk laten zien dat carfilzomib in combinatie met een MCL-1-remmer mogelijk effectief is voor het bestrijden van AML-stamcellen.

Conclusies

In dit proefschrift hebben we laten zien dat azacitidine effectief en goed toepasbaar is in de klinische praktijk als behandeling voor hoog-risico MDS-patiënten en oudere AML-patiënten, inclusief patiënten met hogere blastenpercentages en met *TP53*-mutaties. Azacitidine en decitabine zijn in oudere AML-patiënten geassocieerd met betere uitkomsten dan ondersteunende behandeling alleen. Bovendien lieten we zien dat deze hypomethylerende middelen in oudere AML patiënten vergelijkbare uitkomsten geven, maar minder bijwerkingen hebben vergeleken met intensieve chemotherapie, tenzij de laatste gevolgd werd door een celtransplantatie. Daarnaast toonden we aan dat de tweede-generatie proteasoomremmer carfilzomib de overleving van primitieve AML-celpopulaties remt *in vitro*. Meer onderzoek is nodig om de huidige behandeling met azacitidine en decitabine te verbeteren, om de respons op de behandeling beter te kunnen voorspellen en daarmee meer richting te geven aan behandelkeuzes, en om de *in vivo* en klinische effectiviteit van nieuwe mogelijke behandelstrategieën zoals carfilzomib te bepalen.

Appendix

List of abbreviations

LIST OF ABBREVIATIONS

Allo-HCT	allogeneic hematopoietic cell transplantation
AML	acute myeloid leukemia
APL	acute promyelocytic leukemia
ATRA	all-trans retinoic acid
AZA	azacitidine
BM	bone marrow
BSC	best supportive care
CD	cluster of differentiation
CI	confidence interval
CMML	chronic myelomonocytic leukemia
CR	complete remission
CRi	complete remission with incomplete blood count recovery
DAC	decitabine
DNA	deoxyribonucleic acid
FLT3-ITD	Fms-like tyrosine kinase 3-internal tandem duplication
G-CSF	granulocyte colony-stimulating factor
Hb	hemoglobin
HCT	hematopoietic cell transplantation
HI	hematologic improvement.
HMA	hypomethylating agent, e.g. azacitidine and decitabine
HR	hazard ratio
IC	intensive chemotherapy
IC-50	the half maximal inhibitory concentration
IPSS(-R)	international prognostic scoring system (revised)
ITD	internal tandem duplication
LDH	lactate dehydrogenase
mCR	complete remission of the bone marrow
MD/PhD	Medical Doctor/Doctor of Philosophy
MDS	myelodysplastic syndromes
N	number
NF-κB	nuclear factor kappa-B
NPM1	nucleophosmin 1
NPMc+/ITD-	cytoplasmic NPM1 without FLT3 internal tandem duplication
NR	not reached
OS	overall survival
PR	partial remission
RBC	red blood cell transfusion
PLT	platelet transfusion

SD	stable disease
SE	standard error
TP53	tumor protein 53
WBC	white blood cell count
WHO	World Health Organization

Dankwoord/Acknowledgements

Appendix

De afgelopen jaren waarin ik klinische stages met wetenschappelijk onderzoek heb mogen combineren, gaven mij veel mooie en dierbare momenten, waren bij perioden soms ook druk en stressvol, maar waren bovenal buitengewoon leerzaam en waardevol. Dit proefschrift was natuurlijk niet tot stand gekomen zonder de hulp van velen, die ik hierbij graag wil bedanken voor hun bijdrage en steun.

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Appendix

List of publications
Curriculum vitae

LIST OF PUBLICATIONS

Platelet doubling after the first azacitidine cycle is a promising predictor for response in myelodysplastic syndromes (MDS), chronic myelomonocytic leukaemia (CMML) and acute myeloid leukaemia (AML) patients in the dutch azacitidine compassionate named patient program.
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Annals of Hematology 2016; 95: 191-199.

CURRICULUM VITAE

Lidia Henrieke van der Helm werd geboren op 1 mei 1989 te Apeldoorn. Op haar vijfde verhuisde ze met haar ouders en haar twee jongere zussen naar Zwolle. Hier behaalde zij in 2007 haar gymnasiumdiploma cum laude aan het Carolus Clusius College. In hetzelfde jaar begon ze met de opleiding geneeskunde aan de Rijksuniversiteit Groningen. Al in het eerste jaar van de opleiding werd haar interesse gewekt door het uitdagende programma van de Junior Scientific Masterclass (JSM) en volgde zij diverse extracurriculaire cursussen. Zo kwam ze bij de afdeling hematologie terecht, waar ze in 2010 begon met het doen van wetenschappelijk onderzoek onder begeleiding van prof. dr. G. Huls en prof. dr. E. Vellenga. In datzelfde jaar behaalde zij haar bachelordiploma met een eervolle vermelding voor het succesvol afronden van het JSM Bachelor-'Honours' traject. In het daarop volgende jaar volgde zij een minor filosofie, werkte ze aan haar eerste wetenschappelijke artikel, dat in 2011 gepubliceerd werd, en deed ze haar wetenschappelijke stage bij de experimentele hematologie in het multidisciplinair oncologisch laboratorium. Na een jaar juniorcoschappen in het UMCG startte zij in 2012 met het MD/PhD-traject, waarbij de masteropleiding geneeskunde werd gecombineerd met wetenschappelijk onderzoek onder leiding van prof. dr. G. Huls, prof. dr. E. Vellenga en prof. dr. J.J. Schuringa, wat heeft geresulteerd in dit proefschrift.

Tijdens haar klinische fase liep Lieke seniorcoschappen in het Scheperziekenhuis te Emmen en het Bethesdaziekenhuis te Hoogeveen. Haar semi-artsstage liep zij op de afdeling interne geneeskunde van het Martiniziekenhuis te Groningen. Na het behalen van haar masterbul in augustus 2016 en de verdediging van dit proefschrift zal zij vanaf oktober 2016 werken als arts-assistent in het Martiniziekenhuis.