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Clinical and genetic studies of high-risk myelodysplastic syndromes and acute myeloid leukemia with chromosome 5q deletion

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Cover image: Multicolor FISH showing a complex aberrant karyotype.

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Abstract

Patients with high-risk myelodysplastic syndromes (MDS) with a chromosome 5q deletion (del(5q)) have a poor prognosis and are often associated with a complex karyotype and *TP53* mutations, factors worsening the prognosis. The hypomethylating agent azacitidine (AZA) is the first-line treatment. Lenalidomide (LEN) is an effective therapy for lower-risk MDS with del(5q).

The aim of this thesis were clinical and genetic studies in patients with high-risk MDS and acute myeloid leukemia (AML) with 20-30% marrow blasts with a karyotype including del(5q). In a prospective, multicenter, open-label, randomized phase II study, we studied if AZA + LEN was superior to AZA alone in high-risk MDS and AML with 20-30% marrow blasts with del(5q). Seventy-two patients were included between 2012 and 2017. The overall response rate (ORR) in the treated cohort was 39% for AZA and 44% for AZA + LEN (P=0.63). The addition of LEN to AZA did not improve outcome. In paper II we studied the influence of cytogenetics on treatment response in the study and if specific cytogenetic findings could predict outcome. Patients with del(5q) and complex karyotype or an unbalanced translocation of 5q had a shorter median overall survival (OS) (P=0.004). The aim in paper III was to optimize diagnostic procedures and follow-up assessment with cytomorphology, bone marrow trephine biopsy and immunohistochemistry (IHC) in patients with higher-risk MDS and AML with 20-30% blasts with a karyotype including del(5q). In 18 patients (25%) a higher bone marrow blast percentage was detected by IHC compared to cytomorphology, shifting the diagnosis to either a higher-risk MDS subgroup or AML and is useful for correct subclassification in del(5q) high-risk myeloid disease and for response assessment.

In conclusion, the findings in this thesis show that high-risk MDS with del(5q) is a myeloid disorder with a dismal prognosis. There seems to be a window of molecular response to AZA after 3 months of treatment. Future studies should focus on the therapeutic window as a possibility for allogeneic stem cell transplantation.

Keywords: *Myelodysplastic syndromes, Acute myeloid leukemia, Chromosome 5q deletion, Complex karyotype, TP53 mutation, Clinical trial, azacitidine, lenalidomide, bone marrow trephine biopsy, IHC p53*

To my family

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List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Rasmussen Bengt, Göhring Gudrun, Bernard Elsa, Nilsson Lars, Tobiasson Magnus, Jädersten Martin, Garelius Hege, Dybedal Ingunn, Grønbaek Kirsten, Ejerblad Elisabeth, Lorenz Fryderyk, Flogegård Max, Werenberg Marcher Claus, Öster Fernström Annette, Cavelier Lucia, Papaemmanuil Elli, Ebeling Freja, Olsnes Kittang Astrid, Nørgaard Jan Maxwell, Saft Leonie, Möllgård Lars, Hellström-Lindberg Eva (2022). "Randomized Phase II Study of Azacitidine ± Lenalidomide in Higher-Risk Myelodysplastic Syndromes and Acute Myeloid Leukemia with a Karyotype Including Del(5q)". *Leukemia*, 36:1436-1439.

II. Rasmussen Bengt, Nilsson Lars, Tobiasson Magnus, Jädersten Martin, Garelius Hege, Dybedal Ingunn, Grønbaek Kirsten, Ejerblad Elisabeth, Lorenz Fryderyk, Flogegård Max, Werenberg Marcher Claus, Cavelier Lucia, Ebeling Freja, Olsnes Kittang Astrid, Nørgaard Jan Maxwell, Saft Leonie, Möllgård Lars, Hellström-Lindberg Eva, Schlegelberger Brigitte, Göhring Gudrun (2025). "Influence of cytogenetics on the outcome of patient with high-risk myelodysplastic syndrome including deletion 5q treated with azacitidine with or without lenalidomide". *Genes, Chromosomes and Cancer*, Feb 8;64(2):e70029.

III. Rasmussen Bengt, Nilsson Lars, Tobiasson Magnus, Jädersten Martin, Garelius Hege, Dybedal Ingunn, Grønbaek Kirsten, Ejerblad Elisabeth, Lorenz Fryderyk, Flogegård Max, Werenberg Marcher Claus, Cavelier Lucia, Ebeling Freja, Olsnes Kittang Astrid, Nørgaard Jan Maxwell, Schlegelberger Brigitte, Göhring Gudrun, Möllgård Lars, Hellström-Lindberg Eva, Saft Leonie. "Trephine biopsy and immunohistochemistry are essential tools for diagnostic and therapeutic evaluation in higher-risk myelodysplastic syndrome and acute myeloid leukemia with del(5q)" (*Manuscript*).

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List of abbreviations

AE	Adverse events
Allo-HSCT	Allogeneic hematopoietic stem-cell transplantation
AML	Acute myeloid leukemia
ANC	Absolute neutrophil counts
Ara-C	Low dose cytarabine
AZA	Azacitidine
BCL-2	B-cell leukemia/lymphoma-2
BSC	Best supportive care
CCyR	Complete cytogenetic response
CD	Cluster of differentiation
CDR	Common deletion region
CH	Clonal hematopoiesis
CHIP	Clonal hematopoiesis of indeterminate potential
CI	Comorbidity index
CK	Complex karyotype
CMML	Chronic myelomonocytic leukemia
cnLOH	Copy neutral loss of heterozygosity
CR	Complete response
del	Deletion
EB	Excess blasts
EPO	Erythropoietin
ESA	Erythropoiesis-stimulating agent

E-TD	Time dependent erythrocyte transfusion dependency
EU	European Union
FISH	Fluorescence in situ hybridization
G-CSF	Granulocyte-colony stimulating factor
Hb	Hemoglobin
HI	Hematologic improvement
HMA	Hypomethylating agents
IC	Intensive chemotherapy
ICC	International Consensus Classification
IDA	Idarubicin
IHC	Immunohistochemistry
INT	Intermediate
IPSS	International prognostic scoring system
IPSS-M	International prognostic scoring system molecular
IPSS-R	Revised international prognostic scoring system
ITT	Intention to treat
iv	Intravenous
IWG	International Working Group
LDH	Lactate dehydrogenase
LEN	Lenalidomide
MDS	Myelodysplastic syndromes
mFISH	Multicolor FISH
MPN	Myeloproliferative neoplasms
MRD	Measurable residual disease

NGS	Next-generation sequencing
NoCyR	No cytogenetic response
ORR	Overall response rate
OS	Overall survival
PCyR	Partial cytogenetic response
PLT	Platelet counts
po	Per oral
PR	Partial remission
RAEB	Refractory anemia of excess blasts
RAEB-T	Refractory anemia of excess blasts in transformation
RS	Ring sideroblasts
RBC	Red blood cell
S-	Serum
sc	Subcutaneous
SD	Stable disease
SNP	Single nucleotide polymorphism
T/C	Telomere/Centromere
VAF	Variant allele frequency
VEN	Venetoclax
VOR	Vorinostat
VPA	Valproic acid
WHO	World Health Organization
WPSS	WHO classification-based prognostic scoring system
WT	Wildtype

Myelodysplastic syndromes

During the second part of the 19th century, hematologists discussed their observations of certain patients with acute non-lymphocytic leukemia who had a variety of already-present hematological aberrations; these were often older patients with anemia, evolving during a period of months to years before developing into acute leukemia. In 1982, the group of French, American, and British researchers that had previously established the French-American-British (FAB) classification system for acute leukemia presented myelodysplastic syndrome (MDS) to describe the group of syndromes as a new disease entity(1).

Since then, MDS has been well-established as a disease entity describing a heterogeneous group of malignant hematopoietic stem-cell disorders that present with various grades of peripheral blood cytopenia (due to ineffective hematopoiesis), dysplastic bone-marrow changes, an impendent risk of developing acute myeloid leukemia (AML), and reduced survival(2-4). The international incidence of MDS is reported to be 3-5 per 100,000 in the general population(5). The incidence of MDS in Sweden is estimated at 3.8/100,000 among males and 2.3/100,000 among females, based on the Swedish MDS registry during 2013-2022(6). The data shows that the median age at diagnosis is 76 years, and there is an increasing incidence with age, such that only 27% of patients are under 60 years of age. The actual incidence of MDS may be underestimated due to underreporting to MDS registries(7).

Diagnostics

MDS leads to various grades of peripheral blood cytopenias and the cytopenias should be lasting for at least 2-4 months(3, 8), to exclude other possible causes of cytopenias. According to the World Health Organization (WHO)´s 2016 classification of myeloid malignancies, a valid cytopenia is defined as Hemoglobin (Hb) <10 g/dL, absolute neutrophile counts (ANC) <1.8 x10^9/L, or platelet counts (PLT) <100 x 10^9/L (Table 1). It has been observed that MDS may occur in cases of milder anemia (Hb<13 g/dL (males), 12 g/dL (females), or thrombocytopenia (<150 x 10^9/L)), together with morphologic changes(8), and this threshold for clonal cytopenias is used in WHO´s 5th classification(4) as well as in International Consensus Classification (ICC)(3). The slightly milder grade of cytopenias, may be useful in detecting some early MDS patients; however, it may not be a clinical meaningful cut-off, as the majority cause of cytopenia will be diseases other than hematological malignancies(9).

Name	Dysplastic lineages	Cytopenias*	Ring sideroblasts as % of marrow erythroid elements	BM and PB blasts	Cytogenetics by conventional karyotype analysis
MDS with single lineage dysplasia (MDS-SLD)	1	1 or 2	<15%/<5%†	BM <5%, PB <1%, no Auer rods	Any, unless fulfills all criteria for MDS with isolated del(5q)
MDS with multilineage dysplasia (MDS-MLD)	2 or 3	1-3	<15%/<5%†	BM <5%, PB <1%, no Auer rods	Any, unless fulfills all criteria for MDS with isolated del(5q)
MDS with ring sideroblasts (MDS-RS)					
MDS-RS with single lineage dysplasia (MDS-RS-SLD)	1	1 or 2	≥15%/≥5%†	BM <5%, PB <1%, no Auer rods	Any, unless fulfills all criteria for MDS with isolated del(5q)
MDS-RS with multilineage dysplasia (MDS-RS-MLD)	2 or 3	1-3	≥15%/≥5%†	BM <5%, PB <1%, no Auer rods	Any, unless fulfills all criteria for MDS with isolated del(5q)
MDS with isolated del(5q)	1-3	1-2	None or any	BM <5%, PB <1%, no Auer rods	del(5q) alone or with 1 additional abnormality except −7 or del (7q)
MDS with excess blasts (MDS-EB)					
MDS-EB-1	0-3	1-3	None or any	BM 5%-9% or PB 2%-4%, no Auer rods	Any
MDS-EB-2	0-3	1-3	None or any	BM 10%-19% or PB 5%-19% or Auer rods	Any
MDS, unclassifiable (MDS-U)					
with 1% blood blasts	1-3	1-3	None or any	BM <5%, PB = 1%,‡ no Auer rods	Any
with single lineage dysplasia and pancytopenia	1	3	None or any	BM <5%, PB <1%, no Auer rods	Any
based on defining cytogenetic abnormality	0	1-3	<15%§	BM <5%, PB <1%, no Auer rods	MDS-defining abnormality
Refractory cytopenia of childhood	1-3	1-3	None	BM <5%, PB <2%	Any

*Cytopenias defined as: hemoglobin, <10 g/dL; platelet count, <100 × 10^9/L; and absolute neutrophil count, <1.8 × 10^9/L. Rarely, MDS may present with mild anemia or thrombocytopenia above these levels. PB monocytes must be <1 × 10^9/L

†If *SF3B1* mutation is present.

‡One percent PB blasts must be recorded on at least 2 separate occasions.

§Cases with ≥15% ring sideroblasts by definition have significant erythroid dysplasia, and are classified as MDS-RS-SLD.

Table 1. Peripheral blood and bone marrow findings and cytogenetics of MDS. Adapted from *Arber et al.* 2016(2).

Morphological examinations

In MDS, the cytopenias are accompanied with morphological changes, with dysplasia in ≥10% of cells in at least one myeloid lineage(2-4, 10). Regarding megakaryocytes, a threshold of ≥10% of cells is adequate in case of typical micro-megakaryocytes, while other dysplastic megakaryocytes may require a higher frequency ≥30%(11). A bone marrow biopsy is strongly recommended and gives important information regarding cellularity, bone marrow microarchitecture with cell clusters, megakaryocytopoiesis, and grade of fibrosis(12). Immunohistochemistry (IHC) staining for cluster of differentiation (CD) 34+ blast cells will provide valuable information in diagnosis of MDS(13). In CD34- cells, it is helpful to use IHC staining for KIT/CD117+; thus, IHC staining may include positivity in proerythroblasts and mast cells(14). A strong p53 positive staining may be useful in detection of patients with a *TP53* mutation(15).

Cytogenetics

Approximately 50% of patients with MDS carry a clonal chromosomal aberration(16), and a cytogenetic analysis should be performed on all MDS patients. Cytogenetics provide valuable information for diagnosis and prognosis and can give helpful information in cases with inconclusive morphology(2, 16, 17). Fluorescence *in situ* hybridization (FISH) is a useful tool in cytogenetic analysis and provides information on the size of the aberrant clone(18).

Next-generation sequencing

Since MDS is a clonal disorder, and almost 50% av MDS patients have a normal karyotype, what drives the clonality? Mutations are detected by means of targeted next-generation sequencing (NGS) in nearly 90% of MDS patients(19, 20). It is helpful to know the type of mutation in order to determine the prognosis(19-22); for example, an isolated *SF3B1* mutation is associated with a good prognosis (Figure 1)(23, 24). In WHOs 4th classification of MDS, a *SF3B1* mutation was added to the diagnostic setting of refractory anemia with ring sideroblasts, and the threshold of ring sideroblasts was lowered from

≥15% to ≥5% in patients with both ring sideroblasts and a *SF3B1* mutation(2).

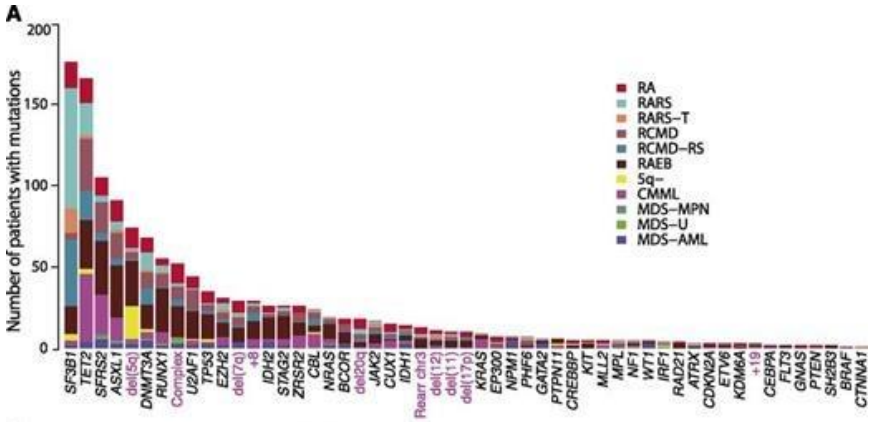


Figure 1. Frequent mutations in MDS subtypes. Adapted from *Papaemmanuil et al.* 2013(19).

Research experience with risk stratification in association with different mutations has influenced new classifications. Cases of MDS patients with a biallelic *TP53* mutations have been taken into consideration in ICC as well as WHO 5th classification and this combination has now been classified as new entity(3, 4). Many mutations are shared among myeloid neoplasms; and in WHO 5th classification, cases of AML with mutations in *ASXL1*, *BCOR*, *EZH2*, *SF3B1*, *SRSF2*, *STAG2*, *U2AF1*, and *ZRSR2* are strongly associated with MDS or MDS/myeloproliferative neoplasms (MPN), leading to the new classification as "AML, myelodysplasia-related"(25). In addition, a greater number of mutations worsens the prognosis(21, 26, 27).

Risk validation

It is important for clinicians to be able to make a reliable estimation of the prognosis for a MDS patient when deciding therapy options. A reliable prognosis is particular essential for the patient, as it allows the patient to understand the severity of the disease and realize how long the respite will be before disease progression and death. The MDS prognostic systems have developed during the research associated

with this thesis. In 1997, Greenberg et al. presented the international scoring system for evaluation of prognosis in MDS (IPSS) based on a study of 816 MDS patients from which prior treatment with intensive chemotherapy and secondary MDS were excluded. The IPSS is based on a synthesis of the number of cytopenias (Hb < 10 g/dL, ANC < $1.5x10^9$/L, PLT < $100x10^9$/L), percentages of bone marrow blasts (<5 to ≤ 30%), and type and number of cytogenetic aberrations(28). After evaluating the overall survival (OS) and 25% risk of AML transformation, patients are divided into four risk categories of *low*, *intermediate* (*INT*)*-1*, *INT-2* or *high* (Table 2). More specifically, these categories are ranked as follows, using the median OS/ risk of AML evolution (Table 2): *low* (5.7/ 9.4 years), *INT-1* (3.5/ 3.3 years), *INT-2* (1.2/ 1.1 years) and *high* (0.2/0.4 years).

	Score Value				
Prognostic Variable	0	0.5	1.0	1.5	2.0
BM blasts (%)	<5	5-10	—	11-20	21-30
Karyotype*	Good	Intermediate	Poor		
Cytopenias	0/1	2/3			

Scores for risk groups are as follows: Low, 0; INT-1, 0.5-1.0; INT-2, 1.5-2.0; and High, ≥2.5.

* Good, normal, −Y, del(5q), del(20q); Poor, complex (≥3 abnormalities) or chromosome 7 anomalies; Intermediate, other abnormalities.

Table 2. IPSS for MDS: Survival and AML Evolution. Adapted from *Greenberg et al.* 1997(28).

In 2012, Greenberg et al. presented a revised IPSS, denoted as the IPSS-R, based on a study of 7012 MDS-patients(8). Rather than the four categories in the IPSS, the IPSS-R divides patients into five risk categories: *very good*, *good*, *intermediate*, *poor* and *very poor*. The depth of cytopenias is taken into consideration in the IPSS-R, while the blast counts are viewed as less important; moreover, the bone marrow blasts are separated from the original < 5% into 0-2% and >2-<5%, and equal value is given to blasts within 10-30%. In addition, more attention is paid to cytogenetic aberrations, which are increased from six

specific aberrations to 16. Additional factors are added for predicting OS: significant but in low grade regarding β_2-microglobulin, lactate dehydrogenase (LDH), serum (S)-ferritin and performance status, and in higher grade, patients age. The IPSS-R different categories are described for a patient with an age of 70 years. The IPSS, the WHO Classification-based Prognostic Scoring System (WPSS), and the IPSS-R have been validated in a study of 1329 patients from the Swedish MDS register (during 2009–2013)(29). No large differences between the scoring systems were observed, although the IPSS-R was significantly superior to the IPSS and WPSS in predicting OS in patients ≤70 years. Patients age, gender (where the risk is lower in females), LDH, bone marrow fibrosis, and type of MDS (i.e. having undergone therapy vs. *de novo* MDS) were added to the IPSS-R as risk factors.

There has been an increasing number of studies regarding the effect on mutational status on outcome, leading to the development of new scoring system that takes mutational status into consideration. Bernard et al. presented the molecular IPSS (IPSS-M)(30), based on a study in which 72 patients from the Nordic MDS Group (NMDSG10B) study constituted 2,4% of a total of 2957 patients. The IPSS-M is further development of the IPSS-R combined with 152 specific gene mutations and a total of 31 mutations for risk stratification of the MDS-patient. Unlike the earlier IPSS and IPSS-R, the IPSS-M is useful in both primary and secondary/ therapy-related MDS patients. The IPSS-M divides patients into six different risk categories of AML transformation and OS: *very low*, *low*, *moderate low*, *moderate high*, *high* and *very high* (Figure 2). An assessment of different mutations may lead to a more clonal and personalized model for establishing a prognosis. An interesting pattern is observed when time-dependent erythrocyte transfusion dependency (E-TD) data are added to IPSS-M. In a study of 677 Swedish MDS patients, originated from the IPSS-M cohort, (E-TD) significantly adds risk in terms of shorter OS independently of IPSS-M(31).

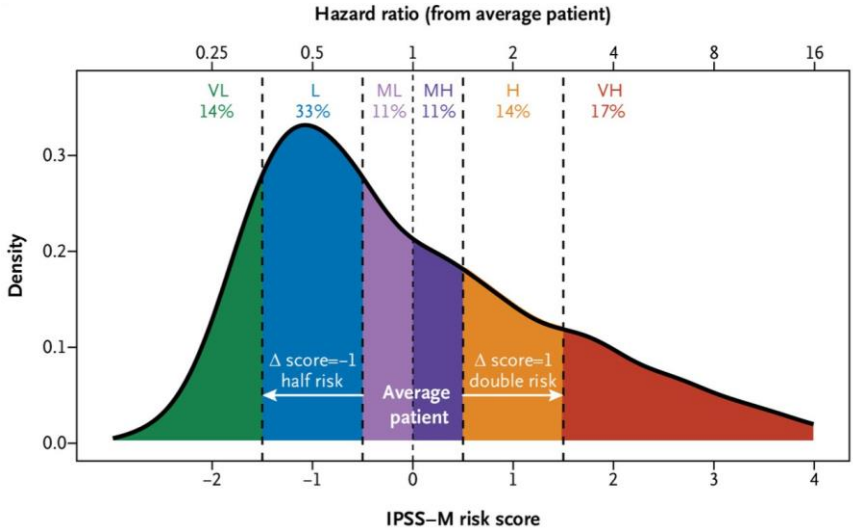


Figure 2. IPSS-M risk score and risk categories. Adapted from *Bernard et al.* 2022(30). Reproduced with permission from (scientific reference citation), Copyright Massachusetts Medical Society

A more convenient method that is used in everyday practice is to divide MDS patients into lower- or higher-risk categories, where *lower-risk* compromises the IPSS categories of low and INT-1; the IPSS-R categories of very low, low and INT with a risk score ≤3.5; and the IPSS-M categories of very low, low and moderate low. In the same way, *higher-risk* comprises the IPSS categories of INT-2 and high; the IPSS-R categories of INT with a risk score >3.5, high, and very high; and the IPSS-M categories of moderate high, high and very high(32, 33).

Other prognostic scoring systems are also used for MDS patients. The WPSS is based on a study of 1165 *de novo* MDS patients, excluding patients with MDS unclassified, chronic myelomonocytic leukemia (CMML) and ≥20% bone marrow blasts. It combines the WHO´s 4th classification of MDS subgroups with cytogenetic abnormalities (the same cytogenetic abnormalities as in the IPSS) and regular red blood cell (RBC) transfusions. The WPSS divides patients in five different risk groups regarding survival and risk of AML transformation(34). In the revised WPSS, which is based on a study of 840 MDS patients, the

subjective category of RBC transfusions is shifted to a more persistent category of anemia, under 9.0 g/dL (males) and 8 g/dL (females) in prognostic assessment(35). The WPSS and the IPSS-R scoring systems have an initial high prognostic power, with loss of power over time(32).

Comorbidity is important in judging an individual patient´s prognosis. In a study of 1344 MDS patients, Della Porta et al. presented a risk stratification based on MDS disease as well as non-hematologic comorbidities, referred to as the MDS-CI. In this classification, the patient's comorbidity (i.e. cardiac, pulmonary, renal, liver or solid tumors) significantly affects the patient´s non-leukemic death and OS without increased blast counts(36). In this study, comorbidity was not found to affect leukemia-free survival.

To summarize, when estimating the status of MDS in order to make a prognosis, the patient´s comorbidity, age and performance status should all be taken into account(37).

MDS with deletion 5q

The incidence of deletion 5q (del(5q)) in MDS patients is 10 -15%, making it the most common single chromosomal aberration in MDS(16, 38, 39). MDS with del(5q) is characterized by a female predominance, macrocytic anemia, often thrombocytosis, bone marrow blast counts <5% (no Auer rods), good prognosis, and low risk of transformation to AML(28, 40). The common deleted region is often situated in 5q32(41, 42). In the WHO´s 2008 classification, del(5q) is described as an isolated deletion(10). In the WHO´s 2016 classification, del(5q) is defined as a single deletion with or without addition of one more abnormality, except monosomy 7 or del(7)(43-45). No changes were added to isolated 5q deletion in the WHO´s 5th classification of MDS, nor in the ICC, except for a change in name to "MDS with del(5q)"(3, 4). The del(5q) clone originates from a pluripotent hematopoietic stem cells (CD34+CD38-) and is an early event in MDS pathogenesis(46). After targeting each of the genes in del(5q) common deletion region (CDR), a haploinsufficiency of the gene coding for the RPS14 ribosomal protein mimics the hematologic phenotype of MDS with del(5q)(47).

The immunomodulating drug lenalidomide (Table 3) is known to be an effective therapy for transfusion-dependent lower-risk MDS patients with a del(5q), reducing the transfusion burden (67%) and inducing complete cytogenetic remissions (45%) in patients(48). Lenalidomide induces apoptosis of del(5q) MDS cells by binding to cereblon (CRBN), a part of the Cul4-based E3 ubiquitin ligase. This leads to ubiquitination and degradation of casein kinase 1A1 protein, which is encoded by the haploinsufficient *CSNK1A1*, a tumor suppressor gene located at del(5q) CDR (5q32), a process depending on functioning p53(49-51).

Variable	Continuous Daily Dosing (N=102)*	21-Day Dosing (N=46)*	All Patients (N=148)
Erythroid response — no. (%)			
Transfusion independence	71 (70)	28 (61)	99 (67)
95% CI			59–74
≥50% decrease in no. of transfusions	8 (8)	5 (11)	13 (9)
95% CI			5–15
Total transfusion response	79 (77)	33 (72)	112 (76)
95% CI			68–82
Time to response — wk			
Median	4.7	4.3	4.6
Range	1–34	1–49	1–49
Hemoglobin — g/dl			
Baseline†			
Median	7.7	8.0	7.8
Range	5.3–10.4	5.6–10.3	5.3–10.4
Response‡			
Median	13.4	13.5	13.4
Range	9.2–18.6	9.3–16.9	9.2–18.6
Increase			
Median	5.4	5.4	5.4
Range	2.2–11.4	1.1–9.1	1.1–11.4

* The daily dose was 10 mg.
† The baseline hemoglobin concentration was the minimum value during the baseline period.
‡ The response hemoglobin concentration was the maximum value during the transfusion-independent response period.

Table 3. Erythroid response to lenalidomide. Adapted from *List et al.* 2006(48). Reproduced with permission from (scientific reference citation), Copyright Massachusetts Medical Society.

The *TP53* mutation - Shifting the deletion of 5q from *good* to *very poor* prognosis

In MDS with del(5q) with good prognosis, the CDR is commonly situated in a distal region, 5q32-33(41, 42). In higher-risk MDS and AML with del(5q), the CDR is situated in a more proximal region 5q31(52). Patients often exhibit a larger deletion, including both regions, and a single nucleotide polymorphism (SNP)-array-based karyotype, which has led to the discovery that patients with a del(5q) involving a centromere and telomere had a more aggressive disease and that includes other additional chromosome aberrations. Patients with extended deletions had a poorer prognosis and associated with del(17p)(53, 54). It is well known that a del(17p) gives rise to a *TP53* mutation(21, 55). About 10% of MDS patients shows a complex karyotype (CK), often involving chromosome abnormalities of 5/5q, 7/7q and 17/17p (Figure 3)(19, 56, 57).

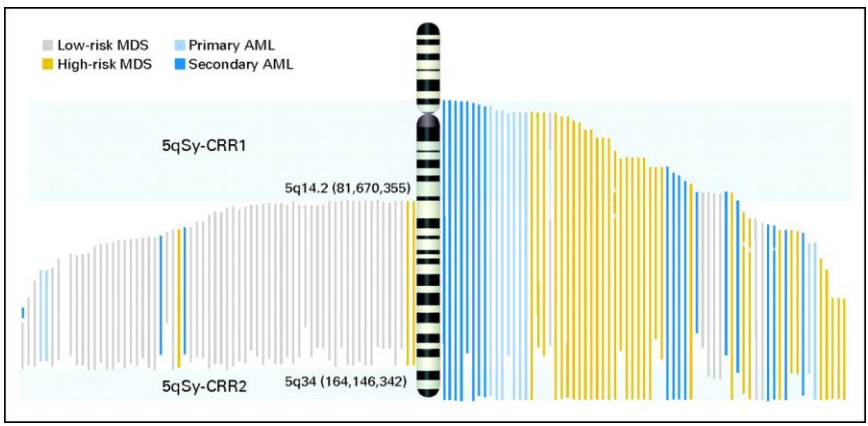


Figure 3. Commonly deletion region and association with different myeloid malignancies. Adapted from *Jerez et al.* 2012(53).

The use of lenalidomide treatment in MDS with del(5q) is a double-edged sword. A new or pre-existing *TP53* clone that has evolved under the pressure of lenalidomide therapy can lead to treatment failure, AML transformation, and a worse outcome(58-61). *TP53* mutations have been shown to have a worse outcome, irrespectively of CDR(54). Higher-risk MDS or AML patients with a CK demonstrates a high

frequency of *TP53* mutation(21), especially patients with the most common recurrent cytogenetic aberrations of deletion in 5q, 7q and 17p(57, 62, 63). A *TP53* mutation was observed in 19% of single del(5q) and 72% of patients with CK and monosomy 5 or del(5q)(64). In 2010, Jasek et al. study of higher-risk MDS and secondary AML patients with a CK including del(5q) and del(7q), noticed a strong association of copy neutral loss of heterozygosity (cnLOH) of 17p, leading to a hemi- or homozygous *TP53* mutations(63). In chromothripsis, a reshuffling of chromosomal material leads to a broad chromosomal rearrangement that is associated with *TP53* mutations in AML(65).

Patients with a CK have a poor prognosis, due to the association with *TP53* mutation(57). *TP53* mutated MDS patients presenting a multi-hit *TP53* lesion, involving a del(17p)(22%), cnLOH (21%) or multiple mutations(24%), have an independently poor prognosis, while those with a mono-allelic status have similar outcome to those with *TP53* wildtype (WT)(66). The poor prognosis among multi-hit *TP53* patients is irrespective of whether the disease is classified as MDS or AML(67), or a therapy-related disease(68).

Strong nuclear staining of p53 by immunohistochemistry is a useful tool as a marker of *TP53* mutation, and the presence of p53 among MDS patients indicates an increased risk of leukemic transformation(15).

AML and high-risk MDS –The same disease with different blast counts?

Is it important to differ higher-risk MDS and AML with 20-29% blasts or is it just a dead end, precluding patients from new treatment options? In 1999, the WHO revised its classification, lowering the threshold of blast percentages between high-risk MDS and AML from 30% to 20% blasts; moreover, refractory anemia with excess blasts in transformation (RAEB-T) no longer had any validity(69). The diagnosis of patients with borderline blast percentages is challenging; in morphologic diagnosis, a difference of 12% between referral and tertiary center was observed(70). Patients with 20-29% marrow blasts showed more similarities with RAEB than AML with >30% blast counts and patients were older, had a lower median white blood

count, an adverse karyotype and the same outcome(71, 72). In younger patients, treatment with intensive chemotherapy led to the same outcomes, despite blast percentages and in older patients, patients with 20-29% had the same outcome as those with <20% but better outcome than patients with AML >30% blasts(72).

Patients with higher-risk MDS and AML with 20-29% blasts share the same genetic profiles. These two patient groups present similar mutations in the genes regulating RNA splicing (*SF3B1, SRSF2, U2AF1* and *ZRSR2)*, chromatin (*ASXL1, BCOR, EZH2, MLL, PHF6* and *STAG2*), DNA methylation-related genes (*DNMT3A* and *TET2*), or transcription (*RUNX1*)(19, 20, 25, 26). Patients with MDS and a *TP53* mutation were not found to acquire additional mutations at disease progression(25). Grob *et al*. studied 2200 *TP53* mutated AML and MDS patients with excess blasts (MDS-EB) and reported a poor outcome, as expected, with no differences in outcome being observed among MDS or AML patients(67). Among both AML and MDS-EB patients, 84% had a CK, often including -5/5q. A single del(5q) was the only cytogenetic abnormality found to be significantly more frequent in MDS-EB versus AML (*P*=0.025)(67). *TP53* mutations were significantly more common in MDS and AML patients with a CK, regardless of blast counts, and was found to be associated with adverse outcome(68).

In the 2008 and 2016 WHO classifications of myeloid neoplasms and acute leukemia, the threshold of blast percentages remains at 20%(2, 10). In the ICC from 2022, Arber *et al*. concluded that there was a biological continuum of MDS and AML and suggested a new classification for patients (≥18 years) who have a blast percentage of 10-19% and whose status is defined as MDS/AML with myelodysplasia-related changes or MDS/AML with myelodysplasia-related cytogenetic abnormalities. The prior category of "AML with myelodysplasia-related changes" is replaced by "AML with myelodysplasia-related cytogenetic abnormalities" and "AML with myelodysplasia-related gene mutations" (*ASXL1, BCOR, EZH2, RUNX1, SFRB1, SRSF2, STAG2, USAF1* or *ZRSR2*). Patients with a *TP53* mutation are categorized as separate entity: MDS/AML and AML with mutated *TP53(3)*.

In the 5th edition of the WHO classification of myeloid neoplasms, also published 2022 by Khoury *et al*, the 20% threshold remaines, even though the boundary between MDS and AML has been softened(4); it is now argued that MDS in patients with increased blasts (MDS-IB2) of 10-19% in bone marrow or 5-19% in peripheral blood (no Auer rods) can be viewed as an AML equivalent, with the intention of making it possible to include these patients in clinical trials and treatment. In addition, a new category has been defined for MDS patients with a multi-hit *TP53,* which is now categorized as MDS with biallelic *TP53* inactivation.

MDS in the light of an aging population

MDS and AML are clonal hematopoietic diseases with increased incidence in an aging population(7). Clonal hematopoiesis (CH) may foreshadow myeloid neoplasms and may also rise in prevalence with increasing age (Figure 4)(73).

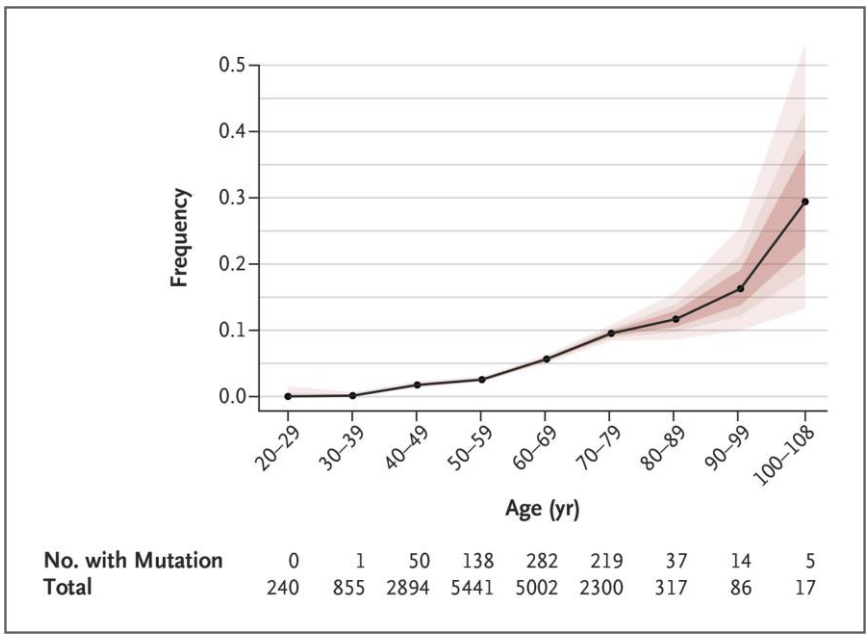


Figure 4. Prevalence of Somatic Mutations, According to Age. Adapted from *Jaiswal et al.* 2014(73). Reproduced with permission from (scientific reference citation), Copyright Massachusetts Medical Society.

The pre-malignant condition, clonal hematopoiesis of indeterminate potential (CHIP), is defined as the occurrence of a somatic mutation in a driver gene associated with a myeloid malignancy (VAF ≥2%) without signs of unexplained cytopenia or absence of myeloid neoplasm(3, 4, 74). However, clonality appears without hematological diseases in the natural aging population, and the majority will never develop MDS or AML. In a study of 12380 Swedes with no known hematological neoplasm, the whole-exome sequencing of DNA from peripheral blood was analyzed, and participants´ medical condition were followed for 2-7 years afterward(75). A clonal hematopoiesis was noticed in 10% of the study population >65 years of age, indicating an increased risk of expansion to a hematological malignancy (Figure 5). A clonal hematopoiesis may also indicate an increased risk of non-hematological diseases, such as cardiovascular diseases(76). Under the pressure of chemotherapy, the existence of a small hematopoietic clone, such as a small *TP53* mutation, may imply an increased risk of developing therapy-resistant MDS or AML(77); in turn, this may indicate the role of extrinsic factors in accelerating the clonal evolution. Different risk scores for CHIP evaluation are published but it still remains a lot of research in this field(78).

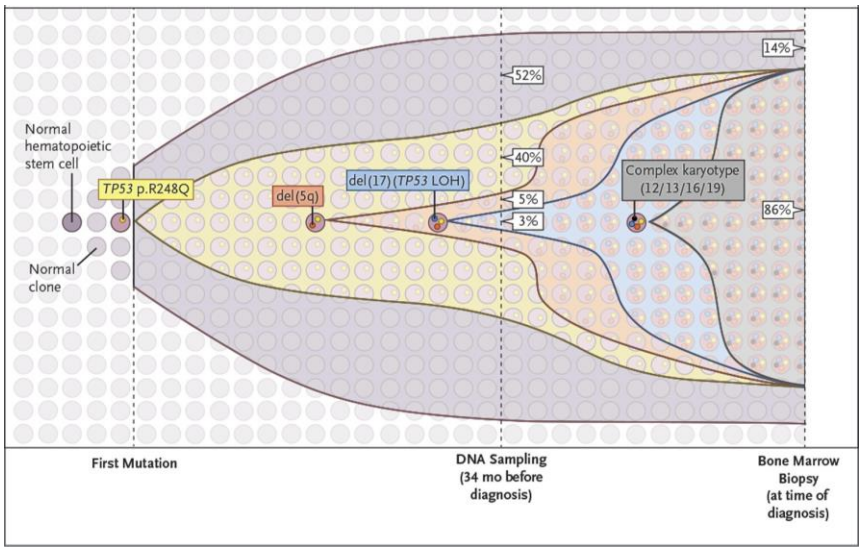


Figure 5. Progression from clonal hematopoiesis to a myeloid malignancy. Adapted from *Genovese et al.* 2014(75). Reproduced with permission from (scientific reference citation), Copyright Massachusetts Medical Society.

Treatment

Erythropoiesis-stimulating agents

Erythropoiesis-stimulating agents (ESAs) are a first-line treatment for anemia in lower-risk (low, INT-1) MDS patients. ESA treatment leads to a significant reduction in RBC transfusions compared with a placebo(79) and is recommended in the Nordic myelodysplastic guidelines and national guidelines(80, 81). Patients with a transfusion need of >2 units/months and s-erythropoietin (S-EPO) over 100-500 U/L have a decreased probability of response to ESA treatment (82-84). RAEB-2 patients have a lower response rate to ESA treatment(83). A large prospective register-based study of the use of ESA treatment for 1696 patients from 17 different countries in European Union with lower-risk MDS (EUMDS) showed a significant prolonged time to first transfusion in patients receiving ESA before or early after transfusion treatment(85). An updated analysis of 2448 patients in the EUMDS registry showed improvement in patients' OS when exposed to ESA treatment(86). The addition of granulocyte-colony stimulating factor (G-CSF) to ESA treatment may improve erythroid response but does not increase the risk of leukemic transformation(87).

Luspatercept

In a randomized, double-blind, placebo-controlled, phase III study of lower-risk (very-low, low, INT according to IPSS-R) MDS with ring sideroblasts (MDS-RS) (n=229), RBC-transfusion dependent patients with a lost response to ESA treatment or ESA treatment naive patients with an S-EPO ≥200 U/L, were treated with luspatercept. Luspatercept is a selective binding recombinant fusions protein that attaches to transforming growth factor β superfamily ligand and decreases the negative effect of the SMAD2-SMAD3 pathway on erythropoiesis. The study showed a modest response of transfusion independence ≥8 weeks in 38% of the lustpatercept arm versus 18% of the placebo arm(88). Although the drug may reduce transfusion dependency, it is too expensive for use in Sweden, according to the New Therapies Council.

Immunosuppressive therapy

One treatment alternative of lower-risk MDS, especially in younger patients with a hypocellular bone marrow without high-risk genetics, is the use of immunosuppressive therapies. An example is treatment with anti-thymoglobulin, whether on its own or in combinations, although it is often in combination with cyclosporin A. Immunosuppressive therapy may reduce the RBC transfusion burden in one-third of treated patients(89, 90).

Chemotherapy

A complete response (CR) after AML-like intensive chemotherapies is achieved in between 40-60% of patients with higher-risk MDS or AML with 20-30% blasts. However, CR is less common in patients with a high-risk karyotype, as the hematological toxicity is high with a prolonged hypoplasia, their response duration is short, and few patients obtain any benefit from consolidation treatment. Intensive chemotherapy is not recommended in elderly MDS patients, although induction therapy treatment may be used for young patients with a low-risk karyotype with the possibility of an allogenic hematopoietic stem-cell transplantation (allo-HSCT) donor(91-93).

A low dose of melphalan (2mg/d) is well tolerated and may lead to a modest response (CR of ~30%) in patients with higher-risk MDS or AML with a multilineage dysplasia, with increased benefit observed in patients with hypo- or normocellular bone marrow and a normal karyotype(94, 95).

Hypomethylating agents

In a randomized phase III trial of 233 higher-risk MDS patients, treatment with a low dose of the DNA hypomethylating agent (HMA) decitabine (5-aza-2-deoxycitabine) was compared to best supportive care (BSC); significantly prolonged progression-free survival was observed among patients treated with decitabine, but no significant change to OS was observed(96). In another randomized controlled study of 191 high-risk MDS patients, azacitidine (5-azacitidine) (AZA), also an HMA agent, was compared to BSC. The AZA treatment resulted in a

significant reduction in leukemic transformation or in death(97). In the randomized phase III study (AZA-001 trial), higher-risk MDS patients and AML patients with 20-30% blasts were treated with AZA (75 mg/m^2/day for 7 days every 28 days for a minimum 6 cycles, sc) or conventional treatments (BSC, low-dose cytarabine (ara-C) or intensive chemotherapy (IC)). AZA prolonged OS by 9 months, 24.5 months versus 15 months(98). The overall response rate (ORR) was significantly higher for AZA compared to BSC or ara-C, but not compared with IC; however, the number of IC-treated patients in the subgroup were low (n=42). In a follow-up study of AZA-001, 91% of responding patients reached a response within 6 cycles ((CR), partial remission (PR) or hematologic improvement (HI)). Among the responders, continuous AZA treatment (median 14 cycles) improved the response category (HI to PR or CR) in 48% of patients(99). Patients with a response to AZA treatment (HI, PR or CR) experienced a reduced risk of death compared with patients responding to conventional care regimes(100). In a "real-world" study of AZA treatment among 1101 patients with higher-risk MDS or AML (>20-30% blasts) (Figure 6), patients were treated with AZA for 7 consecutive days, 6 consecutive days or 5-2-2 (5 days, followed by a 2-day weekend break, followed by AZA for 2 days). The median OS was 11.5 months, which was lower than that in the AZA-001 trial, but no differences were observed in response due to dosing schedule(101).

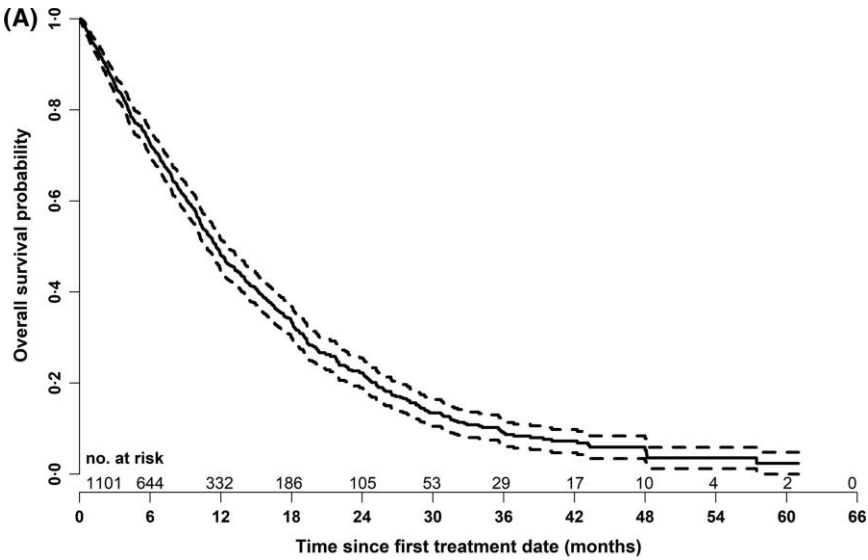


Figure 6. Median OS in a "real-world" study of AZA. Adapted from *Mozessohn et al.* 2018(101).

Lenalidomide in higher-risk MDS and AML treatment

The treatment arsenal of higher-risk myeloid diseases is scanty, making it necessary to find new treatment options. Scientists have hypothesized whether lenalidomide (LEN) could be an effective treatment of high-risk MDS with del(5q), as it is in lower-risk MDS.

In a phase II study of higher-risk MDS patients (n=42) (INT-2, high-risk and RAEB-T) with del(5q) (single del(5q) (19%), +1 additional aberration (23%) or >1 additional aberrations (58%)) treated with LEN (initial dose of 10 mg/day, 21 of 28 days cycle interval), 27% of patients exhibited HI. None of the patients with del(5q) and a CK reached CR(102). The NMDSG phase II study of higher-risk MDS and secondary AML patients with chromosome 5 abnormalities treated with LEN included 28 patients between 2007-2009 who were treated with LEN, with a maximum dose of 30 mg/day. A response was achieved in 35% of patients, but *TP53*-mutated patients responded to a lesser extent, a response of 15% was observed(103).

Chen et al, performed another phase II study of high-risk MDS and refractory/relapsed AML patients (n=27) with del(5q) (89% with a CK). Patients were treated with LEN (5-25 mg/day, 21 of 28 days) until disease progression/unacceptable adverse events (AE). Two AML patients had a CR, with or without platelet recovery, and two MDS patients had a stable disease (SD), but no patients with CK responded(104). In another phase II study of LEN treatment in patients with higher-risk MDS or AML (20-29% blats) refractory to HMA treatment, 24 patients were initially treated with a high dose of LEN (50 mg/d, 28 days for 2 cycles). Patients with a response (CR, PR or HI) proceeded for a total of 12 cycles, and 33% had a marrow CR; however, AE where common, and 50% of the patients had a significant infection(105). In a similar phase II study of relapsed/refractory patients (n=27) with higher-risk MDS and AML with trilineage dysplasia, patients were treated with LEN (15-50 mg/day, days 1-28 for a cycle length of 42 days). No patients had an isolated del(5q), 41% had a CK and two patients had a del(5q) as a part of a CK. The study was terminated due to lack of response among patients treated with 15 mg LEN and high grade of toxicity among patients treated with 50 mg LEN(106).

HMA is the first-line treatment for higher-risk MDS; and in Europe, it is the only licensed therapy for this MDS category. Many phase II studies have combined AZA with other therapies, demonstrating a better response compared with historical data of using AZA alone. In a single-center, phase II study, higher-risk MDS and AML patients (n=35) were treated with a combination of AZA (75 mg/m^2/ day for 7 days in a 42-day cycle, median 2 cycles) plus LEN (50 mg/day on day 8-28 in each cycle). The patients had previously been treated with immunomodulating agents or HMA. The ORR was 25% among the evaluable patients. The combination was well tolerated, and the median OS for responders versus non-responders was 9.8 and 4.0 months, respectively (HR=0.36, *P*=0.016)(107). In a multi-center phase II study of LEN and AZA in combination, 32 patients with higher-risk MDS were treated with AZA (75 mg/m^2/ day for 5 days with a 28-day cycle length) and LEN (10 mg/day, on day 1-21). At inclusion, two patients (6%) had a del(5q). Patients were treated with a median of 5 cycles. The ORR was 72% (CR (42%) and HI (28%)), and median

OS was 13.6 months for the entire cohort, and 37+ months for patients receiving a CR(108). In a multi-center phase II study, 28 patients with higher-risk MDS were treated with AZA (75 mg/m^2/day for 5 of 28 days) and LEN (10 mg/day, on days 6-21 of 28 days) induction therapy, followed by 6 months of consolidation with AZA (75 mg/m^2/day for 5 of 28 days). Consolidation was then followed by 12 months with maintenance LEN (10 mg/day, 1-21 days). Two patients had del(5q) at inclusion. The ORR was 72% (CR (24%), marrow CR (12%) and HI (36%)), and the median OS was 12 months. Thirteen patients completed induction therapy, and two patients completed maintenance treatment(109).

In a multi-center phase II/III North American Intergroup Study (SWOG S117), 277 higher-risk MDS patients were treated with AZA alone (75 mg/m^2/day for 7 or 5-2-2 days of a 28-day cycle) or in combination with either LEN (10 mg/day for 1-21 of 28 days) or vorinostat (VOR) (300 mg x 2/day on days 3-9). The ORR was 38% for AZA, 49% for AZA + LEN (P=0.14) and 27% for AZA + VOR (P=0.16). The median OS was 17 months for the entire cohort, with no significant differences between treatment arms, at 15 months for AZA, 19 months for AZA+LEN, and 17 months for AZA + VOR (Figure 7). Interestingly, regardless of treatment arms, patients with chromosome 5 aberrations had a better ORR (odds ratio, 2.17; P=0.008) than those without, although the OS was worse for patients with a CK including chromosome 5, -7 and 17p. A total of 113 patients had available mutational data, the median number of mutations was two (range, 0-7), and 22 patients had a *TP53* mutation, with a worse response duration (P=0.003) and OS(110).

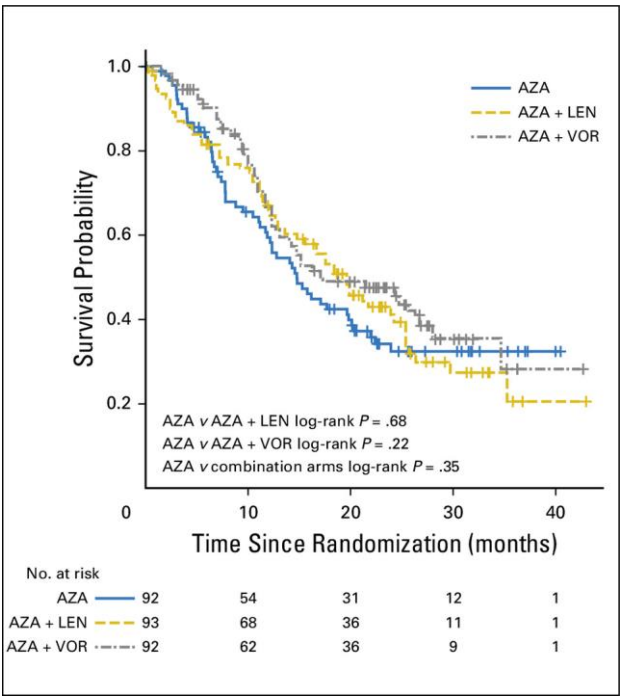


Figure 7. Median OS among patients in randomized phase III study of AZA, AZA + LEN or AZA + VOR. Adapted from *Sekeres et al.* 2017(110).

In a "pick-a-winner" multi-center, randomized, phase II study of 322 patients with higher-risk MDS, CMML or AML (20-30% blasts), patients were treated with AZA alone (75 mg/m^2/day for 7 of 28 days) or in combination of either LEN (10 mg/day, day 1-14), valproic acid (VPA) (50 mg/kg/day on days 1-7 each cycle, 35mg/kg/day in patients >60 years), or idarubicin (IDA) (10 mg/m^2, day 1 of each cycles 1-9) (Figure 8). The patients received a median of 7 cycles of treatment. After 6 cycles of treatment, the ORR was 40% (CR, PR or HI), with no significant difference between the treatment arms, at 42% for AZA, 39% for AZA + LEN, 41% for AZA + VPA, and 38% for AZA+IDA. Median OS for the entire cohort was 19.7 months, with no significant difference in OS between arms (Figure 8). Patients in the combination arms of AZA + LEN and AZA + IDA were hospitalized at a greater extent (P=0.038)(111).

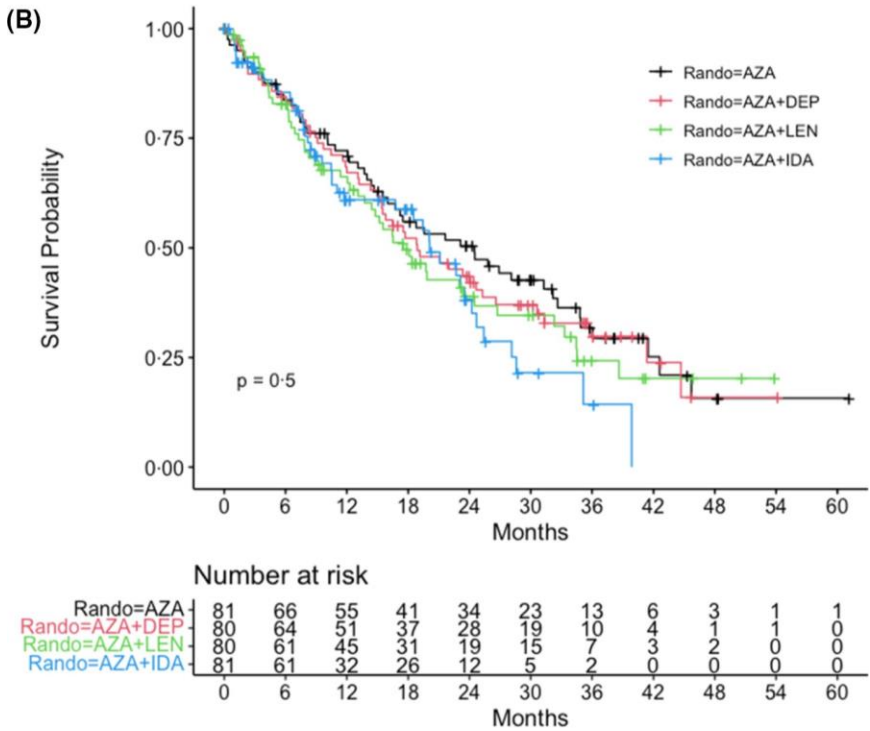


Figure 8. Median OS from a randomized phase III study of AZA, AZA + LEN, AZA + VPA or AZA + IDA. Adapted from *Ades et al.* 2022(111).

Future therapies

The increasing knowledge regarding MDS diagnostics have led to more individualized diagnostics and deeper insight into pathophysiological processes. Is it possible to find new targeted drugs to optimize higher-risk MDS therapy? With the deeper understanding of MDS as a clonal disease, the borderline between MDS and AML is now less distinct, introducing a discussion on whether it is possible to include MDS patients in AML studies or treat patients with 10-30% blast counts with AML regimes(3, 4). This could be a way forward, although a typical MDS patient is older and may experience more side effects from standard therapies.

Venetoclax (VEN) is an antiapoptotic protein B-cell leukemia/lymphoma-2 (BCL-2) inhibitor that targets the increased BCL-2 activity in the malignant myeloid clone inducing apoptosis(112, 113). The combination of AZA (75 mg/m^2/day, 1-7 days, 28-day cycle, sc or iv) and VEN (400 mg, day 1-28, po) led to a higher CR in comparison with treatment with AZA alone (37% vs. 18%), as well as a longer OS (14.7 vs. 9.6 months). The AZA + VEN combination has been approved for treatment of older AML patients(114). The results from a combined phase 1b/III study of 127 AML patients with poor-risk cytogenetics, with or without a *TP53* mutation, who were treated with AZA alone or with AZA + VEN, showed an improved CR rate in the AZA + VEN arm versus the AZA arm (41% vs. 17%). However, remission was short-lasting (6.5 vs. 6.7 months), and the median OS was low (5.2 vs. 4.9 months)(115). In a newly published phase 1b study of safety and efficacy, 107 higher-risk untreated MDS patients were treated with AZA (75 mg/m^2/day, for 7 or 5-2-2 days of a 28-day cycle) in combination with VEN (400 mg, 1-14 days every 28 days, po), with treatment being continued as long as it was tolerated or had any benefit. The combination was well tolerated, with Grade 3 or 4 treatment-emergent AE of neutropenia and thrombocytopenia in 49% and 45%, respectively, and with a CR of 30% and a marrow CR of 50.5%(116). Twenty patients (24%) in the study had a *TP53* mutation, with a CR in five patients (25%).

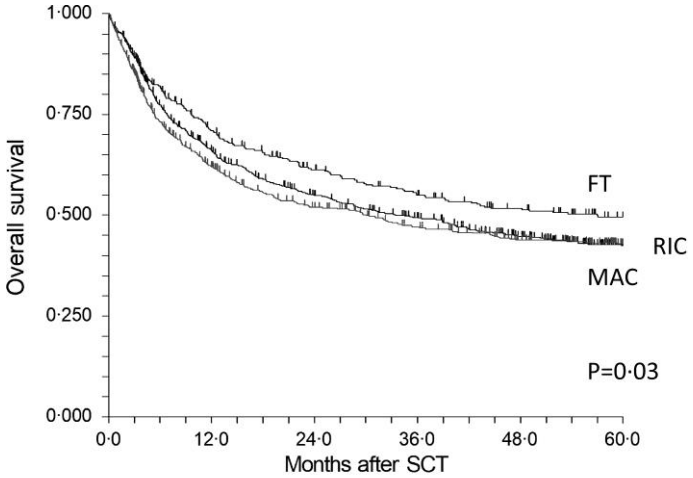
Eprenetapopt (APR-246) acts by reinstating the dysfunctional transcriptional p53 activity in order to induce p53 activity to a function more like that of WT(117). In a phase 1b/II study of 55 MDS, MDS/MPN or AML patients with 20-30% blasts and a *TP53* mutation, patients were treated with eprenetapopt and AZA. The treatment was well-tolerated, with a CR among MDS and AML patients of 50% and 36%, respectively(118). In a French phase II single-arm study of 52 MDS and AML patients with a *TP53* mutation treated with eprenetapopt with AZA, the study reported a CR of 47% among MDS and AML patients with 20-30% blasts(119). In both studies, a significant reduction or disappearance of *TP53* variant allele frequency (VAF) was observed in responding patients, 38% and 73%, respectively. Sadly, in a randomized phase III study of *TP53* mutated patients (n=154) the results of the outcome was negative and the trial could

not show any significant higher CR with the combination of eprenetapot + AZA compared to single AZA, 35% vs. 22% (NCT03745716)(120).

The anti-CD47 monoclonal antibody magrolimab acts as a cancer drug by inhibiting cancer cells overexpressed antiphagocytic signaling. In a phase 1b study of higher-risk, previously untreated MDS patients (*n*=95) (62% with poor-risk karyotype and a *TP53* mutation in about ~1/4 of patients), the patients were treated with a combination of AZA and magrolimab. The treatment was well-tolerated, with a CR of 33% for the whole cohort and a CR of 40% among the *TP53* mutated patients(121). In a follow-up randomized phase III study of *TP53* mutated, untreated AML patients, the patients were treated with either AZA + VEN versus AZA + magrolimab or 7+3 induction therapy versus AZA + magrolimab. At interim analysis, the primary endpoint of the median OS was lower in AZA + magrolimab groups compared with control groups, and the study was terminated early(122).

Allogeneic hematopoietic stem-cell transplantation

Allo-HSCT is the only curative treatment for eligible MDS patients (Figure 9), with a 5-year OS for approximately 40% of patients (Figure 9)(123).



At risk						
RIC	687	411	291	270	217	148
MAC	668	370	287	244	204	162
FT	367	247	199	174	153	130

Figure 9. Overall survival after allo-HSCT by conditioning intensity, MAC, myeloablative conditioning; RIC, reduced-intensity conditioning; FT, fludarabine and treosulfan. Adapted from *Shimoni et al.* 2021(123).

The vast majority of MDS patients are older, and it is important to take a patient´s biological age and comorbidity into consideration in order to optimize long-term survival and non-relapse mortality after allo-HSCT(124). A monosomal karyotype and a high or very high IPSS-R category worsen the OS and increase the risk of relapse(125). If a human leukocyte antigen-matched donor is available, allo-HSCT could be an option in the treatment of MDS patients with higher-risk disease(126). In lower-risk MDS, allo-HSCT may be taken in consideration for fit patients with an extensive transfusion burden, severe cytopenia, high-risk genetics, and no response to non-transplantation therapies(127, 128). It is debated whether patients could benefit from pre-transplant treatment, and, if so, what kind of treatment to use. A common recommendation is to do an upfront allo-HSCT in cases with <10% bone marrow blasts(127). If cytoreductive treatment is indicated as bridging therapy, it is important to take the patient's age

into consideration in order to reduce side-effects prior to transplant. With the aim of reducing pre-transplant toxicity, HMA is used as an alternative to intensive cytoreductive treatment, as it has less toxicity and yields the same outcome after transplantation(129, 130). A reduced-toxicity regime with fludarabine/treosulfan can be an interesting treatment option in MDS and is associated with low relapse rate and improved OS compared with myoablative conditioning or reduced-intensity regimes(123). By optimizing which patients to treat and what conditioning regimes to use, the 3-year OS can be increase to 67% in selected cases(131).

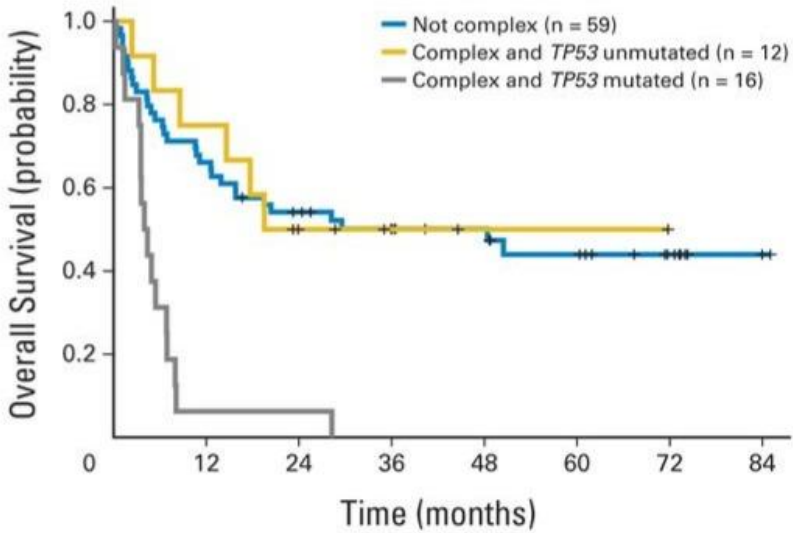


Figure 10. Overall survival among *TP53* mutated patients with complex karyotype compared to *TP53* mutated patients with noncomplex karyotype, treated with allo-HSCT. Adapted from *Bejar et al.* 2014(132).

Is allo-HSCT a therapy alternative even in *TP53*-mutated patients? A *TP53* mutation is associated with an unfavorable outcome after allo-HSCT(132, 133); when this is combined with CK, the outcome is even poorer regarding OS and earlier relapse (Figure 10). In the literatude, >80% of the patients with both a *TP53* mutation and CK died within 2 years; of these, 60% of deaths were due to relapse(132, 134). In a study on the size of the mutational clone among AZA-treated

patients, those patients with a multi-hit *TP53* mutation responded to HMA treatment, but the response was short. A subset of 13 patients with multi-hit *TP53* mutation underwent allo-HSCT, with a median OS of 19 months. Six patients were still alive after 2-years, including all patients (*n*=4) in which the *TP53* clone size decreased with a VAF <10%(135). However, Versluis et al. could not confirm this observation. The 3-year OS was similar among patients (*n*=17) with *TP53* VAF <2% pre-HSCT versus patients (*n*=18) with VAF ≥2% pre-HSCT. The study showed a better OS in *TP53*-mutated patients treated with allo-HSCT compared with those without allo-HSCT (3-years OS 23% ± 7% vs. 11% ± 7%, *P*=0.04), but the benefit of allo-HSCT treatment in *TP53*-mutated patient was modest(136).

In a prospective observational study, Tobiasson el al. investigated the usege of monitoring patient-specific mutations of measurable residual disease (MRD) by NGS and the digital droplet polymerase chain reaction (ddPCR) technique before allo-HSCT and following MRD post-transplant. A total of 266 patients were included; of these, it was possible to follow MRD in 221 patients. The estimated 3-years OS was 64% for the whole cohort; among the patients with MRD positivity at relapse (*n*=42), the MRD was positive at a median of 71 (range, 23-283) days before a clinical relapse. In a multivariate analysis, MRD positivity alone was associated with shorter OS and was found to lead to an early detection of relapse(137). The use of an individualized MRD may lead to an earlier detection of relapse, optimizing post-HSCT treatment and, by the extension, improving OS.

Aims of the thesis

Paper I

To study the efficacy and safety of azacitidine with or without the addition of lenalidomide in high-risk MDS (IPSS INT-2 or high) and AML with multilineage dysplasia and 20-30% marrow blasts with a karyotype including del(5q).

To study the biological effects of treatment on the malignant clone.

Paper II

To study the influence of cytogenetics on the treatment response in the study cohort from the clinical trial.

To study if specific cytogenetic findings can predict patient outcome.

Paper III

To optimize diagnostic procedures and follow-up assessment with cytomorphology, bone marrow trephine biopsy and immunohistochemistry in high-risk MDS and AML with 20-30% marrow blasts with a karyotype including del(5q).

Patients and methods

Data sources

Paper I was a prospective, multi-center, open-label, randomized phase II study of patients with higher-risk MDS (IPSS INT-2 and high) and AML with multilineage dysplasia and 20-29% marrow blasts (former RAEB-T) with a karyotype including del(5q). Refractory and relapsed patient could be included if the fulfilled the inclusion criteria. Patients ≥18 years of age were included. Women of childbearing potential must have had a negative pregnancy test prior starting LEN, using adequate contraceptive methods during LEN treatment. Males had to use barrier contraceptive with women of childbearing potential while on LEN treatment and 28 days after the last dose of LEN. Patient had to signed informed consent. Patients exclusion criteria were; eligible for upfront allo-HSCT without prior induction chemotherapy or AZA, pregnancy or lactating females, prior therapy with > 1 cycle of AZA, prior therapy with LEN, expected survival less than 2 months, acute promyelocytic leukemia, central nervous system leukemia, serum creatinine >2.0 mg/dL, serum aminotransferase >3.0 x upper limit of normal or serum total bilirubin >1.5 mg/dL, prior allergic reaction to thalidomide or uncontrolled systemic infection. Prior therapy with AZA was not allowed.

During the first 23 months of the study, five of 32 patients, who were subject to screening, increased their blast counts to ≥30% between the local and the subsequent screening bone marrow sample and were thus not eligible for the study. This was a problem since primary hospitals were invited to identify these rare patients and refer them to a study center. We experienced that the study cohort had a more progressive nature than higher-risk MDS patients in general. An amendment was therefore approved, allowing one cycle of AZA between the diagnostic and the screening bone marrow sample. Prior treatment with one cycle of AZA was allowed after contact with study center, in those patients where the physician determined that the patient needed treatment before randomization process was completed. This AZA treatment cycle was not counted as one of the six cycles in the study protocol.

Eligible patients from Sweden, Denmark, Norway and Finland were included in the study. The estimated inclusion rate, for the Nordic countries, was approximately 10 patients/year. The initial plan to increase inclusion rate, was to include a patient cohort from hematological center in Leeds, Great Britain. However, it was not doable, due to the higher cost per patient of inclusion in Great Britain. The reason was caused by a higher cost for National Pharmaceutical Insurance compared to the Nordic countries.

In the end, 22 centers, from different university- or county hospitals in Sweden, Denmark, Norway and Finland, participated in the study. University hospitals are normally those centers involved in treatment of patients with higher-risk myeloid diseases. This was a factor to increase the probability of finding eligible patients for the study. Ninety-one patients from 13 different centers in the Nordic countries, were assessed eligible for the study and were screened during March 2012 and January 2017. Seventy-two patients were included in the study and 19 patients were excluded due to high blast counts (≥30%) in eight patients, no detectable del(5q) in six patients, centrally revised IPSS score INT-1 in two patients, clinical judgement due to rapid disease progression in two patients and one patient withdrew consent.

The study-cohort in **Paper II** and **Paper III** consists of the same 72 patients that were included in **Paper I**.

Methodological considerations

The hypomethylating agent AZA is first-line treatment in higher-risk MDS, and in Europe the only licensed therapy for this MDS category. LEN is an effective therapy for lower-risk MDS with del(5q) with cytogenetic remission induced in about 45% of patients(48). LEN selectively induces apoptosis of del(5q) MDS cells through ubiquitination of *CSNK1A1*, located on chromosome 5(49, 50). Del(5q) is an early genetic event in patients with combinations of other chromosomal aberrations(46). Between 2007 and 2009 NMDSG performed a phase II study treating 28 patients with higher-risk MDS and secondary AML with chromosome 5 abnormalities with LEN as monotherapy, showing therapeutic response in 35% of patients(103). The rational of

the study was to investigate if the combination of drugs, with different mechanisms of action, could improve the outcome of del(5q) myeloid malignancies.

Patients in **Paper I** were centrally randomized in blocks, generated with the use of www.randomization.com in a 1:1 ratio, to AZA or AZA + LEN.

Patients were treated with AZA 5-2-2 (75mg/m^2/day, *sc*, for 5 days, followed by a 2-day weekend break, followed by 2 days of treatment, 28-day cycle length, for 6 cycles). A prolongation of cycle interval, to a maximum of 8 weeks, was allowed due to hematologic toxicity (neutropenia and/or thrombocytopenia) according to predefine criteria. A bone marrow examination was done in cases with persistent cytopenia after 6 weeks, to differentiate bone marrow hypoplasia due to treatment from disease progression.

Two dose levels of LEN were used in the study. The initial dose of LEN was 10 mg, po, day 1-21 in each cycle. The dose was escalated to 25 mg, in cycle 4, if no toxicity occurred leading to a prolonged AZA cycle interval for more than 5 weeks. Patients were treated for a total of 24 weeks + additional weeks caused by prolonged cycle intervals. Dose modification of LEN was performed due to hematological toxicity (neutropenia and/or thrombocytopenia). The dose of LEN was interrupted if toxicity occurred during day 1-21, and if a recovery occurred before day 21 the dose of LEN was resumed to the same dose level if the patient was on 10 mg or lowered to 10 mg if the patient was on 25 mg. At resumption of LEN treatment during day 1-21, the patient proceeded to day 21 without addition of extra days of LEN treatment. If no recovery occurred the cycle interval of AZA was prolonged to a maximum of 8 weeks. A bone marrow sample was taken in cases with persistent cytopenia after 6 weeks.

Celgene Corporation provided LEN capsules during the treatment. The study drug was sent to a central pharmacy, responsible for the distribution of LEN to the study-centers. LEN was supplied in individual and labeled bottles containing 21 days of dosing. LEN was stored at the study center in a locked room at room temperature.

Unused medication was returned to the pharmacy for destruction. AZA was supplied as a commercial drug.

Peripheral blood samples for effect evaluation of Hb, white blood cell count, a differential, ANC and PLT, were taken weekly. The number of blood product transfusions were registered.

Responding patients, eligible for allo-HSCT treatment, could leave the study after cycle 3, 4 or 5 and in that case be subject for final assessment.

It was allowed to use G-CSF, in doses according to investigators judgement, to patients with neutropenia and an infection or if ANC $<0.5 \times 10^9$/L, this to keep ANC $\geq 0.5 \times 10^9$/L. Prophylactic medication were used according to local routines. ESA treatment was not allowed during the study. Treatment with steroids was not allowed during the study, except for treatment of inflammatory disorder with prednisolone ≤ 25 mg, po. All concomitant medication was recorded.

The primary endpoint was response according to International Working Group (IWG) criteria, 2006, for MDS (Table 4 and Table 5)(138). Response evaluation was performed during the first week after six cycles of AZA or AZA + LEN, or at end of treatment if it occurred at an earlier time point. Patients were followed once yearly from start of treatment for 3 years, follow-up included survival, AML transformation, MDS specific treatment and secondary primary malignancies. Response was assessed by two independent observers.

Secondary endpoints in **paper I** were safety, AZA cycle intervals between groups, mutational status, relapse and survival from time of randomization to death. Responding patient were allowed to continue with AZA after cycle six, according to standard practice.

Hematologic improvement*	Response criteria (responses must last at least 8 wk)†
Erythroid response (pretreatment, < 11 g/dL)	Hgb increase by ≥ 1.5 g/dL Relevant reduction of units of RBC transfusions by an absolute number of at least 4 RBC transfusions/8 wk compared with the pretreatment transfusion number in the previous 8 wk. Only RBC transfusions given for a Hgb of ≤ 9.0 g/dL pretreatment will count in the RBC transfusion response evaluation†
Platelet response (pretreatment, < 100×10^9/L)	Absolute increase of ≥ 30×10^9/L for patients starting with > 20×10^9/L platelets Increase from < 20×10^9/L to > 20×10^9/L and by at least 100%†
Neutrophil response (pretreatment, < 1.0×10^9/L)	At least 100% increase and an absolute increase > 0.5×10^9/L†
Progression or relapse after HI‡	At least 1 of the following: At least 50% decrement from maximum response levels in granulocytes or platelets Reduction in Hgb by ≥ 1.5 g/dL Transfusion dependence

Deletions to the IWG response criteria are not shown.
To convert hemoglobin levels from grams per deciliter to grams per liter, multiply grams per deciliter by 10.
Hgb indicates hemoglobin; RBC: red blood cell; HI: hematologic improvement.
*Pretreatment counts averages of at least 2 measurements (not influenced by transfusions) ≥ 1 week apart (modification).
†Modification to IWG response criteria.
‡In the absence of another explanation, such as acute infection, repeated courses of chemotherapy (modification), gastrointestinal bleeding, hemolysis, and so forth. It is recommended that the 2 kinds of erythroid and platelet responses be reported overall as well as by the individual response pattern.

Table 4. Hematologic improvement according to IWG 2006 for MDS. Adapted from *Cheson et al*. 2006(138).

Category	Response criteria (responses must last at least 4 wk)
Complete remission	Bone marrow: ≤ 5% myeloblasts with normal maturation of all cell lines*
	Persistent dysplasia will be noted*†
	Peripheral blood‡
	Hgb ≥ 11 g/dL
	Platelets ≥ 100 × 10^9/L
	Neutrophils ≥ 1.0 × 10^9/L†
	Blasts 0%
Partial remission	All CR criteria if abnormal before treatment except:
	Bone marrow blasts decreased by ≥ 50% over pretreatment but still > 5%
	Cellularity and morphology not relevant
Marrow CR†	Bone marrow: ≤ 5% myeloblasts and decrease by ≥ 50% over pretreatment†
	Peripheral blood: if HI responses, they will be noted in addition to marrow CR†
Stable disease	Failure to achieve at least PR, but no evidence of progression for > 8 wks
Failure	Death during treatment or disease progression characterized by worsening of cytopenias, increase in percentage of bone marrow blasts, or progression to a more advanced MDS FAB subtype than pretreatment
Relapse after CR or PR	At least 1 of the following:
	Return to pretreatment bone marrow blast percentage
	Decrement of ≥ 50% from maximum remission/response levels in granulocytes or platelets
	Reduction in Hgb concentration by ≥ 1.5 g/dL or transfusion dependence
Cytogenetic response	Complete
	Disappearance of the chromosomal abnormality without appearance of new ones
	Partial
	At least 50% reduction of the chromosomal abnormality
Disease progression	For patients with:
	Less than 5% blasts: ≥ 50% increase in blasts to > 5% blasts
	5%-10% blasts: ≥ 50% increase to > 10% blasts
	10%-20% blasts: ≥ 50% increase to > 20% blasts
	20%-30% blasts: ≥ 50% increase to > 30% blasts
	Any of the following:
	At least 50% decrement from maximum remission/response in granulocytes or platelets
	Reduction in Hgb by ≥ 2 g/dL
	Transfusion dependence
Survival	Endpoints:
	Overall: death from any cause
	Event free: failure or death from any cause
	PFS: disease progression or death from MDS
	DFS: time to relapse
	Cause-specific death: death related to MDS

Deletions to IWG response criteria are not shown.

To convert hemoglobin from grams per deciliter to grams per liter, multiply grams per deciliter by 10.

MDS indicates myelodysplastic syndromes; Hgb, hemoglobin; CR, complete remission; HI, hematologic improvement; PR, partial remission; FAB, French-American-British; AML, acute myeloid leukemia; PFS, progression-free survival; DFS, disease-free survival.

*Dysplastic changes should consider the normal range of dysplastic changes (modification).[41]

†Modification to IWG response criteria.

‡In some circumstances, protocol therapy may require the initiation of further treatment (eg, consolidation, maintenance) before the 4-week period. Such patients can be included in the response category into which they fit at the time the therapy is started. Transient cytopenias during repeated chemotherapy courses should not be considered as interrupting durability of response, as long as they recover to the improved counts of the previous course.

Table 5. Response criteria according to IWG 2006 for MDS. Adapted from *Cheson et al*. 2006(138).

Cytogenetic analysis

Karyotyping

Karyotyping for **paper I, II** and **III** was performed at baseline and at final assessment at week 25. A volume of five ml bone marrow aspirate, in heparin flask were sent to department of Human Genetics, Hannover, starting the analyzing process before lunch the following day. In cases of dry tap the karyotyping was made on peripheral blood. Unstimulated short-term cultures (24-48h) were set up from bone marrow. Chromosome preparation and fluorescence R-banding were performed(139). Twenty metaphases were analyzed when possible and karyotypes were described according to the International System for Chromosome Nomenclature (ISN, 2016). Karyotype without FISH was sufficient to categorize the patient.

Patient with less than 10 metaphases and no clonal aberrations were excluded from the studies.

FISH Analysis

FISH analyses in **paper I, II** and **III** were performed at inclusion, after 3 cycles of treatment (week 13) and at final assessment using a dual color probe for the locus 5q31 (Vysis EGR1/5p14 FISH Probe Kit-Abbott, Weisbaden, Germany). At inclusion and final assessment, FISH was prepared on fixed cells prepared for classical banding analysis. In cases with dry tap, FISH analyses were made from bone marrow imprint. FISH at cycle 13 was performed on bone marrow slides. FISH was used to detect the del(5q), especially in cases with a complex karyotype, and as a quantitative method to analyze the clone size. For each sample 200 interphase nuclei were analyzed. The cutoff level for the probe used in the study was evaluated by analyzing 1000 interphase nuclei from 10 healthy donors. The cutoff level for fixed cells was set at 8% and for bone marrow slides 6%. At inclusion, an abnormal clone could be identified by less than 15 metaphases. During follow-up, less than 15 metaphases were adequate if a known clonal aberration was detected again. Less than 15 metaphases without aberrations during follow-up were not sufficient, unless FISH was available and del(5q) was identified in the major clone.

Multicolor FISH

Analysis with multicolor FISH (mFISH) was used to find cryptic aberrations in patients with uncertain cytogenetic aberrations. Metaphases for mFISH analysis were prepared from heparinized bone marrow aspirate. The analysis was carried out using 24XCyte mFISH (Metasystems, Altlussheim, Germany) and analysis was performed according to manufacturer´s instructions. The ISIS software was used for analysis (Metasystems, Atlslussheim, Germany). Whenever possible five metaphases were analyzed for each sample.

Telomere/Centromere FISH

For telomere/centromere FISH (T/C-FISH), metaphase preparations from heparinized bone marrow aspirates were made according to standard procedure. Whenever possible, 10 metaphases were examined from each sample after combined karyotyping and T/C-FISH analysis. Telomere PNA FISH Kit (Dako, Glostrup, Denmark) was used for analysis. The centromere probe of chromosome 2 was developed by Dako. For an area of 22x22mm we used 9μL telomere probe and 1 μL centromere probe(140). The ISIS-Telomere module (Metasystems, Altlussheim, Germany was used for analysis(141). The software calculates a T/C value for each individual chromosome arm and the mean value of metaphase was calculated.

For each patient in **paper II** a cohort of five healthy individuals (adapted) (age matched) as control for telomere length measurement.

Cytogenetic Response

In **paper I, II** and **III** cytogenetic response was evaluated with FISH, after three and six cycles and with karyotype after six cycles. A complete cytogenetic response (CCyR) was defined as disappearance of the del(5q) abnormality and any other chromosomal aberrations present at baseline. A partial cytogenetic response (PCyR) was defined as a 50% reduction (vs. baseline) in the number of aberrant cells. Persistent clones or reduction <50% defined a lack of cytogenetic response (NoCyR). Cytogenetic relapse was defined as the reappearance of aberrations or ≥50% increase in abnormal metaphases after achievement

of a PCyR. Cytogenetic progression was defined as the appearance of previously undetected aberrations in the sense of clonal evolution or development of new independent clones.

Bone marrow analysis

Bone marrow trephines and smears from bone marrow and peripheral blood, **in paper I, II** and **III,** were assessed centrally and blinded by an experienced hematopathologist at inclusion and final assessment at department of Clinical Pathology, Division of Hematopathology, Karolinska University Hospital, Solna, Stockholm. Bone marrow samples were classified according to WHO 2008 and re-classified, **in paper III**, according to the revised WHO 2016 classification(2, 10). The percentage of blasts was calculated in bone marrow smears and/or imprints and correlated to the percentage of blasts in biopsies by using CD34+ staining.

Bone marrow histology

Bone marrow cellularity and grade of marrow fibrosis were documented according to European consensus guidelines(142). IHC was performed according to manufacture guidelines including the monoclonal antibodies p53 DO-7, CD43, glucophorin A and CD61 (all Ventana/Roche) using the automated Ventana Bench Mark XT system. The number of CD34+ staining cells was assessed in randomly selected fields based on a total of at least 500 hematopoietic cells (excluding lymphocytes and lymphoid aggregates) at a magnification (40x objective). The presence of CD34+ clusters was documented separately, with a cluster being defined as a group of three or more positive cells as described(12). Sections stained with p53 were assessed for presence of cells with strong p53 nuclear staining.

Cytomorphology

Bone marrow smears and/or biopsy imprints stained with May-Grunewald-Giemsa were assessed for the percentage of blasts and ring sideroblasts and the type and degree of dysplasia. The percentage of blasts was calculated based on a 500-cell count of total bone marrow nucleated cells and a 200-leukocyte differential count in the

peripheral blood according to WHO 2016 guidelines(2). Morphological features used for the definition of myeloblasts were those proposed by International Working Group on Morphology of MDS(143).

Next generation sequencing

Mononuclear cells or CD34+ cells from bone marrow and peripheral blood were isolated by using Lymphoprep™ and genomic DNA was separated from these cells by using GeneElute DNA extraction kit (Sigma Aldrich). Forty-two different genes were analyzed, in Memorial Sloan Kettering Cancer Center, New York, at screening phase (*ASXL1*, *ATRX*, *BCL10*, *BCOR*, *BCORL1*, *BRAF*, *CALR*, *CDKN2A*, *CDKN2C*, *CEBPA*, *CSF1R*, *CSNK1A1*, *CTCF*, *DDX41*, *DDX54*, *DNMT3A*, *EGFR*, *EP300*, *EZH2*, *FLT3*, *GATA2*, *GNB1*, *IDH1*, *IDH2*, *JAK2*, *KMT2C*, *KRAS*, *LUC7L2*, *NF1*, *NRAS*, *PHIP*, *PPM1D*, *PTPN11*, *RAD50*, *ROBO1*, *RUNX1*, *SF3B1*, *SH2B3*, *SRSF2*, *TET2*, *TP53* and *U2AF1*). Gene mutations, at screening phase as well as *TP53* mutational status were analyzed after the end of study by deep targeted sequencing(66). VAF was determined in all samples with a sensitivity of ≥2%. Following failure of initial sequencing six patients were analyzed by TruSight™ Myeloid Sequencing panel. TruSight™ analyzed 54 different genes (*ABL1*, *ASXL1*, *ATRX*, *BCOR*, *BRORL1*, *BRAF*, *CALR*, *CBL*, *CBLB*, *CDKN2A*, *CEBPA*, *CBLC*, *CSF3R*, *CUX1*, *DNMT3A*, *ETV6*, *EZH2*, *FBXW7*, *FLT3*, *GATA1*, *GATA2*, *GNAS*, *HRAS*, *IDH1*, *IDH2*, *IKZF1*, *JAK2*, *JAK3*, *KDM6A*, *KIT*, *KMT2A*, *KRAS*, *MPL*, *MYD88*, *NPM1*, *NRAS*, *NOTCH1*, *PRGFRA*, *PTEN*, *PTPN11*, *PHF6*, *RAD21*, *RUNX1*, *SMC1A*, *SETBP1*, *SF3B1*, *SMC3*, *SRSF2*, *STAG2*, *TET2*, *TP53*, WT, *U2AF1* and *ZRSR2*). VAF was determined in all samples with a sensitivity of ≥5%. To evaluate the effect of treatment, cryopreserved separated MNC or CD34+ cells were analyzed by TruSight™ for analyze of the presence of residual disease.

Statistical analyses

The study in **Paper I** was designed to detect an improvement in efficacy of ≥20%, as defined as ≥20% of patients reaching the primary endpoint. We used the Simon Two-Stage Adaptive Design. With an unacceptable response probability p_0=0,30, with a α=0,05 and

power=0,90, the sample size was 35 for each group. In the case, the first stage consisted of 18 patients and if there was up to two responses the trial was terminated; otherwise continued to a total of 35 patients. This scenario implied two cohorts of 35 patients, 70 in total. The randomization process was not stratified by any patient characteristics.

In paper **I, II** and **III** were continuous data described by mean and median (range) values depending on the distribution data. X^2- or Fisher´s exact tests were used to measure the difference between responders and non-responders. Continuous variables regarding mutational status, cycle interval, response, clone size of FISH analyses and blast percentages was used univariate analysis by Mann-Whitney *U* test or T-test independent analysis. Comparison of differences in blast percentages and *TP53* VAF% changes were analyzed by paired *t*-test. McNemar´s test was used to compare binary data of the detection of del(5q) with classical banding analysis vs. FISH. Survival estimates were calculated by Kaplan-Meier method, and test for differences in survival were done using log-rank test. Survival was calculated from day of randomization to death, censoring date was the date patient was last known to be alive.

Ethical considerations

All three studies, in this thesis, were conducted in accordance with the Declaration of Helsinki(144). The studies were approved by National Ethical committees in Sweden, Denmark, Norway and Finland and were approved in Stockholm (2011/1109-31/1), with an amendment (2016/2321-32) regarding transfer and storage of coded biological material to a laboratory in New York, USA, for genetical analyses of bone marrow sampling and the agreement of destruction of the material after analysis and an amendment (2016/2404-32) of a new informed consent, regarding the shipment of biological material, was not deemed necessary, due to the risk of adding emotional stress among relatives to deceased patients in the study.

Paper I was registered at www.clinicaltrials.gov NTC01556477 and received an EudraCT number 2011-001639-21. Patients provided written informed consent and received the information of the participation in the study was completely voluntary and that it would not affect patient-physician relationship. All information related to the patients was handled confidential. The investigators were detailed informed and knew the characteristics of the drugs used in the clinical trial. The clinical study, **paper I**, was performed in accordance with the study protocol and in accordance with Good Clinical Practice, EU-directive (2001/20/EC) and regulatory requirements. All study-patients were covered by respectively countries National Pharmaceutical Insurance Pool. Each patient received a country specific study number and each study site kept a record with a key linking study number ant the patient id.

Biological material from **paper I-III** were stored with study number code at Stockholm biobank 914. Cytogenetic and FISH, **paper I-III**, were analyzed at Hannover Institute of Cell and Molecular Pathology and the material were stored with the study number code. Bone marrow material for morphological analysis, **paper I-III**, were performed at Department of Pathology at Karolinska University Solna and material related to the study were stored with a study specific number in the Karolinska University Hospital biobank (no 406).

Results and discussion

Paper I

Main findings

The median age of the 72 eligible patients was 71.5 years (range, 35-84 years). Fifty-four (75%) were diagnosed with MDS and 18 (25%) with AML. Eleven (15%) received one course of AZA before inclusion. Most of the patients (83%) had a complex karyotype, and 53 patients (76%) had a *TP53* mutation, whereof 49 (92%) were multi-hit. Thirty-six patients were randomized to each arm. Thirty-two patients (44%) terminated therapy prior to protocol plan; 15 patients (42%) in the AZA arm and 17 patients (47%) in the AZA + LEN arm (P=0.64). The reasons were encompassed disease progression in 10 patients (6 AZA, 4 AZA + LEN (P=0.50)), adverse events in 18 patients (7 AZA, 11 AZA + LEN (P=0.28)) and subject request in two patients in each arm (Figure 11). Seventeen patients with early termination died (6 AZA, 11 AZA + LEN (P=0.17)). The cause of death was disease progression in eight patients, four in each arm, infection in five patients, one in the AZA arm, four in the AZA + LEN arm (P=0.36), CNS hemorrhage in two cases, one in each arm and heart failure in two patients (P=0.49). The overall rate of infections did not differ between the arms, with one exception, eight patients (22%) in AZA + LEN arm and one patient in AZA arm had an adverse event grade 1 or 2 unspecified infection (P=0.028). Serious adverse events (SAE) were similar in the two treatment arms. One-hundred and eighty-eight SAE´s in 54 patients (75%) were reported. The most frequent criterion being hospitalization. Seventeen patients had ≥5 SAE´s, which accounted for 60% of total SAE´s. No suspected unexpected serious adverse reaction was observed. Six patients (8%) were withdrawn from study during the pretreatment period, three in each arm.

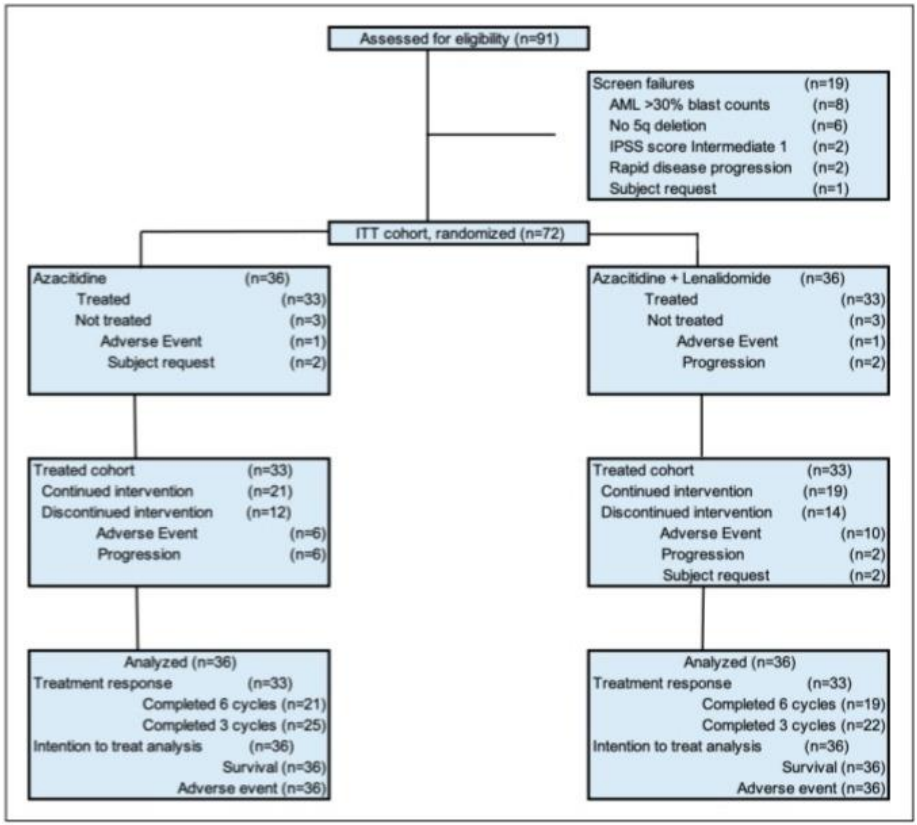


Figure 11. CONSORT diagram.

Treatment response was analyzed in intention to treat (ITT) cohort. Forty-seven of these 72 treated patients (65%) completed three cycles, and 40 patients (56%) completed all six cycles. The median length on treatment was 24 weeks in booth arms (P=0.87). The 4 weeks cycle interval was extended, due to the protocol, with additional time with a median of 1.5 weeks (range, 0-7) in the AZA group and 2.5 weeks (range, 0-10) in the AZA + LEN group (P=0.25). In the AZA + LEN arm, 7 out of 33 patients (21%) increased LEN dose to 25 mg/day.

ORR in the treated cohort was 39% for AZA and 44% for AZA + LEN arm (P=0.63) and CR was 17% and 28%, respectively (P=0.086) (Table 6). Four patients (11%) in both arms had a HI. There were no

significant differences in erythroid, neutrophil or platelet responses. Eleven patients received study treatment as bridge to allo-HSCT, six in the AZA arm and five in the AZA + LEN arm (P=0.74), with a median of 6.5 months from study enrollment to transplantation. Responding patients had a shorter pre-treatment disease duration than non-responders, 1.6 vs. 2.4 months (P=0.048). No other pre-treatment variables were significantly associated with ORR.

Variable, No. (%)	Total	AZA	AZA + LEN	AZA *vs*AZA + LEN
	n = 72	*n* = 36	*n* = 36	*P*
ORR	30 (42)	14 (39)	16 (44)	0.63
CR	6 (8)	4 (11)	2 (6)	0.67
Marrow CR	16 (22)	6 (17)	10 (28)	0.086
PR	0	0	0	1.0
HI	8 (11)	4 (11)	4 (11)	1.0
No response	42 (58)	22 (61)	20 (56)	0.63
Stable disease	8 (11)	5 (14)	3 (8)	0.71
Failure or treatment interrupted due to AE or subject request	34 (47)	17 (47)	17 (47)	1.0
Cytogenetic CR, final assessment	11 (15)	4 (11)	7 (19)	0.18
Cytogenetic PR, final assessment	2 (3)	2 (6)	0	0.49
Cytogenetic response, CR or PR, 3 cycles (FISH)	30 (42)	13 (36)	17 (47)	0.063
No cytogenetic response	28 (39)	16 (44)	12 (33)	0.51
Allogeneic transplantation	11 (15)	6 (17)	5 (14)	0.74

Table 6. Response to treatment in Paper I.

The median follow-up for all patients was 11.5 months. At follow-up 36 months (range, 0-36 months) after the last patient completed the trial, 60 patients (83%) were dead, and 12 patients (17%) were alive. The median survival was 11.5 months for the entire study population, 13.6 months in the AZA arm and 10.8 months in the AZA + LEN arm (P=0.43) (Figure 12). As expected, a diagnosis of AML (P=0.002), *TP53* mutations, any type (P=0.0001), and no response (P=0.047) were associated with shorter overall survival. Eighteen patient (25%) continued with AZA, after final assessment, nine patients in each arm. The median survival was 21.1 months for responding patients treated with allo-HSCT and 14.5 months for responding patients not treated with allo-HSCT (P=0.92).

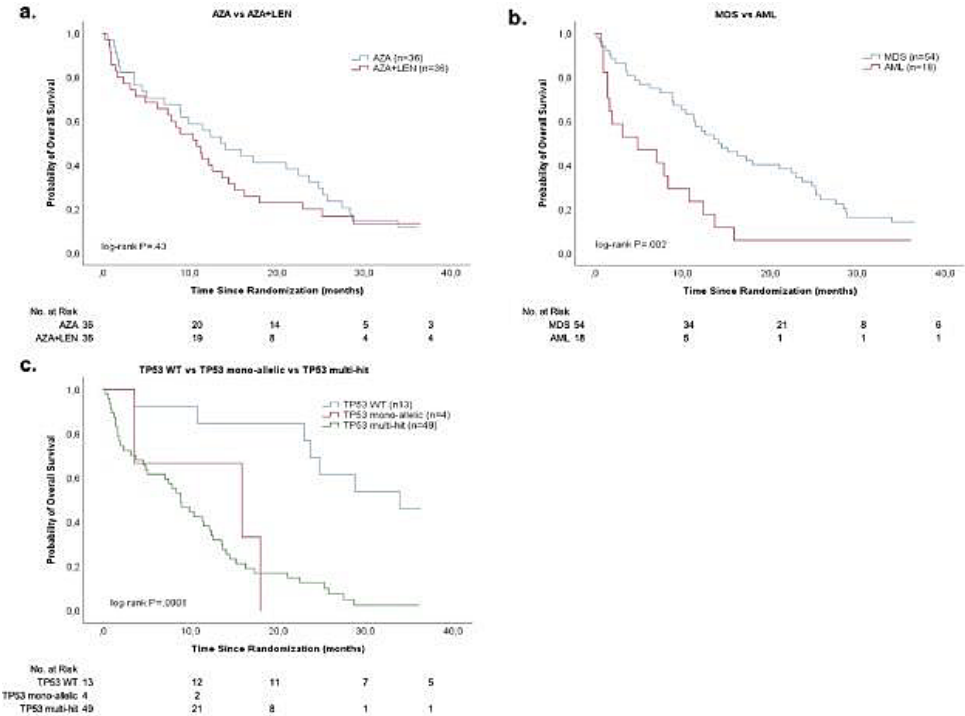


Figure 12. Overall Survival in Paper I. **a.** Survival in patients treated with AZA vs. AZA + LEN (log-rank *P*=0.43). **b.** Survival comparison between patients with AML or MDS at inclusion (log-rank *P*=0.002). **c.** Survival among *TP53* mutation subgroups; *TP53* mono-allelic vs. multi-hit vs. *TP53* WT (log-rank *P*=0.0001).

Limitations

The choice of a patient cohort consisting of higher-risk myeloid neoplasms with a karyotype including del(5q), had the consequence of an unexpected selection bias with a study cohort with even worse prognosis due to a high grad of coexisting CK and multi-hit *TP53* mutations. This was knowledge from studies presented after inclusion had started(66), as well as from early data from our own study. The high grade of *TP53* mutations limited the effect of LEN(58-61).

The study cohort consisted of 72 patients, 36 patients in each arm. This is a relatively small patient cohort. The statistical model in the study was a pick-a-winner model, Simon two step design test(145).

This model is designed to test if the effect of a phase II study drug and if it is worth to do any further phase III studies. The drug will be rejected if the overall response rate is <10% and continue if overall response rate is >30%, with α=0.05 and power=0.90, allowing 35 patients in each arm. The statistical design will answer if the two treatment arms are possible to take to the next level in the study. However, it is not designed to do any direct comparison between the two groups. Another way to demonstrate the superiority of AZA + LEN vs. AZA, would have been to use a model with two group continuity corrected χ^2 test with a 0.05 one-side significance level and a power=0.90 to detect the difference between a proportion of 20% and a proportion of 40% (odds ratio of 2.667) with the total sample size of 171 patients. A two-sided test would have been preferable due to it was not known if AZA + LEN treatment was better or worse compared to AZA alone. One limitation with the study cohort of high-risk MDS with del(5q) was the low prevalence which affected the recruiting rate (approximately 10 patients/year). If we had chosen the later statistical model, the study had not been ethical defensible because of too long inclusion time needed to answer the scientific question. Involving more sites, might have been a way forward, but it was associated with other problems like high cost per patient due to different patient insurance system outside the Northen countries. The study cohort was a small study, but it can give valuable information together with other similar studies.

The response of primary endpoint was according to 2006 IWG criteria, an efficacy measurement with some limitations. One limitation, in our study population treated with chemotherapy, was response evaluation of hematologic improvement, regarding grade of cytopenia and transfusion burden. According to IWG 2006, a transitory cytopenia and transfusion are accepted as part of the myelosuppressive treatment, if there is a recovery back to baseline. Other accepted causes are infections, hemolysis and gastrointestinal bleeding. This makes HI response more subjective assessment, but it might be diminished if HI is reported as a whole entity and not as changes in single cytopenia. HI response evaluation is more suitable in lower-risk disease. HI response is recommended to make after 8 weeks, but in higher-risk diseases after 4 weeks, to keep up cycle intervals. Baseline

transfusion pattern is difficult in a study with 13 different study-centers in four different countries. Marrow response evaluation is also difficult regarding the assessment of acceptable changes due to chemotherapy and remaining dysplastic changes due to dysplastic diseases(146). Response assessment according to IWG 2006 for MDS had a further limitation since the study cohort consisted of both MDS and AML patients. It is preferable to have the same assessment for the entire study cohort and decision was made on basis of the former classification of AML with myelodysplastic changes as RAEB-T. The new revised IWG 2023 response criteria for higher-risk MDS(33) have an intention to harmonize MDS and AML response criteria, e.g. changing the threshold for MDS to <5% instead of ≤5%. The threshold for Hb has changed from <15 g/dL to <10 g/dL and is more adequate in relation to transfusion need.

The study cohort showed a high degree of risk factors with a high age, AML at diagnosis, marrow fibrosis, CK, therapy related disease and a vast majority with multi-hit *TP53* mutations. This negatively affected response rates and OS. It also affected the rate of patients failing inclusion criteria due to rising bone marrow blast counts and the rate of patients withdrawn from study due to SAE´s or progression. Nevertheless, an important advantage of our study is that our cohort probably is representative for this particular patient population.

The median OS was 11.5 months for the entire study population, 13.6 months in the AZA and 10.8 months in the AZA + LEN arm (P=0.48). The worse outcome compared with AZA-001(98) showing a median overall survival of 24.5 months for AZA treated patients is probably explained by the high-risk baseline characteristics in **paper I**.

Cytogenetic analysis

Main findings

Forty-six patients (64%) of the study cohort from NMDSG10B had sufficient cytogenetics at inclusion, after three cycles of treatment and at final assessment and were analyzed in **paper II.** Baseline characteristic of the 26 patients without follow-up cytogenetics were more unfavorable with more transfusion dependency, low platelet counts,

marrow fibrosis, prior treatment with chemotherapy and more frequent *TP53* mutations. Karyotyping was performed in 70 patients (95%) at inclusion, and in 41 patients (95%) at final assessment. In two patients no metaphases were possible to analyze due to low number of cells in the bone marrow aspirate. At inclusion, mFISH was performed in 43 patients and in 40 patients (93%) several uncertain aberrations were characterized more clearly which has not been identified by karyotyping (Figure 13).

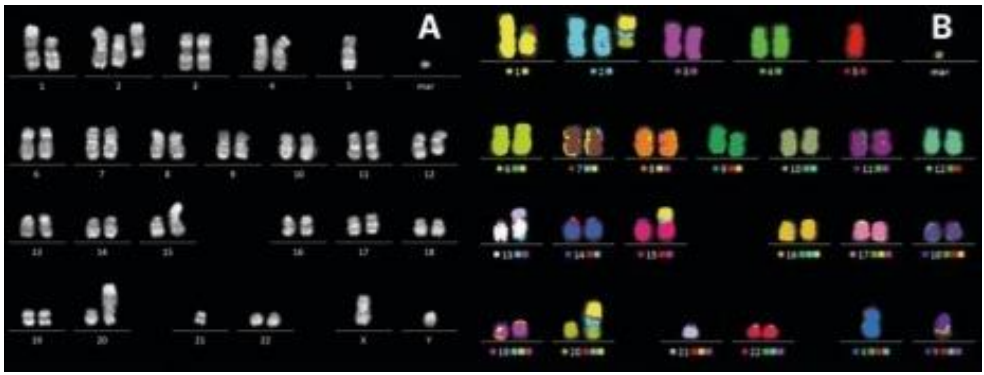


Figure 13. **(A)** Karyogram after FISH showing a complex aberrant karyotype. **(B)** mFISH of the same patient detecting cryptic aberrations.

Fifty-nine out of 70 karyotyped patients (84%) had a CK at inclusion, 12 patients (20%) with three to four aberrations and 47 patients (80%) with ≥5 aberrations. Seventeen patients (24%) had a karyotype including 17p aberrations. The number of aberrations at inclusion was associated with survival but not with response to treatment. The median OS was 11.4 months for the entire study population, 9.9 months in patients with a complex karyotype and 25.2 months in patients with less than three aberrations (log rank P=0.004) (Figure 14).

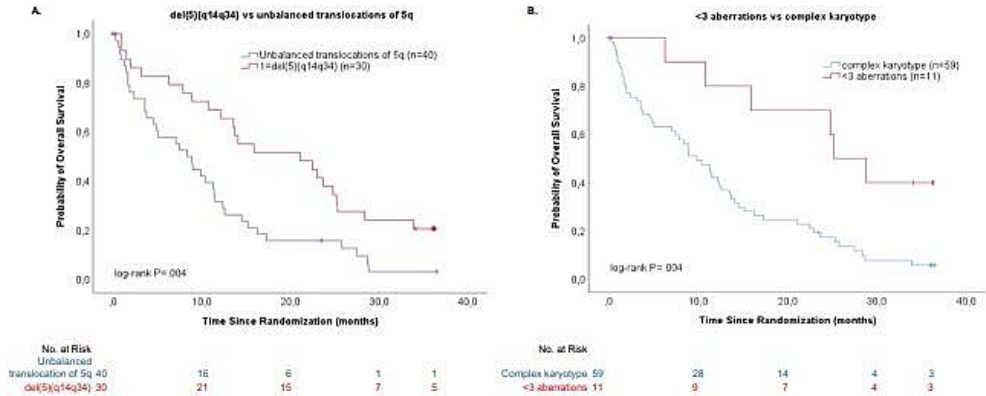


Figure 14. OS according to the type of chromosome 5 abnormality and karyotype complexity. **(A)** OS in patients with del(5)(q14q34) vs. unbalanced translocations of 5q deletion (log-rank *P*=0.004). **(B)** OS according to number of aberrations at inclusion, <3 vs. CK (log-rank *P*=0.004).

At inclusion, del(5q) was detected by karyotyping and FISH in all cases. At final assessment, the classical banding analysis was significantly more sensitive to detect the deletion in smaller clones compared to FISH, 34 patients (97%) vs. 27 patients (77%) (*P*=0.027). The aberrant clone was identified by karyotyping, but not by FISH in eight patients (23%), *versus* in only one patient (3%) the aberrant clone was detected by FISH, but not by karyotyping.

A CCyR by karyotyped was achieved in four patients (11%) in the AZA arm and seven patients (19%) in the AZA+LEN arm (*P*=0.18) and a PCyR was achieved in two patients (6%) in the AZA arm (*P*=0.49). Twelve patients (43%) showed a cytogenetic progression at final assessment, six patients (38%) in the AZA arm, and six patients (50%) in the AZA + LEN arm (*P*=0.60). A clonal evolution was detected in eight patients (67%), five patients (63%) in the AZA arm and three patients (37%) in the AZA + LEN arm (*P*=0.70). Four patients (33%) developed a new independent clone, one patient (25%) in the AZA arm and three patients (75%) in the AZA + LEN arm (*P*=0.29).

Forty-three of 46 patients (93%) had sufficient cytogenetics after three cycles of treatment. A CCyR (FISH) was achieved in 25 patients (58%), a PCyR was achieved in 6 patients (14%), and no CyR in 12

patients (28%). A reduction of del(5q) by FISH was observed after cycle three and final assessment, without significant difference between treatment arms. Eleven patients (44%) lost their CCyR after three cycles of treatment, seven patients due to clonal progression, involving five patients (45%) with a clonal evolution and two patients (18%) with a new independent clone, one patient (8%) showed a PCyR at final assessment and two patients (8%) stopped the study due to AE. One patient (4%) was transplanted after three cycles and cytogenetics from week 13 was used as final assessment. Importantly, FISH positivity increased between 3 months and end of study in several patients, indicating that the tumor-inhibiting effect of treatment may be short-lasting (Figure 15).

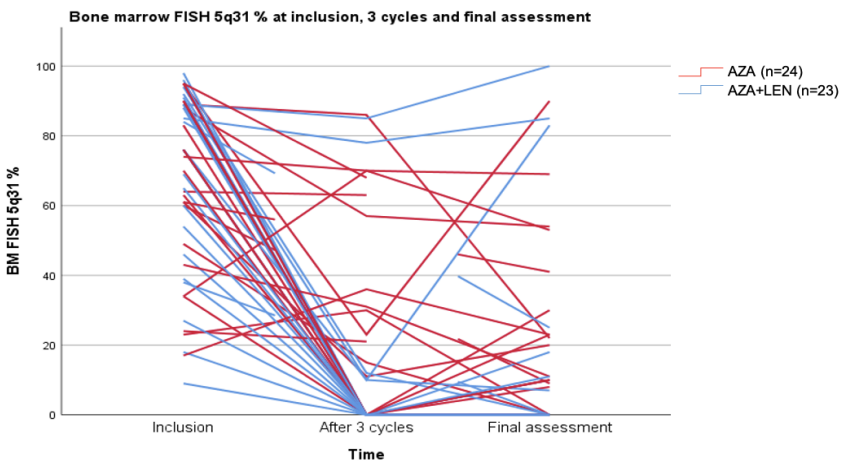


Figure 15. Bone marrow FISH 5q31% at inclusion, after 3 cycles and final assessment.

Seventy patients were analyzed with karyotype and FISH at inclusion. Thirty patients (57%) had a del(5q)(q14q34) and 40 patients (57%) had del(5q) due to other abnormalities, nearly always due to unbalanced translocations leading to partial loss of 5q.

Complex karyotype was more frequent in patients with unbalanced translocations of 5q compared to del(5q)(q14q34), 39 patients (98%) vs. 20 patients (67%) (P<0.001). Notably, a CK with ≥5 aberrations

was more frequent among patients with unbalanced translocation of 5q compared to patients with del(5)(q14q34), 33 patients (83%) vs. 13 patients (43%) (P<0.001). Therapy-related MDS or AML were more common in patients with unbalanced translocation of 5q compared to del(5)(q14q34), 14 patients (35%) vs. three patients (10%) (P=0.019). Five patients were classified as IPSS-R low or intermediate, all of those had a del(5)(q14q34) (P=0.006). The ORR was 38% among patients with unbalanced translocations of 5q and 43% for patients with del(5)(q14q34) (P=0.62) and there were no other significant differences in responses to treatment between the two groups. However, patients with del(5)(q14q34) vs. unbalanced translocations of 5q showed a longer OS, 21.1 months vs. 8.4 months (P=0.004) (Table 7).

Deletion 5q at inclusion	Total *n*=70	del(5)(q14q34) *n*=30	Unbalanaced translocations of 5q *n*=40	*P*
Age	72 (35-84)	73,5 (35-84)	70.0 (37-82)	0.096
complex karyotype	59 (84)	20 (67)	39 (98)	**<.001**
complex karyotype, chr 17 excluded	58 (81)	19 (63)	39 (98)	**<.001**
complex, > 4 abnormalities	46 (66)	13 (43)	33 (83)	**<.001**
Other abnormalities				
Chromosome 17	17 (24)	5 (17)	12 (31)	0.18
monosomy 7/del(7q)	22 (31)	7 (23)	15 (38)	0.21
inv3/t(3q)/del(3q)	3 (4)	1 (3)	2 (5)	1.0
Chromosome 9	12 (17)	5 (17)	7 (18)	0.93
number of oncogenic gene mutations	1 (0-5)	1 (0-5)	1 (0-4)	**0.002**
TP53 mutation present	52 (74)	16 (53)	36 (90)	**<.001**
TP53 mutation mono-allelic	3 (4)	2 (7)	1 (2.5)	0.57
TP53 mutations multi-hit	49 (70)	14 (47)	35 (88)	**<.001**
TP53 1 mutation + del	15 (21)	5 (17)	10 (25)	0.41
TP53 1 mutation + cnloh	16 (23)	5 (17)	11 (28)	0.29
TP53 >1 mutations	18 (26)	4 (13)	14 (35)	**0.041**
TP53, WT	12 (17)	10 (33)	2 (5)	**0.002**
another drivermutation (*TET2, SF3B1, ASXL1,RUNX1, SRSF2, BCOR, CBL*)	19 (28)	13 (45)	6 (15)	**0.007**
TET2	3 (4)	1 (3)	2 (5)	1.0
SF3B1	9 (13)	8 (29)	1 (3)	**0.008**
ASXL1	6 (9)	3 (11)	3 (9)	1.0
RUNX1	1 (1)	1 (4)	0	0.45
SRSF2	1 (1)	1 (4)	0	0.45
BCOR	1 (1)	0	1 (4)	1.0
CBL	1 (1)	1 (4)	0	0.45
elevated blasts over 5 percents	45 (67)	21 (72)	24 (63)	0.42
severe thrombocytopenia less 50	39 (58)	13 (45)	26 (68)	0.052
therapy related state	17 (25)	3 (10)	14 (35)	**0.019**
IPPS-R, low+ intermediate	5 (10)	5 (25)	0	**0.006**
IPSS-R, poor	14 (27)	5 (25)	9 (28)	0.81
IPSS-R, very poor	33 (63)	10 (50)	23 (72)	0.11
AML	18 (26)	10 (33)	8 (20)	0.21
Enough cytogenetics final assessment	44 (63)	22 (73)	22 (55)	0.12
Cytogenetic progression (n=38)	12 (32)	8 (38)	4 (24)	0.33
Clonal evolution (n=38)	8 (21)	4 (19)	4 (24)	1.0
Independent clone (n=38)	4 (11)	4 (19)	0	0.11
EGR1 % inclusion (n=29/39)	76 (0-98)	70 (18-98)	79 (0-95)	0.33
RPS14 %, inclusion (n=22/25)	74 (8-97)	79.5 (19-96)	71 (8-97)	0.13

Table 7: Del(5)(q14q34) versus unbalanced translocation of 5q.

Limitations

Our aim was to investigate the efficacy after adding LEN to AZA treatment in higher-risk MDS patients and AML patients with

myeloid dysplasia and 20-30% blasts with a karyotype including del(5q). In **paper I, II** and **III** we used karyotyping and FISH to find the 5q deletion. mFISH was used to identify cryptic aberrations and sort out the cytogenetic complexity. This might have helped us to identify patients fulfilling inclusion criteria. On the other hand, the possibility to find patients with karyotype including del(5q) among other aberrations, selected patients with CK in high extent, which might have been a negative selection bias to the results **paper I**.

A reduction of del(5q) by FISH was observed after three cycles but increased between cycle 3 and end of study in several patients. This data support more frequent monitoring. However, usage of FISH as only method to detect the del(5q) clone is difficult. In **paper II** we observed that karyotyping was significantly more sensitivity to detect del(5q) compared to FISH. This finding has also been shown previously in MDS-004 study(147). Small clones, below FISH detection, might have gone under the radar and progressed later, affection the response negatively.

The additive value of cytogenetic methods in prognosis settings has been questioned, since different analyzing methods for gene mutation have been cheaper and are more frequently used in clinical practice. However, MDS del(5q) remains still an entity in the WHO's 5th classification as well as the ICC, and is treated with specific therapy. Cytogenetic response evaluation remains important in clinical studies, and an improved OS was seen in AZA treated patients with a cytogenic response(148). This was also noticed in **paper II**, patients with CK had a shorter median OS compared to patients with less than three aberrations, 9.9 months vs. 25.2 months (log rank P=0.004).

Morphological analysis

Main findings

An adequate bone marrow trephine biopsies were available in 70 of 72 patients, while the assessment was based on smears only in two patients. In seven patients, bone marrow smears were inadequate and/or not representative due to extremely low cellularity and/or

hemodilution, therefore assessment was primarily based on corresponding biopsy material. Morphological classification according to the revised WHO 2016 was as follow, 53 (74%) MDS patients and 19 (26%) AML patients. In ten of 19 (53%) AML patients bone marrow smears were either hemodiluted (three patients) or had blast counts below 20% (7 patients), therefore definite diagnosis was established only by histological examination of trephine biopsy. Significant (grade 2-3) marrow fibrosis was present in three of these patients.

Comparison of manual blast counts in bone marrow smears/imprints and the frequency of blast cells in the biopsy using IHC (CD34) was calculated for groups using cut-off levels according to the WHO 2016 classification. In 38 of 72 (53%) cases, the percentage of blasts was comparable, thus assigning the patients to the same subgroup. In 18 of 72 (25%) samples, significant higher blast percentages were detected in the bone marrow trephine, shifting the diagnosis to a higher MDS subgroup (13 patients) or AML (five patients) ($P<0.001$), including nine MDS patients with initial <5% blasts by cytomorphology ($P=0.011$) and four MDS patients with initial 5-<10% blasts by aspirate (P=0.43); five MDS patients with excess blasts between 10-<20% by aspirate ($P=0.047$) were eventually diagnosed as AML based on IHC findings (Figure 16). In seven cases (10%) bone marrow smears were of poor quality and assessment was therefore primarily based on biopsy. In six patients (8%), the blast percentage was higher in bone marrow smears compared to CD34+ blast cells in the biopsy, indicating a fraction of blasts lacked CD34-positivity. Three patients with AML and 20-30% blasts cells in smears had sheets of blasts cells exceeding the 30% threshold in the biopsy material.

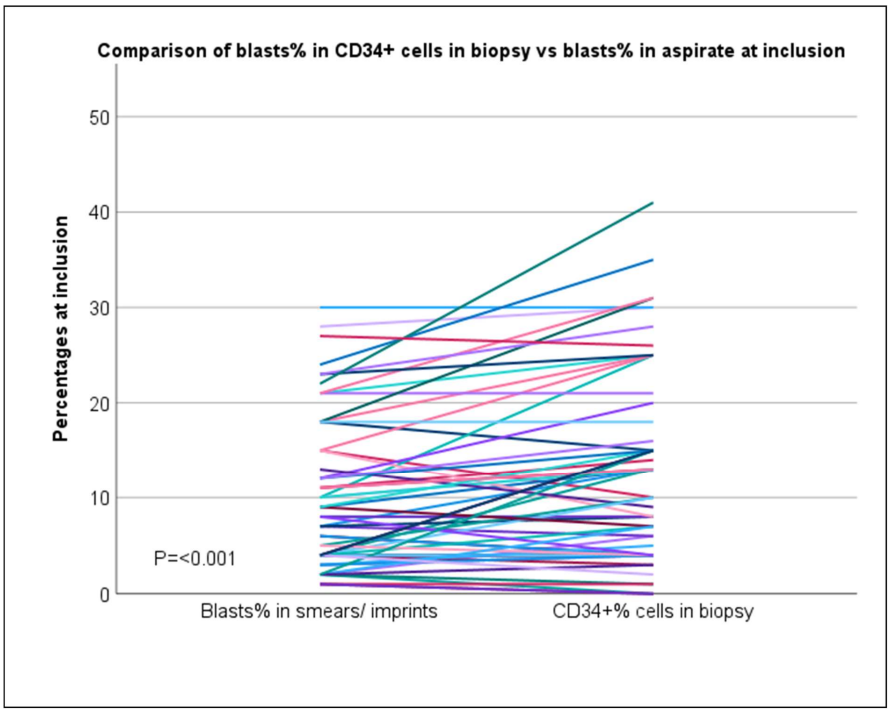


Figure 16. Comparison of blast% in CD34+ cells in biopsy vs. blast% in aspirate at inclusion.

Patients with blast percentages within the same range by both methods had a significant lower rate of CR compared to cases were the percentages of blast cells differed by bone marrow biopsy vs. cytomorphology, six out of 26 patients (23%) vs. 10 out of 17 patients (59%) (P=0.0018).

At inclusions, 51 patients (74%) had no or only grade 1 fibrosis and 18 patients (26%) had significant (grade 2 or 3) marrow fibrosis. The effect of marrow fibrosis was assessed with respect to blast percentages in bone marrow aspirate vs. the trephine biopsy. In 18 patients the blast percentages were higher in the bone marrow biopsy vs. aspirate smears: 16 patients with no or only mild fibrosis and one patient had significant fibrosis.

In **paper I** 16 patients (22%) showed a marrow CR in total, six patients (17%) in the AZA arm and ten patients (28%) in the AZA + LEN arm, respectively (*P*=0.086).

In conclusion, our study in **paper III** demonstrates higher blast percentages in bone marrow biopsy compared to cytomorphology in a significant number of cases independent of marrow fibrosis underscoring the additive value of a histological examination for correct diagnosis and risk assignment, in high-risk MDS and borderline forms, both in routine diagnostics and in the frame of clinical trials.

Limitations

Higher-risk MDS and AML patients with a karyotype including del(5q) have a dismal prognosis. This was observed in our study cohort with a low number of patients reaching final assessment. Patients who ended the study earlier, due to disease progression or adverse events, had a higher grade of risks factors including fibrosis grade 2 or 3. A bone marrow at final assessment was made in low extent in patients who stopped the study earlier. This might have influenced the cohort at final assessment, since patients reaching final assessment belonged to a better risk group compared to all included. However, the ORR and marrow CR was low in the study cohort with high-risk features. This further might have reduced the possibility to see any morphological differences in the small study cohort at final assessment. We demonstrated a significant higher blast percentage in the bone marrow trephine compared bone marrow smears, affecting finals classification of 18 patients (25%) in the cohort, shifting the diagnosis of 13 patients to a higher subclassification of MDS and five patients to AML. However, this did not affect the response rate, likely because most of the patients had very poor risk disease. There is no difference in risk classification, according to IPSS-R, in patients with marrow blasts of 10-20% or 20-<30%(149). A fixed blast percentage may be less important regarding outcome compared to genetic and clinical characteristics(150). The usage of marrow CR without HI in clinical trials has been diminished in the revised IWG 2023 response assessment(33) and it is only recommended for assessment before allo-HSCT.

Mutational analysis

Main findings

We detected 37 different mutations in 70 patients at inclusion (Table 8). The median number of mutations was two (range, 0-6). There was no significant difference in ORR according to mutational status. Fifty-three patients (76%) carried a *TP53* mutations and 49 (92%) of these were multi-hit. In patients with multi-hit *TP53* mutations, 15 (31%) patients had one mutation + del(17p), 16 (33%) patients had one mutation + cnLOH, and 18 (37%) patients had bi-or triallelic mutations.

Mutation	Incidence, NGS n=70 (%)	No. Mutated AZA, AZA+LEN
ASXL	6 (9)	3, 3
BCL10	1 (1)	1, 0
BCOR	1 (1)	0, 1
BCROL1	2 (3)	2, 0
CALR	1 (1)	0, 1
CBL	1 (1)	1, 0
CEBPA	1 (1)	1, 0
CSF1R	1 (1)	0, 1
CSF3R	1 (1)	1, 0
CSNK1A1	3 (4)	2, 1
DDX41	2 (3)	1, 1
DDX54	1 (1)	0, 1
DNMT3A	12 (17)	7, 5
EGFR	1 (1)	1, 0
EP300	2 (3)	0, 2
EZH2	6 (9)	3, 3
GATA2	3 (4)	1, 2
GNB1	1 (1)	0, 1
IDH1	1 (1)	1, 0
IDH2	1 (1)	1, 0
KMT2C	1 (1)	1, 0
KRAS	1 (1)	1, 0
LUC7L2	1 (1)	1, 0
NF1	3 (4)	1, 2
NRAS	2 (3)	1, 1
PPM1D	2 (3)	1, 1
PTPN11	2 (3)	1, 1
RAD50	1 (1)	1, 0
ROBO1	2 (3)	1, 1
RUNX1	1 (1)	1, 0
SF3B1	10 (14)	3, 7
SH2B3	2 (3)	1, 1
SRSF2	1 (1)	1, 0
TET2	3 (4)	0, 3
TP53	53 (76)	27, 26
TP53 1 mutation	4 (8)	2, 2
TP53 multi-mutations	49 (92)	25, 24
TP53 1 mutation + del	15 (31)	10, 5
TP53 1 mutation + cnloh	16 (33)	8, 8
TP53 >1 mutations	18 (37)	7, 11
TP53 WT	13 (19)	8, 5
ZBTB33	1 (1)	1, 0
No mutations	2 (3)	3, 1

Table 8. Mutations analyzed by NGS at inclusion

In **paper I** the ORR in patients carrying a *TP53* mutation was 47% (*P*=0.99). Responding patients had a median VAF at final assessment of 0% in the single patient in mono-allelic group and 5.7% (0-93%) in

the multi-hit group (*P*=0.21). After treatment an interesting pattern was observed in *TP53* (VAF%). In 12 of 15 patients with any response, VAF was significantly reduced (*P*=0.0001) at final assessment, with no significant differences of the change in VAF% between treatment arms (*P*=0.49) (Figure 17). Patients with a *TP53* mutation, any type, were associated with a shorter median OS (log-rank *P*=0.0001).

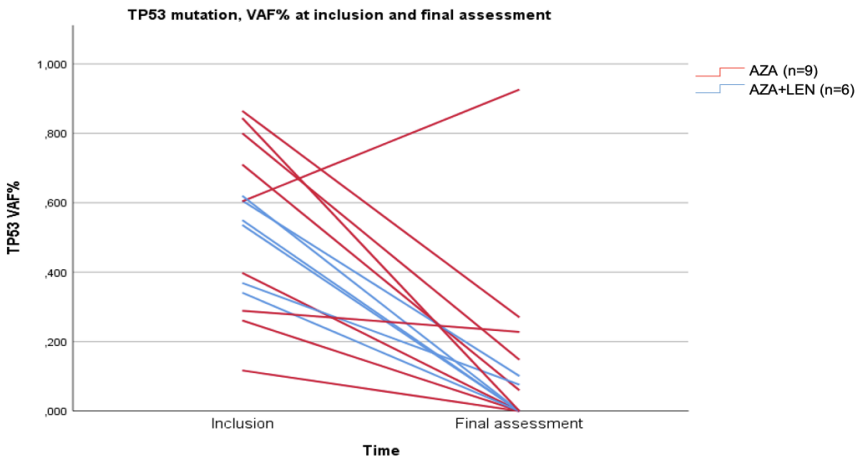


Figure 17. *TP53* mutation VAF% in responding patients at inclusion and at final assessment.

In **paper II**, 36 patients (90%) with unbalanced translocation of 5q carried a *TP53* mutation vs. 16 patients (53%) with del(5q) (*P*<0.001). Multi-hit *TP53* alterations (*P*<0.001) and among those biallelic *TP53* mutations (*P*=0.041) were more common in the group with unbalanced translocation of 5q vs. del(5)(q14q34). Ten patients (33%) with del(5)(q14q34) were *TP53* WT vs. two patients (5%) among patients with unbalanced translocation of 5q (*P*=0.002). The median number of oncogenic mutations was 1 (range, 0-5) in patients with del(5q)(q14q34) and 1 (range, 0-4) in patients with unbalanced translocation of 5q (*P*=0.002). *TP53* mutations with another driver mutation (*ASXL*, *BCOR*, *CBL*, *RUNX1*, *SF3B1* and/or *TET2*) were more common in patients with del(5)(q14q34), 13 patients (45%) vs. 6

(15%) in patients with unbalanced translocations of 5q (P=0.007). *SF3B1* mutations were observed in eight patients (29%) with del(5)(q14q34) and one patients with an unbalanced translocation of 5q (P=0.008).

Correlation with p53 staining status and *TP53* mutation

Assessment of *TP53* mutational status is essential for prognostic assessment in high-risk myeloid disease and particular in patients with del(5q)(31, 66). p53 staining status gives additive information of the occurrence of *TP53* mutations. In **paper III**, 49 patients (70%) had evidence of multi-hit TP53 mutation and a strong p53 staining in erythroid and/or myeloid precursors was noted in 57 patients (80%) at diagnosis. The p53 staining was negative in 14 patients at inclusion, while molecular analysis discovered biallelic *TP53* mutations in six patients and monoallelic *TP53* mutation in one patient. Screening for *TP53* mutation failed in two patients (4%) who had strong positive p53 staining ranging between 5-<20%. Stainable p53 was detected in 10 patients (14%) without proven *TP53* mutation. In AML a strong p53 staining was observed in 18 of 19 patients. A strong p53 staining showing a frequency of >20% was significantly more common in AML patients compared to MDS patients, 12 patients (63%) vs. 17 patients (32%), respectively (P=0.021). Forty-two patients (58%) were positive in p53 with a *TP53* multi-hit, 16 AML patients (84%) compared to 26 MDS patients (49%) (P=0.009).

Forty-one patients reached final assessment, and 20 patients were analyzed both by p53 IHC and *TP53* sequencing. Six patients (30%) still showed *TP53* mutations sequencing and were positive by IHC. Patients showed the same complementary pattern at final assessment as inclusion with p53 staining and *TP53* mutations. Four (20%) and three (15%) patients, respectively, were positive only by sequencing or IHC. The four patients with only positive sequencing, two of those patients had lost their p53 positivity since inclusion and two had same status as inclusion. The three patients with a positive IHC, two of those patients were IHC positive and had *TP53* mutations at inclusion and one showed the same status as inclusion. Seven patients were negative by sequencing and IHC. In conclusion, bone marrow

trephine biopsy including IHC p53 provides complementary information to *TP53* mutational status. A reduction of strong p53 staining was observed at final assessment (Figure 18). The reduction of *TP53* (VAF%) was also noted in 12 of 15 patients with any response (*P*=0.0001). Results from two different studies indicates allowing results in outcome after allo-HSCT in *TP53* mutated higher-risk MDS patients, if initial treatment markedly reduces *TP53* mutated clone size(135, 137).

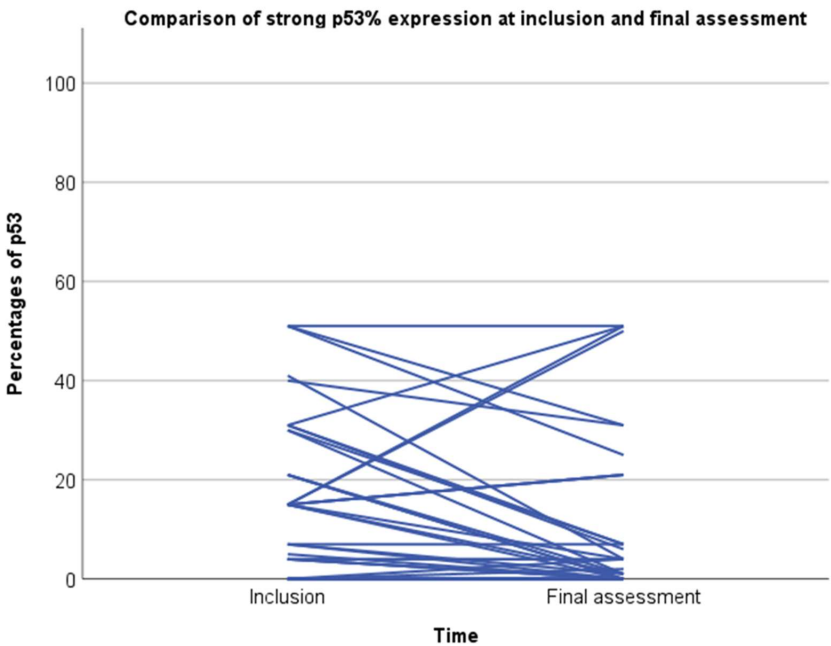


Figure 18. Comparison of changes in percentages of strong p53 cells (IHC) at inclusion and at final assessment.

At final assessment 25 patients (61%) showed strong p53 staining and 16 patients (39%) did not show strong p53 staining. A CR was significantly more frequent in patients with no p53 expression vs. those with strong p53 expression, five patients (31%) vs. one patient (4%) (*P*=0.026). CCyR was significantly higher in patients without strong p53 staining vs. those with strong p53 staining, eight patients (53%)

vs. three patients (13%) (P=0.010). NoCyR was more frequent in patients with a strong p53 staining vs. those without, 19 patients (83%) vs. six patients (40%) (P=0.005). The median OS was 16.3 months for patients reaching final assessment, 13.6 months in patients with a remaining strong p53 and 25.2 months in patients without (log-rank 0.050).

Limitations

Patients with higher-risk myeloid neoplasm with a CK including del(5q) and especially with a multi-hit *TP53* mutation have a resistant to different therapies, and the relapse risk after allo-HSCT is very high (151). At inclusion, most patients in our study cohort had a strong p53 staining and a confirmed *TP53* mutations, often multi-hit. Forty-one patients (57%) reached final assessment. It may be difficult to compare cohort at inclusion and final assessment since patients reaching final assessment may have more favorable prognosis compared to the whole study cohort at inclusion. *TP53* WT was significantly higher in patients reaching final assessment compared to those patients who stopped treatment before final assessment. The relatively low number of patients reaching final assessment with few numbers of mutational analyzes plus the low response rate in the patient cohort with a severe outcome, might also have affected the possibility make conclusions from the study population since the differences are small. However, patients with a response to treatment showed reduction of p53 by clone size and had a longer survival.

One limitation with the mutational analysis was the usage of different gene panels at inclusion and final assessment, Lymphoprep™ (42 genes) and TruSight™ (54 genes). However, the study cohort consisted of a high grade of multi-hit *TP53* mutated patients which may have reflected the low numbers of co-mutations. This is known from other studies showing patients with a multi-hit *TP53* mutation have low numbers of other driver mutations compared to patients with a mono-allelic *TP53* mutation(66). Both gene panels included *ASXL1*, *BCOR*, *JAK2*, *RUNX1*, *SF3B1, SRSF2* and *TET2*, except from *CBL* which was only analyzed by TruSight™, genes which are important to analyze since they are more frequent in *TP53* mono-allelic

compared to multi-hit(66). This might have been reflected in in the group with unbalanced translocation of 5q vs. del(5)(q14q34). Multi-hit *TP53* alterations and biallelic *TP53* mutations were more frequent in patients with an unbalanced translocation of 5q compared to del(5)(q14q34). Del(5)(q14q34) had more frequent *TP53* WT, higher number of driver mutations and *TP53* mutations with other driver mutations, especially *SF3B1*.

The gene panels had different sensitivity of VAF% at inclusion and final assessment. This might have been a limitation in our studies since TruSight™ have a sensitivity for VAF% of ≥5% compared to Lymohoprep™ with a VAF of ≥2%. Our agreement with Memorial Sloan Kettering Cancer Clinic, New York, did not include follow-up, this was the reason for why we used TruSight™ analyzed at Science for Life Laboratory, Uppsala.

Del(5q) FISH positivity decreased after three cycles of treatment but increased thereafter. The p53 staining detected in small evolving or increasing fraction of cells with strong nuclear staining in sequential biopsies, indicating expansion of an underlying *TP53* mutated subclone, even in cases that showed morphological response by rutin parameters. Patient with strong p53 staining at final assessment also had significant lower rate of CCyR. Patients with high-risk myeloid malignancies have a dismal prognosis and within this group those with CK including del(5q) and *TP53* mutations have even worse prognosis. The treatment effect on *TP53* mutated cells have a short-lasting duration and do not translate into improved patient outcome.

Conclusions

- The findings in the clinical study show that high-risk MDS with del(5q) is a myeloid disorder with a dismal prognosis.

- The addition of LEN to AZA treatment did not improve the outcome of patients with high-risk MDS or AML with 20-30% marrow blasts with a karyotype including del(5q).

- There seems to be a window of molecular response to AZA after 3 months of treatment. A reduction of del(5q) by FISH was observed after cycle three and at final assessment, without significant difference between treatment arms. FISH positivity increased between 3 months and end of study in several patients, indicating that the tumor-inhibiting effect of treatment may be short-lasting.

- In 12 of 15 patients with any response, *TP53* VAF% was significantly reduced at final assessment, with no significant differences of the change in VAF% between treatment arms.

- Both complex karyotype and multi-hit *TP53* alterations were more frequent in patients with unbalanced translocations of 5q compared to del(5q)(q14q34).

- The morphological study demonstrates higher blast percentages in bone marrow biopsy compared to cytomorphology in a significant number of cases independent of marrow fibrosis underscoring the additive value of a histological examination for correct diagnosis and risk assignment, in high-risk MDS and borderline forms, both in routine diagnostics and in the frame of clinical trials.

- Both IHC p53 and *TP53* sequencing analysis provide complementary information on *TP53* mutational status thereby contributing to prognostic assessment.

Future perspectives

What will be a way forward to solve the gordian knot represented by higher-risk myeloid malignancies with a karyotype including del(5q)? To deliver an Alexander cut might not be a solution since many MDS patients are elderly.

Even if the combination of AZA + LEN did not improve the outcome of the higher-risk myeloid malignancies with del(5q) with extreme high-risk characteristics, important lessons can be drawn from our studies.

It is important to design prospective studies to enable detailed assessment of biological characteristics in defined patients group treated with target therapies. However, the incidence of MDS patients with a specific characteristic is rare. There is a need of broader collaborations to facilitate gathering a study cohort large enough to show statistically significant differences and to optimize the inclusion rate. A way forward of making it easier would be a model like the Nordic patient insurance system, adopted in a broader extent in Europe. This to enable international academic studies across national borders. MDS patients are often elderly and due to different exclusion criteria have these patients a reduced possibility to be included in phase III clinical trials. One of the exclusion criteria in our clinical study was expected survival less than two months, sometimes difficult to estimate. On the other hand, we did not exclude patients according to performance status and patients were recruited from 13 different study centers which normally treat high-risk MDS patients, which might have made the study more “real-world” cohort. Eligible criteria have a tendence to be similar in phase I and phase III studies and does not necessary reflect safety associated with the study drug. A concordance of 13% between safety data from phase I studies reflected in exclusion criteria in phase III studies was presented in a review study analyzing the eligible criteria in 191 clinical trials of MDS patients registered at ClinicalTrials.com between 2000 until 2023 (152). Real-world studies, without excluding older patients, are essential to give realistic expectations and will minish selection bias data from a selected study cohort.

It is important to include molecular analysis in clinical trials in assessment of residual disease(119, 137) and be aware of the increased number of clonal hematopoiesis of indeterminate potential with the older MDS patients (73).

Corroborating the findings of other investigators hypomethylating agents has an anti-tumoral effect on *TP53* mutated cells, even though it is temporary and do not improve patients outcome. Timing of treatment is important and future therapeutic studies on patients with CK and *TP53* mutated MDS should be of shorter duration, with molecular follow-up after each cycle, and in suitable patients a rapid planning for allo-HSCT(135, 137).

The question of finding specific targeted therapies in MDS are tricky. The MDS-patient cohort is heterogenic *per se* and within the subgroup of high-risk MDS with a karyotype including del(5q) the patient group have several risks to take in consideration when choosing the target. The additional effect of LEN to AZA might have been overcome by the vast majority of multi-hit *TP53* mutated patients with a CK. Finding a combination which is targeting different aspect of the disease is difficult but also the only way forward. Different phase I/II clinical trials of restoring the transcriptional activity of p53 with eprenetapopt in combination with AZA have initially shown promising responses(118, 119) but the phase III trial was negative and the combination of eprenetapopt + AZA was not superior to AZA alone(120). The effect of LEN requires a functioning p53(49-51). An alternative combination of LEN might be with a study drug capable of restoring the mutant p53 back to p53 WT. A third generation thiosemicarbazone, COTI-2, with an effect of transforming mutated p53 in the direction to WT(153) would be an interesting combination with LEN.

The borderline between MDS and AML is arbitrary, and the diseases are often driven by specific biological characteristics like *TP53*-mutations. To increase available studies for MDS-patients would be a closer cooperation with different AML-groups to investigate if MDS-patients are assessed eligible to include in clinical AML-studies would be useful. Both the WHO´s 5th classification as well as the ICC arguing of include patients with 10-30% blasts in AML-like therapies(3, 4). This

would also make more patients available for inclusion in studies. If more MDS patients were to be included, it would be important to consider different exclusion criteria and management of prolonged cytopenia.

At last, it is important to have in mind what benefit the MDS-patient most. What should be a meaningful ORR of the treatment in relation to a cost-benefit analyses, also in relation to different biological age-categories. What is natural aging and what can we prevent?

Populärvetenskaplig sammanfattning

En aprildag våren 2010 låg det annars så hektiska Arlanda öde och tomt. Askmolnet från Eyjafjallajökull´s vulkanutbrott hade spridits över stora delar av norra Europa och lamslagit flygtrafiken. Samtidigt samlades den nordiska MDS gruppen (NMDSG) till sitt återkommande forskningsmöte på Arlanda. Bertil Uggla hade tagit med mig till denna livaktiga forskningsgrupp. Under ledning av Eva Hellström-Lindberg, diskuterades behovet av att finna nya behandlingar för patienter med högrisk myelodysplastiskt syndrom (MDS) som har en mycket dålig prognos. Hypoteser framfördes om det var möjligt ett lägga till ytterligare behandling till azacitidin, som utgjorde förstahandsbehandlingen. Kunde en riktad terapi med lenalidomid leda till förbättrade resultat för patienter med högrisk MDS som saknade en del av långa armen på kromosom 5 (del(5q))? Kunde läkemedlet ha samma effekt vid högrisk MDS som vid lågrisk MDS? En ny klinisk studie (NMDSG10B) tog form under ledning av Lars Möllgård.

MDS är en typ av blodcancer som drabbar den ”myeloida” cellinjen som normalt bildar röda blodkroppar, vita blodkroppar och blodplättar. Det uppkommer olika grader av blodbrist beroende på var mognadsstörningen sker. Detta kan yttra sig i en brist som enbart påverkar utmognaden av en cellinje, till exempel brist på röda blodkroppar (anemi). I andra fall, kan störningen ske tidigt i utmognaden och påverka flera eller samtliga ”myeloida” cellinjer. Patienter kan då få en uttalad blodbrist, blödningsbenägenhet samt ökad infektionskänslighet. Patienter med MDS löper en risk att försämras och har en förhöjd risk att utveckla akut myeloisk leukemi (AML). Den vanligaste åldern för att insjukna i MDS är 76 år i Sverige och män har en ökad risk att insjukna i MDS jämfört med kvinnor.

MDS är ett syndrom med varierande grad av sjukdomsbild och prognos. Patienter med goda prognosmarkörer ”lågrisk” MDS har en förväntad överlevnad på flera år. Däremot är prognosen betydligt sämre för patienter med ”högrisk” MDS med en förväntad överlevnad på några månader. Prognosen påverkas av flera faktorer, bland annat av förekomsten av omogna celler ”blaster”. Vid en tidig mognadstörning ses en högre andel blaster. Gränsdragningen mellan högrisk MDS och

AML är svår och gränsen har ändrats över tid, från tidigare 30% blaster till idag 10–20% blaster, beroende på klassificeringssystem. Prognosen påverkas även av kromosomförändringar och ca 50% av patienterna med MDS har olika former av kromosomavvikelser. Den vanligast kromosomförändringen är del(5q) (patienten saknar del av långa armen på kromosom 5) och motsvarar ca 10% av fallen. Antalet kromosomförändringar har också betydelse och patienter med tre eller fler kromosomförändringar (komplex karyotyp) har en dålig prognos. Vid MDS ses i 90% av patientfallen någon eller flera mutationer i de sjukdomsalstrande cellerna. *TP53* mutationer kan förekomma vid MDS. *TP53* mutationen påverkar celldöden på ett ogynnsamt sätt vilket leder till en försämrad prognos.

Behandlingen av patienter med lågrisk MDS utgörs av understödjande, symptomlindrande terapi. Detta sker med läkemedel som stimulerar bildandet av röda blodkroppar (erytrocyter) erytrocytstimulerande läkemedel eller med regelbundna blodtransfusioner. Lågrisk MDS med del(5q) kan behandlas med lenalidomid, vilket är ett immunmodulerande läkemedel. Effekten av läkemedlet försämras vid en samtidig förekomst av *TP53* mutation.

Högrisk MDS behandlas med azacitidin (AZA), vilket är det enda godkända läkemedlet i Europa. AZA fungerar genom att det påverkar ”låsfunktionen” hos DNA, ett ”hypometylerande” läkemedel. Effekten av AZA är bromsande, med en förlängd överlevnad på 15 månader, men behandlingen är inte botande. Den enda botande behandling som finns är benmärgstransplantation, då patienten får blodbildande stamceller från en donator, ”allogen stamcellstransplantation”. Behandlingen är krävande för patienten och hänsyn behöver tas dels till patientens biologiska ålder och dels till samsjuklighet.

Avhandling bygger på tre studier. Första studien är en jämförande studie där vi studerande effekten av kombinationsbehandlingen med AZA +LEN jämfört med enbart AZA hos patienter med högrisk MDS och AML med 20–30% blaster i benmärgen och en samtidig kromosomförändring med del(5q). 72 patienter från 13 olika center i Sverige, Danmark, Norge och Finland ingick i studien. Patienterna lottades till att få behandling med antingen enbart AZA eller AZA + LEN.

Behandlingen varade i sex månader. Effekten av behandlingen studerade vi genom att bedöma grad av ”remission” i form av kvarvarande omogna celler, kromosomförändringar och förbättrade blodvärden. Vi studerade om kombinationsbehandlingen var säker genom att följa biverkningar. Antalet patienter som ingick i studien var relativt få, varför det inte gick att dra några direkta slutsatser avseende överlevnad även om vi hade det som ett av våra effektmått. Tyvärr kunder studien inte påvisa någon ytterligare effekt med tillägget av LEN till AZA jämfört med enbart AZA. Patienter med högrisk MDS med del(5q) har en generellt dålig prognos. Därtill är det vanligt att högrisk MDS patienter med del(5q) samtidigt har en komplex karyotyp och *TP53* mutationer, vilket försämrar möjligheten till att svara på behandlingen och försämrar prognosen ytterligare. Detta är kunskap som framkommit under senare år och inte var känd när studien startades. Däremot kunde vi se i studien att den *TP53* muterade klonen minskade hos de patienter som svarade på behandlingen.

I studie II undersökte vi vilken betydelse kromosomförändringar har vid högrisk MDS, deras betydelse för hur patienten svarade på behandling och möjligheten till att förutse behandlingssvar. Studien gjordes i samarbete med forskare vid ett cytogenetiskt laboratorium i Hannover, Tyskland. Vi kunde inte utifrån kromosomavvikelser se några avgörande skillnader i hur patienter svarade på behandlingen med AZA + LEN eller AZA. Däremot såg vi att hos en del patienter minskade del(5q) klonen efter tre behandlingsomgångar, mätt med Fluorescence *in situ* hybridisering, för att sedan åter försämras vid slutbedömningen efter sex månader. Vi såg även en kortare överlevnad hos de patienter som hade en komplex karotyp samt hos de patienter där deletionen på kromosom 5 var lokaliserad på en annan plats än den vanligt förekommande platsen för deletionen.

Delarbete III var en morfologisk studien där vi studerade diagnostiken och om den skiljdes åt mellan benmärgsbiopsi och benmärgsaspirat. Benmärgsbiopsin utgörs av benmärg samt omkringliggande stödjestrukturer medan benmärgsaspiratet innehåller blodbildande celler som aspirerats från benmärgen med hjälp av en grov nål. Vi studerade även, med hjälp av immunohistokemisk infärgning, olika benmärgsstrukturer som blaster och förekomsten av starkt infärgad p53 och

jämförde det med förekomsten av samtidig *TP53* mutation. Studien visade på att 18 patienter (25%) av totalt 72 patienter fick en annan klassificering av MDS/AML vid undersökning med benmärgsbiopsi jämfört med aspirat. Vi såg, såväl vid diagnos som vid uppföljande prov, nyttan med både infärgning av p53 samt *TP53* mutationsanalys då proverna kompletterar varandra och därmed kan påvisa förekomst av *TP53* mutation, vilket är en värdefull pusselbit för bedömning av prognosen.

I våra studier av patienter med högrisk MDS med del(5q) har vi kunnat se en effekt av AZA behandling även om den varit kortvarig. Behovet kvarstår att finna nya behandlingsstrategier som förbättrar överlevnaden för denne grupp av patienter med mycket dålig prognos.

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