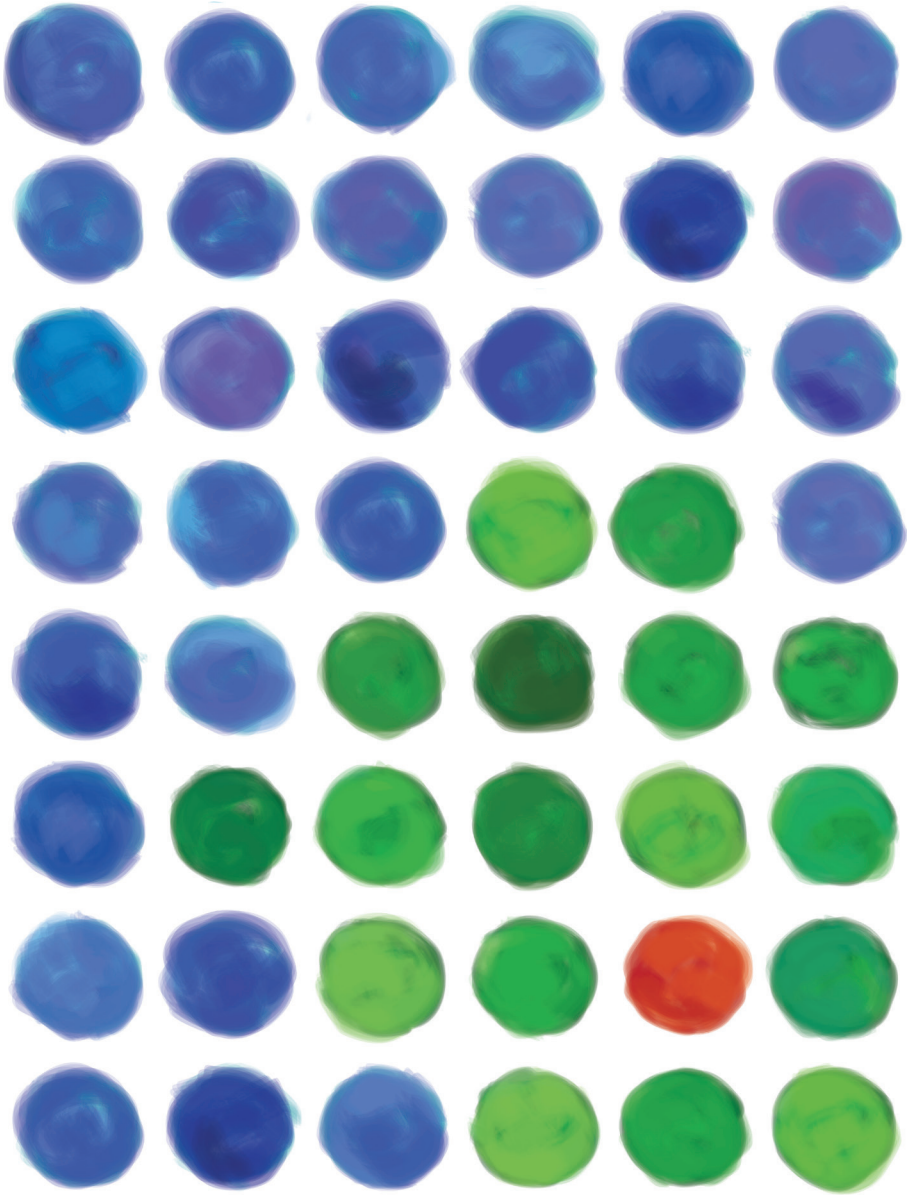
Molecular Stratification and Residual Disease Detection in Acute Myeloid Leukemia



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Molecular Stratification and Residual Disease Detection in Acute Myeloid Leukemia

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in acute myeloïde leukemie

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INTRODUCTION

ACUTE MYELOID LEUKEMIA

Acute myeloid leukemia (AML) is a malignant disease of the hematopoietic stem cell in the bone marrow.[1] In normal hematopoiesis the hematopoietic stem cell gives rise to all mature blood cells by differentiation. AML cells or blasts are characterized by abnormal differentiation, increased proliferation and reduced sensitivity to apoptosis. As a result blasts will accumulate in the bone marrow and repress normal blood cell production, resulting in anemia, thrombocytopenia and immature dysfunctional leukocytes, which are all contributing to the cardinal manifestations of AML.

Disease and Risk Classification

AML is diagnosed when morphologically ≥20% of blasts can be detected in the peripheral blood or bone marrow. The discovery of chromosomal translocations and inversions has revealed the genetic pathophysiology of AML.[2,3] These chromosomal aberrations initiate the formation of fusion genes and proto-oncogenes that target key hematopoietic transcription factors and epigenetic modulators that induce leukemic transformation.[4,5] Ongoing studies deciphering the mechanisms of abnormal differentiation and proliferation in AML with polymerase chain reaction (PCR) resulted in the identification of gene mutations in *FLT3*, *CEBPA*, and *NPM1*.[6-8] Subsequent large cohort studies have established the molecular heterogeneity and prognostic importance of the genetic variants in AML, which formed the basis of the refined European Leukemia Network (ELN) risk classification (Table 1).[9-15] The implementation of the risk classification is essential in current AML treatment protocols.

Treatment

The treatment strategy of AML consists of the induction phase and the consolidation phase. The aim of the induction phase is to eradicate leukemic cells and to achieve a complete morphological remission (CR). For half a century induction chemotherapy is comprised of two cycles of cytarabine combined with an anthacycline drugs such as daunorubicin or idarubicin.[1,16] However, relapse is inevitable without post-remission therapy such as additional chemotherapy and autologous transplantation or allogeneic transplantation. The clinical decision regarding post-remission therapy for AML is guided by the comparative assessment of the estimated relapse risk according to the ELN risk classification and treatment-related mortality.[15,17] In HOVON protocols, AML patients with favorable risk receive autologous transplantation, whereas intermediate and adverse risk AML patients are considered for allogeneic transplantation.[18,19] In recent years a multitude of new drugs has been tested to increase the depth and duration of remission.[20,21] Recently, the addition of the FLT3 inhibitor midostaurin to induction chemotherapy prolonged survival outcomes and got approved for standard treatment of AML patients with *FLT3* mutations.[22] Among others, inhibitors targeting mutant *IDH1*, *IDH2* and second generation FLT3 inhibitors combined with induction chemotherapy and/or maintenance therapy are currently under investigation (e.g., HOVON 150 and 156).

Table 1. ELN 2017 risk stratification

Risk category	Genetic aberration
Favorable	t(8;21)(q22;q22.1); *RUNX1-RUNX1T1* inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11* Mutated *NPM1* without *FLT3*-ITD or with *FLT3*-ITD^{low}† Biallelic mutated *CEBPA*
Intermediate	Mutated *NPM1 and FLT3*-ITD^{high}† Wild-type *NPM1* without *FLT3*-ITD or with *FLT3*-ITD^{low}† (without adverse risk genetics) t(9;11)(p21.3;q23.3); *MLLT3-KMT2A* ‡ Cytogenetic abnormalities not classified as favorable or adverse
Adverse	t(6;9)(p23;q34.1); *DEK-NUP214* t(v;11q23.3); *KMT2A* rearranged t(9;22)(q34.1;q11.2); *BCR-ABL1* inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2*,*MECOM* (*EVI1*) -5 or del(5q); -7; -17/abn(17p) Complex karyotype,§ monosomal karyotype* Wild-type *NPM1* and *FLT3*-ITD^{high}† Mutated *RUNX1*◊ Mutated *ASXL1*◊ Mutated *TP53*

† Low allelic ratio (<0.5); high allelic ratio (>0.5); *FLT3*-ITD alleles divided by *FLT3* wild-type alleles

‡ The presence of t(9;11)(p21.3;q23.3) takes precedence over concurrent adverse cytogenetics

§ Three or more unrelated chromosome abnormalities in the absence of one of the recurrent translocations or inversions in this table

* The presence of one single monosomy in association with at least 1 additional monosomy or structural chromosome abnormality (excluding CBF or loss of X or Y).

◊ These markers should not be used as an adverse prognostic marker if they co-occur with favorable risk AML subtypes

Measurable Residual Disease

Although the majority of AML patients (>80%) attain CR, relapse remains the major cause of failure in AML. Remission assessment after treatment is traditionally determined by morphological examination of the bone marrow and CR is achieved when less than 5% of blasts can be detected.[23,24] However, morphologic examination of the bone marrow has low sensitivity (~1%) and cannot distinguish blasts from normal hematopoietic stem cells. Detection of measurable residual disease (MRD) during treatment is a quantitative method to evaluate the remission status with increased sensitivity to improve relapse estimation. Established techniques for MRD detection are multiparameter flow cytometry (MFC) and quantitative real-time quantitative polymerase chain reaction (RQ-PCR).[25] MRD detection by MFC uses combinations of fluorescent antibodies for blast-specific surface

molecules, or leukemia-associated immunophenotypes (LAIP), that can be detected in 90% of AML patients.[26] In contrast, MRD detection with RQ-PCR is applicable for individual gene mutations and this technology requires multiple assays to cover all gene mutations in AML. The best studied example in AML are gene mutations in *NPM1*. Multiple studies have shown that the detection of MFC MRD or mutant *NPM1* MRD in AML are strongly correlated with increased risk of relapse.[27-37] These results have paved the way for the incorporation of MRD negative CR as a distinct treatment response entity and accelerated the use of MRD guided post-remission therapy.[15,38] In the recently completed HOVON 132 and ongoing HOVON AML trials intermediate risk AML patients without MFC or mutant *NPM1* MRD are considered for autologous transplantation instead of allogeneic transplantation.[19] However, regardless of the MRD technique used, MRD detection is imperfect. Not all AML patients with MRD develop relapse, while some AML patients without MRD relapse. Furthermore, considering the complex molecular heterogeneity of AML, both techniques have limitations. MFC solely indicate the presence or absence of MRD and RQ-PCR MRD is restricted to single gene mutations in selected AML subsets that often necessitates multiple assays for individual patients.[39] Along with a genomic driven classification, it is imperative to investigate MRD with multiple gene mutations in parallel with next-generation sequencing (NGS).

Acute Myeloid Leukemia in the Era of Next-Generation Sequencing

NGS is a powerful platform to sequence millions of DNA molecules in a single assay.[40] Many different NGS strategies are available, ranging from whole-genome sequencing (WGS) or whole-exome sequencing (WES) to targeted sequencing for a selection of smaller genomic regions of interest. The first AML genome was sequenced by NGS in 2008 and other sequencing strategies in AML have led to the discovery of gene mutations in *DNMT3A*, *ASXL1*, *TET2* and *IDH1*.[41-46] Ongoing advances in NGS technology have revolutionized our understanding of the molecular genetics and pathophysiology of AML, including the identification of molecular drivers and insights in disease ontogeny and clonal evolution patterns.

Identification of Molecular Drivers

For many decades it was thought that initiating events that induces genomic instability were causing tumor formation and progression.[47] Although for AML this seems to be unlikely regarding the high prevalence of normal karyotypes, the origin and evolution of the disease remained obscure. WGS experiments comparing gene mutations in paired normal skin and leukemic blast cells from 24 AML patients uncovered that the majority of mutations were random passenger events that were acquired as a result of aging rather than leukemia initiators that cause genomic instability.[48]

To comprehensively study the molecular basis of AML, the cancer genome atlas research network performed WGS or WES and RNA sequencing of 200 *de novo* AML patients.[49] This consortium identified important leukemic driver mutations with on average only 5 recurrently mutated genes per AML patient. Categorizing AML patients by cytogenetics, aberrant cellular pathways and mutation patterns of mutual exclusivity and co-occurrence, nine functional related groups with a distinct pathophysiology emerged (Table 2), including transcription factor gene fusions (e.g., *CBFB-MYH11, RUNX1-RUNX1T1*), mutant *NPM1*, mutations in DNA methylation genes (e.g., *DNMT3A, TET2, IDH1/2*), chromatin modifier genes (e.g., *ASXL1*, *EZH2*, *KDM6A*) or cohesion and spliceosome genes (e.g. *U2AF1, SMC1A, SMC3*), mutated myeloid transcription factors (e.g., *RUNX1*, *CEBPA*), activated signaling genes (e.g., *FLT3, KIT, KRAS, NRAS*) and mutations in tumor suppressors (e.g., *TP53, WT1, PHF6*). Besides identification of specific functional subgroups other important observations were made. Concurrent mutations differed in their variant allele frequencies (VAF) in individual AML patients, suggesting that AML consist of multiple subclones. The transcription factor gene fusions or mutations in DNA methylation genes were found at higher VAFs and never co-occurred. This implies that gene mutations in these functional groups originate early and possibly represent leukemia initiating events. In contrast, mutations in activated signaling genes were found at lower VAFs and present in all functional groups. It is conceivable that these mutations arise later, indicating a cooperating or transforming character.[50,51] However, functional evidence for these observations was lacking.

Table 2. Functional categories of genes that are frequently mutated in acute myeloid leukemia.

Functional category	Gene mutation	Frequency (%)
Transcription factor fusions	*MYH11-CBFB*, *RUNX1-RUNX1T1*	18
DNA methylation	*DNMT3A*, *IDH1*, *IDH2*, *TET2*	44
Chromatin modification	*ASXL1*, *BCOR*, *EZH2*, *KDM6A*, *KMT2A*	30
Cohesin complex	*RAD21*, *SMC1A*, *SMC3*, *STAG2*	13
Spliceosome complex	*SF3B1*, *SRSF2*, *U2AF1*, *ZRSR2*	14
Nucleophosmin	*NPM1*	27
Transcriptional deregulation	*CEBPA*, *CUX1*, *ETV6*, *GATA2*, *IKZF1*, *RUNX1*	22
Activated signaling	*BRAF*, *CBL*, *CSF3R*, *FLT3*, *JAK2*, *KIT*, *KRAS*, *MPL*, *NF1*, *NRAS*, *NOTCH1*, *PTPN11*	59
Tumor suppressor	*PHF6*, *PTEN*, *TP53*, *WT1*	16

Order of Mutation Acquisition and Clonal Evolution

Several research groups attempt to understand the biological significance of gene mutations by investigating clonal evolution patterns in AML patients. Targeted sequencing of FACS purified AML and residual hematopoietic stem cells along with single cell experiments provided the first insights for the existence of preleukemic gene mutations in otherwise normal hematopoietic stem cells that precede frank leukemia.[52-54] These preleukemic

mutations mostly affected genes involving DNA methylation and epigenetic regulation, including mutations in *DNMT3A* and *TET2*.[53-55] Complementary findings were obtained from AML studies comparing the mutational landscape of paired diagnosis and relapse samples.[56-58] These studies revealed that initiating mutations were present in both diagnostic and relapse samples, whereas others were lost or gained at the time of relapse. The authors concluded that the initiating clone could not be eradicated by therapy and gained additional mutations, which allowed the founding clone to evolve to relapse. Strikingly, mutations in the founding clone were identical to the preleukemic mutations found in non-leukemic cells. This suggested that the cells with mutations that were lost or gained at relapse, represent the transforming descendants of the cells carrying the initiating gene mutations. It became clear that AML consists of multiple subclones, which acquire gene mutations according to an evolutionary hierarchy and possess distinct biological behavior driven by the mutation status and their complex interactions. Large AML cohort studies investigating patterns of co-occurrence and mutual exclusivity of gene mutations and their clonal composition inferred from VAFs, confirmed and validated these findings.[13,14]

Age-Related Clonal Hematopoiesis

Supported by the presence of preleukemic mutations in normal hematopoietic stem cells in patients with AML, three independent studies used NGS to sequence the genome of peripheral blood of over 10 thousands of people without prior hematological disease or cytotoxic treatment.[59-62] The incidence of gene mutations among these otherwise healthy individuals appeared to be unexpectedly high and increasing with age. Although very rare in people in their forties, gene mutations could be detected in over 10% of the individuals above the age of 70. The mutations persisted for many years and were most frequently detected in genes involved in DNA methylation and epigenetic regulation. About 80% of the gene mutations are found in *DNMT3A*, *TET2* and *ASXL1*. Although the presence of these clones was associated with a 10-fold risk of progression, only less than 1% of the individuals eventually developed a hematological malignancy, including AML.[61,63,64] Following these results, another group investigated 50-60 year old healthy individuals with improved NGS platforms that enabled mutation detection over 60 times more sensitive. They confirmed the presence of mutations in *DNMT3A* and *TET2* in virtually every healthy individual (95%).[65] It is thought that the persistence and expansion of clones over time is sustained by enhanced fitness of the hematopoietic stem cells, however, the exact mechanisms of leukemic initiation and progression are not fully understood.[66-68] Corroborating the findings of clonal evolution studies, this entity is entitled clonal hematopoiesis of indeterminate potential (CHIP) or age-related clonal hematopoiesis (ARCH).[69]

Acute Myeloid Leukemia Ontogeny

Regarding its clinical ontogeny several forms of AML can be distinguished. When AML arises from antecedent malignancies (e.g., myelodysplatic syndrome or MDS), the condition is defined secondary AML, whereas AML without prior disease is considered *de novo* AML.[70] Certain molecular abnormalities, such as the core binding factor (CBF) gene fusions, are disease defining for AML and never occur in MDS.[71,72] Therefore, the detection of CBF is sufficient to confirm AML, including in patients with a blast percentage below 20%.[70] NGS studies comparing gene mutations in clinically defined *de novo* and secondary AML patients revealed high specificity of CBF and mutant *NPM1* for *de novo* AML and *ASXL1*, *BCOR*, *EZH2*, *SF3B1*, *SRSF2*, *STAG2*, *U2AF1* and *ZRSR2* gene mutations for secondary AML.[73-75] This indicates that AML ontogeny can be retrieved on the basis of gene mutations and implies that *de novo* AML and secondary AML are distinct disease entities, irrespective of a fixed blast percentage. However, in contrast to other gene mutations, *TP53* mutations are found in both *de novo* and secondary AML. Despite distinct clinical and molecular features of AML with mutated *TP53*, the clinical ontogeny and molecular characteristics of mutant *TP53* AML and MDS remains elusive and requires further investigation.[13,14,49,76]

Molecular Residual Disease by Next-Generation Sequencing

Considering the genomic driven disease classification and complex molecular clonal architecture of AML, there is growing interest to investigate molecular MRD by NGS during treatment. However, current NGS platforms are not designed for mutation detection at low levels for sufficient MRD analysis. There is only one initial study that subjected remission samples of 50 AML patients to targeted sequencing.[77] Although this is the first proof of principle study that revealed the feasibility of molecular MRD by NGS in AML, large scale application needs to be investigated. High quality clinical data and available patient samples from HOVON trials afforded a unique opportunity to study molecular MRD detection with NGS on a large scale.

AIMS AND OUTLINE OF THIS THESIS

The objective of this thesis was to further refine risk classification at diagnosis (aim 1) and investigate the value of molecular MRD detection by NGS during treatment (aim 2), with the ultimate goal to improve relapse prediction in AML.

In **Chapter 2** we addressed the first aim and studied the clinical and molecular characteristics of mutant *TP53* in a large cohort of 2,200 newly diagnosed AML and MDS-EB. We performed next-generation sequencing to assess the *TP53* mutant allelic status (mono- of bi-allelic), the number of *TP53* mutations, *TP53* clone size, concurrent mutations and cytogenetics and associate these characteristics with overall survival. By comparing the characteristics of mutant *TP53* AML and MDS-EB we assessed differences and commonalities between these myeloid malignancies.

For the second aim of this thesis we assessed the applicability and prognostic value of molecular MRD by NGS. In **Chapter 3**, we performed NGS on 482 AML patients at diagnosis and in CR and studied the rate of persistence of mutations and their allele frequencies. We investigated the incidence of residual ARCH in CR and its distinction with mutations that represent residual leukemia by examining the association with relapse risk. Subsequently, we investigated the prognostic value for relapse of mutations that occur late in leukemogenesis in a subset of AML patients with *FLT3*-ITD in **Chapter 4**. To this end, we assessed the stability of *FLT3*-ITD by comparing AML samples at diagnosis and during relapse. We performed NGS-based deep sequencing of mutant *NPM1* and *FLT3*-ITD in CR and comprehensively investigate the impact of *FLT3*-ITD MRD on treatment outcome in AML. Finally, we aimed to identify mutant *TP53* AML patients with better outcomes by correlating mutant *TP53* deep sequencing in CR with outcome. These results are presented in **Chapter 2**.

In the last chapter of this thesis the results are summarized and discussed. There will be special focus on the applicability and implementation of this work in molecular diagnostics and molecular MRD detection during treatment in clinical practice with directions for future research.

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2

MOLECULAR CHARACTERIZATION OF MUTANT *TP53* ACUTE MYELOID LEUKEMIA AND HIGH-RISK MYELODYSPLASTIC SYNDROME

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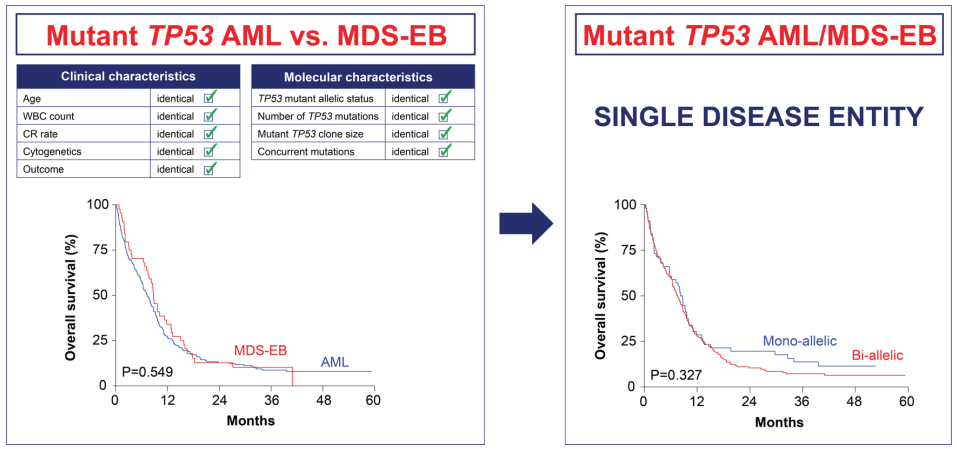
ABSTRACT

Substantial heterogeneity within mutant *TP53* acute myeloid leukemia (AML) and myelodysplastic syndrome with excess of blast (MDS-EB) precludes the exact assessment of prognostic impact for individual patients. We performed in-depth clinical and molecular analysis of mutant *TP53* AML and MDS-EB to dissect the molecular characteristics in detail and determine its impact on survival. We performed next-generation sequencing on 2200 AML/MDS-EB specimens and assessed the *TP53* mutant allelic status (mono- or bi-allelic), the number of *TP53* mutations, mutant *TP53* clone size, concurrent mutations, cytogenetics, and mutant *TP53* molecular minimal residual disease and studied the associations of these characteristics with overall survival. *TP53* mutations were detected in 230 (10.5%) patients with AML/MDS-EB with a median variant allele frequency of 47%. Bi-allelic mutant *TP53* status was observed in 174 (76%) patients. Multiple *TP53* mutations were found in 49 (21%) patients. Concurrent mutations were detected in 113 (49%) patients. No significant difference in any of the aforementioned molecular characteristics of mutant *TP53* was detected between AML and MDS-EB. Patients with mutant *TP53* have a poor outcome (2-year overall survival, 12.8%); however, no survival difference between AML and MDS-EB was observed. Importantly, none of the molecular characteristics were significantly associated with survival in mutant *TP53* AML/MDS-EB. In most patients, *TP53* mutations remained detectable in complete remission by deep sequencing (73%). Detection of residual mutant *TP53* was not associated with survival. Mutant *TP53* AML and MDS-EB do not differ with respect to molecular characteristics and survival. Therefore, mutant *TP53* AML/MDS-EB should be considered a distinct molecular disease entity.



INTRODUCTION

Mutations in *TP53* are present in approximately 10% of patients with acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) and represent a unique subtype with poor outcome.[1-5] *TP53* is located on chromosome 17p13 and is essential for cell cycle control and DNA damage response.[6] Although the exact mechanism of leukemogenesis for mutant *TP53* AML remains unknown, it has been shown that some *TP53* mutations drive a dominant negative effect and typically occur in founding clones that expand after cytotoxic stress.[7,8] Mutant *TP53* is strongly associated with large structural and complex chromosomal aberrations, as illustrated by the co-occurrence of complex karyotypes (CK), which is associated with reduced overall survival in myeloid malignancies.[9-11] In line with the observed poor outcome, mutant *TP53* AML is assigned to the adverse risk category of the 2017 European LeukemiaNet (ELN) risk classification and is recommended to receive intensive consolidation treatments.[11] Although patients with mutant *TP53* AML in complete remission (CR) generally receive allogeneic hematopoietic stem cell transplantation (HSCT), relapse rates remain considerably high.[12]

Recent findings in MDS assign additional prognostic value to the molecular characteristics of mutant *TP53*, including *TP53* mutant allelic status (mono- or bi-allelic) and *TP53* clone size.[4,5,13] However, recent studies exploring the link between these molecular characteristics and outcome in AML were limited and inconclusive.[14,15] Furthermore, it is currently unknown whether mutant *TP53* high risk MDS with excess of blast (MDS-EB) and AML differ in molecular makeup and response to treatment and should be considered as separate entities.

Here, we present an in-depth characterization of a large cohort of newly diagnosed mutant *TP53* AML and MDS-EB in relation to survival. We performed next-generation sequencing (NGS) to assess the molecular characteristics of mutant *TP53* AML/MDS-EB in detail, including *TP53* mutant allelic status (mono- or bi-allelic), the number of *TP53* mutations, mutant *TP53* clone size, concurrent mutations, cytogenetics, and molecular minimal/measurable residual disease (MRD).

METHODS

Patients and Samples

In total, 2200 patients with AML and MDS-EB (international prognostic scoring system [IPSS] ≥ 1.5 or revised IPSS > 4.5) were assessed for eligibility and treated in the Haemato-Oncology Foundation for Adults in the Netherlands and Swiss Group for Clinical Cancer Research (HOVON-SAKK) clinical trials between 2001 and 2017 (supplemental Figure 1). All patients received standard induction chemotherapy and were consolidated according to the HOVON-SAKK study protocols. Details of treatment protocols were described previously (www.hovon.nl).[16-21] All trial participants provided written informed consent in accordance with the Declaration of Helsinki. DNA was isolated from diagnostic bone marrow samples of 2200 patients with AML/MDS-EB and 537 CR samples (supplementary Methods). In 33 patients with AML/MDS-EB carrying *TP53* variants with a variant allele frequency (VAF) >40%, DNA from saliva was available to verify the germline status.

Cytogenetics and SNP Array Analyses

Cytogenetic analysis was carried out at the local reference centers using standard protocols. These data, including karyotypes and FISH, were centrally peer-reviewed by clinical genetics laboratory specialists. The clonal structural and numerical chromosomal abnormalities were reported in accordance with the International System for Human Cytogenetic Nomenclature and the ELN 2017 recommendations.[11] CK was defined by 3 or more unrelated chromosome abnormalities in the absence of one of the World Health Organization–designated recurring translocations or inversions, that is, t(8;21), inv(16) or t(16;16), t(9;11), t(v;11) (v;q23.3), t(6;9), inv(3) or t(3;3), AML with *BCR-ABL1*.[11] Single nucleotide polymorphism (SNP) array was performed according to the manufacturer's instructions using Illumina Infinium GSA+MD-24 version 3.0 BeadChip (Illumina, Inc., San Diego, CA) on 134 of 230 mutant *TP53* AML samples (110 bi-allelic and 24 mono-allelic mutants). The array was scanned with the Illumina iScan Control. Genome studio version 2.1 and Nexus Discovery version 10.0 (Biodiscovery, El Segundo, CA) were used for data analysis.

Targeted NGS and *TP53* Deep Sequencing

The TruSight Myeloid Sequencing panel (Illumina) was used to detect the presence of driver mutations at diagnosis. Details were described previously.[22] Only pathogenic *TP53* variants were included as defined by occurrence in the COSMIC and IARC TP53 database as well as by analyses in silico with programs such as Polyphen-2, SIFT, FATHMM, MetaSVM, MetaLR, CADD, DANN, and ClinVar. The limit of detection was VAF 1% at diagnosis. To detect *TP53* mutations in CR, we used Illumina-based deep sequencing (supplementary Methods). The limit of detection in the follow-up samples was VAF 0.001% (variable depending on *TP53* mutation type). In the case of multiple *TP53* mutations, the highest VAF was chosen for MRD analysis. Of note, patients with *TP53* germline mutations were excluded from MRD assessment (n = 2).

Allocation of Patients Based on *TP53* Mutant Allelic Status

Patients with mutant *TP53* AML/MDS-EB were considered bi-allelic when (1) 2 or more *TP53* gene variants were detected, regardless of the VAF; (2) at least 1 *TP53* gene variant co-occurred with a cytogenetic aberration involving chromosome 17p (eg, abnormality of 17p or monosomy 17); or (3) *TP53* mutations were detected with a VAF >55% (supplemental Figure 2). The allocation to the bi-allelic mutant *TP53* group by a VAF threshold of >55% was confirmed in all 15 of 110 patients with bi-allelic mutant *TP53* AML that could be evaluated for copy number alterations by SNP array analyses (ie, either loss of the wild-type allele or segmental uniparental disomy of the mutant *TP53* allele).

Statistical Analysis

Associations between variables were tested by the Fisher's exact test for categorical variables and by the Mann-Whitney *U* test for continuous variables. The primary endpoint of the study was overall survival, defined as death from any cause. Survival time was calculated from the start of induction chemotherapy until the event of interest or censoring. Of note, the survival time in the analysis evaluating allogeneic HSCT started at the date of transplant. To compare the survival distributions, we used the log-rank test and the Cox proportional hazards model. The proportional hazards assumption was tested by interaction with time. All *P* values were two sided, and *P* values <.05 were considered statistically significant. Statistical analyses were executed with Stata Statistical Software, Release 16.0 (College Station, TX).

RESULTS

Molecular Characteristics of Mutant *TP53* AML and MDS-EB

We detected 283 *TP53* mutations in 230 of 2200 (10.5%) patients with AML/MDS-EB by NGS (Table 1; supplemental Figure 1). Of 230 patients with AML/MDS-EB, 44 (19%) were diagnosed with MDS-EB. No significant difference in age, sex, white blood cells, remission rate, and consolidation treatment was present between AML and MDS-EB (Table 1). Deletion 5q was the only cytogenetic aberration significantly more frequently present in MDS-EB (P = .025). Of note, in 112 patients with mutant *TP53* AML/MDS-EB, concurrent chromosomal aberrations involving *TP53* (eg, abnormality 17p or loss of chromosome 17) were detected.

Two or more *TP53* mutations were found in 49 AML/MDS-EB cases (Table 2; supplemental Figure 2). In total, 206 missense, 16 nonsense, 38 insertion/deletion, and 23 splice-site mutations were detected (supplemental Figure 3). Nearly all missense mutations occurred in the TP53 DNA binding domain (supplemental Figure 3). In total, 56 of the 230 patients with *TP53* mutant AML/MDS-EB (24.3%) were considered mono-allelic and 174 (75.7%) were bi-allelic (Table 2; supplemental Figure 2). The mutant *TP53* clone size was normally distributed with a median VAF of 47% (supplemental Figure 4A). Concurrent mutations were detected in only 113 (49%) patients with mutant *TP53* AML/MDS-EB (Figure 1). The most frequent concurrent mutations were detected in *DNMT3A*, *TET2*, *ASXL1*, *RUNX1*, and *SRSF2* (Figure 1; Table 2). The *TP53* mutant allelic status, number of *TP53* mutations, *TP53* clone size, and concurrent mutations at diagnosis did not significantly differ between mutant *TP53* AML and MDS-EB (Table 2).

Of note, most (84%) patients with mutant *TP53* AML/MDS-EB have CK, and many associations between CK and the different molecular characteristics were observed (Table 1; supplemental Table 1). CK was detected in most patients with bi-allelic mutant *TP53* (97%), in patients with multiple *TP53* mutations (94%), and in patients with larger *TP53* clones (94% in VAF >40%) (supplemental Table 1; supplemental Figure 4B). Concurrent mutations were enriched in AML/MDS-EB marked by non-CK, yet the most prevailing mutated genes (*DNMT3A* and *TET2*) were not significantly associated with CK (supplemental Table 1).

Table 1. Patient characteristics of AML/MDS-EB with mutated *TP53*.

	AML (n=186)	MDS-EB (n=44)	AML/MDS-EB (n=230)	
Age - yr				p=0.820
Median	62	63	62	
Range	18-80	35-73	18-80	
Sex - no. (%)				p=0.736
M	111 (60)	25 (57)	136 (59)	
F	75 (40)	19 (43)	94 (41)	
WBC at diagnosis - no. (%) *				
≤100	183 (99)	44 (100)	227 (99)	p=1.000
>100	2 (1)	0 (0)	2 (1)	
Last treatment before first CR - no. (%)				p=0.106
Refractory	70 (38)	10 (23)	80 (35)	
Cycle I	90 (48)	29 (66)	119 (52)	
Cycle II	26 (14)	5 (11)	31 (13)	
Consolidation therapy - no. (%)				p=1.000
No allogeneic HSCT	137 (74)	33 (75)	170 (74)	
Allogeneic HSCT	49 (26)	11 (25)	60 (26)	
Cytogenetics - no. (%)†				
Monosomy 5	51 (28)	11 (27)	62 (28)	p=1.000
Deletion 5q	78 (44)	26 (63)	104 (47)	p=0.025
Monosomy 7	58 (32)	14 (34)	72 (33)	p=0.855
Monosomy 17	71 (40)	10 (24)	81 (37)	p=0.075
Abnormality 17p	33 (18)	6 (15)	39 (18)	p=0.656
Complex karyotype	148 (83)	37 (90)	185 (84)	p=0.343
Monosomal karyotype	139 (78)	35 (85)	174 (79)	p=0.394

*Numbers may not sum to 230 because of missing values.
†Cytogenetics failed in 10 patients.

Table 2. Molecular characteristics of mutant *TP53* AML/MDS-EB.

	AML (n=186)	MDS-EB (n=44)	AML/MDS-EB (n=230)	
***TP53* mutant allelic status - no. (%)**				p=0.241
Mono-allelic	42 (23)	14 (32)	56 (24)	
Bi-allelic	144 (77)	30 (68)	174 (76)	
Number of *TP53* mutations - no. (%)				p=0.153
Single	150 (81)	31 (70)	181 (79)	
Multiple	36 (19)	13 (30)	49 (21)	
Mutant *TP53* clone size - VAF (%)				p=0.409
Median	48	41	47	
Range	1-97	3-91	1-97	
Mutation at diagnosis - no. (%)				
any concurrent	95 (51)	18 (41)	113 (49)	p=0.244
DNMT3A	25 (13)	6 (14)	31 (13)	p=1.000
TET2	17 (9)	3 (7)	20 (9)	p=0.773
ASXL1	10 (5)	2 (5)	12 (5)	p=1.000
RUNX1	10 (5)	1 (2)	11 (5)	p=0.695
SRSF2	11 (6)	1 (2)	12 (5)	p=0.471

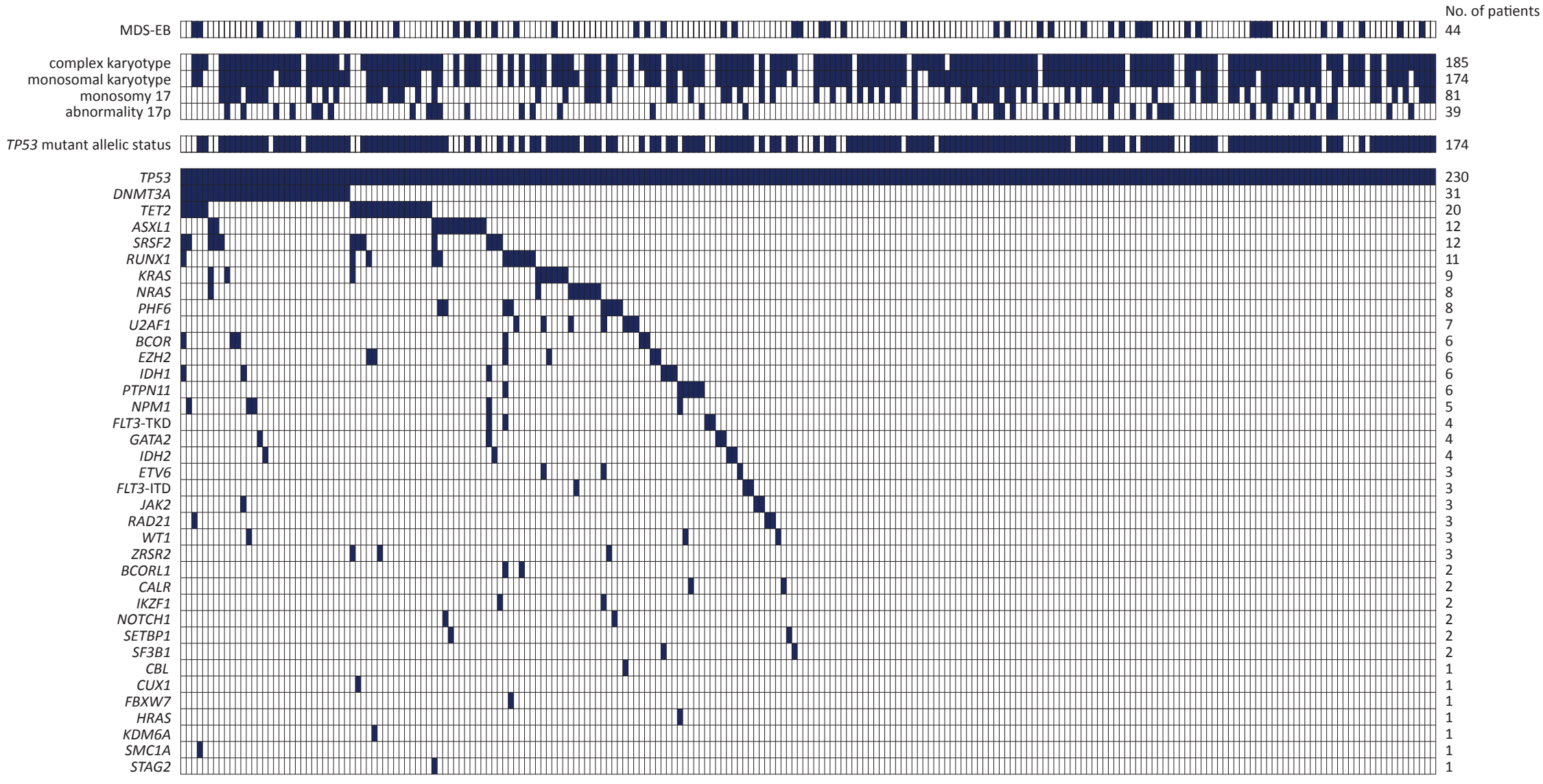


Figure 1. Overview of cytogenetic aberrations and concurrent mutations in mutant *TP53* AML/MDS-EB (n=230). Each column represents an invidual patient and the presence of the aberration is indicated in blue. The upper panel shows the cytogenetic aberrations and the lower panel the concurrent mutations. MDS-EB patients or bi-allelic *TP53* mutant status is also indicated in blue. In case of failed cytogenetics the cytogenetic aberrations were considered negative.

Association of Mutant *TP53* Characteristics and Outcome in AML and MDS-EB

We next compared outcome of patients with mutant *TP53* AML/MDS-EB in relation to the established ELN 2017 prognostic subgroups. Mutant *TP53* strongly associated with reduced survival in the context of the ELN 2017 adverse risk category (2-year overall survival, 12.8% *TP53* mutant vs 42.5% *TP53* wild-type; $P < .001$) (Figure 2A). Because of the molecular homogeneity of mutant *TP53* AML and MDS-EB, we investigated whether the AML or MDS-EB status associated with survival. No difference in outcome was observed between the AML and MDS-EB mutant *TP53* subgroups ($P = .549$) (Figure 2B). All our findings indicate that mutant *TP53* AML/MDS-EB represents a homogeneous group and is therefore considered a singular entity in the following analysis.

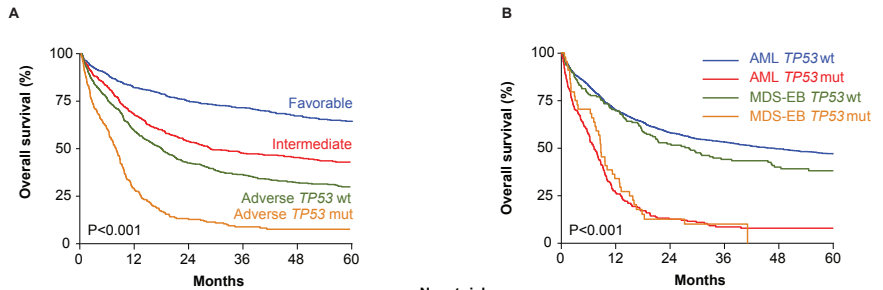


Figure 2. Overall survival of AML/MDS-EB patients by the ELN 2017 risk classification (n=2,200).
Patient in the adverse risk category are segregated by *TP53* wild type and *TP53* mutant. Overall survival of AML and MDS-EB disease classification at diagnosis stratified by *TP53* wild type and *TP53* mutant patients (B).

We performed survival analysis to evaluate the relationship of molecular characteristics and cytogenetic aberrations to outcome in mutant *TP53* AML/MDS-EB. Mono-allelic mutant *TP53* AML/MDS-EB had a similar dismal survival compared with its bi-allelic counterpart ($P = .327$) (Figure 3A; supplemental Figure 5). Neither the number of *TP53* mutations (Figure 3B) nor aberrations involving chromosome 17 (supplemental Figure 6) associated with altered outcome in patients with mutant *TP53* AML/MDS-EB. Concurrent mutations conferred limited but detectable survival benefit (Figure 3C), whereas the presence of specific concurrent mutations provided no further survival advantage (supplemental Figure 7). Clone size, realized by taking decreasing *TP53* mutation VAF thresholds and continuous modeling per 10% VAF, was investigated for impact on outcome. None of the mutant *TP53* VAF thresholds significantly associated with survival: VAF 50% ($P = .990$); VAF 40% ($P = .257$); VAF 30% ($P = .064$); VAF 20% ($P = .189$); VAF 10% ($P = .161$); and VAF 5% ($P = .226$) (supplemental Figure 8A-F) (hazard ratio per 10% VAF, 1.04; 95% CI, 0.99-1.09; $P = .141$). Hence, the molecular characteristics of mutant *TP53* AML/MDS-EB did not evidently relate to treatment outcome.

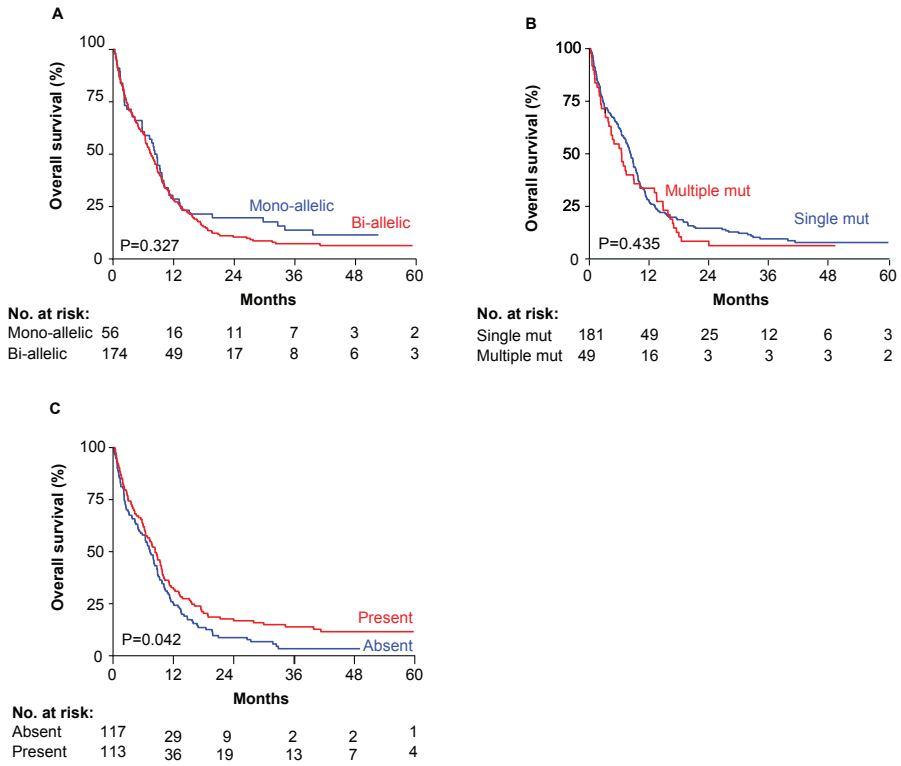


Figure 3. Overall survival of molecular characteristics in mutant *TP53* AML/MDS-EB (n=230).
Overall survival of *TP53* mutant allelic status (mono-allelic versus bi-allelic) (A), the number of *TP53* mutations (B) and the presence or absence of concurrent mutations (C).

In line with previous work, we confirmed that CK associates with reduced survival in *TP53* mutant AML/MDS-EB (2-year overall survival, 9% CK vs 34% non-CK; $P = .002$), regardless of type of consolidation therapy (Figure 4).[9,10] However, the overall survival of patients with non-CK *TP53* mutant AML/MDS remains poor. Because of strong association of CK with all mutant *TP53* molecular characteristics, no further stratification was feasible among patients with AML/MDS-EB with CK (supplemental Table 1). Of note, CK AML/MDS-EB with wild-type *TP53* appeared to have a significantly improved outcome in our cohort of 2200 AML/MDS-EB cases as compared with CK in the context of mutant *TP53* (Figure 4C), indicating that the presence of mutant *TP53* at diagnosis defines a separate CK entity.

Sensitivity analysis, performed to identify potential treatment modification within trial protocols, yielded no significant interactions. Similar results were obtained when elderly patients with AML were excluded (data not shown).

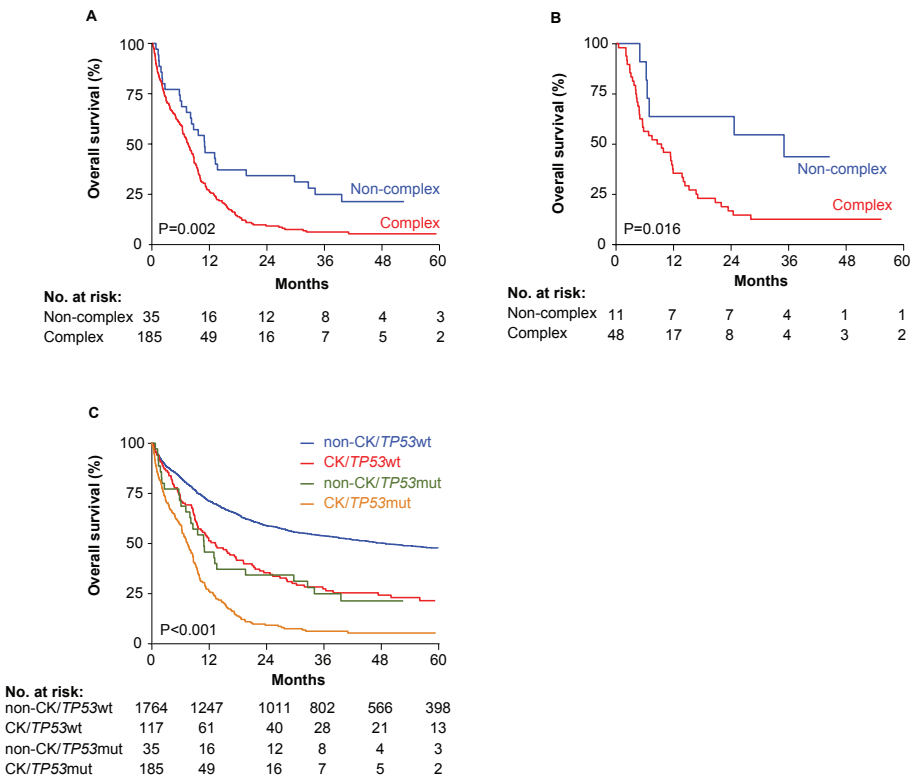


Figure 4. Overall survival of patients with AML/MDS-EB by CK.
Overall survival of mutant *TP53* AML/MDS-EB patients by complex karyotype (A) and of mutant *TP53* AML/MDS-EB by complex karyotype in patients who received allogeneic hematopoietic stem cell transplantation (B). Of note, the survival time starts at the date of transplant. Overall survival AML/MDS-EB patients by CK and mutant *TP53* status (C).

Molecular Minimal Residual Disease in Mutant *TP53* AML

Detection of molecular MRD is an important prognostic marker in AML.[22-24] We performed deep targeted sequencing on complete morphological remission bone marrow samples from 62 patients with mutant *TP53* AML to assess molecular MRD. Mutant *TP53* is often the only suitable marker for molecular MRD detection because the prevalence of concurrent mutations at diagnosis is relatively low and most concurrently mutated genes may associate with antecedent clonal hematopoiesis (*DNMT3A*, *TET2*, and *ASXL1*) rather than residual leukemia (Figure 1). In total, 45 of 62 patients with AML/MDS-EB had detectable *TP53* mutations in CR, for which the status did not associate with overall survival (*P* = .653) (Figure 5).

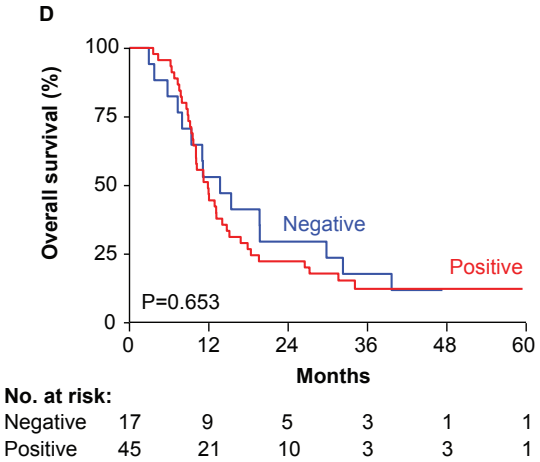


Figure 5. Overall survival of AML/MDS-EB by *TP53* mutations detected in complete remission (n=62).

DISCUSSION

Substantial heterogeneity within the mutant *TP53* AML/MDS-EB subgroup on a clinical and molecular level precludes the exact assessment on prognostic impact for individual patients with AML/MDS-EB. Here, we report the detailed molecular characterization of mutant *TP53* in a large cohort of patients with AML and MDS-EB. No significant differences in the distribution of *TP53* molecular characteristics and outcome between patients with AML and MDS-EB were observed. In fact, the 5-year overall survival of patients with mutant *TP53* AML and MDS-EB in our study is similar to others.[13] Mutant *TP53* AML/MDS-EB represents a molecular homogeneous group with distinct clinicopathologic characteristics and outcomes. Therefore, we propose that mutant *TP53* MDS-EB and AML should be considered a single entity, regardless of the requisite blast percentage at diagnosis.

Recent studies revealed important associations of *TP53* mutant allelic status and mutant *TP53* clone size with a more favorable outcome for patients with MDS and AML.[5,13,15] These studies established significant associations and interactions of *TP5*3 mutant allelic status and mutant *TP53* clone size with CK. Remarkably, in our study based on a substantial number of patients with mutant *TP53* AML and MDS-EB undergoing standard induction chemotherapy, we did not reveal an association between any of the *TP53* molecular characteristics and survival. Although the distribution of molecular characteristics and outcome of mutant *TP53* AML and MDS-EB in HOVON-SAKK clinical trials is comparable to other clinical trials, our analysis did not include low-risk MDS patients who often associate with non-CK.[5,13] It is thought that the presence of wild-type *TP53* is critical for maintaining chromosomal stability. During progression from MDS to high-risk MDS-EB or AML, mutant *TP53* clones often become bi-allelically mutated and genomically unstable, which is reflected by the strong association between bi-allelic *TP53* mutants and CK in our study. However, some patients with mono-allelic mutant *TP53* AML/MDS-EB also had CK. In 8 (2 non-CK and 6 CK) of 24 patients with mono-allelic mutant *TP53* AML/MDS-EB in whom high-quality DNA was available, we indeed confirmed, by SNP array analyses, the presence of uniparental disomy or focal 17p deletions that had been missed with conventional cytogenetics. Those patients can easily be misclassified as having mono-allelic *TP53* mutation. Reallocation of these 8 patients with mono-allelic mutant *TP53* AML/MDS-EB to the mutant *TP53* bi-allelic group did not affect our results (data not shown). Additional studies are required to investigate whether other (epigenetic) mechanisms are affecting the wild-type *TP53* allele in mono-allelic cases without copy number alterations. Altogether, our results indicate that further stratification by the molecular characteristics of mutant *TP53* appears to be less relevant when patients have progressed to MDS-EB or AML.

Although molecular MRD has prognostic value for predicting impending relapse in AML/MDS-EB,[22-24] we did not observe such association in mutant *TP53* AML/MDS-EB. Despite using deep sequencing, which revealed MRD in most cases, molecular MRD detection in mutant *TP53* AML/MDS-EB did not yield prognostic value. It is conceivable that all patients with mutant *TP53* AML/MDS-EB achieving CR have MRD, sometimes at levels undetectable with current NGS approaches. In fact, the high relapse rates in patients with AML/MDS-EB without detectable mutant *TP53* MRD in CR illustrates the critical role of mutant *TP53* in chemotherapeutic response and implies that small refractory clones are present below our NGS detection limit.[7] Although concurrent mutations are present in mutant *TP53* AML/MDS-EB, mutant *TP53* itself appeared to be exclusive in half of the patients. Of note, most concurrent mutations are known contributors of age-related clonal hematopoiesis, in which we and others previously showed lack of prognostic significance.[25,26] Although the applicability of molecular MRD detection in patients with mutant *TP53* AML/MDS-EB in our study is limited, future clinical trials with new drugs and other quantified MRD endpoints may benefit from molecular MRD detection based on mutant *TP53*.

Although very poor, better overall survival is observed in a minority of patients with AML/MDS-EB with non-CK mutant *TP53*. Possible explanations for the improved outcome in selected cases may be the enrichment of single *TP53* mutations with low VAFs as well as higher frequencies of concurrent mutations in this group, indicating that mutant *TP53* in these cases may represent clonal hematopoiesis rather than subclonal disease. Previous work in therapy-related AML indicates that mutant *TP53* will eventually be the founding clone[7]; however, additional studies, including those involving relapse of patients with non-CK mutant *TP53*, are needed to demonstrate that mutant *TP53* may be responsible for early relapse. Nevertheless, whether patients with mono-allelic non-CK mutant TP53 AML/MDS-EB have better outcome requires additional investigations on larger numbers of patients.

In conclusion, from a clinical and molecular perspective, we propose to consider mutant *TP53* AML/MDS-EB a distinct disease entity.

ACKNOWLEDGEMENTS

The authors thank all the participating centers of the Dutch–Belgian Cooperative Trial Group for Hematology–Oncology (HOVON) and Swiss Group for Clinical Cancer Research (SAKK), where the clinical trials that formed the basis for this study were conducted; Eric Bindels for performing next-generation sequencing; Remco Hoogenboezem for assisting with bioinformatics; and Egied Simons for assisting with preparation of the figures (Erasmus University Medical Center). This work was supported by grants from the Dutch Cancer Society “Koningin Wilhemina Fonds” (2020-12507). A.S.A.A.H. is a recipient of a PhD scholarship from the Ministry of Health of the Sultanate of Oman.

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SUPPLEMENTARY METHODS

Patient Samples and DNA Isolation

Follow up samples were taken at least 21 days after the start of the last induction cycle. If additional CR samples were available, the sample preceding consolidation therapy was chosen. When patients did not receive any consolidation therapy, the last sample within four months after the start of the last induction cycle was selected.

The majority of AML samples were purified by Ficoll-Hypaque centrifugation (Nygaard, Oslo, Norway), cryopreserved and subsequently lysed in RLT buffer (Qiagen, Venlo, the Netherlands). High quality DNA was extracted using the QIASymphony DSP DNA Mini Kit according to the manufacturer's instructions (Qiagen, Venlo, the Netherlands). DNA concentration was measured by Qubit Fluorometric Quantitation (Thermo Fisher Scientific, Wilmington, DE).

Targeted NGS and Data Analysis

The TruSight Myeloid Sequencing Panel libraries were paired-end sequenced (2x221bp) on an Illumina HiSeq 2500 System (Illumina, San Diego, CA) in Rapid Run mode or on a Illumina MiSeq System (Illumina, San Diego, CA). The vast majority of amplicon target regions were completely paired-end sequenced. Overlap-based error-correction was utilized to attenuate any form of strand-specific error biases. Error-corrected paired-end reads aligned to the human genome version 19 (hg19) with BBMAP3 followed by quality control to determine cases with insufficient number of reads for adequate variant calling. Single nucleotide variants (SNVs) and insertions-deletions (indels) at diagnosis were determined by MuTect, Samtools, GATK, Varscan, Indelocator and Pindel. Variant allele frequencies (VAF) of mutations detected at diagnosis were calculated as the ratio between the number of mutant and total reads. Pathogenic *TP53* variants were defined by occurrence in the COSMIC and IARC TP53 database as well as by analyses in silico with programs, such as Polyphen-2, SIFT, FATHMM, MetaSVM, MetaLR, CADD, DANN and ClinVar.

TP53 Deep Sequencing

For deep sequencing template specific primers for *TP53* mutation NGS analysis were designed by using the Ion AmpliSeq Designer software (ThermoFisher Scientific, Bleiswijk). The *TP53* specific primers (see below) were adapted for Illumina-based sequencing by adding an Illumina forward (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3'-[locus-specific sequence] or reverse (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3')-[locus-specific sequence] overhang adapter sequence to the *TP53* specific primers.

In the second PCR, by means of these adapter sequences, sample-specific dual indices for sample identification and Illumina sequencing adapters are attached by using unique dual index primers from the IDT for Illumina Nextera DNA UD Index Kit (Illumina, San Diego, CA, USA). Two multiplex reactions were carried out (see below). The *TP53* loci of interest were amplified by multiplex PCR on 100ng genomic DNA using the Roche FastStart High Fidelity PCR System (Roche) containing 1× Buffer with 1.8mM MgCl2, 0.2mM dNTP, ~0.4µM each primer, 0.1U FastStart Taq DNA polymerase. Amplification was performed using the following thermocycling conditions: 95°C for 5 minutes, 25 cycles of 30 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C and a final extension for 7 minutes at 72°C. Amplicons from the first step PCR were purified using the Agencourt AMPure XP bead purification kit (Beckman Coulter, Fullerton, CA, USA).

The second step PCR was performed with primers from the IDT for Illumina Nextera DNA UD Index Kit (Illumina, San Diego, CA) using the KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Wilmington, MA, USA) with the following thermocycling conditions: 95°C for 3 minutes, 10 cycles of 20 seconds at 95°C, 30 seconds at 55°C, and 30 seconds at 72°C and a final extension for 5 minute at 72°C.

The library pool was purified with Agencourt AMPure XP beads and normalized for Illumina-based sequencing, according to the manufacturer's protocol (Illumina, San Diego, CA). The NGS libraries were paired-end sequenced (2×221-bp) on the MiSeq System (Illumina, San Diego, CA) according to manufacturer's recommendation (Illumina, San Diego, CA). NGS data analysis to detect variants at a low detection level is performed as described before (Jongen-Lavrencic et al., 2018). The maximum limit of detection in the follow up samples was VAF 0.001% (dependent on type of *TP53* mutation).

SUPPLEMENTARY FIGURES

Table S1. Molecular characteristics of mutant *TP53* AML/MDS-EB by complex karyotype.

	Complex karyotype (n=185)	Non-complex karyotype (n=35)	
***TP53* mutant allelic status - no. (%)**			p<0.001
mono-allelic	20 (11)	30 (86)	
bi-allelic	165 (89)	5 (14)	
No. of *TP53* mutations - no. (%)			p=0.044
Single	140 (76)	32 (91)	
Multiple	45 (24)	3 (9)	
***TP53* clone size - VAF (%)**			p<0.001
Median	53	11	
Range	2-97	1-97	
Cytogenetics - no. (%)			
monosomy 5	62 (34)	0 (0)	p<0.001
deletion 5q	101 (55)	3 (9)	p<0.001
monosomy 7	68 (37)	4 (11)	p=0.003
monosomy 17	80 (43)	1 (3)	p<0.001
abnormality 17p	39 (21)	0 (0)	p=0.001
monosomal karyotype	171 (92)	3 (9)	p<0.001
Mutation at diagnosis - no. (%)			
any concurrent	84 (45)	26 (74)	p=0.003
DNMT3A	25 (14)	6 (17)	p=0.597
TET2	16 (9)	4 (11)	p=0.534
ASXL1	6 (3)	5 (14)	p=0.017
RUNX1	6 (3)	5 (14)	p=0.017
SRSF2	4 (2)	7 (20)	p<0.001

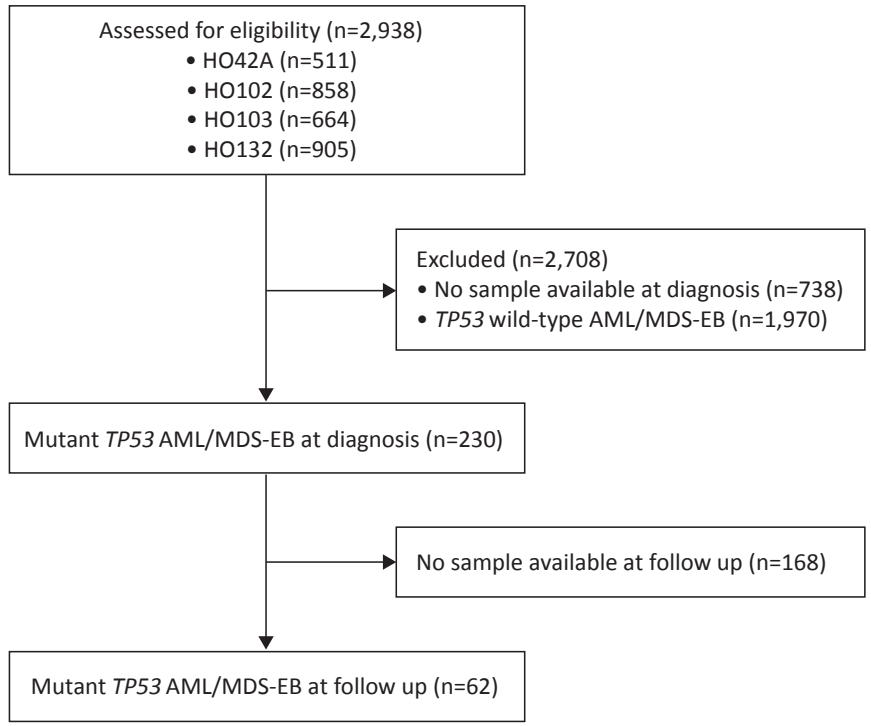

Figure S1. Consort diagram of mutant *TP53* study.
Abbreviations: HO, HOVON-SAKK, Dutch-Belgian Hemato-Oncology Cooperative Group and the Swiss Group for Clinical Cancer Research.

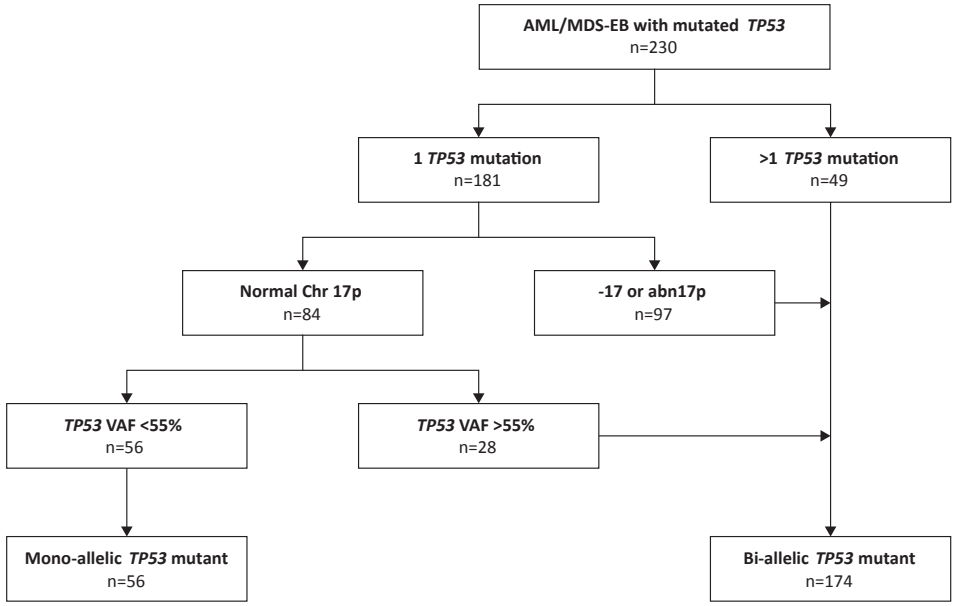

Figure S2. Consort diagram of the mutant *TP53* allelic status of 230 AML/MDS-EB patients.
AML/MDS-EB patients with multipele *TP53* mutations or with mutant *TP53* combined with concurrent chromosome 17 aberrations (eg. monosomy 17 of abnormality 17p) were assigned as bi-allelic *TP53* mutant. In *TP53* AML/MDS-EB without chromosome 17 aberrations, patients with a mutant *TP53* variant allele frequency above 55%, were also considered bi-allelic *TP53* mutant. In 10 patients cytogenetic data was missing. Of note, 4 patients with missing cytogenetics were considered bi-allelic *TP53* mutant based on the presence of multiple *TP53* mutations or a variant allele frequency above 55%.

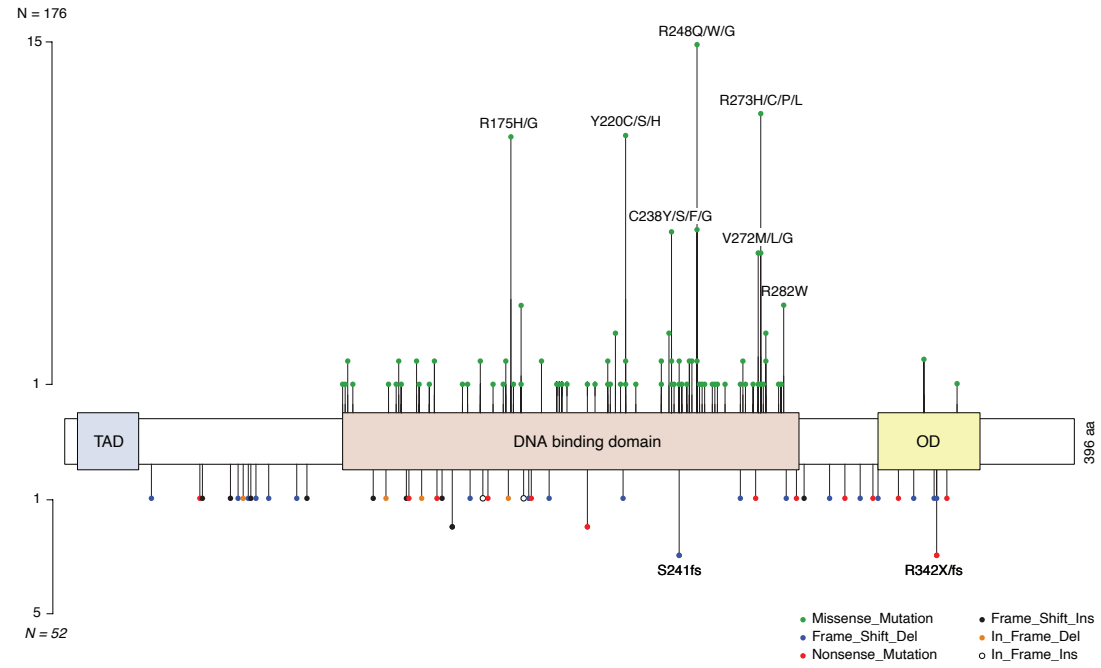

Figure S3. Lollipop plot illustrating the distribution of 283 *TP53* gene variants in 230 mutant *TP53* AML/MDS-EB patients.
The variants shown are depicted by using the NM_000546 transcript reference sequence. Functional protein domains are indicated in purple (TAD: Trans-activation domain), orange (DNA binding domain) and yellow (oligomerization domain). The upper panel shows the distribution of missense mutations (green) and the lower panel the nonsense and indel mutations. The Y-axis represent the number of detected gene variants. Splice-site mutations (n=23) are not reported and 32 gene variants are out of scope of the reference sequence.

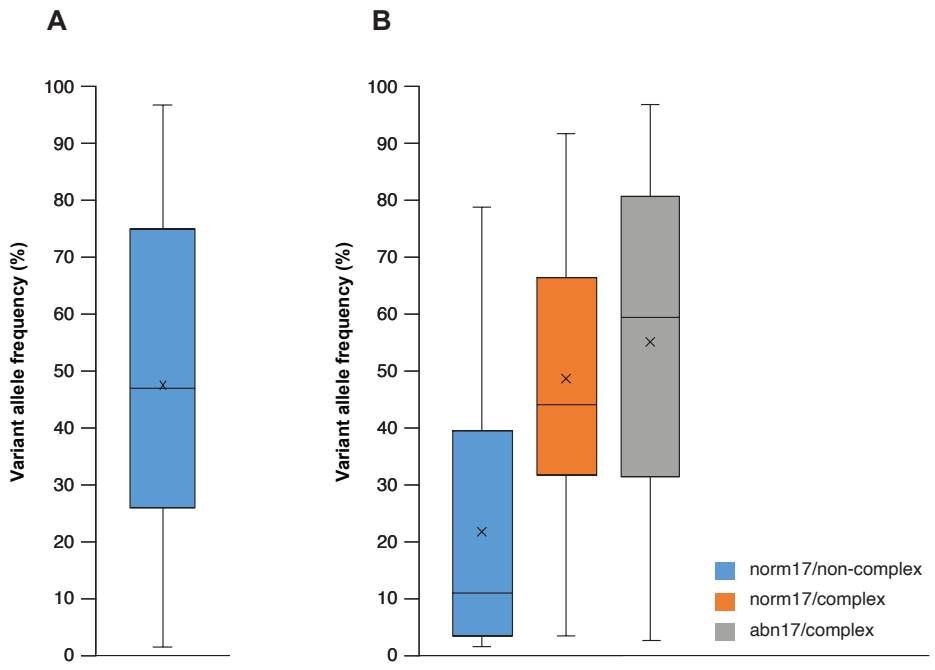

Figure S4. Variant allele frequency of *TP53* mutations.
Variant allele frequency of *TP53* mutations (A) and *TP53* variant allele frequency in relation to chromsome 17 aberrations and complex karyotype (B). *TP53* mutant AML/MDS-EB patients without an abnormality involving chromosome 17 (norm17) or complex karyotype (blue); *TP53* mutant AML/MDS-EB patients with norm17 and complex karyotype (orange) and *TP53* mutant AML/MDS-EB patients with an abnormality involving chromosome 17 (abn17) and complex karyotype (gray).

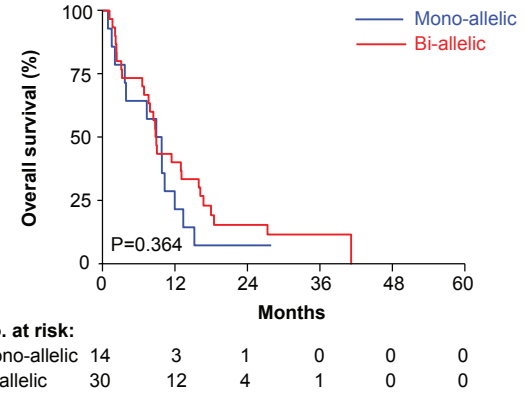

Figure S5. Overall survival of *TP53* mutant allelic status (mono-allelic versus bi-allelic) in MDS-EB patients (n=44).

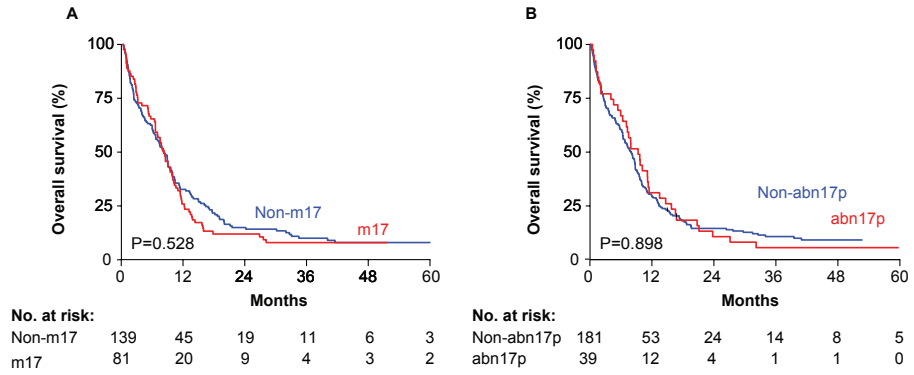

Figure S6. Overall survival of mutant *TP53* AML/MDS-EB by chromosome 17p aberrations.
Monosomy 17 (A) and abnormality chromosome 17p (B).

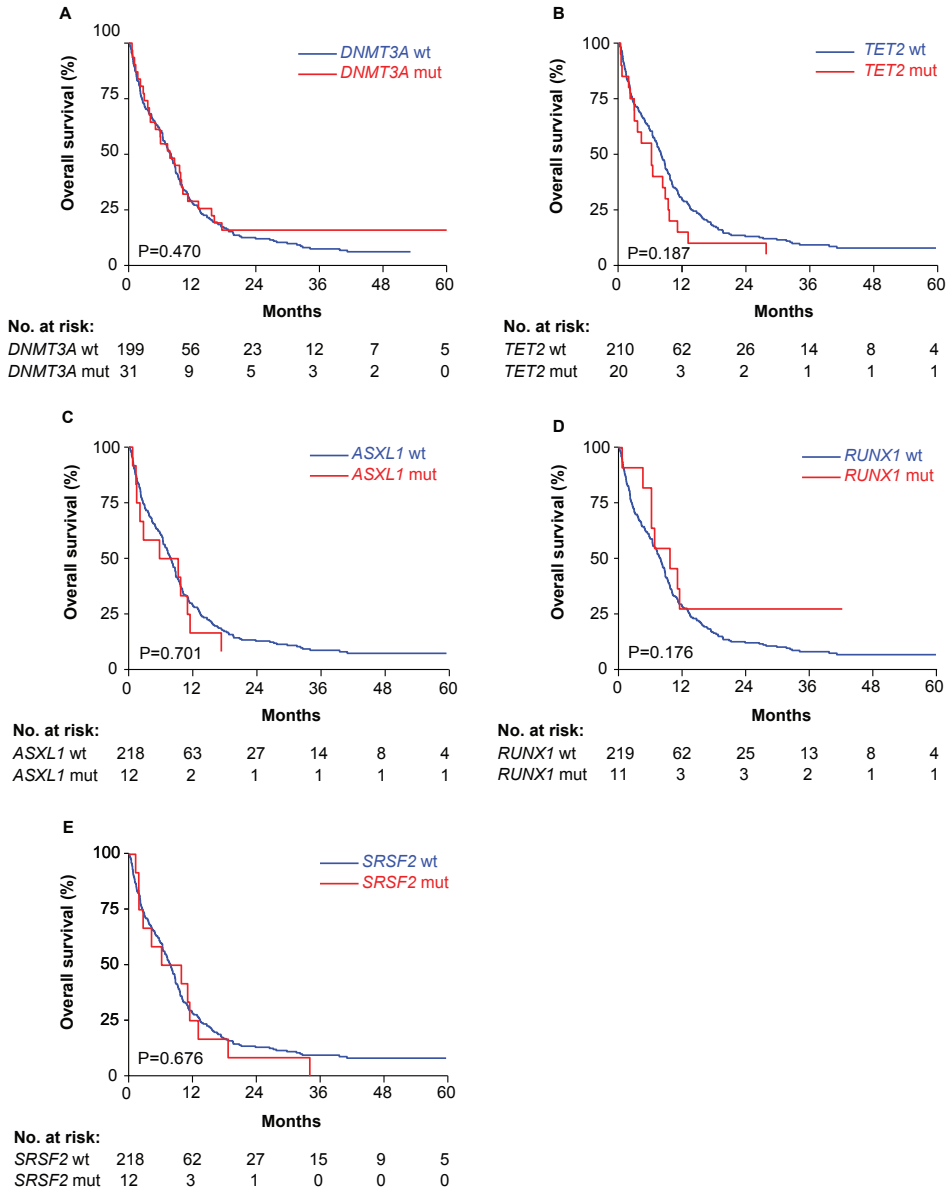

Figure S7. Overall survival of mutant *TP53* AML/MDS-EB by individual concurrent mutations.

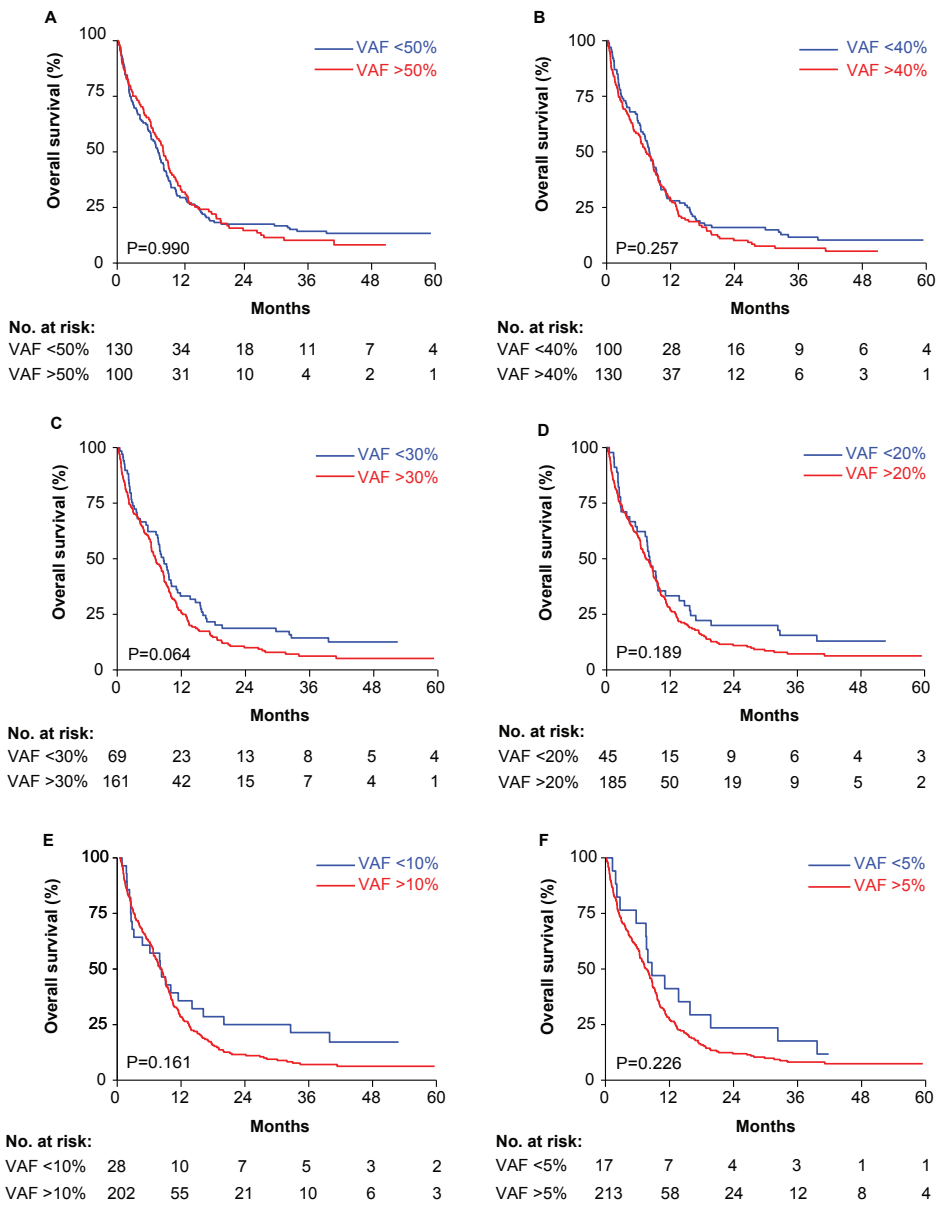

Figure S8. Overall survival by mutant *TP53* variant allele frequency cut off.

3

MOLECULAR MINIMAL RESIDUAL DISEASE IN ACUTE MYELOID LEUKEMIA

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ABSTRACT

BACKGROUND

Patients with acute myeloid leukemia (AML) often reach complete remission, but relapse rates remain high. Next-generation sequencing enables the detection of molecular minimal residual disease in virtually every patient, but its clinical value for the prediction of relapse has yet to be established.

METHODS

We conducted a study involving patients 18 to 65 years of age who had newly diagnosed AML. Targeted next-generation sequencing was carried out at diagnosis and after induction therapy (during complete remission). End points were 4-year rates of relapse, relapse-free survival, and overall survival.

RESULTS

At least one mutation was detected in 430 out of 482 patients (89.2%). Mutations persisted in 51.4% of those patients during complete remission and were present at various allele frequencies (range, 0.02 to 47%). The detection of persistent *DTA* mutations (i.e., mutations in *DNMT3A*, *TET2*, and *ASXL1*), which are often present in persons with age-related clonal hematopoiesis, was not correlated with an increased relapse rate. After the exclusion of persistent *DTA* mutations, the detection of molecular minimal residual disease was associated with a significantly higher relapse rate than no detection (55.4% vs. 31.9%; hazard ratio, 2.14; P<0.001), as well as with lower rates of relapse-free survival (36.6% vs. 58.1%; hazard ratio for relapse or death, 1.92; P<0.001) and overall survival (41.9% vs. 66.1%; hazard ratio for death, 2.06; P<0.001). Multivariate analysis confirmed that the persistence of non-*DTA* mutations during complete remission conferred significant independent prognostic value with respect to the rates of relapse (hazard ratio, 1.89; P<0.001), relapse-free survival (hazard ratio for relapse or death, 1.64; P=0.001), and overall survival (hazard ratio for death, 1.64; P=0.003). A comparison of sequencing with flow cytometry for the detection of residual disease showed that sequencing had significant additive prognostic value.

CONCLUSIONS

Among patients with AML, the detection of molecular minimal residual disease during complete remission had significant independent prognostic value with respect to relapse and survival rates, but the detection of persistent mutations that are associated with clonal hematopoiesis did not have such prognostic value within a 4-year time frame.

INTRODUCTION

Acute myeloid leukemia (AML) is a heterogeneous group of clonal hematopoietic stem-cell disorders with a variable response to therapy.[1-3] Although the majority of patients with newly diagnosed AML have morphologic complete remission after they are treated with intensive induction chemotherapy, relapse rates remain high.[2] Decisions about the choice of postremission therapy in patients with AML currently depend on the identification of a selected set of genetic markers at diagnosis and the detection of residual disease with multiparameter flow cytometry.[2,4] Quantitative molecular evaluation during complete remission could further improve prognostication of outcomes in patients with AML.

The potential of the detection of molecular minimal residual disease after treatment to predict disease relapse in patients with AML has been explored, but assessment of molecular minimal residual disease is not widely established in clinical practice. Previous studies have dealt with only a few leukemia-specific genetic aberrations.[5-11] Next-generation sequencing enables comprehensive, simultaneous detection of somatic mutations that are often patient-specific, both at diagnosis and during treatment.[5,12] Initial studies showed the complex dynamics of residual mutations after induction therapy and the possible association between the persistence of certain somatic mutations and risk of relapse.[12,13]

In determining whether molecular monitoring may be applicable in patients with AML, the phenomenon of age-related clonal hematopoiesis (also known as clonal hematopoiesis of indeterminate potential),[14-17] a condition characterized by the recurrence of gene mutations (allele frequency, >2%) in healthy persons with no evidence of hematologic disease, has added an extra layer of complexity. Persons with age-related clonal hematopoiesis have a slightly increased risk of developing hematologic cancers over time.[14,15,18] Mutations in the epigenetic regulators *DNMT3A*, *TET2*, and *ASXL1* (i.e., *DTA* mutations) are most common in persons with age-related clonal hematopoiesis.[14-19] Residual leukemia-specific mutations that are present in the bone marrow during complete remission may represent either residual leukemic cells or age-related clonal hematopoiesis.[14,15,17] Whether posttreatment persistence of genetic mutations associated with age-related clonal hematopoiesis in the bone marrow from patients with AML has an effect on the disease course remains unclear.

We evaluated a large cohort of patients with AML to investigate whether targeted molecular monitoring with next-generation sequencing could add clinical value for predicting the recurrence of leukemia.

METHODS

Study Design

The study was designed by the first two and the last two authors, who wrote the manuscript with input from the other authors. The authors vouch for the completeness and accuracy of the data and analysis. No one who is not an author contributed to the manuscript. There was no commercial support for the study.

Patients and Cell Samples

Between 2001 and 2013, we obtained samples of bone marrow or peripheral blood from 482 patients, between the ages of 18 and 65, who had a confirmed diagnosis of previously untreated AML (428 patients) or had refractory anemia with excess of blasts, with a score on the Revised International Prognostic Scoring System of more than 4.5, indicating a high or very high risk of relapse (54 patients). To be included in the study, patients had to be in either complete remission or complete remission with incomplete hematologic recovery (defined according to the European Leukemia Net recommendation; hereafter collectively referred to as complete remission), with less than 5% blast cells in the bone marrow,[2,4] after receiving two cycles of induction chemotherapy (Fig. 1).

Patients were treated according to the clinical protocol of either the Dutch–Belgian Cooperative Trial Group for Hematology–Oncology (HOVON)[20] or the Swiss Group for Clinical Cancer Research (SAKK). The treatment protocols and patient eligibility criteria have been described previously.[21,22] The median follow-up of the was 39.7 months and the residual disease status was not available to the clinical investigator and did not influence the choice of consolidation therapy.

Bone marrow aspirations or peripheral blood samples were taken after informed consent. Among 430 out of 482 patients in whom at least one mutation was detected at diagnosis, follow-up bone marrow samples in CR were taken at least 21 days after the start of the second induction cycle. If additional samples were available, the most recent sample prior to start of consolidation therapy was selected. In case no consolidation therapy was given, the last sample that was available within a four month interval from start of the second induction cycle was selected. The probabilities of relapse in AML patients with or without available samples did not differ (p=0.281).

DNA and RNA Isolation

Blasts and mononuclear cells at diagnosis were purified by Ficoll-Hypaque (Nygaard, Oslo, Norway) density gradient centrifugation and cryopreserved. Of all 430 AML cases reaching CR, white blood cells were isolated after induction treatment in 385 cases and mononuclear cells were subsequently purified in 45 cases. After thawing, cells were lysed in RLT solution with the addition of DTT (Qiagen, Venlo, The Netherlands). High quality DNA was extracted using the QIAsymphony (Qiagen, Venlo, The Netherlands). DNA concentration was measured by Qubit Fluorometric Quantitation (Thermo Fisher Scientific, Wilmington, DE). RNA was isolated with RNA-Bee following the protocols of the manufacturer (Bio-Connect BV, Huissen, The Netherlands). *CBFB-MYH11*, *RUNX1-RUNX1T1*, *FLT3* internal tandem duplication (ITD) and *CEBPA* mutations were determined as described previously[23,24].

Targeted Next-Generation Sequencing and Data Analysis

To detect the mutations in 54 genes that are often present in patients with hematologic cancers, we used targeted next-generation sequencing with the Illumina TruSight Myeloid Sequencing Panel (Illumina), following the manufacturer's protocol. The NGS libraries were paired-end sequenced (2x221bp) on an Illumina HiSeq 2500 System (Illumina, San Diego, CA) in Rapid Run mode. Overlap-based error-correction was utilized to attenuate any form of strand-specific error biases. Error-corrected paired-end reads aligned to the human genome version 19 (hg19) with BBMAP[25] followed by quality control to determine cases with insufficient number of reads for adequate variant calling. Single nucleotide variants (SNVs) and insertions-deletions (indels) at diagnosis were determined by MuTect[26], Samtools[27], GATK[28], Varscan[29], Indelocator[30] and Pindel[28]. Variant allele frequencies (VAF) of mutations detected at diagnosis were calculated as the ratio between the number of mutant and total reads. Quantile normalization of the calculated VAFs was performed per flow cell to mitigate the effect of qualitative differences amongst samples. Since CBF fusion transcripts, *CEBPA* mutations and *FLT3*-ITDs were not reliably assessed, we excluded these molecular aberrations from the analyses.

A follow-up background error model was determined by calculating the VAF for each potential SNV within the set of target genes across all follow-up samples from patients who did not carry that specific mutation at diagnosis. The persistence of the SNV in the follow-up sample from the patient of interest was confirmed when the VAF was an outlier compared to the background VAF distribution according to the Thompson-Tau test. A one-sided p-value <0.01 was considered statistically significant.

Multiparameter Flow Cytometry

Detection of residual disease with multiparameter flow cytometry was performed as described previously.[31] The residual disease percentage was defined as the number of leukemia-associated immunophenotype (LAIP) cells within the total white blood cell compartment. The threshold between residual and no residual disease based on flow cytometry was established and validated on 0.1%.[31] Multiparameter flow cytometry was carried out in a subset of 340 of the 430 AML cases analyzed by NGS, which did not differ significantly from the total series of cases regarding clinical, cytogenetic and molecular characteristics (data not shown).

Statistical Analysis

The 430 patients in whom at least one mutation was detected at diagnosis were randomly assigned to either a training cohort (283 patients) or a validation cohort (147 patients). Differences in clinical, cytogenetic and molecular characteristics of the training and validation cohorts or NGS and flow cohorts were tested using Fisher's exact test for categorical variables and Mann-Whitney U test for continuous variables and were not significantly different (Table 1, and Fig. 1 and Table S1 in the Supplementary Appendix online). The primary end point was the 4-year cumulative incidence of relapse, and the secondary end points were the 4-year rates of overall survival and relapse-free survival (defined according to the European Leukemia Net recommendation[4]). Relapse and survival time was calculated from the sampling date until the date of the event of interest or censoring. Within each cohort, the difference in the incidence of relapse between patients in whom residual disease was detected and those in whom residual disease was not detected was evaluated with the use of the method of Gray and the Fine and Gray model for competing risks.[32] The log-rank test and the Cox proportional-hazards model were used for overall survival and relapse-free survival analyses. The proportional hazards assumption was tested by interaction with time and the interactions were evaluated in a standard way. The effect of allogeneic stem cell transplantation on CIR and OS was investigated in both multivariable models as a time-dependent covariate.[33] A two-sided P value of 0.05 or less was considered to indicate statistical significance. Statistical analyses were performed with Stata Statistical Software, Release 14.1 (Stata, College Station, TX).

Table 1. Clinical, cytogenetic and molecular characteristics (n=430).

Age		
Median	51	
Range	18-66	
Sex		
M	216	50%
F	214	50%
WBC at diagnosis (x10^9/l)		
≤ 100	387	90%
>100	43	10%
ELN 2017 risk classification		
Favorable	204	48%
Intermediate	113	26%
Adverse	113	26%
Number of cycles to CR		
One cycle	360	84%
Two cycles	70	16%
Consolidation therapy		
None	46	11%
Chemotherapy	117	27%
Autologous HSCT	78	18%
Allogeneic HSCT	189	44%
Cytogenetics*		
t(8;21)	27	6%
inv(16)	24	6%
Complex karyotype	38	9%
Monosomal karyotype	30	7%
Mutation at diagnosis		
ASXL1	31	7%
CEBPA-DM	19	4%
DNMT3A	141	33%
FLT3-TKD	53	12%
FLT3-ITD (low ratio)	40	9%
FLT3-ITD (high ratio)	51	12%
NPM1	168	39%
RUNX1	50	12%
TET2	48	11%

*Karyotyping failed in 13 cases

RESULTS

Detection of Mutations at Diagnosis

We performed targeted next-generation sequencing to detect gene mutations at diagnosis in samples obtained from 482 patients with AML (Fig. 1). We detected an average of 2.9 mutations per patient; at least 1 single mutation, which could potentially serve as a marker of residual disease, was present in 430 (89.2%) of the patients. Mutations in *NPM1*, *DNMT3A*, *FLT3*, and *NRAS* were among the most common detectable mutations at diagnosis (Table 1 and Fig. 1, and Table S1 in the Supplementary Appendix).

Detection of Mutations during Complete Remission

We then performed targeted next-generation sequencing to detect persistent mutations after induction therapy in samples of bone marrow obtained from 430 patients who were in complete remission. Persistent mutations were detected in 51.4% of the patients (Fig. 2, and Fig. 3). The rate at which mutations persisted was highly variable across genes. *DTA* mutations were most common, persisting at rates of 78.7% for *DNMT3A*, 54.2% for *TET2*, and 51.6% for *ASXL1* (Fig. 2). In contrast, the majority of mutations in genes related to the RAS pathway were cleared after induction therapy, with mutations in *NRAS*, *PTPN11*, *KIT*, and *KRAS* persisting at rates of 4.2%, 7.0%, 13.5%, and 12.5%, respectively.

Of note, the allele frequencies of the mutations that persisted during complete remission ranged from 0.02 to 47% (Fig. 2). This finding suggests that residual mutation-bearing cells could constitute a minor population of the cells or perhaps even a majority of the cells. An allele frequency of 50% is consistent with the presence of a heterozygous mutation in all cells. Thus, although the patients were in morphologic complete remission, which would typically imply that heterozygous mutations are present at allele frequencies lower than 2.5% (the equivalent of <5% blast cells in the bone marrow), the samples that were obtained during remission often contained mutations with much higher allele frequencies (Fig. 2).

Mutations that persisted after induction therapy at allele frequencies higher than 2.5% were often *DTA* mutations (Fig. 2, and Fig. 3 and S1 in the Supplementary Appendix). In contrast, mutations in *IDH1*, *IDH2*, *STAG2*, *TP53*, and other genes only occasionally persisted after induction therapy at allele frequencies higher than 2.5%, and thus the allele frequencies of these mutations were typically consistent with the state of morphologic complete remission (<5% blast cells in the bone marrow).

Because *DTA* mutations have been established as the most common gene mutations in persons with age-related clonal hematopoiesis,[14-19] the persistent *DTA* mutations might have represented nonleukemic clones that repopulated the bone marrow after induction therapy. Among patients who had both *DTA* mutations and non-*DTA* mutations at diagnosis, non-*DTA* mutations were generally cleared after induction chemotherapy, whereas *DTA* mutations often remained detectable during complete remission and were the only persistent mutations in 90 of 133 (67.7%) of those patients (Fig. S2). These observations are consistent with the notion that residual cells bearing *DTA* mutations after induction therapy represent nonleukemic clones rather than persistent malignant disease.

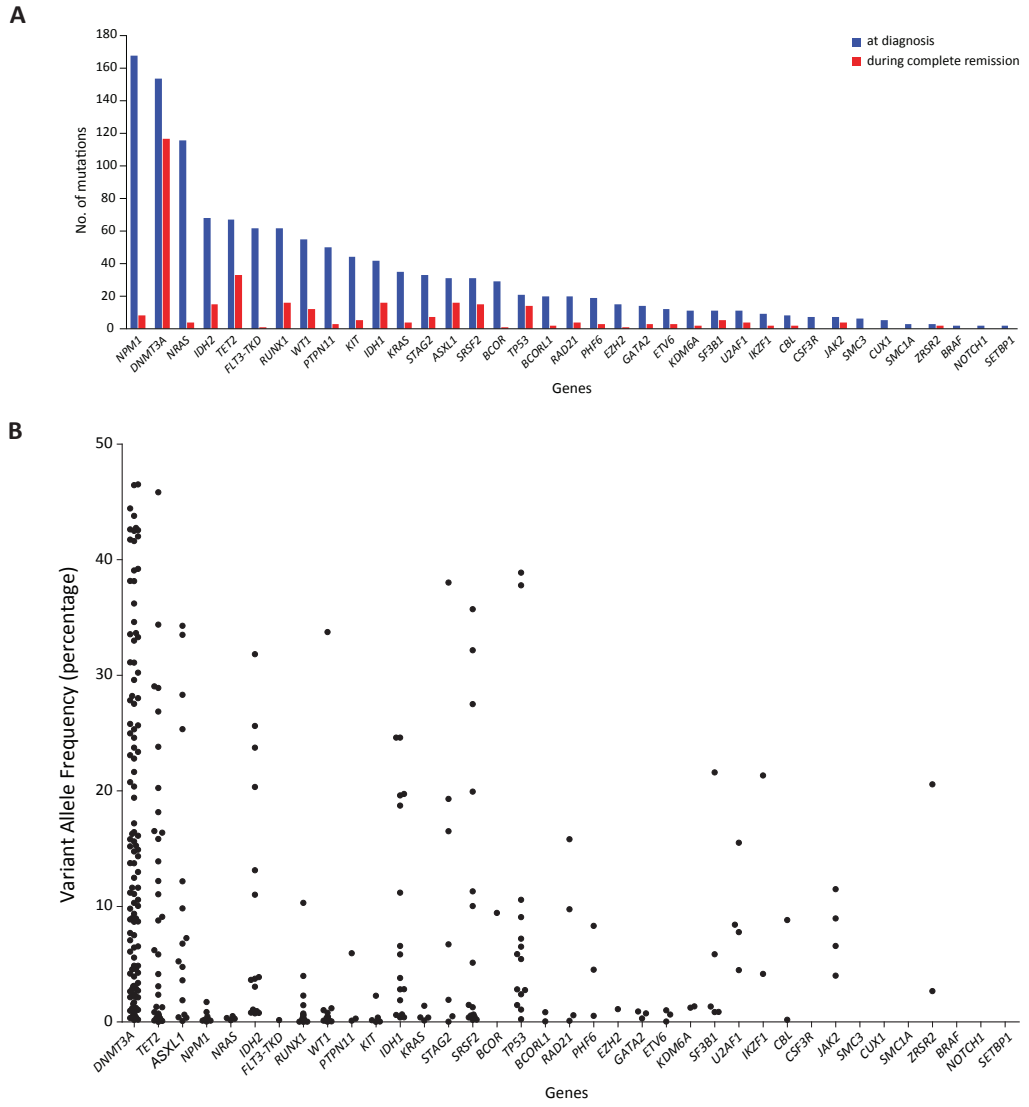

Figure 1. Detection of Mutations at Diagnosis and during Complete Remission and Allele Frequency of Mutations Detected during Complete Remission.

Panel A shows the number of mutations in each leukemia-associated gene, both at diagnosis of acute myeloid leukemia and during complete remission, in 430 patients. Panel B shows the allele frequency of each mutation in each gene during complete remission in 430 patients. In male patients, the variant allele frequencies for *PHF6*, *KDM6A*, *ZRSR2*, *BCOR*, *BCORL1*, and *STAG2* (on the X chromosome) were divided by 2.

Relapse and Survival End Points

In the training cohort (283 patients), we found that the detection of any persistent mutation during complete remission was associated with an increased risk of relapse (4-year relapse rate, 48.2% with detection vs. 32.4% with no detection; P=0.03) (Fig. 4A). We then imposed various thresholds for allele frequency to determine whether the prognostic value of the persistent mutations would improve after the exclusion of mutations with a high allele frequency, which could indicate a state of clonal hematopoiesis. The correlation of persistent mutations with an increased relapse risk appeared to be independent of allele frequency. A correlation with relapse risk generally remained present when we excluded persistent mutations with allele frequencies at or above the following thresholds: 30% (P=0.09), 20% (P=0.11), 10% (P=0.01), 5% (P=0.04), 2.5% (P=0.007), and 1% (P=0.07) (Fig. 4). The exclusion of persistent mutations with certain allele frequencies had no clear effect on the relationship between persistent mutations and an increased relapse risk, thus precluding the identification of a threshold for allele frequency that could be used to distinguish populations at higher or lower risk for relapse. As we mentioned previously, the patients with persistent mutations at high allele frequencies were enriched for *DTA* mutations (Fig. 2).

We next determined whether persistent *DTA* mutations, which are associated with age-related clonal hematopoiesis, might be correlated with an increased relapse risk. We observed that the detection of persistent *DTA* mutations was not significantly associated with a higher 4-year relapse rate than no detection (P=0.29). The absence of a correlation was independent of allele frequency. No significant correlation of persistent *DTA* mutations with an increased relapse risk was apparent when we excluded persistent *DTA* mutations with allele frequencies at or above the following thresholds: 30% (P=0.91), 20% (P=0.66), 10% (P=0.89), 5% (P=0.82), 2.5% (P=0.53), and 1% (P=0.92) (Fig. 5). In contrast, among patients who had persistent *DTA* mutations during complete remission, coexisting persistent non-*DTA* mutations had high prognostic value with respect to relapse (4-year relapse rate, 66.7% with detection vs. 39.4% with no detection; P=0.002) (Fig. 6A). Thus, in patients with persistent *DTA* mutations, the presence of residual disease that specifically included coexisting non-*DTA* mutations represented a predictor of impending relapse.

We next assessed whether persistent non-*DTA* mutations might be correlated with an increased relapse risk. The detection of persistent non-*DTA* mutations at any allele frequency was strongly associated with an increased relapse risk (4-year relapse rate, 55.7% with detection vs. 34.6% with no detection; P=0.001) (Fig. 6B), as well as with reduced relapse-free survival (4-year rate of relapse- free survival, 56.7% with detection vs. 36.6% with no detection; P=0.006) (Fig. 6C) and reduced overall survival (4-year rate of overall survival, 65.3% with detection vs. 43.7% with no detection; P=0.01) (Fig. 6D).

To assess the reproducibility of these results, we evaluated the effect of sequencing-based detection of persistent non-*DTA* mutations during complete remission on the rates of relapse, relapse-free survival, and overall survival in the validation cohort (147 patients). The rates with detection versus no detection were as follows: 4-year relapse rate, 55.1% versus 26.5% (P<0.001); 4-year rate of relapse-free survival, 60.6% versus 35.6% (P<0.001); and 4-year rate of overall survival, 67.6% versus 37.1% (P<0.001) (Fig. 6B, 6C, and 6D). The results in the validation cohort confirmed the significant findings in the training cohort.

In the combined training and validation cohorts (a total of 430 patients), persistent non-*DTA* mutations were detected during complete remission in 28.4% of the patients. Detection of these mutations was associated with a significantly higher 4-year relapse rate than no detection (55.4% vs. 31.9%; hazard ratio, 2.14; 95% confidence interval [CI], 1.57 to 2.91; P<0.001), as well as with lower 4-year rates of relapse-free survival (36.6% vs. 58.1%; hazard ratio for relapse or death, 1.92; 95% CI, 1.46 to 2.54; P<0.001) and overall survival (41.9% vs. 66.1%; hazard ratio for death, 2.06; 95% CI, 1.52 to 2.79; P<0.001) (Fig. 6E, 6F, and Fig. 6G).

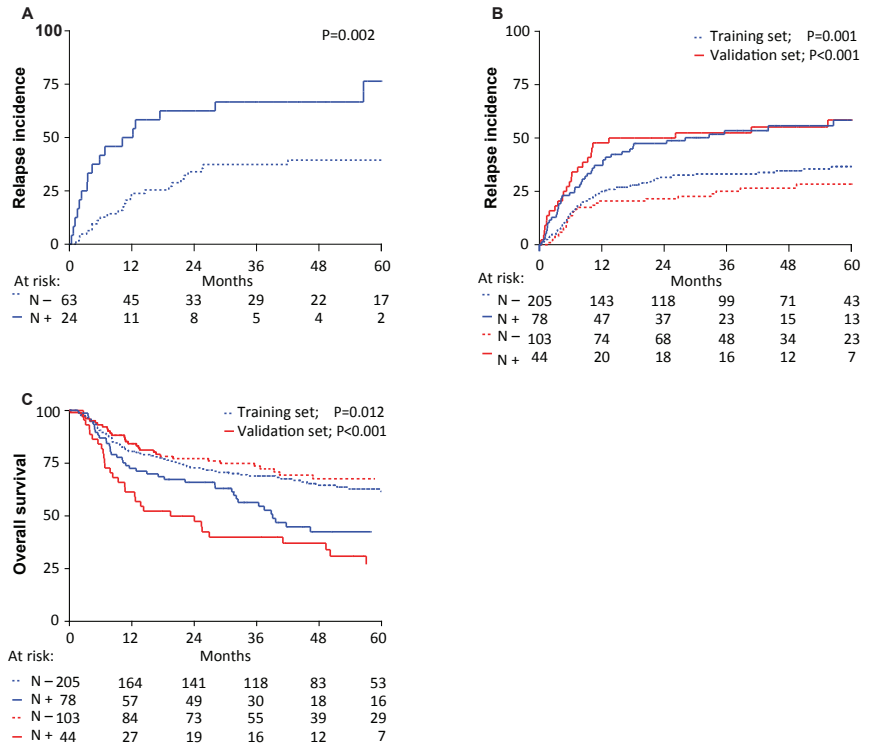

Figure 2. Rates of Relapse and Overall Survival.
Shown is the cumulative incidence of relapse among patients in the training cohort with persistent *DTA* mutations, according to the detection of coexisting persistent non-*DTA* mutations during complete remission (Panel A), and among all patients in the training and validation cohorts, according to the detection of any persistent non-*DTA* mutations during complete remission (Panel B). Panel C shows the rate of overall survival among all patients in the training and validation cohorts, according to the detection of any persistent non-*DTA* mutations during complete remission. *DTA* mutations are mutations in *DNMT3A*, *TET2*, and *ASXL1*.

Multivariate and Sensitivity Analyses

We performed multivariate analyses that accounted for the major established relevant prognostic factors, including age, white-cell count, 2017 European Leukemia Network risk classification, and the number of cycles of induction chemotherapy needed to attain complete remission. Sequencing-based detection of non-*DTA* mutations maintained significant independent prognostic value with respect to the rates of relapse (hazard ratio, 1.89; 95% CI, 1.34 to 2.65; P<0.001), relapse-free survival (hazard ratio for relapse or death, 1.64; 95% CI, 1.22 to 2.20; P=0.001), and overall survival (hazard ratio for death, 1.64; 95% CI, 1.18 to 2.27; P=0.003) (Table 2). No significant interactions were apparent between the detection of residual disease and the other prognostic factors in the multivariate model, type of consolidation therapy, or disease entity (AML vs. refractory anemia with excess of blasts) (data not shown).

In sensitivity analyses involving correction for variation in the time at which bone marrow specimens were obtained for sequencing analysis (within the remission period of 21 days to 4 months after the second treatment cycle), the prognostic value of sequencing-based detection of non-*DTA* mutations with respect to the rates of relapse, relapse-free survival, and overall survival remained unaffected (Table S2 in the Supplementary Appendix). In addition, an analysis that included postremission treatment with allogeneic stem cell transplantation as a time-dependent variable conferred no effect on the prognostic value of the detection of residual disease (Table 3 in the Supplementary Appendix).

Table 2. Multivariable analysis.

Prognostic factor	Relapse incidence			Relapse-free survival			Survival		
	SHR	95% CI	p-value	HR	95% CI	p-value	HR	95% CI	p-value
Molecular residual disease (Present vs. Absent)	1.89	1.34-2.65	<0.001	1.64	1.22-2.20	0.001	1.64	1.18-2.27	0.003
Age per year	1.01	0.99-1.03	0.212	1.02	1.00-1.03	0.009	1.03	1.01-1.04	0.001
WBC at diagnosis (>100 vs. ≤100)	2.16	1.31-3.56	0.003	2.03	1.34-3.08	0.001	2.02	1.27-3.21	0.003
ELN2017 risk classification									
Intermediate vs. Favorable	1.67	1.12-2.49	0.012	2.01	1.42-2.83	<0.001	2.53	1.72-3.72	<0.001
Adverse vs. Favorable	1.83	1.26-2.66	0.002	2.21	1.58-3.10	<0.001	2.67	1.83-3.92	<0.001
Number of cycles to CR (2 cycles vs. 1 cycle)	2.17	1.50-3.15	<0.001	2.43	1.74-3.39	<0.001	2.96	2.09-4.21	<0.001

Detection of Residual Disease with Multiparameter Flow Cytometry

Multiparameter flow cytometry is an increasingly used method for predicting relapse in patients with AML who are in complete remission.[7,34] We compared next-generation sequencing for the detection of persistent non-*DTA* mutations with flow cytometry for the detection of residual disease in a representative subgroup of 340 patients, from whom sufficient samples were obtained for both analyses. Concordant results (either detection or no detection on both assays) were found in 69.1% of the patients (30 patients with detection and 205 with no detection), whereas persistent non-*DTA* mutations were detected only on sequencing in 64 patients and only on flow cytometry in 41 patients. The 4-year relapse rate was 73.3% among patients in whom both assays were positive, 52.3% among those who had residual disease on sequencing but not on flow cytometry, 49.8% among those who had residual disease on flow cytometry but not on sequencing, and 26.7% among those in whom both assays were negative (Fig. 7). In a multivariate analysis that combined the results of sequencing and flow cytometry, the combined use of the two assays for the detection of residual disease conferred independent prognostic value with respect to the rates of relapse (P<0.001), relapse-free (P<0.001), and overall survival (P=0.003) (Table 4).

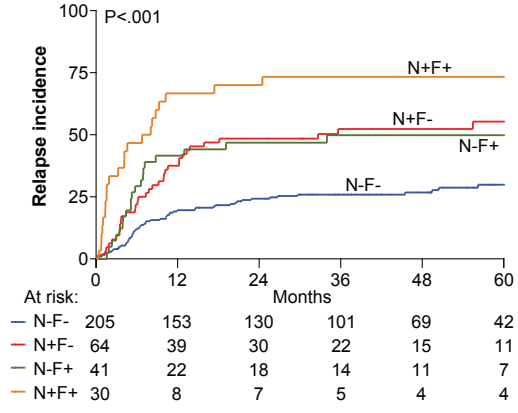

Figure 3. Rate of Relapse According to Results of Next-Generation Sequencing and Multiparameter Flow Cytometry.

Shown is the cumulative incidence of relapse, according to the presence of positive (+) or negative (−) results for the detection of persistent non-*DTA* mutations during complete remission on next-generation sequencing (NGS) and on multiparameter flow cytometry (MFC).

DISCUSSION

In addition to the presence of genetic abnormalities at diagnosis, the continued presence of particular gene mutations during or after treatment carries prognostic information for certain genetically defined AML subtypes.[5-11] This applies, for example, to AML associated with a mutation in *NPM1*, for which the detection of a residual mutation in *NPM1* transcripts during complete remission is indicative of an increased probability of relapse.[8,9] However, this example is only representative of a single-gene approach. We report the results of a systematic study that involved a large number of patients with AML, in which we used a genomewide approach to evaluate the persistence of multiple gene mutations for the effect on treatment outcomes. Patients were treated with intensive chemotherapy regimens and attained morphologic complete remission, with a median follow-up exceeding 3 years.

Of note, age-related clonal hematopoiesis,[14-17] which is characterized by recurrent somatic mutations in leukemia-associated genes in persons with no apparent hematologic disease, adds a challenge in the detection of residual disease. Our study showed that the persistence of mutations that are most commonly associated with age-related clonal hematopoiesis (i.e., *DTA* mutations [mutations in *DNMT3A*, *TET2*, and *ASXL1*]) during complete remission did not contribute to a measurably increased risk of relapse within a follow up period of 4 years in adults with AML who were younger than 65 years of age. This appeared to be true for mutations that were present at various allele frequencies, which suggests that the clone size in age-related clonal hematopoiesis yields no prognostic value with respect to the end points defined in this study.

The cells bearing *DTA* mutations appeared to persist and possess a selective clonal advantage over normal stem cells when they repopulated the bone marrow after induction therapy. This finding is consistent with the competitive clonal advantage of hematopoietic stem cells with deficiencies and mutations in *DNMT3A* and *TET2*, an advantage that has been reported previously.[35-37] The proliferative advantage of hematopoietic stem cells with *DTA* mutations and their capacity to withstand chemotherapy because of inherent resistance may explain why persistent premalignant *DTA* mutations were not correlated with an increased probability of relapse and thereby did not constitute a reliable molecular biomarker for the assessment of relapse risk.

It is possible that gene mutations other than *DTA* mutations also partially reflect clonal hematopoiesis. However, at this time, we cannot rigorously verify the possibility that gene mutations associated with age-related clonal hematopoiesis also reside as subfractions among the other gene abnormalities in leukemia cells. In addition, mutations in *TP53*, *IDH1*, and *IDH2*, along with genes related to the RAS pathway and spliceosome genes, have been shown to have distinct biologic features in the context of AML pathogenesis.[38-41] Therefore, in this study, we collectively considered non-*DTA* mutations to be abnormalities that are unrelated to clonal hematopoiesis.

Our study had a median follow-up of almost 40 months. Among patients with AML who have complete remission, most relapses generally occur within the first 4 years. We found that the continued persistence of *DTA* mutations was not associated with an increased relapse risk, and thus these residual cells may not need to be eliminated to prevent relapse. However, the limited follow-up of 40 months does not rule out the possibility that persistent *DTA* mutations represent an increased risk of relapse at a later time point.

Although sequencing-based detection enables assessment for residual disease in virtually all patients with AML, it is imperfect in two ways. First, not all patients with residual mutation-bearing cells have a relapse. Second, some patients with no measurable residual disease have a relapse. It is conceivable that relapse estimation can be improved with the development of technological variations of sequencing-based approaches that have greater sensitivity or a broader scope (e.g., those with molecular barcoding, exome sequencing, or whole-genome sequencing) or with the identification of additional molecular and phenotypic markers so that quantitative minor clones or subclones associated with the leukemia are captured by the assay. In this respect, it is of particular interest that the use of multiparameter flow cytometry[7,34] — which identifies patients with AML who have an increased risk of relapse according to an entirely different approach that is based on a residual leukemia-associated immunophenotype[31,42] — can increase the yield of identification of residual leukemia during complete remission.

In this study, gene sequencing and multiparameter flow cytometry each had independent and additive prognostic value with respect to rates of relapse and survival in patients with AML. The detection of residual leukemia with both methods is associated with an excessively high probability of relapse (approximately 75%), and the absence of detection of residual disease with both methods is correlated with a relatively low probability of relapse (approximately 25%). Thus, the combined use of sequencing and flow cytometry during complete remission warrants further development and evaluation in clinical practice.

In conclusion, targeted sequencing-based detection of molecular minimal residual disease during complete remission was associated with an increased risk of relapse or death in patients with AML. However, over a 4-year follow-up period, the risk of relapse or death was not influenced by the persistence of genetic lesions that are associated with age-related clonal hematopoiesis.

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SUPPLEMENTARY METHODS

Patients and Cell Samples

Bone marrow aspirations or peripheral blood samples at diagnosis were taken after informed consent. Follow-up bone marrow samples of 430 out of 482 AML or RAEB patients in CR with mutations at diagnosis (Figure S1) were taken at least 21 days after the start of the second induction cycle. If additional samples were available, the most recent sample prior to start of consolidation therapy was selected. In case no consolidation therapy was given, the last sample that was available within a four month interval from start of the second induction cycle was selected. The probabilities of relapse in AML patients with or without available samples did not differ (p=0.281). The median follow-up of the 430 AML cases was 39.7 months and the residual disease status was not available to the clinical investigator and did not influence the choice of consolidation therapy. Blasts and mononuclear cells at diagnosis were purified by Ficoll-Hypaque (Nygaard, Oslo, Norway) density gradient centrifugation and cryopreserved. Of all 430 AML cases reaching CR, white blood cells were isolated after induction treatment in 385 cases and mononuclear cells were subsequently purified in 45 cases. After thawing, cells were lysed in RLT solution with the addition of DTT (Qiagen, Venlo, The Netherlands).

DNA and RNA Isolation

High quality DNA was extracted using the QIAsymphony (Qiagen, Venlo, The Netherlands). DNA concentration was measured by Qubit Fluorometric Quantitation (Thermo Fisher Scientific, Wilmington, DE). RNA was isolated with RNA-Bee following the protocols of the manufacturer (Bio-Connect BV, Huissen, The Netherlands). *CBFB-MYH11*, *RUNX1-RUNX1T1*, *FLT3* internal tandem duplication (ITD) and *CEBPA* mutations were determined as described previously [1,2].

Targeted NGS

The NGS libraries were paired-end sequenced (2x221bp) on an Illumina HiSeq 2500 System (Illumina, San Diego, CA) in Rapid Run mode. Since CBF fusion transcripts, *CEBPA* mutations and *FLT3* ITDs cannot be reliably assessed with NGS on DNA, these molecular aberrations were excluded from the analyses.

NGS Data Analysis

The vast majority of amplicon target regions were completely paired-end sequenced. Overlap-based error-correction was utilized to attenuate any form of strand-specific error biases. Error-corrected paired-end reads aligned to the human genome version 19 (hg19) with BBMAP[3] followed by quality control to determine cases with insufficient number of reads for adequate variant calling. Single nucleotide variants (SNVs) and insertions-deletions

(indels) at diagnosis were determined by MuTect[4], Samtools[5], GATK[6], Varscan[7], Indelocator[8] and Pindel[6]. Variant allele frequencies (VAF) of mutations detected at diagnosis were calculated as the ratio between the number of mutant and total reads. The persistence of mutations at follow-up, previously detected at diagnosis, requires the detection of mutations at exceptionally low VAFs. A follow-up background error model was determined by calculating the VAF for each potential SNV within the set of target genes across all follow-up samples.

The detection of variants at low detection level is primarily reserved for highly discriminative insertion or deletion mutations sequenced at sufficient depth in both the follow-up samples of interest and the control set of remission samples. The strength of a site-specific error model is that it models the unique site-and-variant- specific noise profile based on a large set of remission samples from patients who did not carry that specific mutation at diagnosis and thereby enables the assessment whether the variant remains persistent, defined as a statistical outlier, in the follow-up sample from the patient of interest. Since more complex insertion or deletion mutations are particularly distinct and the odds of detecting such variants as a consequence of sequencing or alignment errors is exceptionally low, such variants can be detected at higher sensitivity. The detection sensitivity of other mutations is variable and highly dependent on the average coverage for that specific locus for all samples, the observed error variance of the site-specific variant in the control set (a high variance results in decreased detection sensitivity) and the number of control sample available. The unique combination of patient-specific mutations, the application of a site-specific error model and strict detection criteria minimizes the odds that variants are erroneously called to persist.

Quantile normalization of the calculated VAFs was performed per flow cell to mitigate the effect of qualitative differences amongst samples. All SNVs detected across the diagnostic samples were compiled and the background VAF distribution was determined for each individual SNV from follow-up samples lacking this SNV in the matched diagnostic sample. For the remaining follow-up samples the persistence of the SNV was considered confirmed when the VAF was an outlier compared to the background VAF distribution according to the Thompson-Tau test. A one-sided p- value <0.01 was considered statistically significant. Indels were processed and compared similarly, except for quantile normalization as there are infinitely many possible indel-configurations per locus.

Multiparameter Flow Cytometry

Residual disease detection by MFC was performed as described previously [9]. The residual disease percentage was defined as the number of leukemia-associated immuno phenotype (LAIP) cells within the total white blood cell compartment. The threshold between residual and no residual disease based on flow cytometry was established and validated on 0.1%[9]. Multi-parameter flow cytometry was carried out in a subset of 340 of the 430 AML cases

analyzed by NGS, which did not differ significantly from the total series of cases regarding clinical, cytogenetic and molecular characteristics (data not shown).

Statistical Analyses

The complete cohort of 430 AML patients was randomly split using Stata into a training (n=283) and validation cohort (n=147) (Table 1, Figure S1 and Table S1). Each patient received a pseudorandom number from a uniform distribution from 0 to 1. The random numbers generated were shuffled by sorting, allowing for random allocation of patients to the training or validation set. Differences in clinical, cytogenetic and molecular characteristics of the training and validation cohorts or NGS and flow cohorts were tested using the Fisher's exact test for categorical variables and Mann-Whitney U test for continuous variables. Clinical, cytogenetic and molecular characteristics of the training and validation cohorts were not significantly different (Table S1). The primary endpoint of the study was the cumulative incidence of relapse (CIR). Competing-risks regression analysis was performed for relapse with adjustment for non-relapse mortality according to the method of Gray and the Fine & Gray model[10]. The secondary endpoints were relapse free (RFS) and overall survival (OS) which were analyzed using the log-rank test and the Cox proportional hazards model. Relapse and survival time was calculated from the sampling date until the date of the event of interest or censoring. RFS was defined from date of sampling to death, relapse or censoring, whichever came first. All statistical tests were two-sided and p-values <0.05 were considered statistically significant. The proportional hazards assumption was tested by interaction with time and the interactions were evaluated in a standard way. The effect of allogeneic stem cell transplantation on CIR and OS was investigated in both multivariable models as a time-dependent covariate[11]. All p-values are two sided and p-values <0.05 were considered statistically significant. Statistical analyses were performed with Stata Statistical Software, Release 14.1 (Stata, College Station, TX).

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SUPPLEMENTARY FIGURES

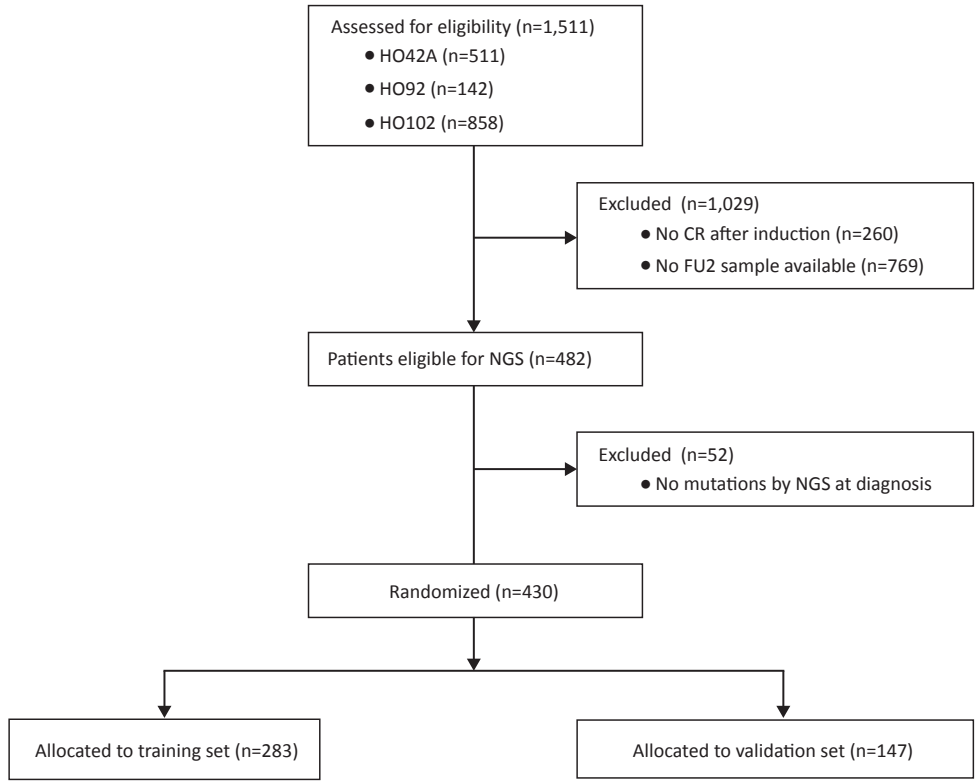

Figure S1. Consort diagram molecular residual disease study
Abbreviations: **HO**, HOVON-SAKK, Dutch-Belgian Hemato-Oncology Cooperative Group and the Swiss Group for Clinical Cancer Research; **CR**, Complete morphological Remission; **FU2**, Follow-up after induction cycle II; **NGS**, Next Generation Sequencing.

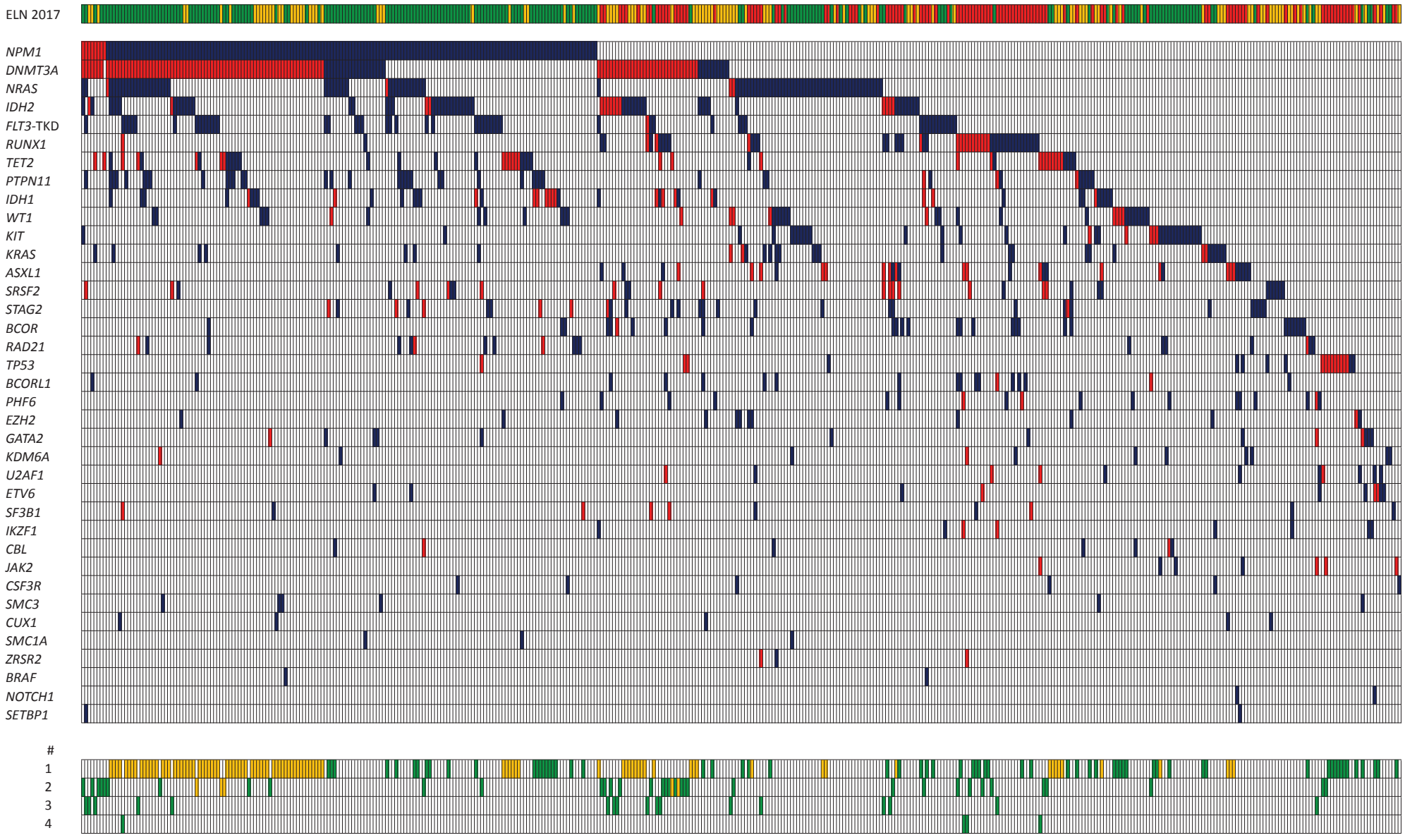

Figure S2A. Mutation status at diagnosis and after induction cycle II.
Overview of the mutation status at diagnosis (present: blue) and after induction cycle II (present: red). Each column represents an individual patient. The upper panel indicates the ELN 2017 risk category (green: favorable risk, orange: intermediate risk, red: adverse risk). The total number of mutations present at diagnosis and the number remaining after induction cycle II are indicated on the right. The total number of residual mutations after induction treatment are summarised in the bottom figure (green: number of residual mutations present after induction treatment; orange: number of residual mutations exclusively in *DNMT3A*, *TET2* and/or *ASXL1*). Abbreviations: ELN, European LeukemiaNet. VAF, Variant Allele Frequency.

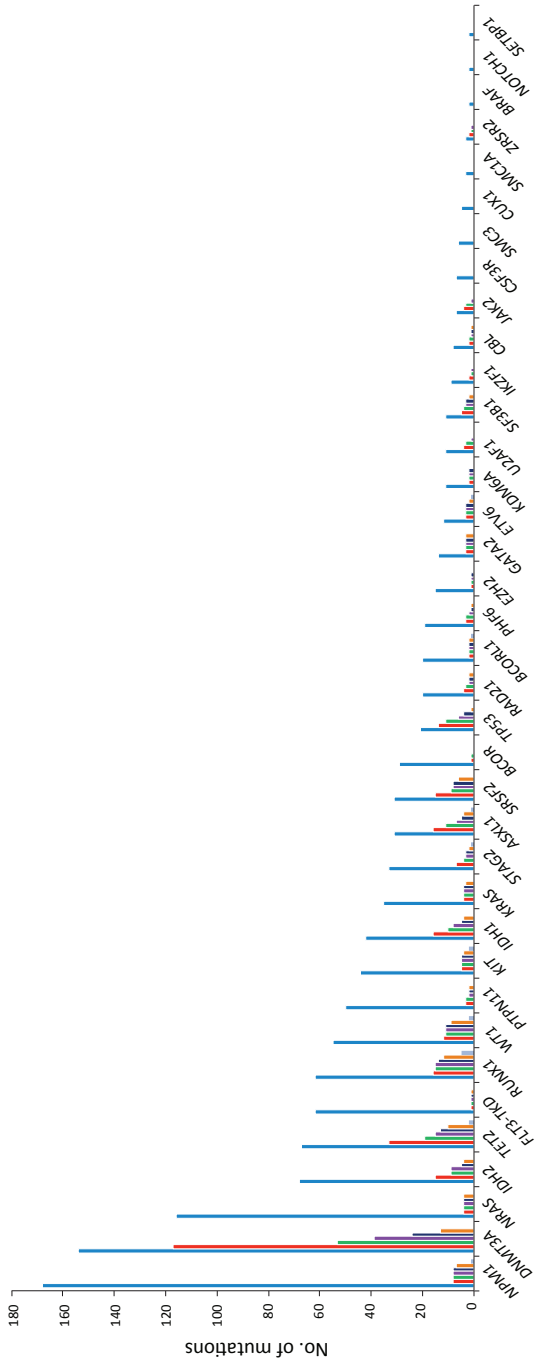

Figure S3. Total number of mutations at diagnosis and after induction cycle II with various VAF cut-offs.
(blue: at diagnosis, red: without cut off, green: cut off 10%, purple: cut off 5%, black: cut off 2.5%, orange: cut off 1% and light blue: cut off 0.1%).

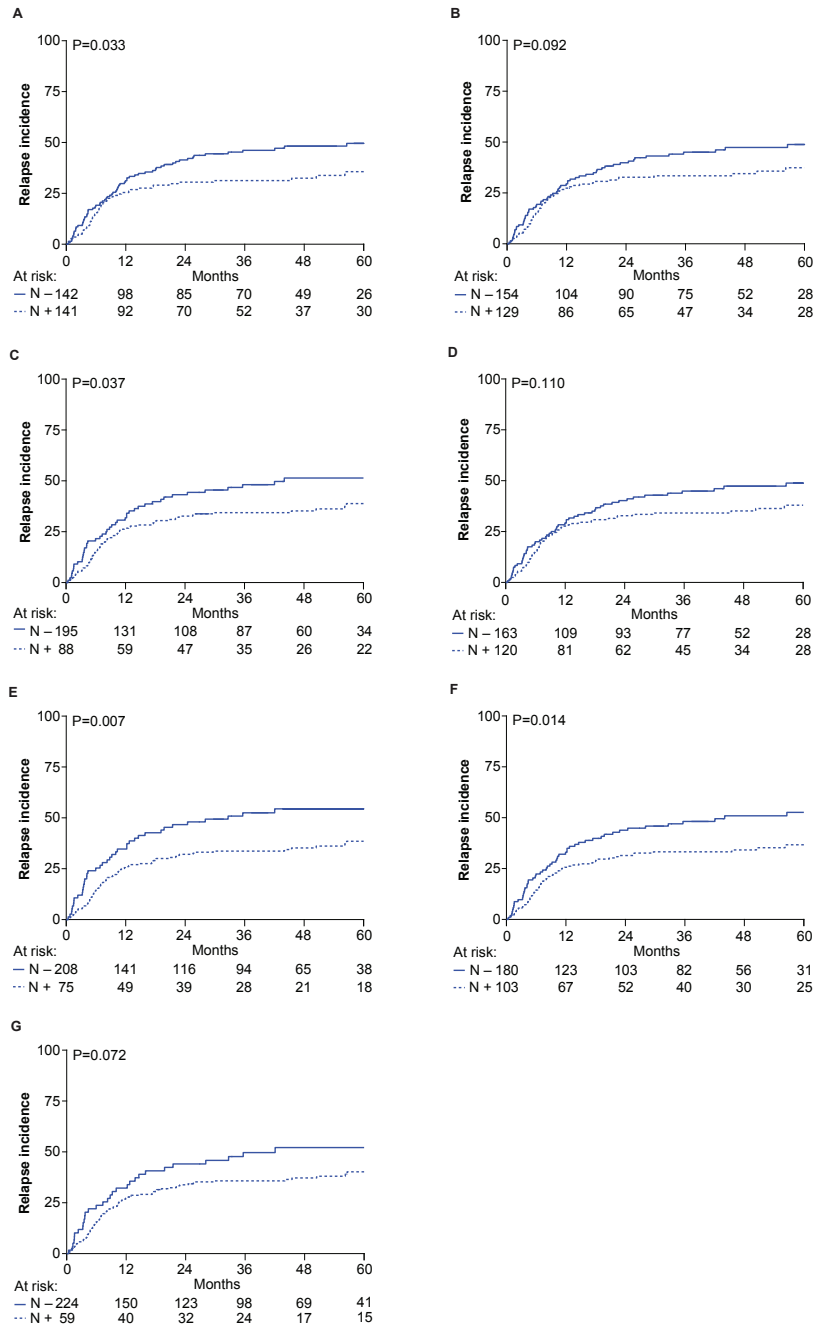

Figure S4. Cumulative incidence of relapse by any detectable mutation in CR with various VAF cut offs in AML patients allocated to the training set.
No cut off (A), ≤30% (B), ≤20% (C), ≤10% (D) ≤5% (E), ≤2.5% (F) and ≤1% (G); solid line: mutations detectable in CR (N+); dashed line: mutations not detectable in CR (N-).

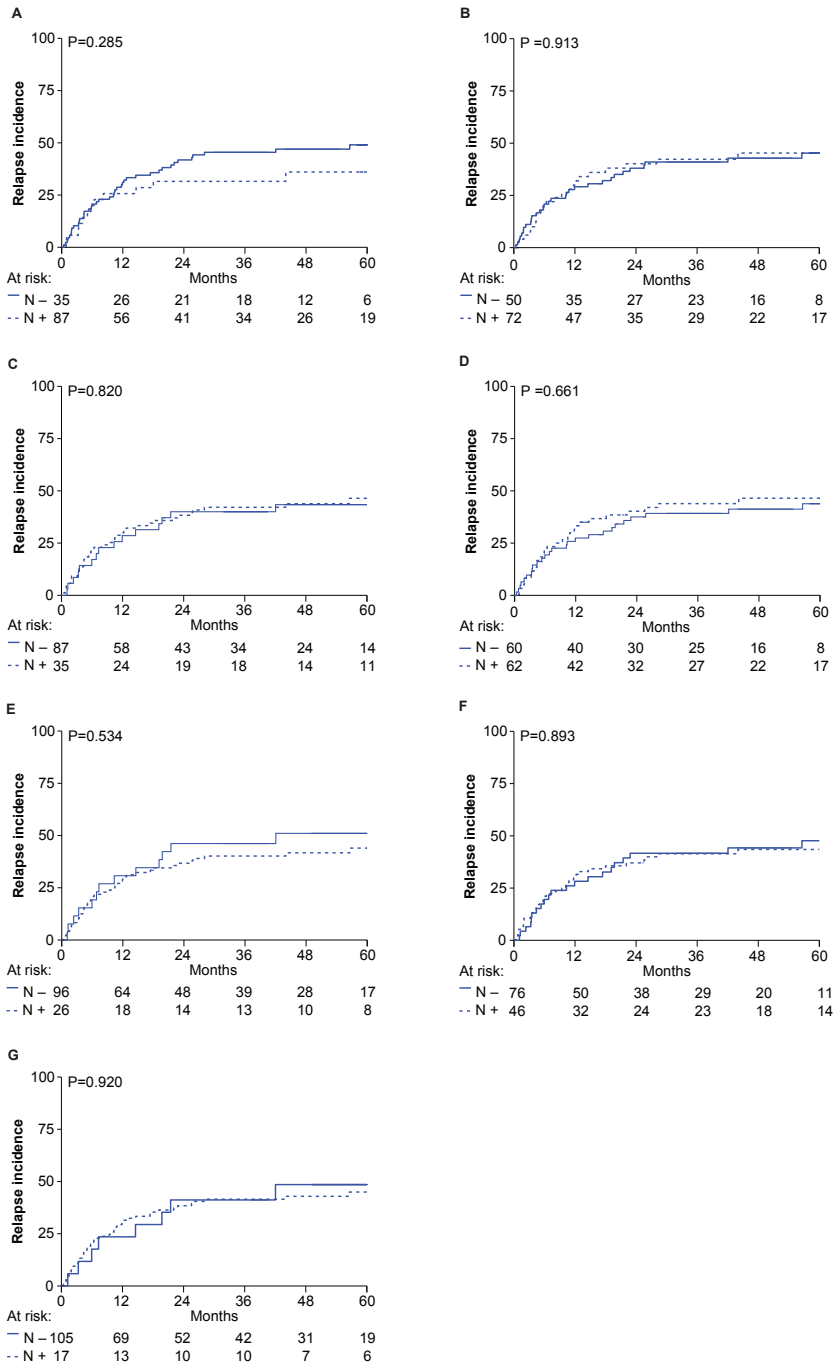

Figure S5. Cumulative incidence of relapse by detectable *DNMT3A, TET2* and/or *ASXL1* mutations in CR with VAF cut offs in *DNMT3A*, *TET2* and/or *ASXL1* mutant AML patients allocated to the training set.
No cut off (A), ≤30% (B), ≤20% (C), ≤10% (D), ≤5% (E), ≤2.5% (F) and ≤1.0% (G); solid line: DTA mutations detectable (N+); dashed line: DTA mutations not detectable (N-).

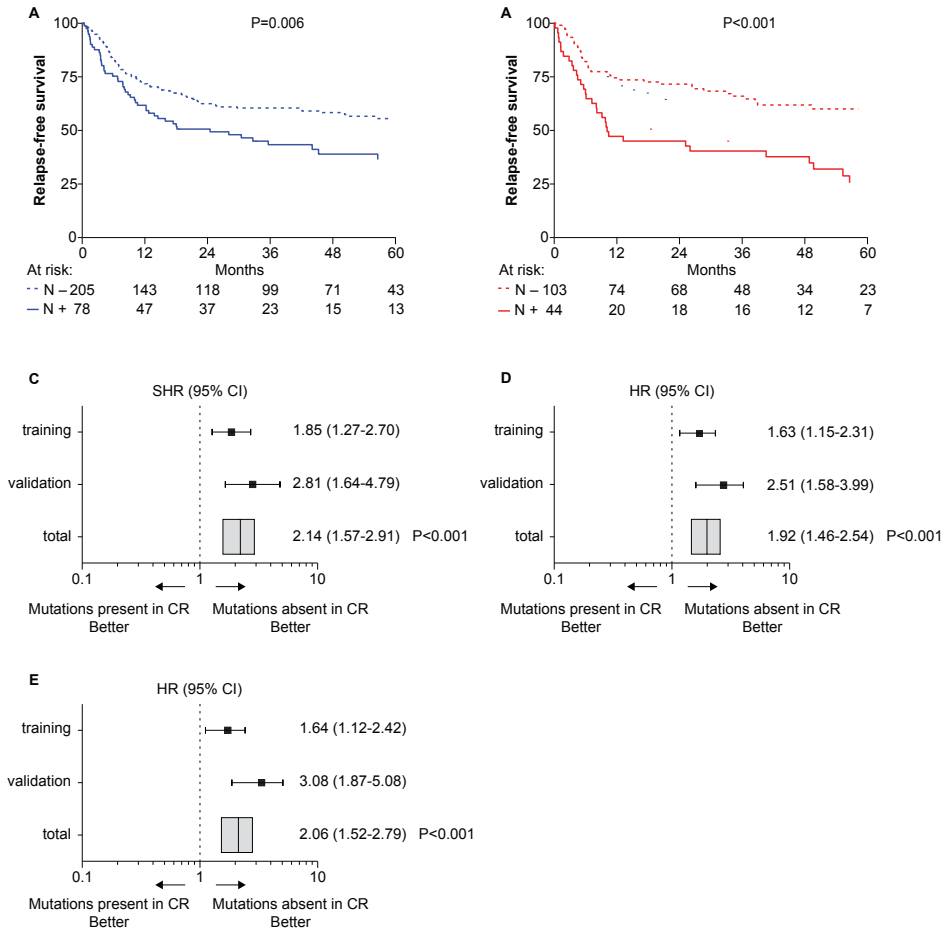

Figure S6. Relapse free survival analyses of non-DTA persisting mutations in CR in AML patients allocated to the training AML cohort and the validation cohort.
Cumulative incidence of relapse curves of (A) relapse free survival training cohort and (B) relapse free survival validation cohort. Solid line: non-DTA mutations detectable (N+); dashed line: non-DTA mutations not detectable (N-). Forest plots of (C) relapse, (D) relapse-free survival and (E) overall survival, including training, validation and summary measures. In Figures S6A-E all DTA persisting mutations are excluded at any VAF.

Table S2. Multivariable analysis of prognostic factors with correction for sampling time.

Prognostic factor	Relapse Incidence		Relapse-free Survival		Overall survival	
	Hazard ratio (95% CI)	P value	Hazard ratio (95% CI)	P value	Hazard ratio (95% CI)	P value
Molecular residual disease (Detection vs. No detection)	1.92 (1.37-2.70)	<0.001	1.66 (1.24-2.23)	0.001	1.66 (1.20-2.30)	0.002
Age (per year)	1.01 (1.00-1.03)	0.114	1.02 (1.01-1.03)	0.004	1.03 (1.01-1.04)	<0.001
WBC at diagnosis (x10^9/l) (>100 vs. ≤100)	2.22 (1.37-3.59)	0.001	2.08 (1.37-3.16)	0.001	2.04 (1.28-3.24)	0.003
ELN 2017 risk classification						
Intermediate vs. Favorable	1.72 (1.15-2.55)	0.008	2.04 (1.45-2.87)	<0.001	2.58 (1.75-3.80)	<0.001
Adverse vs. Favorable	1.81 (1.25-2.63)	0.002	2.21 (1.57-3.09)	<0.001	2.67 (1.82-3.91)	<0.001
No. of cycles to CR (2 cycles vs. 1 cycle)	2.10 (1.44-3.06)	<0.001	2.41 (1.73-3.36)	<0.001	2.97 (2.09-4.22)	<0.001
Sampling time (in days)	0.99 (0.98-1.00)	0.021	0.99 (0.99-1.00)	0.121	0.99 (0.99-1.00)	0.094

Table S3. Multivariable analysis with time-dependent correction for allogeneic HSCT.

Prognostic factor	Relapse incidence			Relapse-free survival			Overall survival		
	SHR	95% CI	P value	HR	95% CI	P value	HR	95% CI	P value
Molecular residual disease (Detection vs. No detection)	1.88	1.34-2.64	<0.001	1.64	1.22-2.21	0.001	1.64	1.18-2.27	0.003
Age per year	1.00	0.99-1.02	0,556	1.02	1.00-1.03	0.024	1.03	1.01-1.04	0.001
WBC at diagnosis (x10^9/l) (>100 vs. ≤100)	2.20	1.34-3.63	0,002	2.04	1.35-3.10	0.001	2.02	1.27-3.20	0.003
ELN 2017 risk classification									
Intermediate vs. Favorable	2.31	1.52-3.51	0.001	2.28	1.58-3.28	<0.001	2.56	1.70-3.84	<0.001
Adverse vs. Favorable	2.44	1.64-3.62	0.001	2.51	1.75-3.59	<0.001	2.71	1.81-4.04	<0.001
Number of cycles to CR (2 cycles vs. 1 cycle)	2.07	1.42-3.03	<0.001	2.39	1.72-3.33	<0.001	2.95	2.08-4.20	<0.001
Post-remission therapy (Allo vs. no Allo)	0.45	0.31-0.65	<0.001	0.73	0.54-0.99	0.043	0.97	0.70-1.34	0.848

4

PROGNOSTIC VALUE OF *FLT3*-ITD RESIDUAL DISEASE IN ACUTE MYELOID LEUKEMIA

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ABSTRACT

PURPOSE

The applicability of *FLT3*-internal tandem duplications (*FLT3*-ITD) for assessing measurable residual disease (MRD) in acute myeloid leukemia (AML) in complete remission (CR) has been hampered by patient-specific duplications and potential instability of *FLT3*-ITD during relapse. Here, we comprehensively investigated the impact of next-generation sequencing (NGS)–based *FLT3*-ITD MRD detection on treatment outcome in a cohort of patients with newly diagnosed AML in relation to established prognostic factors at diagnosis and other MRD measurements, ie, mutant *NPM1* and multiparameter flow cytometry.

METHODS

In 161 patients with de novo *FLT3*-ITD AML, NGS was performed at diagnosis and in CR after intensive remission induction treatment. *FLT3*-ITD MRD status was correlated with the cumulative incidence of relapse and overall survival (OS).

RESULTS

NGS-based *FLT3*-ITD MRD was present in 47 of 161 (29%) patients with AML. Presence of *FLT3*-ITD MRD was associated with increased risk of relapse (4-year cumulative incidence of relapse, 75% *FLT3*-ITD MRD *v* 33% no *FLT3*-ITD MRD; $P < .001$) and inferior OS (4-year OS, 31% *FLT3*-ITD MRD *v* 57% no *FLT3*-ITD MRD; $P < .001$). In multivariate analysis, detection of *FLT3*-ITD MRD in CR confers independent prognostic significance for relapse (hazard ratio, 3.55; $P < .001$) and OS (hazard ratio 2.51; $P = .002$). Strikingly, *FLT3*-ITD MRD exceeds the prognostic value of most generally accepted clinical and molecular prognostic factors, including the *FLT3*-ITD allelic ratio at diagnosis and MRD assessment by NGS-based mutant *NPM1* detection or multiparameter flow cytometry.

CONCLUSIONS

NGS-based detection of *FLT3*-ITD MRD in CR identifies patients with AML with profound risk of relapse and death that outcompetes the significance of most established prognostic factors at diagnosis and during therapy, and furnishes support for *FLT3*-ITD as a clinically relevant biomarker for dynamic disease risk assessment in AML.

INTRODUCTION

Acute myeloid leukemia (AML) is a heterogeneous disease that arises from the sequential acquisition of specific driver mutations in the leukemic stem cell.[1] Internal tandem duplications (ITD) in the *FMS-like tyrosine kinase 3* (*FLT3*) receptor gene are among the most common genetic molecular abnormalities in patients with AML.[2,3] In *FLT3*-ITD AML, the FLT3 kinase is constitutively activated resulting in uncontrolled proliferation of leukemic blasts.[4] *FLT3*-ITDs are generally considered late-event mutations in leukemogenesis and are frequently preceded by the appearance of mutations in *DNMT3A* and *NPM1*.[2,3] *FLT3*-ITD has been suggested to characterize an aggressive leukemic phenotype with early relapse and inferior treatment outcome.[3-5] Several features present at diagnosis have been postulated to modify the prognostic effect of *FLT3*-ITD, including the presence of concurrent mutant *NPM1* and the *FLT3*-ITD clone size, ie, allelic ratio.[5]

Increasing evidence indicates that treatment outcome prediction can be improved by assessing the kinetics and depth of response during therapy by detection of measurable residual disease (MRD).[6] Currently, MRD detection in *FLT3*-ITD AML is carried out by a combination of multiparameter flow cytometry (MFC), mutant *NPM1* real-time quantitative polymerase chain reaction (RQ-PCR), and next-generation sequencing (NGS).[7] In the past, *FLT3*-ITD MRD detection by RQ-PCR and NGS was hampered by the variety of patient-specific *FLT3*-ITD, ie, sequence, position, and length. Advances in sequencing technology now enable accurate molecular detection of *FLT3*-ITD MRD.[8-10]

Clonal evolution studies revealed that late-event mutations in activated signaling genes, such as those in *FLT3*, are often subclonal and can be unstable during relapse in up to 25% of patients with AML, which may disqualify these markers for MRD detection.[11-16] Systematic studies evaluating the applicability of *FLT3*-ITD MRD detection as a prognostic biomarker in AML are therefore lacking. Here, we present a comprehensive study investigating the impact of NGS-based *FLT3*-ITD MRD detection on treatment outcome in a cohort of patients with newly diagnosed AML, enrolled in multicenter prospective phase III HOVON-SAKK clinical trials, in relation to various other established baseline and MRD prognostic markers.

METHODS

Patients and Samples

In total, 161 treatment-naive patients with de novo *FLT3*-ITD AML out of 2,274 patients with AML were included (Fig 1). Patients were enrolled in Dutch-Belgian Cooperative Trial Group for Hematology-Oncology (HOVON) or the Swiss Group for Clinical Cancer Research (SAKK) clinical trials HO42A AML, HO102 AML, and HO132 AML (Fig 1). Trial protocols were approved by the ethics committees at each participating site and performed in accordance with the Declaration of Helsinki and after obtaining patient written informed consent. All patients had achieved complete remission (CR; < 5% blast cells in the bone marrow) after two cycles of induction chemotherapy. Treatments protocols and inclusion criteria have been described previously.[17-19] Of note, in a subset of patients with AML included in the HOVON 132 trial, the residual disease status by MFC and/or mutant *NPM1* RQ-PCR assay was available to the clinical investigator before consolidation therapy to enable subsequent treatment choice.[19] Patient samples were taken at diagnosis and during CR after two cycles of standard induction chemotherapy. Details about patient and sample selection are provided in the Data Supplement.

FLT3-ITD and Mutant *NPM1* Detection by Targeted NGS

The *FLT3*-ITD status at AML diagnosis was assessed with both capillary fragment length analysis and the NGS TruSight Myeloid Sequencing Panel. Patients were excluded when the presence of *FLT3*-ITD at diagnosis could not be confirmed by NGS or when the *FLT3*-ITD was located in exon 15 (Fig 1). High and low allelic *FLT3*-ITD allelic ratios were determined by capillary fragment length analysis and defined as ≥ 0.5 or < 0.5, respectively.[5,20] *NPM1* mutations at AML diagnosis were determined by NGS. In CR, *FLT3*-ITD and mutant *NPM1* MRD detection was performed with a single-amplicon NGS library panel, covering exon 14 of *FLT3* or exon 12 of *NPM1*, for targeted deep sequencing analysis. The limit of detection of the *FLT3*-ITD MRD assay in CR ranges between variant allele frequencies of 0.01% and 0.001% (Data Supplement). In case of insufficient read coverage in CR (< 300,000 reads), the patient samples were excluded (Fig 1). All patients with *FLT3*-ITD AML were considered for MRD analysis, irrespective of *FLT3*-ITD ratio and/or number of *FLT3*-ITDs detectable by NGS at diagnosis. The NGS libraries were paired-end sequenced (2 x 221-bp) on Illumina NGS platforms according to manufacturer's recommendation (Illumina, San Diego, CA). We used our in-house data analysis pipeline for variant calling as previously described.[21] Details on the experimental procedures, limit of detection, and bioinformatic analyses are available in the Data Supplement.

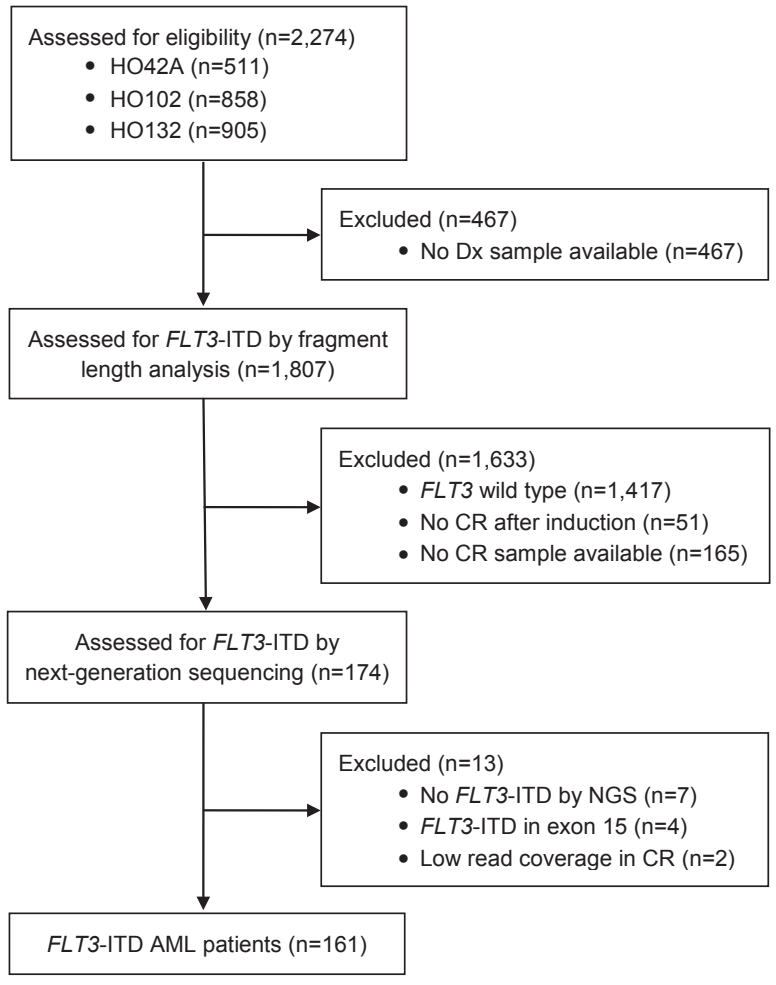

Figure 1. Consort diagram of *FLT3*-ITD MRD study.

Multiparameter Flow Cytometry

Detection of MFC MRD was performed as recommended.[7] The residual disease percentage was defined as the number of leukemia-associated immunophenotype cells within the total white blood cell compartment. The threshold between residual disease and no residual disease on the basis of flow cytometry was established at 0.1%. MFC MRD was carried out in 138 out of 161 patients with *FLT3*-ITD AML.

Statistical Analysis

The primary end point of the study was the cumulative incidence of relapse (CIR). Relapse and survival time were calculated from the date when CR bone marrow samples were taken until the date of the event of interest or censoring set at the date last known alive. Competing-risk analysis was performed for relapse with adjustment for nonrelapse mortality according to the method of Gray[22] and the Fine and Gray[23] model. The secondary end point was overall survival (OS), defined by death from any cause. Survival curves were estimated using the Kaplan-Meier method and differences in survival were assessed with the log-rank test. Multivariable modeling was performed with the Cox proportional hazards model. The proportional hazards assumption was tested by including the interaction with time-varying coefficients. Differences in patient or molecular characteristics were tested using the Fisher's exact test for categorical variables and Mann-Whitney U test for continuous variables. All statistical tests were two-sided, and P values < .05 were considered statistically significant. Statistical analyses were executed with Stata Statistics and Data Science software, Release 17.0 (Stata, College Station, TX).

RESULTS

FLT3-ITD Detection at Diagnosis, Relapse and in Complete Remission

NGS-based *FLT3*-ITD detection was carried out in a cohort of 161 patients with AML, with a median survival time of 29.6 months (Table 1 and Fig 1). At diagnosis, 74 out of 161 (46%) patients had *FLT3*-ITD low allelic ratios and 87 (54%) patients had high allelic ratios. Patients with *FLT3*-ITD AML carried a variety of concurrent gene mutations (Table 1 and Data Supplement). The most frequent coexisting mutations were found in *NPM1* (57%) and *DNMT3A* (47%). At relapse, identical *FLT3*-ITDs were observed in 88% of patients with AML (n = 25). Two patients with AML had gained mutations in different genes at relapse that had been wild-type at diagnosis (Data Supplement).

Table 1. Patient characteristics of *FLT3*-ITD AML by detection of *FLT3*-ITD MRD.

	no *FLT3*-ITD MRD (n=114)	*FLT3*-ITD MRD (n=47)	Total (n=161)	
Age - yr				p=0.247
Median	51	53	51	
Range	23-66	19-65	19-66	
Sex - no. (%)				p=0.863
M	59 (52)	23 (49)	82 (51)	
F	55 (48)	24 (51)	79 (49)	
WBC at diagnosis - no. (%)				p=0.069
≤100x10^9/L	103 (90)	37 (79)	140 (87)	
>100x10^9/L	11 (10)	10 (21)	21 (13)	
ELN 2017 risk classification - no. (%)				p<0.001
Favorable	42 (37)	4 (9)	46 (28)	
Intermediate	51 (45)	18 (38)	69 (44)	
Adverse	21 (18)	25 (53)	46 (28)	
Last treatment before first CR - no. (%)				p<0.001
Cycle I	101 (89)	26 (55)	127 (79)	
Cycle II	13 (11)	21 (45)	34 (21)	
Consolidation therapy - no. (%)				p=0.075
None	14 (12)	9 (19)	23 (14)	
Chemotherapy	14 (12)	4 (9)	18 (11)	
Autologous HSCT	24 (21)	3 (6)	27 (17)	
Allogeneic HSCT	62 (55)	31 (66)	93 (58)	
Cytogenetics - no. (%)†				p=0.040
Normal karyotype	90 (81)	30 (65)	120 (76)	
Aberrant karyotype	21 (19)	16 (35)	37 (24)	
Mutations at diagnosis - no. (%)				
FLT3				p=0.121
Internal tandem duplication, low ratio	57 (50)	17 (36)	74 (46)	
Internal tandem duplication, high ratio	57 (50)	30 (64)	87 (54)	
NPM1				p<0.001
wild type	36 (32)	34 (72)	70 (43)	
mutant	78 (68)	13 (28)	91 (57)	
DNMT3A				p=1.000
wild type	61 (54)	25 (53)	86 (53)	
mutant	53 (46)	22 (47)	75 (47)	

†Cytogenetics failed in 4 patients.

FLT3-ITD MRD was detected in 47 out of 161 (29%) patients with AML, with a median variant allele frequency of 0.008% (range, 0.00031%-3.10%; Table 1 and Data Supplement). All *FLT3*-ITDs found in CR were identical (ie, sequence, position, and length) compared with diagnosis and multiple *FLT3*-ITD MRD clones were detected in five patients with AML. *FLT3*-ITD MRD was significantly associated with aberrant cytogenetics at diagnosis (35% *FLT3*-ITD MRD *v* 19% no *FLT3*-ITD MRD; *P* = .040) and patients with *NPM1* wild-type AML (72% *FLT3*-ITD MRD *v* 32% no *FLT3*-ITD MRD; *P* < .001; Table 1). Interestingly, *FLT3*-ITD MRD was observed in 22% of patients with triple-mutant AML (*DNMT3A*/*NPM1*/*FLT3*-ITD), whereas none of the patients with double–mutant AML (*NPM1*/*FLT3*-ITD) showed *FLT3*-ITD MRD. Although significantly higher *FLT3*-ITD ratios were observed in patients with *FLT3*-ITD MRD AML (*P* = .035), the association of *FLT3*-ITD MRD with the *FLT3*-ITD allelic ratio status at diagnosis was not significant (*P* = .121). Detection of *FLT3*-ITD MRD was significantly more frequent in patients with AML who needed two cycles rather than one induction cycle to attain CR (45% *FLT3*-ITD MRD *v* 11% no *FLT3*-ITD MRD; *P* < .001; Table 1).

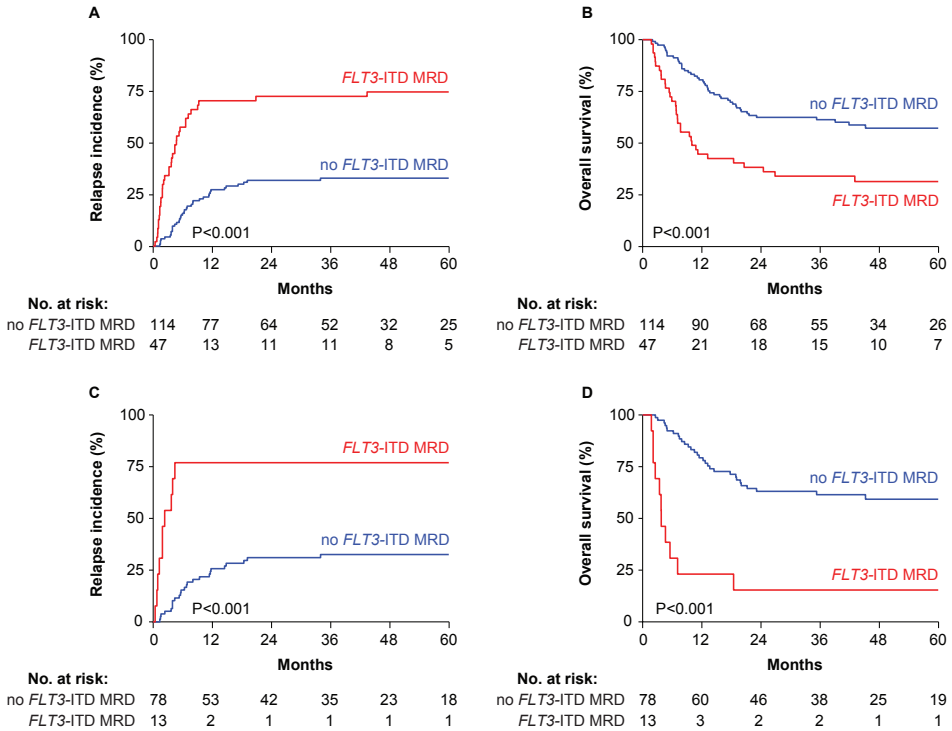

Figure 2. Survival outcome of *FLT3*-ITD MRD.
Relapse incidence (A) and overall survival (B) of *FLT3*-ITD AML according to *FLT3*-ITD MRD in complete remission (n=161). Relapse incidence (C) and overall survival (D) according to *FLT3*-ITD MRD in mutant NPM1 *FLT3*-ITD AML patients (n=91).

FLT3-ITD Residual Disease and Outcome

We next assessed whether *FLT3*-ITD MRD was prognostic for relapse and OS. *FLT3*-ITD MRD was associated with an increased risk of relapse (4-year CIR, 75% *FLT3*-ITD MRD *v* 33% no *FLT3*-ITD MRD; hazard ratio [HR], 3.70; 95% CI, 2.31 to 5.94; *P* < .001) and reduced OS (4-year OS, 31% *FLT3*-ITD MRD *v* 57% no *FLT3*-ITD MRD; HR, 2.47; 95% CI, 1.59 to 3.84; *P* < .001; Figs 2A and 2B). To increase the sensitivity of the *FLT3*-ITD MRD assay, we compared 100 ng and 500 ng DNA input in a selected subset of patients with AML for whom sufficient DNA was available (n = 122). Although the number of patients with *FLT3*-ITD MRD AML increased (n = 13) at very low levels (< 0.01%), the association with relapse did not improve (Data Supplement). The *FLT3*-ITD MRD clone size, as indicated by the variant allele frequency in remission, was directly correlated with the risk of relapse (Data Supplement). The number of persisting *FLT3*-ITD MRD clones did not associate with relapse (Data Supplement). Within the mutant *NPM1* *FLT3*-ITD AML subset, the prognostic value for relapse (4-year CIR, 77% *FLT3*-ITD MRD *v* 33% no *FLT3*-ITD MRD; HR, 4.87; 95% CI, 1.92 to 12.3; *P* < .001) and survival (4-year OS, 15% *FLT3*-ITD MRD *v* 59% no *FLT3*-ITD MRD; HR, 5.36; 95% CI, 2.65 to 10.8; *P* < .001) was preserved (Figs 2C and 2D).

To assess whether the detection of *FLT3*-ITD MRD serves as an independent prognostic factor, we performed univariate analysis and multivariate analysis. In univariate analysis, significantly increased risk of relapse and reduced OS were observed among patients with a high white blood cell count at diagnosis (> 100×10^9/L), late CR (ie, achieved after two cycles of induction chemotherapy), and patients with a high *FLT3*-ITD allelic ratio at diagnosis (Data Supplement) and within the ELN intermediate and adverse risk classification (Data Supplement). In multivariable modeling, *FLT3*-ITD MRD confers profound independent prognostic significance with respect to the relapse rate (HR, 3.55; 95% CI, 1.92 to 6.56; *P* < .001) and OS (HR, 2.51; 95% CI, 1.42 to 4.43; *P* = .002; Table 2). Besides *FLT3*-ITD MRD, only a high white blood cell count and late CR appeared to be independently associated with relapse and OS. Remarkably, the *NPM1* mutation status and the *FLT3*-ITD allelic ratio at diagnosis lost their prognostic value for relapse and survival when *FLT3*-ITD MRD was taken into account (Table 2). In sensitivity analysis, no significant clinically relevant or treatment-related interactions were observed. The prognostic value of *FLT3*-ITD MRD was unaffected in a correction for variation in sampling time (Data Supplement).

Table 2. Multivariable analysis of prognostic factors for relapse and overall survival.

Prognostic factor	Relapse Incidence		Overall Survival	
	Hazard ratio (95% CI)	P Value	Hazard ratio (95% CI)	P Value
***FLT3*-ITD MRD** (detection vs. no detection)	3.55 (1.92-6.56)	<0.001	2.51 (1.42-4.43)	0.002
Age (per 10 years)	1.05 (0.86-1.29)	0.621	1.20 (0.97-1.49)	0.087
WBC at diagnosis (>100x109/L vs. ≤100x109/L)	2.96 (1.73-5.07)	<0.001	1.89 (1.05-3.43)	0.035
***NPM1* mutation at diagnosis** (mutant vs. wild type)	1.21 (0.68-2.16)	0.522	1.30 (0.75-2.23)	0.348
***FLT3*-ITD ratio at diagnosis** (high vs. low)	1.76 (1.00-3.09)	0.050	1.60 (0.96-2.67)	0.070
Number of cycles to attain complete remission (two cycles vs. one cycle)	1.84 (1.10-3.09)	0.020	1.73 (0.98-3.06)	0.058

FLT3-ITD Residual Disease and Allogeneic Transplantation

Regarding the poor risk features of *FLT3*-ITD AML, many patients will undergo allogeneic transplantation. Therefore, we explored the prognostic value of *FLT3*-ITD MRD in the 93 patients who underwent allogeneic transplantation. In total, 30 patients with AML received myeloablative conditioning (MAC) and 63 patients received reduced-intensity conditioning (RIC). Although the overall risk of relapse is reduced in the transplanted *FLT3*-ITD AML patients, we demonstrate increased relapse incidence and inferior outcome of patients with *FLT3*-ITD MRD AML (Fig 3). The prognostic value of *FLT3*-ITD MRD is comparable in both MAC and RIC groups (P = .858). However, the risk of relapse is lower in patients with *FLT3*-ITD MRD AML who had received MAC conditioning. No significant differences for nonrelapse mortality between the RIC and MAC groups were observed, resulting in improved OS for patients with AML with residual disease after MAC conditioning (Fig 3). In a time-dependent correction for allogeneic hematopoietic stem-cell transplant, the prognostic significance of *FLT3*-ITD MRD is maintained (Data Supplement).

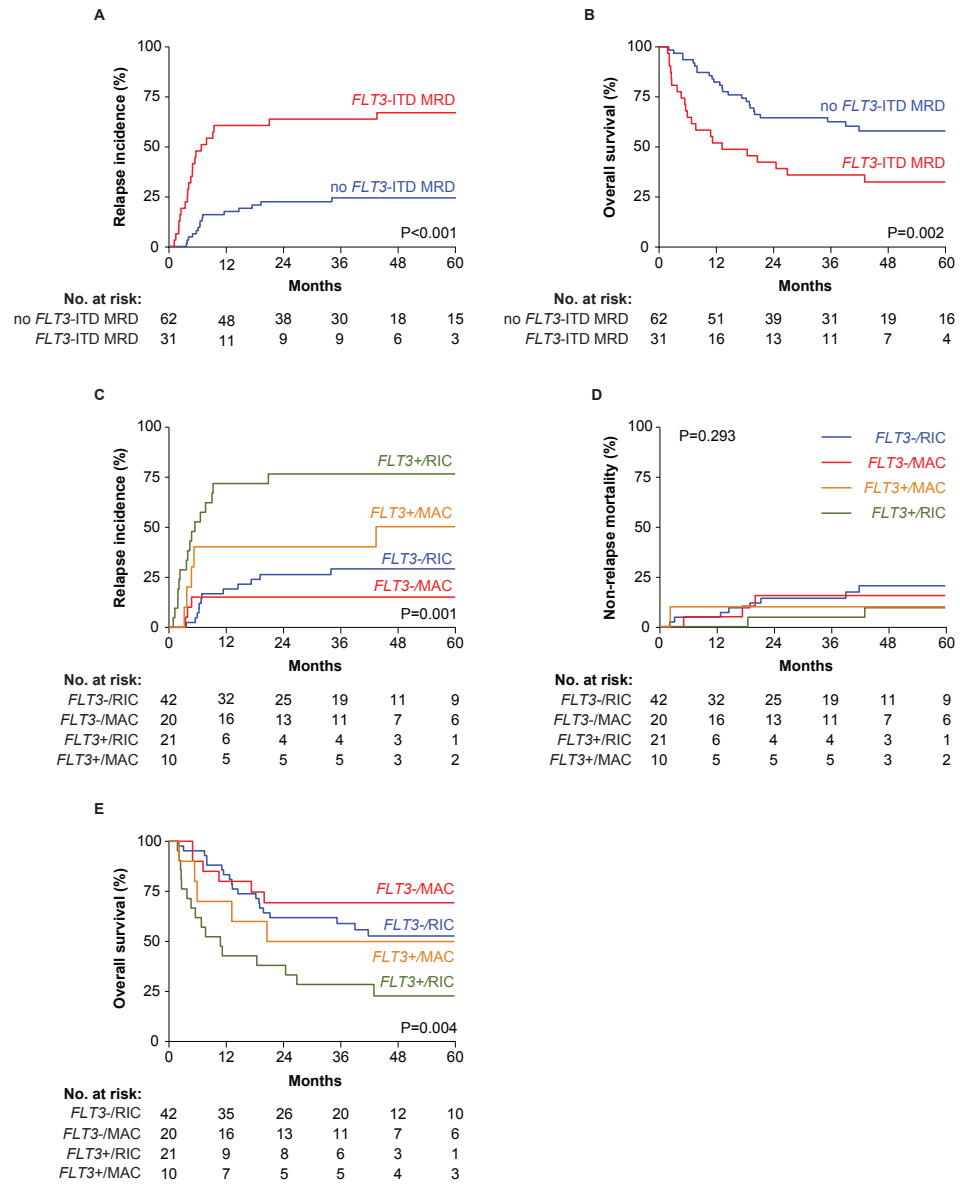

Figure 3. Survival outcome of *FLT3*-ITD MRD and allogeneic transplantation.
Relapse incidence (A) and overall survival (B) of *FLT3*-ITD MRD in AML patients that received allogeneic transplantation (n=93). The lower panel depicts the relapse incidence (C), non-relapse mortality (D) and overall survival (E) of *FLT3*-ITD MRD in AML patients stratified by reduced intensity (RIC) or myeloablative (MAC) conditioning regimens.

Residual Disease Detection by Mutant *NPM1* and MFC

MFC MRD and mutant *NPM1* MRD are recommended methods for relapse prediction in patients with AML in CR.[7] We compared the assessment of *FLT3*-ITD MRD with available NGS-based mutant *NPM1* and MFC residual disease measurements in 91 and 138 patients with AML, respectively.

Interestingly, *FLT3*-ITD MRD significantly associated with high relapse risk and adverse OS irrespective of the mutant *NPM1* MRD or MFC MRD status (Fig 4). Increased risk of relapse and inferior survival was observed in patients with AML without *FLT3*-ITD MRD but with persistent mutant *NPM1* MRD; however, the association was not significant (P = .081 and P = .236). By contrast, MFC MRD without *FLT3*-ITD MRD appeared to confer limited prognostic value for relapse and survival (Figs 4C and 4D).

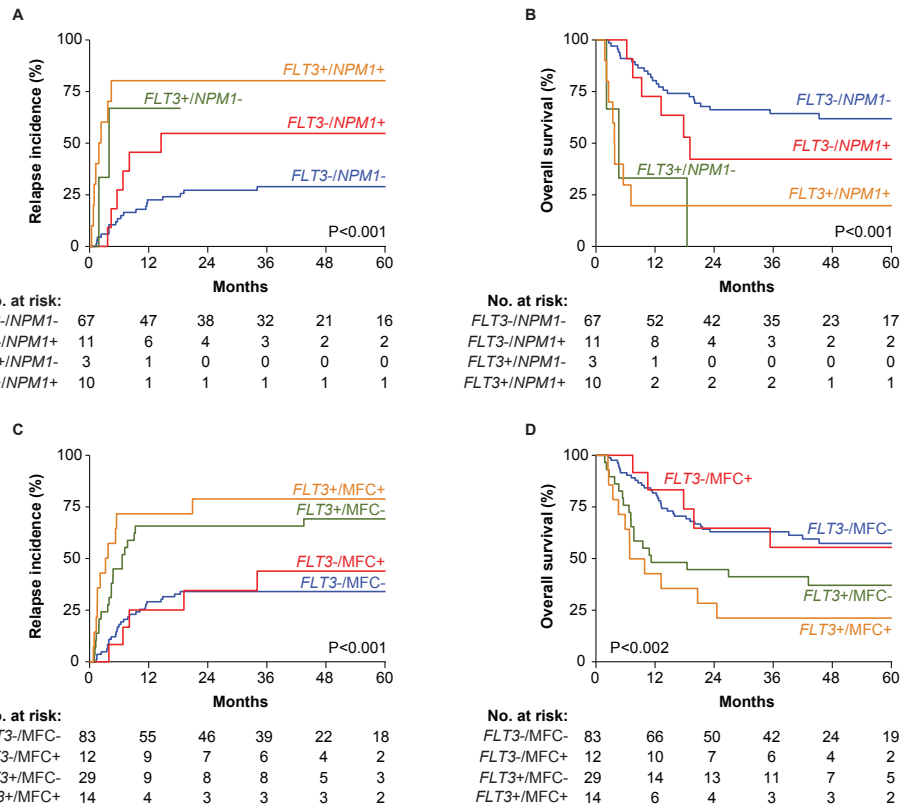

Figure 4. Survival outcome of *FLT3*-ITD MRD and NGS-based mutant *NPM1* and MFC MRD.
Relapse incidence (A) and overall survival (B) of *FLT3*-ITD MRD and mutant *NPM1* MRD combined (n=91). Panel C and D depicts the relapse incidence and overall survival of both *FLT3*-ITD MRD and multiparameter flow cytometry (MFC) MRD (n=138).

DISCUSSION

Current risk classification and management of *FLT3*-ITD AML relies on the assessment of gene mutations at diagnosis and dynamic residual disease response measurements during therapy.[5] However, the prognostic significance of *FLT3*-ITD has been subject of ongoing debate as it may depend on allelic burden and the presence of other gene mutations, in particular mutant *NPM1*.[5] Here, we performed a comprehensive study in *FLT3*-ITD AML investigating the impact of persistence of *FLT3*-ITD in CR after induction chemotherapy and treatment outcome. Our results reveal *FLT3*-ITD MRD as a strong independent prognostic factor that identifies patients with AML with profound risk of relapse and death. Furthermore, *FLT3*-ITD MRD outcompetes the impact of other currently established prognostic factors in *FLT3*-ITD AML, including the *NPM1* mutation status and *FLT3*-ITD allelic ratio at diagnosis and residual disease measurements in CR by NGS-based mutant *NPM1* detection or MFC.

Mutations in activated signaling genes, such as *FLT3*-ITD, generally represent late events in AML development and can be lost or gained at relapse.[11-16] Therefore, residual disease detection of these late mutations have previously been considered to be of limited prognostic value, in contrast to some of the more stable leukemic driver mutations, such as mutant *NPM1*.[7,16] The results of the current study, however, indicate stability of *FLT3*-ITD during relapse in the majority of patients with AML and substantiate residual *FLT3*-ITD in CR as a clinically useful indicator for relapse. We showed that leukemic clones with residual *FLT3*-ITDs that were present at baseline carry high impact on the risk of relapse, whereas AML clones with persistent mutant *NPM1* without *FLT3*-ITD had limited prognostic value for relapse. This implies that, within *FLT3*-ITD AML with concurrent mutant *NPM1*, residual disease characterized by *FLT3*-ITD better identifies patients with AML for relapse than mutant *NPM1* MRD detection alone. Future studies exploring the comparison of DNA- and RNA-based mutant *NPM1* and *FLT3*-ITD MRD testing as well as using peripheral blood may further improve MRD monitoring of these genetically defined AML subtypes.

FLT3-ITD MRD failed to identify all cases of AML relapse. Although NGS-based sequencing was carried out at substantial depth, *FLT3*-ITDs at ultralow levels (< 0.001%) may have been missed. The *FLT3*-ITD assay may even further improve prediction of relapse by the ability to sequence at even higher depth or by increased DNA sample input, as well as the capability to sequence larger *FLT3*-ITDs and those in exon 15 of the *FLT3* gene. Of note, we studied *FLT3*-ITD MRD at a single time point. With respect to the correlation of *FLT3*-ITD MRD clone size with relapse, it will be of interest to examine how longitudinal monitoring may support relapse prediction or guide different treatment strategies and allogeneic conditioning regimens for *FLT3*-ITD AML.

We have studied *FLT3*-ITD MRD in patients treated with intensive chemotherapy only. It will be interesting to investigate the prognostic value of *FLT3*-ITD MRD in the context of treatments that include the use of FLT3 inhibitors, since these patients have been shown

to acquire novel *FLT3*-ITD mutations or mutations in other activated signaling genes during relapse.[24-26] From this perspective, it will be important to expand NGS-based MRD detection to additional relevant late-event mutations in activated signaling genes and investigate the efficiency of novel and more specific FLT3 inhibitors on the eradication of residual *FLT3*-ITD cells and the prevention of impending relapse in AML.

In conclusion, NGS-based detection of *FLT3*-ITD MRD in CR identifies patients with AML with profound relapse risk and death, and outweighs the prognostic factors that are currently used in AML risk stratification. Therefore, we propose to incorporate *FLT3*-ITD MRD in AML treatment protocols.

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SUPPLEMENTARY METHODS

Patients and Samples

FLT3-ITD was detected in 174 AML patients at diagnosis with fragment length analysis (Fig. S1). Patients were excluded when the presence of *FLT3*-ITD at diagnosis could not be confirmed by NGS (n=7) or in case the *FLT3*-ITD was located in exon 15 (n=4). Two patients were excluded because of insufficient read coverage in complete remission (<300.000 reads) (Fig. S1). In total 4 out of 161 patients classified as high-risk myelodysplastic syndrome with excess blasts (MDS-EB) with IPSS ≥1.5 or IPSS-R >4.5. All patients achieved complete morphological remission (CR). Only 1 patient sample was taken in complete remission with incomplete blood count recovery (CRi; <5% blasts, absolute neutrophil count >1.0x10^9/L and thrombocytes >100 x10^9/L). Remission samples were taken 21 days after the start of the second cycle of induction chemotherapy and prior to consolidation therapy. In patients without consolidation therapy the last sample within a four-month time interval was selected. The mean and median time of sample collection calculated form the first day of starting the second induction cycle was 7.3 weeks and 6.5 weeks respectively. In 5 of the 161 patients only peripheral blood remission samples were available. In a subset of samples, blasts and mononuclear cells at diagnosis and complete remission were purified by Ficoll-Hypaque (Nygaard, Oslo, Norway) density gradient centrifugation and cryopreserved. After thawing, cells were lysed in RLT solution with the addition of DTT (Qiagen, Venlo, The Netherlands) or lysed in RNA-bee (Bio-Connect, Huissen, The Netherlands). High quality DNA was extracted using the QIAsymphony (Qiagen, Venlo, The Netherlands) or by RNA-bee DNA isolation (Bio-Connect, Huissen, The Netherlands) according to the manufacturer's instructions. DNA concentration was measured by Qubit Fluorometric Quantitation (Thermo Fisher Scientific, Wilmington, DE).

FLT3-ITD Capillary Fragment Length Analysis and Allelic Ratio

FLT3-ITD analysis at AML diagnosis was performed on DNA using capillary fragment length analysis as previously described.[1,2] Mutations with a calculated *FLT3*-ITD allelic ratio (AR) of <0.01 were considered wild-type. *FLT3*-ITD allelic ratio (AR) = total mutant peak area / wild-type peak (WT) area as determined by capillary fragment length analysis. High and low allelic *FLT3*-ITD allelic ratios were defined as ≥0.5 or <0.5, respectively, similarly to the ELN 2017 risk classification. The size of each *FLT3*-ITD clone as detected with capillary fragment length analysis was matched with the size of each clone detected with NGS at AML diagnosis.

Targeted Next-Generation Sequencing at Diagnosis and Relapse

NGS analysis at AML diagnosis was performed using the TruSight Myeloid Sequencing Panel (Illumina, San Diego, CA) designed to achieve full exonic coverage of 15 genes plus targeting exonic hotspots of an additional 39 genes recurrently mutated in myeloid malignancies

including *FLT3* exon 14 and 15 and *NPM1* exon 12. Libraries were generated with 50ng DNA input according to manufacturer's instructions and paired-end sequenced (2x221bp) using a MiSeq Reagent Kit v3 or HiSeq Rapid SBS Kit v2 (Illumina, San Diego, CA). In 7 patients the *FLT3*-ITD was missed because of extensive ITD length (>123bp) (Fig. S1).

Limit of Detection

To determine the limit of detection in CR we performed serial dilution of *FLT3*-ITD AML samples at diagnosis by spiking in *FLT3* wild type DNA. Patients with several ITD lengths (24, 45, 69, 81 and 117 base pairs) were selected and all samples were analyzed in duplicate. *FLT3*-ITDs can be detected at very low levels with variant allele frequencies ranging between 0.01% and 0.001% (Fig. S2). Quantification of *FLT3*-ITD MRD was linear in all AML patients irrespective of the ITD length with a goodness of fit close to 1 (R^2 value).

Targeted Next-Generarion Deep Sequencing in Complete Remission

In a subset of patients, the *FLT3*-ITD MRD (n=12) and mutant *NPM1* MRD (n=4) status was determined present by conventional analysis using the TruSight Myeloid Sequencing Panel (Illumina, San Diego, CA). The remaining remission samples were subjected to NGS deep sequencing. Primers for the target selection of *FLT3*-ITD and mutant *NPM1* MRD NGS deep sequencing analysis were designed using the Ion AmpliSeq Designer software (ThermoFisher Scientific, Bleiswijk). *FLT3*-ITD: FLT3_ex14_fw: 5'-CTCTATCTGCAGAACTGCCTATTCC-3' and FLT3_ex14_rv: 5'-TGCAAAGACAAATGGTGAGTACGT-3'. *NPM1* mutations: NPM1_ex12-fw: 5'-GTTAACTCTCTGGTGGTAGAATGAAAAATAGA-3' and NPM1_ex12-rv: 5'-GATATCAACTGTTACAGAAATGAAATAAGACG-3'. The *FLT3*-ITD and mutant *NPM1* specific primers were adapted for Illumina-based sequencing by adding an Illumina TruSeq Custom Amplicon Index Kit compatible Rd1 (forward) (5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3') or Rd2 (reverse) (5'-TCGCGAGTTAATGCAACGATCGTCGAAATTCGC-3') overhang adaptor sequence to the gene specific primers. Alternatively the Illumina Nextera DNA Unique Dual Indexes Set (Illumina, San Diego, CA, USA) compatible overhang adapter sequence Rd1 (forward) (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3') or Rd2 (reverse) (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3') are used to add the overhang adaptor sequence to the gene specific primers. In the second PCR, by means of these overhang adaptor sequences, sample-specific dual indices for sample identification and Illumina sequencing adaptors were attached by using primers from the Illumina TruSeq Custom Amplicon Index Kit or the Illumina Nextera DNA Unique Dual Indexes Set (Illumina, San Diego, CA, USA).

Target regions *FLT3* exon 14 and *NPM1* exon 12 were amplified by PCR on 100ng genomic DNA using the Roche FastStart High fidelity PCR System (Roche) containing 1× Buffer with 1.8mM MgCl2, 0.2mM dNTP, 0.4µM each primer, 0.1U FastStart Taq DNA polymerase.

Amplification was performed using the following PCR condition: 95°C for 5 minutes, 25 cycles of 30 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C and a final extension for 7 minutes at 72°C. Amplicons from the first step PCR were purified using the Agencourt AMPure XP bead purification kit (Beckman Coulter, Fullerton, CA, USA). The second step PCR was performed with primers from the Illumina TruSeq Custom Amplicon Index Kit or Illumina Nextera DNA Unique Dual Indexes Set using the KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Wilmington, MA, USA) with the following thermocycling condition: 95°C for 5 minutes, 10 cycles of 20 second at 98°C, 30 seconds at 66°C (TruSeq) / 55°C (Nextera), and 30 seconds at 72°C and a final extension for 1 minutes at 72°C. The library pool was purified with Agencourt AMPure XP beads and normalized for Illumina-based sequencing, according to the manufacturer's protocol (Illumina, San Diego, CA).

The final *NPM1* and *FLT3*-ITD single amplicon libraries were subjected to deep sequencing and paired-end sequenced (2x221bp) by the manufacturer's instructions using a HiSeq Rapid SBS Kit v2 or NovaSeq 6000 SP Reagent Kit (Illumina, San Diego, CA).

Details *FLT3*-ITD MRD deep sequencing analysis (n=149)	
FLT3 total reads range CR_FU2:	384020 – 3577012
FLT3 total reads mean CR_FU2:	1117137
FLT3-ITD reads range CR_FU2	6 – 87865
FLT3-ITD reads mean CR_FU2 [*FLT3*-ITD detectable]:	3138
FLT3-ITD VAF range CR_FU2:	0.00031 – 3.1%
FLT3-ITD VAF mean CR_FU2 [*FLT3*-ITD detectable]:	0.11%

Details mutant *NPM1* MRD deep sequencing analysis (n=87)	
NPM1 total reads range CR_FU2:	271210 – 1878150 (n=87)
NPM1 total reads mean CR_FU2:	849112
Mutant *NPM1* reads range CR_FU2:	8 – 2468 (n=17)
Mutant *NPM1* reads mean CR_FU2 [mut *NPM1* detectable]:	221
Mutant *NPM1* VAF range CR_FU2:	0.0018- 0.22%
Mutant *NPM1* VAF mean CR_FU2 [mut *NPM1* detectable]:	0.027%

Sensitivity and DNA Input

To assess whether the sensitivity of the *FLT3*-ITD MRD assay could be improved with loading more DNA at the start we performed additional experiments. Of 122 *FLT3*-ITD AML patients, of whom sufficient DNA was available, we compared 100ng DNA input versus 500ng DNA input and determined *FLT3*-ITD MRD by NGS. Congruent results were obtained in 109 out of 122 (89%) *FLT3*-ITD AML patients. In 13 AML patients *FLT3*-ITD MRD was detected when using 500ng DNA input, while *FLT3*-ITD MRD was undetectable when using 100ng DNA input. The latter 13 cases had detectable *FLT3*-ITD MRD at very low levels (<0.01%). By

using 500ng DNA input the prognostic value of *FLT3*-ITD MRD at low levels (<0.01%) was not significant (P=0.082) (Fig. S6). These findings imply that using more DNA at the start or deep sequencing strategies do not necessarily improve the prognostic value of *FLT3*-ITD MRD.

Bioinformatic Analyses

Overlap-based error-correction was utilized to attenuate any form of strand-specific error biases. Error-corrected paired-end reads were aligned to the human genome version 19 (hg19) with BBMAP followed by quality control to determine cases with insufficient number of reads for adequate variant calling.[3] Single nucleotide variants (SNVs) and insertions-deletions (indels) at diagnosis were determined by MuTect[4], Samtools[5], GATK[6], Varscan[7], Indelocator[8] and Pindel[9]. Single nucleotide variants (SNVs) and insertions/deletions (indels) were called for each sample by combining the results produced by MuTect (SNVs), SAMtools (SNVs & indels), GATK (SNV & indels), VarScan (SNVs & indels), Indelocator (indels) and Pindel (more complex indels). In brief, the union of all variants called across the different variant callers was taken and only variants with a variant allele frequency (VAF) greater than 0.015 (1.5%) were considered candidates. Differences between variant callers in call sets were not considered and a call from a single variant caller was sufficient.

The variant allele frequency (VAF) of both single nucleotide variants (SNVs) and insertions/deletions (indels) were determined by AnnotateBAMStatistics (https://github.com/MathijsSanders/AnnotateBAMStatistics) for all variant caller output. VAFs estimated by the different callers were not considered. AnnotateBAMStatistics is a light-weight multi-threaded application that is paired-end aware. Mutations irrespective of type must be present in both mates when overlapping at the genomic position of interest. In case of discrepancy, the base with the highest base call quality score is selected for SNVs. VAFs of mutations detected at diagnosis were calculated as the ratio between the number of mutant and total reads.

For *FLT3*-ITD MRD and mutant *NPM1* MRD detection by ultra-deep NGS, we aligned the sequencing reads derived from complete remission (CR) samples to hg19 with BBMAP.[3] The amplicon is positioned such that the canonical *NPM1* mutations (i.e., type A, B, etc.) are situated at its midpoint and the mutation is present in the overlap of paired-end reads. The MRD status was considered positive if the *NPM1* mutation present at diagnosis was detected in both mates of one or more paired-end reads. The criteria for the mutation to be present in both mates was implemented to attenuate the impact of index hopping or other possible artefacts produced by sequencing in a multiplex setting.

In case multiple *FLT3*-ITDs were present at diagnosis, all ITDs were considered for MRD analysis. Pindel was used for short insertion or tandem insertion detection in CR. MRD status was considered positive if the identical ITD was detected in one or more reads.

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SUPPLEMENTARY FIGURES

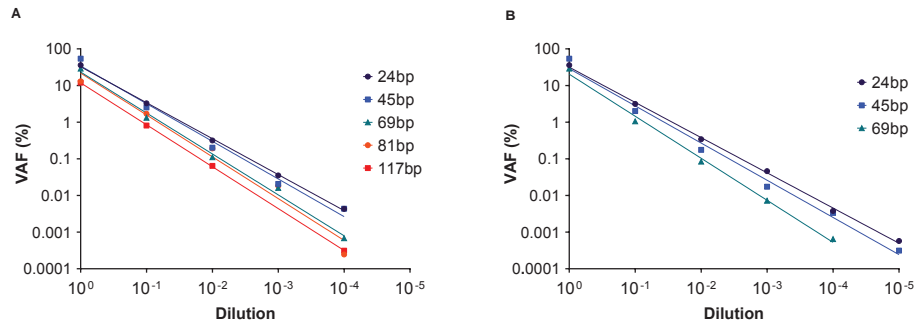

Figure S1. *FLT3*-ITD limit of detection by serial dilution of diagnosis samples with several ITD lengths.
Shown are the results with 100ng DNA input (A) and 500ng DNA input (B). R^2 values for (A) 24bp, 0.999; 45bp, 0.986; 69bp, 0.994; 81bp, 0.972; 117bp, 1.000 and R^2 values for (B) 24bp, 0.999; 45bp, 0.990; 69bp, 0.996.

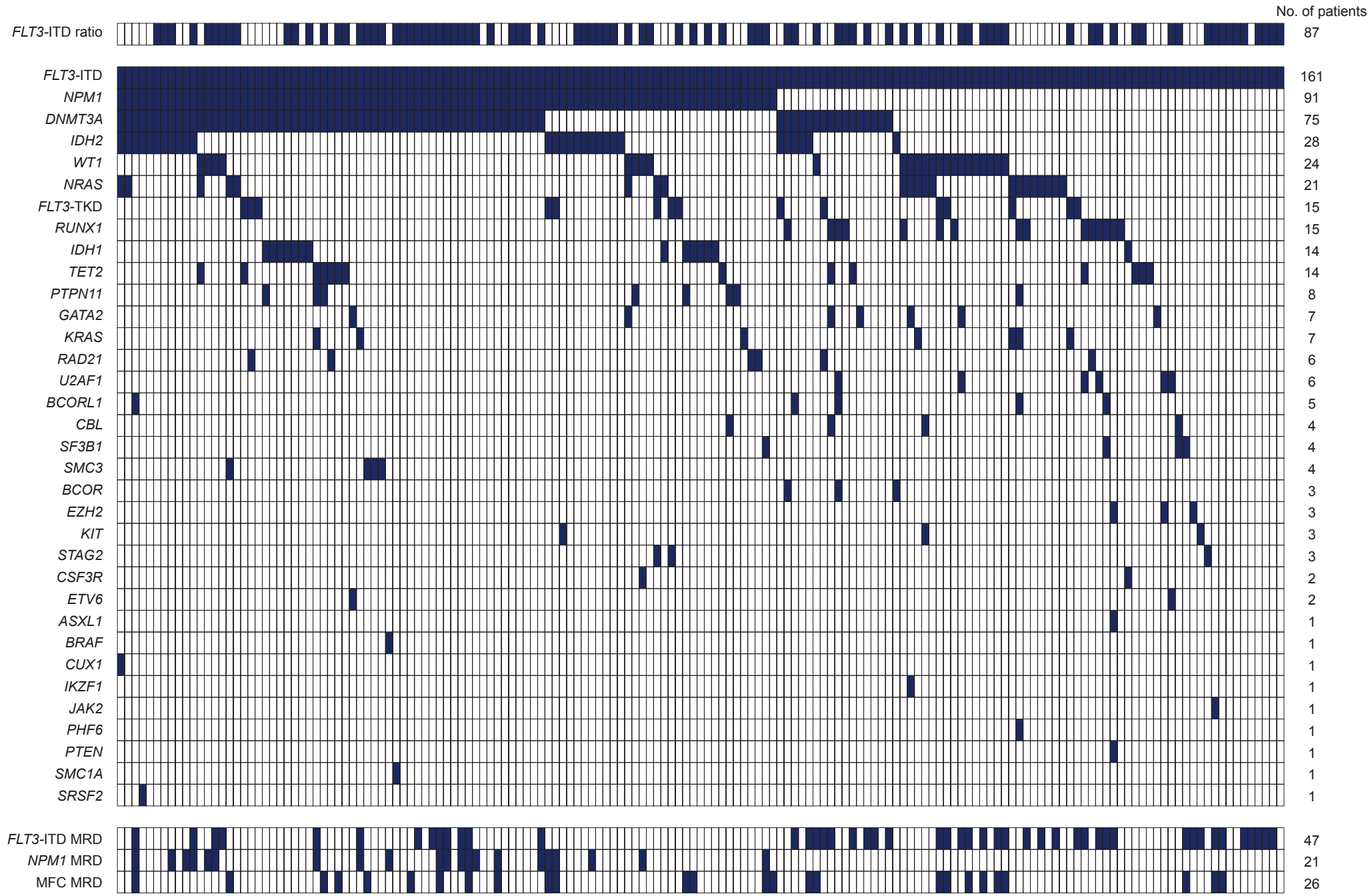

Figure S2. Molecular profile of concurrent mutations in *FLT3*-ITD AML (n=161).
Each column represents an individual patient and the presence of the aberration is indicated in blue. The upper panel shows the presence of *FLT3*-ITD high allelic ratios at diagnosis. The bottom panel shows the detection of *FLT3*-ITD MRD, mutant *NPM1* MRD and MFC MRD after induction therapy.

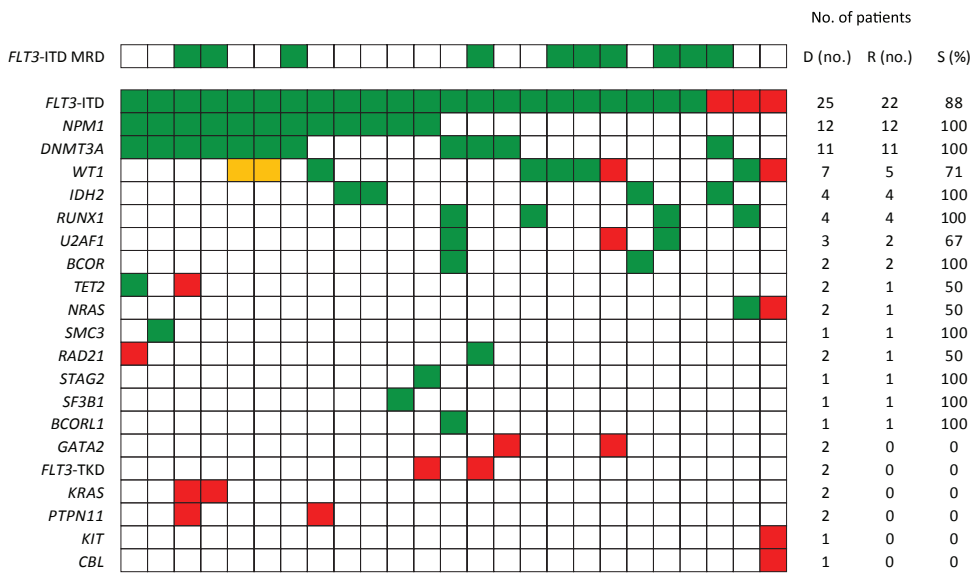

Figure S3. Molecular profile of *FLT3*-ITD AML during relapse (n=25).
Each column represents an individual patient. The presence of the aberration at both diagnosis and relapse is indicated in green. Mutations that are present at diagnosis and lost at relapse are depicted in red. The upper panel shows the *FLT3*-ITD MRD status. Please note that only 2 patients gained mutations in different genes at relapse that had been wild type at diagnosis (orange). Abbreviations D (diagnosis), R (relapse), S (stability at relapse).

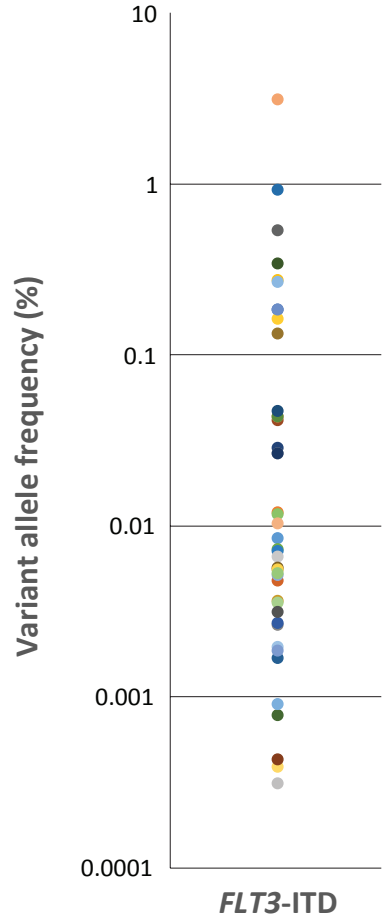

Figure S4. Variant allele frequency of *FLT3*-ITD MRD (n=47).

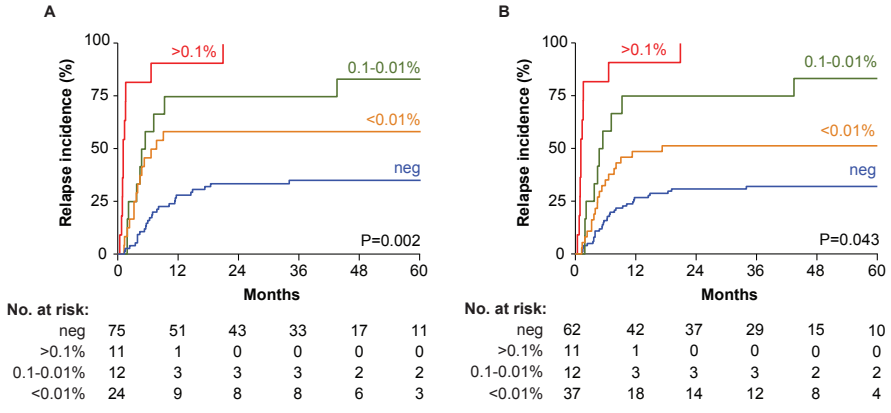

Figure S5. Relapse incidence of *FLT3*-ITD MRD at various *FLT3*-ITD variant allele frequency cut offs.
Compared are 100ng DNA input (A) and 500ng DNA input (B) (n=122).

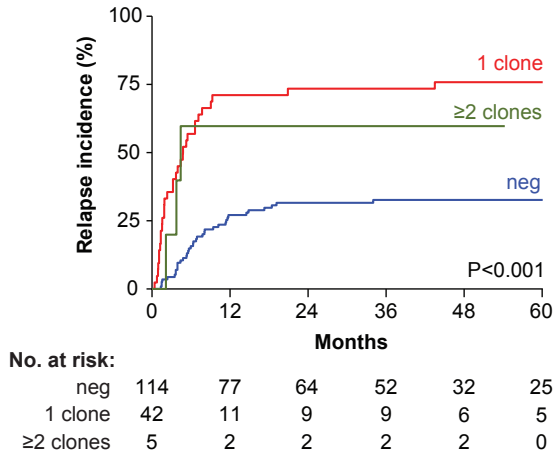

Figure S6 Relapse incidence of *FLT3*-ITD MRD by the number of *FLT3*-ITD MRD clones (n=161).

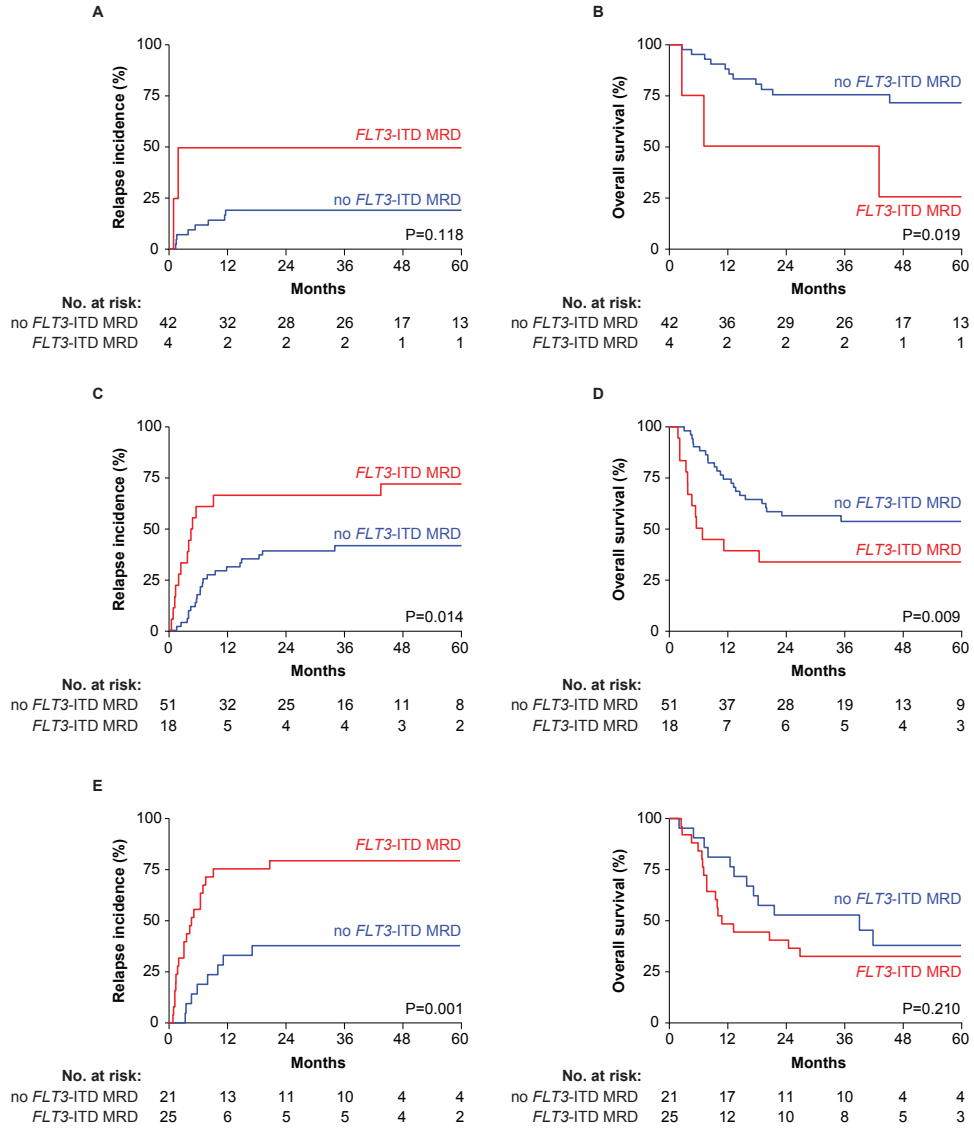

Figure S7. Relapse incidence and overall survival of *FLT3*-ITD MRD across ELN 2017 risk groups.
Favorable risk (A, B), intermediate risk (C, D) and adverse risk (E, F).

5 SUMMARY AND DISCUSSION

SUMMARY

Acute myeloid leukemia (AML) is a heterogeneous disease with a variable response to treatment. Although the majority (>80%) of AML patients with age <60 years achieve a complete morphologic remission (CR) after induction chemotherapy, relapse remains the major cause of death in AML. Evaluation of relapse risk and subsequent post-remission therapy relies on a genetic driven classification at diagnosis and the detection of measurable residual disease (MRD) by multiparameter flow cytometry (MFC) in CR. Molecular MRD monitoring has been evaluated and implemented in clinical care for single gene mutations by real-time quantitative polymerase chain reaction (RQ-PCR). Advances in next-generation sequencing (NGS) technology allow for the detection of multiple gene mutations in a single assay. In recent years NGS has been widely established to determine the presence of clinically relevant genetic markers at diagnosis, however, the application of NGS-based MRD detection in CR is still under investigation. The objective of this thesis was to further refine risk classification at diagnosis and investigate the value of molecular MRD detection by NGS during treatment, with the ultimate goal to improve relapse prediction in AML.

Patients with AML and myelodysplastic syndrome with excess blasts (MDS-EB) with mutated *TP53* have a very poor outcome. Substantial heterogeneity within these genetically defined subtypes of AML and MDS-EB precluded the exact assessment of prognostic impact for individual patients. Therefore, we performed in-depth molecular characterization of a large cohort of mutant *TP53* AML (>20% blasts) and high-risk MDS-EB (5-19% blasts) **(Chapter 2)**. We revealed that the molecular characteristics (such as *TP53* mutant allelic status (mono- or bi-allelic), the number of *TP53* mutations, mutant *TP53* clone size, concurrent mutations and cytogenetics) did not differ between mutant *TP53* AML and MDS-EB. Furthermore, the molecular characteristics of mutant *TP53* AML/MDS-EB, and in particular mutant *TP53* allelic status (mono- or bi-allelic), did not identify patients with more favorable responses. These findings indicate that **mutant *TP53* AML/MDS-EB represents a homogeneous group that should be considered a single disease entity.**

In an unprecedentedly large AML cohort, we showed that NGS-based assessment of residual disease by persistence of gene mutations in CR is applicable to the great majority (>95%) of newly diagnosed adults with AML **(Chapter 3)**. We revealed that mutations in genes associated with clonal hematopoiesis, i.e., in *DNMT3A*, *TET2* or *ASXL1,* abbreviated by *DTA*, frequently persist in CR at high variant allele frequencies (VAF) and that persistence of mutations in *DTA* in CR is not related to increased relapse risk. In contrast, **persistence of molecular residual leukemia as defined by the presence of non-*DTA* mutations in CR is a powerful and independent predictor for relapse and survival.** A comparison between molecular and MFC-based MRD detection and risk of relapse revealed additive prognostic value for molecular residual disease detection by NGS.

After investigating molecular MRD with a broad panel of genes that are frequently mutated in myeloid malignancies, we evaluated molecular MRD in specific AML subgroups with *FLT3*-ITD aberrations or mutated *TP53*. In Chapter 2, deep targeted sequencing experiments focusing on persistence of mutant *TP53* in AML/MDS-EB marrows in CR revealed detectable mutant *TP53* residual disease in the majority of cases that did not associate with survival. In Chapter 4 we comprehensively investigated the impact of *FLT3*-ITD residual disease detection on treatment outcome in *FLT3*-ITD AML. We showed that **detection of *FLT3*-ITD residual disease identifies AML patients with profound relapse risk and death that outweighs currently used prognostic factors at diagnosis as well as NGS-based mutant *NPM1* and MFC MRD detection in CR.** In addition, we provided evidence that intensification of allogeneic transplant conditioning regimens in AML patients with *FLT3*-ITD residual disease may reduce the risk of relapse and improve survival.

DISCUSSION

The research summarized in the preceding paragraphs have enhanced our understanding of the molecular aspects of myeloid malignancies with mutant *TP53* and has demonstrated the successful application of NGS-based molecular MRD detection during treatment in AML in general and in AML subsets with mutated *TP53* or *FLT3*-ITD to estimate the risk of relapse. The interpretation of these results will be discussed in the following sections, focusing on the applicability and implementation of molecular diagnostics (part I) and molecular MRD detection during treatment (part II) in clinical practice with directions for future research.

PART I – REFINING THE CLASSIFICATION AT DIAGNOSIS

1.1 AML/MDS-EB with Mutant *TP53*

From a historical perspective, AML is classified based on morphologic features at diagnosis with a stringent distinction between MDS (<20% blasts) and AML (>20% blasts).[1] In recent years, AML classification has evolved towards schemes purely based on genetic abnormalities.[2,3] With the exception of AML patients that carry core binding factor (CBF) abnormalities, the dichotomy of MDS and AML based on the blast percentage has been retained for many years. However, the blast percentage to define myeloid malignancies is currently under debate.[4] Compared to other gene mutations observed in myeloid malignancies, *TP53* mutations have a distinct role in the pathogenesis of AML and MDS that involves cell cycle control and DNA damage response mechanisms and associate with complex karyotypes, a paucity of concurrent mutations and very poor survival.[5] Because of this dominant clinical phenotype, mutant *TP53* AML patients have been assigned to the adverse risk category of the ELN 2017 risk classification, but remained separated from MDS by the 20% blast count.[3] In Chapter 2 we examined high-risk MDS-EB (>5% blasts) with mutated *TP53* and AML patients with mutated *TP53* and investigated molecular differences and commonalities between MDS-EB and AML. Remarkably, we didn't observe any differences among clinical and molecular characteristics (e.g. CR rate, overall survival, *TP53* mutant allelic status (mono- or bi-allelic), the number of *TP53* mutations, mutant *TP53* clone size, concurrent mutations and cytogenetics) between mutant *TP53* MDS-EB and AML. We concluded that from a clinical and molecular perspective MDS-EB and AML with mutated *TP53* should be considered as a single entity.

Moving forward, our study confirmed the prognostic value of complex karyotypes (CK) within mutant *TP53* AML (Chapter 2).[2,6] *TP53*-mutated AML/MDS-EB patients with CK have inferior response to treatment than those without CK. However, in contrast to others, we did not find an independent association of *TP53* mutant allelic status or mutant *TP53* clone size with outcome in AML/MDS-EB.[7-11] Within all MDS and AML studies there appears a

strong association between mutant *TP53* and CK since nearly all bi-allelic mutant *TP53* AML/MDS-EB cases have become genomically unstable resulting in CK in 97% of patients. Although mono-allelic mutant *TP53* patients with CK have a relatively poor outcome, long term overall survival was observed in selected mono-allelic non-CK AML/MDS-EB patients that generally carry very small mutant *TP53* clones (VAF ≤10%) and concurrent mutations at higher VAFs. This may imply that mutant *TP53* in those cases may not be part of the AML driving clone but could represent a separate mono-allelic non-CK initiating leukemic clone or possibly clonal hematopoiesis. Additional single cell experiments are required to reveal the clonal compositions in these cases and distinguish mutant *TP53* leukemia from mutant *TP53* clonal hematopoiesis.

For the first time, this study demonstrated that mutant *TP53* AML/MDS-EB represent a single disease entity. Based on our results and subsequent confirmation by others, the international consensus classification (ICC) of myeloid neoplasms and acute myeloid leukemias and the ELN working party modified the AML classification and risk stratification (Table 1).[12-14] In these classifications AML or MDS patients (10-20% blasts) with any somatic *TP53* mutation (VAF >10%) are now considered AML/MDS with mutated *TP53*, irrespective of the *TP53* mutant allelic status. MDS patients with <10% blasts with bi-allelic mutant *TP53* or any somatic *TP53* mutation (VAF >10%, both mono- and bi-allelic) with CK are classified as MDS with mutated *TP53*. However, the exact quantitation of the *TP53* mutant bi-allelic status is technically challenging, inaccurate and has low prognostic yield because of the strong association with CK. Therefore, I propose to stratify mutant *TP53* AML and MDS patients by CK alone.

Table 1. Myeloid neoplasm with mutated *TP53*

Type	Cytopenia	Blasts	Genetics
MDS with mutated *TP53*	Any	0-9% bone marrow and blood blasts	Multi-hit *TP53* mutation* or *TP53* mutation (VAF >10%) and complex karyotype often with loss of 17p†
MDS/AML with mutated *TP53*	Any	10-19% bone marrow and blood blasts	Any somatic *TP53* mutation (VAF >10%)
AML with mutated *TP53*	Not required	≥20% bone marrow and blood blasts or meets criteria for pure erythroid leukemia	Any somatic *TP53* mutation (VAF >10%)

* Defined as 2 distinct *TP53* mutations (each VAF >10%) OR a single *TP53* mutation with (1) 17p deletion on cytogenetics; (2) VAF of >50%; or (3) Copy-neutral LOH at the 17p *TP53* locus.
† If *TP53* locus LOH information is not available.

PART II – MOLECULAR RESIDUAL DISEASE DETECTION DURING TREATMENT

Considering the complex clonal architecture of AML, it is essential to investigate multiple gene mutations in parallel to understand the dynamics of these mutations during treatment and to determine the prognostic value for relapse. Initial studies have explored the potential for molecular MRD detection by NGS and the association with outcome in patients with AML.[15,16] However, our study presented in Chapter 3 of this thesis is the first major study with sufficient number of patients that has demonstrated the feasibility and prognostic value of NGS-based molecular MRD detection in AML. Thereafter, multiple groups have validated and confirmed our results in independent AML cohorts.[17-22] Because of our large AML cohort (Chapter 3) and by studying molecular MRD in specific subgroups of AML with mutated *TP53* (Chapter 2) or *FLT3*-ITD (Chapter 4), we revealed strong evidence to investigate and implement molecular MRD monitoring in the context of genetically-defined subtypes of AML and their clonal hierarchy. AML is an oligoclonal disease that consists of multiple leukemic clones at diagnosis with mutations that are acquired early (*DNMT3A*, *TET2*, *ASXL1*, in short *DTA* mutations) or late (*NPM1*, *FLT3*-ITD, *NRAS*, *KRAS*) in leukemogenesis (Fig. 1). In Chapter 2 we revealed that induction chemotherapy reduced the overall leukemic burden in CR significantly, but clones with late mutations were eradicated more frequently compared to the clones with early mutations (Fig. 1). Strikingly, we found no prognostic association with relapse for early mutations in CR (Chapter 3) and established profound prognostic relevance for residual late mutations (Chapter 3 and 4). In contrast, we observed that residual *TP53* mutations persisted in nearly every AML patient who had poor survival regardless of the molecular MRD status (Chapter 2). These findings imply that each functional category of mutations in CR contains unique molecular features and relapse potential that is related to the hierarchical mutation acquisition in leukemic evolution. In the following section, the interpretation and prognostic relevance of these mutation categories in CR will be deliberated.

2.1 Selecting Mutations Eligible for Molecular Detection in AML

2.1.1 Early Mutations and MRD Detection

Perhaps one of the most important and unexpected findings in this thesis was the identification of persistent clonal hematopoiesis in CR that appeared not to be related to increased risk of AML relapse (Chapter 3). Mutations that have been detected in age-related clonal hematopoiesis (ARCH) frequently persisted in CR at high VAFs, i.e., significantly higher than one would expect in CR (blast count <5%, expected VAF <2.5%). In fact, in some cases ARCH mutations were observed to be present in all cells. Clearly, these mutations are not constrained to the blast compartment in CR and confer a clonal advantage to these cells over normal hematopoietic stem cells when repopulating the bone marrow after induction chemotherapy, resembling early mutations and clonal expansion

found in clonal hematopoiesis in healthy individuals. Several independent research groups confirmed our finding that residual mutations associated with ARCH lack prognostic value for relapse.[17,21,23] Nevertheless, a considerable number of AML patients that only carry ARCH mutations in CR will relapse. This implies that mutations resembling residual AML, or non-*DTA* mutations, have not been detected because they persist at low levels below the detection limit at the time of molecular MRD assessment in those patients or were not included in our gene panel. Future studies with improved NGS MRD protocols, comprehensive gene panels as well as longitudinal monitoring at different time points are needed to dissect residual clonal hematopoiesis from residual leukemia to improve relapse prediction in these cases.

In our study we excluded *DTA* mutations in the definition of molecular MRD since these mutations are the most prominent in ARCH in the healthy population. However, mutations in isocitrate dehydrogenase (*IDH1*, *IDH2*) and spliceosome factor genes (*SRSF2*, *SF3B1*, *U2AF1* and *ZRSR2*) are also observed in ARCH and persist at high variant allele frequencies in CR in selected cases (Chapter 3).[24-27] Because of the relatively low prevalence of these mutations at diagnosis, the AML cohort used for the studies described in this thesis was not large enough to determine the prognostic value of these mutations in CR.

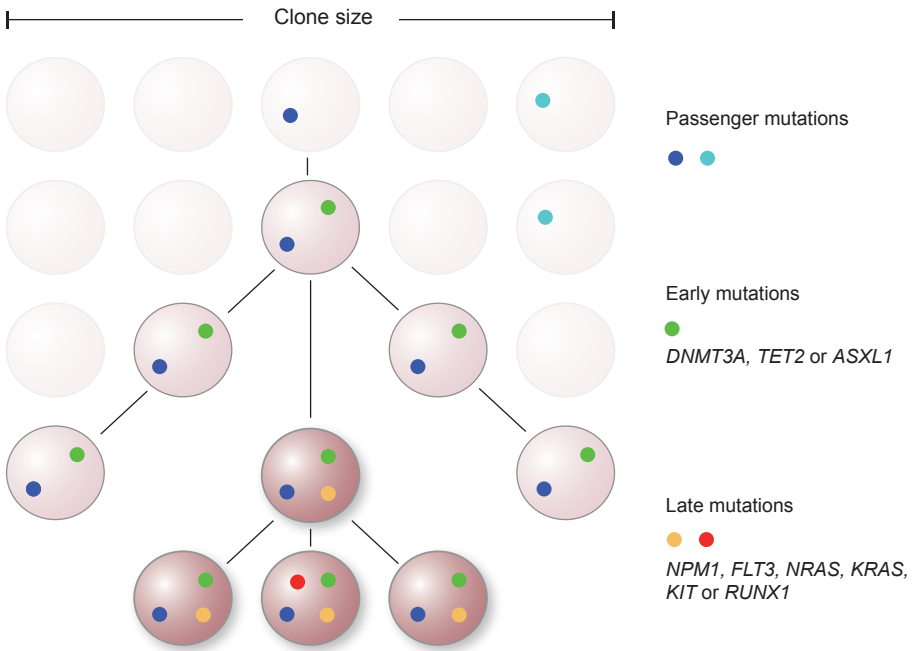

Figure 1. Clonal architecture and acquisition of mutations in AML.
Passenger mutations without proliferative advantage occur in normal hematopoietic progenitor cells and do not contribute to leukemogenesis (blue). Mutations that are acquired early are frequently found in age-related clonal hematopoiesis, but are not associated with increased relapse risk when detected in complete remission (green). In contrast, mutations in activated signaling genes are never observed in healthy individuals and considered late events (yellow, red). Detection of late mutations in CR is associated with profound relapse risk.

2.1.2 Late Mutations and MRD Detection

In contrast to mutations in ARCH, mutations in activated signaling genes are considered late events because they are never observed in healthy individuals, frequently subclonal and associated with disease progression. Because of this subclonality and potential instability during relapse, the ELN MRD working party recommended that late mutations should not be considered as optimal residual disease markers.[28] However, in Chapter 3 of this thesis we show that late mutations are frequently cleared and persist at low VAFs in CR. It can be presumed that these residual late mutations in CR represent fully developed AML cells that are capable to initiate relapse. We investigated this hypothesis in a subtype of AML with *FLT3*-ITD, a late event in leukemogenesis, described in Chapter 4. For the first time, we demonstrated that residual *FLT3*-ITD in CR after induction chemotherapy is associated with early relapse and excessively high relapse rates. These findings have recently been confirmed by other AML research groups.[29,30] Importantly, we demonstrated that the prognostic value of residual *FLT3*-ITD exceeded other commonly used prognostic factors at diagnosis and residual disease measurements by MFC and NGS-based mutant *NPM1*. Altogether, based on these results I propose to introduce *FLT3*-ITD MRD monitoring in routine care of *FLT3*-ITD AML patients.

The most striking contribution of the residual *FLT3*-ITD work to the field is the refute of a longstanding misconception concerning the reliability of late mutations for relapse prediction in AML. It is conceivable that residual late event mutations in genes associated with activated signaling and uncontrolled cell proliferation other than *FLT3*-ITD (e.g. *NRAS*, *FLT3*-TKD, *PTPN11*, *KIT* and *KRAS* mutations) in CR may also be associated with high relapse rates. Investigating this hypothesis requires a larger AML cohort with sufficient number of AML patients and thorough evaluation of the current sequencing depth of late mutations. In addition, it should be noted that the absence of residual late mutations in CR doesn't preclude AML relapse since novel late mutations may be acquired during treatment. This is often observed in AML patients that receive treatment with new inhibitors and especially relevant for longitudinal monitoring of molecular MRD.[31] Therefore, it will be important to study all relevant late mutations in parallel using a late event gene panel that is currently being developed and tested in our research group. Furthermore, this panel will shed new light on the leukemic evolution and prognostic value of residual late mutations in AML patients treated with FLT3 inhibitors.

2.1.3 Mutations in *TP53* and MRD Detection

In addition to the ARCH mutations discussed previously, mutations in *TP53* are also found in healthy individuals and are considered high risk ARCH since many of the individuals with mutant *TP53* ARCH develop hematological malignancies.[26,27] At diagnosis mutant *TP53* AML is considered adverse risk regarding the very poor outcome of these AML patients.[3] However, it was not known whether mutant *TP53* MRD measurements could stratify the relapse risk in

these AML patients. Therefore, we attempted to gain insights into the relapse risk of mutant *TP53* AML by investigating their molecular MRD status by determining persistence of *TP53* mutations using NGS (Chapter 2). By assessing MRD by deep sequencing of bone marrow cells from mutant *TP53* AML patients that achieved CR after induction chemotherapy, we showed persistence of mutant *TP53* leukemic clones in the majority of AML patients (73%). Residual mutant *TP53* in CR was not associated with relapse or survival, irrespective of the mutant *TP53* clone size. These data suggest that mutant *TP53* persist in all AML patients that have received chemotherapy and that only few residual mutant *TP53* cells are capable to induce relapse. Along with the observation that mutant *TP53* itself is often the single known driver mutation or co-occurs with ARCH related mutations, molecular MRD markers in this subtype of AML are lacking. Considering that the majority of mutant *TP53* AML patients carry residual *TP53* mutations in CR and have a very poor survival, residual *TP53* mutations perturb molecular MRD algorithms in favor of detectable molecular MRD. From this regard, I recommend to consider mutant *TP53* AML patients as a separate entity in future MRD studies. Because of the dismal outcome of mutant *TP53* AML many new agents are currently tested with the aim to improve the outcome of these AML patients. Whether mutant *TP53* will be a suitable residual disease marker in AML patients treated with these new therapeutic agents (e.g. Eprenetapopt) needs to be verified.

2.1.4 Mutations that Require Further Investigation

Following the preceding paragraphs, it is evident that the eligibility of residual disease mutations varies for individual mutations and depends on patterns of concurrent mutations as well as the order of mutation acquisition in AML development. These patterns can also vary between mutated variants within a single gene. In fact, certain mutations in the same gene may be considered early or late mutated variants. A typical example are mutations in *IDH2*. Clonal evolution analysis by using VAFs has demonstrated that *IDH2* R140 is an early mutation that is frequently followed by *NPM1* mutations, whereas the *IDH2* R172 mutation is mutually exclusive with mutant *NPM1* and is considered a distinct AML subtype.[2,32] Indeed, the finding of variable VAFs in CR for persistent *IDH2* mutations reported in Chapter 3 indicates a mix of early and late mutations. This is further corroborated by work from others that compared residual *IDH2* R140 and *IDH2* R172 mutations in CR. In contrast to *IDH2* R172 mutations, residual *IDH2* R140 mutations had generally higher VAFs in CR that may indicate a state of clonal hematopoiesis rather than residual leukemia.[33] Obviously, accurate relapse prediction in those AML patients requires independent analyses for residual *IDH2* R140 and *IDH2* R172 in CR.

In addition to *IDH* mutations, there are many other mutations for which the eligibility for molecular MRD detection in AML needs to be investigated. This group consists of mutations in *RUNX1*, *SF3B1*, *SRSF2*, *STAG2*, *U2AF1* and *ZRSR2*. Along with mutations in *DTA* and *IDH,* these mutations have been detected in healthy individuals with clonal hematopoiesis and

frequently persist with variable VAFs in CR (Chapter 3). Preliminary results of molecular MRD by NGS in elderly AML patients show no prognostic value for these residual mutations in CR, even when we excluded *DTA* mutations (data not shown). Except for the fact that some of these mutations in CR are associated with residual clonal hematopoiesis, there are other plausible explanations for the lack of prognostic value for residual *RUNX1*, *SF3B1*, *SRSF2*, *STAG2*, *U2AF1* and *ZRSR2* mutations in CR. In fact, these mutations are highly prevalent in patients with MDS. Research in our group (manuscript in preparation) and by others have shown that the clinical and molecular characteristics of patients with these mutations are disease defining for both AML and MDS patients.[34-38] Similar to mutant *TP53* AML and MDS described in Chapter 2, AML and MDS patients with mutations in *RUNX1*, *SF3B1*, *SRSF2*, *STAG2*, *U2AF1* and *ZRSR2* are considered a single molecular disease entity and classified as AML with myelodysplasia-related gene mutations.[14] Compared to AML with recurrent genetic abnormalities, AML patients with myelodysplasia-related gene mutations have adverse outcomes and appear to have a distinct molecular etiology. AML with myelodysplasia-related gene mutations have significantly more mutations at diagnosis that are acquired early, however, very few late mutations in activated signaling genes (data not shown). In fact, the order of mutation acquisition in AML with myelodysplasia-related gene mutations has not been fully elucidated and therefore it remains elusive which mutations are early or late events.[35] Furthermore, some mutations may be acquired early in AML with recurrent genetic abnormalities and late in AML with myelodysplasia-related gene mutations. Further research should reveal whether molecular MRD algorithms can be improved by unraveling the order of mutation acquisition in AML with myelodysplasia-related gene mutations and by the assessment of residual gene mutations in this group separate from AML patients with recurrent genetic abnormalities.

Another layer of complexity for molecular MRD monitoring in AML with myelodysplasia-related gene mutations are potential changes in the clonal composition caused by emerging mutations during treatment that were not present at diagnosis. This may indicate patterns of (treatment induced) branching evolution that may mask the eligible mutation to be monitored. Indeed evidence revealed that AML with spliceosome factor gene mutations arises from multiple independent leukemic driver clones that are subjected to branching evolution.[39-42] As a consequence and beyond their association with ARCH, it will be a daunting task to interpret the molecular MRD status in those AML patients because it is unclear which leukemic clone will eventually progress towards relapse. A better understanding of the evolutionary trajectories of mutations by studying relapse samples of these patients will uncover whether these phenomena are related to (persistent) ARCH or branching evolution of independent leukemic clones.

2.1.5 Molecular Residual Disease Markers other than Gene Mutations

When selecting molecular MRD markers it is important to choose the event that ultimately results in leukemic transformation, such as late non-*DTA* mutations. However, it is possible that in some AML patients the mechanisms of transformation are not exclusively driven by acquisition of gene mutations, but could be induced by distinct gene expression patterns, epigenetic changes or cell extrinsic properties instead. One such mechanism that is prevalent in myeloid malignancies are aberrations resulting in gene haploinsufficiency. Typical examples are AML patients with allele-specific *GATA2* expression due to chromosome 3q26 rearrangements.[43] For this matter, experiments investigating cytogenetic aberrations at low levels or methylation profiling could provide new insights into the association of alternative definitions of MRD and outcome.

2.2 Sequencing Techniques and Depth for Molecular MRD Detection

Advances in sequencing technology enables mutation detection in AML with profound sensitivity.[44] The gene panel NGS MRD experiments described in Chapter 3 of this thesis were performed with the Illumina Myeloid sequencing panel. Although this panel was not designed for low level molecular MRD detection, we were able to achieve an average sequencing depth of only 0.1 to 1% in our CR samples. This detection limit is largely due to random sequencing errors that results in background noise. To detect mutations in CR we used computational outlier testing to discriminate residual mutations from background noise. To increase sequencing specificity and sensitivity several research groups are implementing error-corrected sequencing with unique molecular identifiers that reduce the background noise. Although a direct comparison of these approaches is on the way, the first result show few discrepancies.[45] However, another point of attention is that computational outlier testing depends on the mutations present at diagnosis. This method requires stability of mutations over time and doesn't take into account novel mutations in upcoming leukemic clones. For the NGS MRD studies provided in this thesis we used CR samples after two cycles of induction chemotherapy. The results in Chapter 3 show few new mutations for this time point that did not affect the prognostic value for relapse. However, the investigation of additional time points during the disease course requires sequencing approaches that are not dependent on mutations at diagnosis. This is especially important for AML patients treated with new inhibitors, since residual leukemic clones in these patients can acquire novel mutations over time.[31] From this regard, I propose to implement error-corrected sequencing rather than sequencing with outlier testing.

Along with error-corrected sequencing there are several other possibilities to increase sensitivity of sequencing to determine MRD. Examples are increasing the amount of DNA input or CD34/CD117 positive selection of hematopoietic and leukemic stem cells with magnetic beads or MFC.[46] This approach is of particular interest for the detection of point mutations with low allele frequencies in CR (e.g. *FLT3*-TKD, *NRAS*, *KRAS*) because they are currently difficult to distinguish from background sequencing noise in bulk AML. Although

it will be useful to develop these approaches, it could be debated whether sequencing even deeper will result in improved relapse prediction. We provided evidence that deep sequencing will not necessarily improve prognostic yield as shown in in Chapter 4. In this study, the relapse probability was directly related to the size of residual *FLT3*-ITD leukemic clones by means of several VAF cut offs. We were able to show that large residual leukemic clones in CR result in high relapse rates, whereas the relapse risk was significantly reduced in AML patients with *FLT3*-ITD clones at low allele frequencies (<0.01%). This observation has recently been confirmed by others.[30] Furthermore, the correlation between clone size and relapse risk has been demonstrated for residual *NPM1* mutation levels in CR measured by RT-PCR as well.[47,48] Therefore the ELN working party now applies a threshold for residual mutant *NPM1* expression levels in CR.[28] Whether this threshold is applicable for residual *NPM1* mutations measured by NGS on DNA and cDNA are subject of ongoing studies in our group. In sum, it is possible that advances in technological sensitivity for deep sequencing exceeds the threshold of clinical relevance. Therefore, it may be more useful to monitor residual leukemic clones longitudinally and assign the clinical relevance based on the increment in the level of mutations over time.

2.3 Flow Cytometry in the Era of Molecular MRD by NGS

In the past decade MFC has been the preferred technique for the detection of residual disease in AML. Therefore, we performed a direct comparison between MFC MRD and molecular residual disease detection using NGS (Chapter 3). We showed that the combination of both techniques provided additive prognostic value for relapse than when using a single technique alone. Overall, 41 out of 340 AML patients (12%) had detectable MRD based on MFC but lacked molecular MRD. A post hoc analysis of this group demonstrated that this group predominantly consists of AML patients that carry mutations in *NPM1* and/or *FLT3*. In our earlier studies (Chapter 3), we were unable to sequence mutant *NPM1* and *FLT3*-ITD sufficiently deep. Improving the assay with NGS-based mutant *NPM1* and *FLT3*-ITD deep sequencing in Chapter 4 resulted in better identification of AML patients with high relapse risk. In fact, there appears to be limited prognostic value for MFC MRD in the context of mutant *NPM1* and *FLT3*-ITD deep sequencing (Chapter 4). Another subset of AML patients with discrepant MRD tests frequently have core binding factor (CBF) abnormalities. Interestingly, all of these CBF patients with MFC MRD have concurrent late mutations in *KIT* or *NRAS*. In fact, it is conceivable that those single nucleotide mutations are currently not detected at sufficient depth and require further optimization of sequencing procedures. These insights largely explain the discrepancies of MFC and molecular MRD observed in Chapter 3. Along with a genomic driven classification and improvements in molecular MRD detection, the role of MFC MRD seems to become limited. Novel MFC markers in AML patients with specific molecular abnormalities are required for the utility of MFC MRD in the near future.

2.4 Towards Molecular MRD Tailored Treatment

MRD measurements allow for better assessment of treatment response. AML patients that have achieved CR with MRD have higher relapse rates compared to patients without MRD. Irrespective of the MRD technique used, the prognostic value of MRD testing in AML has been widely established.[49] In recognition of these results, the European leukemia working group modified the AML treatment response criteria to CR with and without MRD, also including molecular MRD testing by NGS. Our study in Chapter 3 of this thesis contributed significantly to these modifications. However, despite the fact that MFC and RT-PCR MRD testing has been performed for more than a decade, there are no prospective randomized controlled trials that investigated MRD testing to guide treatment. Evidence for improved outcome of MRD guided treatment has been retrospectively derived from the GIMEMA and HOVON-SAKK trials.[50,51] In these trials, intermediate risk AML patients with MRD were directed towards allogeneic transplant, whereas intermediate risk AML patients without MRD were considered more favorable relapse risk and received less intensive non-allogeneic transplant therapies such as autologous transplantation or chemotherapy. Survival analysis of intermediate risk AML patients revealed comparable overall survival between patients with and without MRD. This observation supports the consideration of treatment intensification with allogeneic transplantation for AML patients with MRD and justifies deintensification with non-allogeneic transplant therapies for AML patients without MRD. However, new insights in the revised ELN 2022 classification let to substantial adjustments in the molecular risk group allocation of AML patients.[14] In fact, the intermediate risk group is now predominated by AML patients with *FLT3*-ITD. Therefore, our *FLT3*-ITD MRD results described in Chapter 4 are of profound clinical relevance for this subset of AML patients in whom MRD tailored treatment is deliberated. From our *FLT3*-ITD MRD work it is clear that MFC MRD confers limited prognostic value and in my opinion MFC MRD should not be preferred in *FLT3*-ITD AML. But, can we make *FLT3*-ITD MRD guided clinical decisions based on the results from this thesis and contributions from others?

2.4.1 Treatment Intensification

A significant number of AML patients with *FLT3*-ITD receive allogeneic transplantation.[52] In our *FLT3*-ITD MRD cohort, we observed reduced relapse rates in AML patients that received allogeneic transplantation (Chapter 4). However, despite allogeneic transplantation the relapse rate of AML patients with *FLT3*-ITD MRD is considerably higher than those without *FLT3*-ITD MRD. One possible strategy to prevent relapse is to aggravate treatment by intensifying transplant conditioning regimens. In Chapter 4, we investigated the impact of allogeneic transplant conditioning regimens on relapse and survival of AML patients with *FLT3*-ITD abnormalities.

There are several types of conditioning regimens available that can roughly be separated into reduced-intensity regimens (RIC) and myeloablative regimens (MAC). Depending on non-relapse mortality risk scores and the likelihood of AML relapse, the conditioning regimen is determined. Several studies have shown that the MRD status before allogeneic transplant can guide the choice of conditioning regimen.[20,53,54] Our results show a high relapse rate for AML patients with *FLT3*-ITD MRD who received RIC, whereas the relapse rate is significantly lower with MAC that results in better overall survival. Meanwhile, these results have been confirmed in independent external cohorts.[29,30] It is evident that the presence of *FLT3*-ITD before allogeneic transplantation often indicates the presence of aggressive leukemic clones that have been insufficiently treated. In addition to intensifying the conditioning regimen, several other strategies are possible to reduce relapse risk such as earlier tapering of immunosuppression or donor lymphocyte infusion (DLI) after transplant. There is accumulating evidence that maintenance therapy with a FLT3 inhibitor post-transplant reduces relapse risk.[55,56] Currently, there are no studies showing that additional post-induction treatment for achieving *FLT3*-ITD MRD clearance results in a lower relapse rate. Studies with interphase therapy with new agents (e.g. new FLT3 inhibitors, immunotherapy) will have to verify this.

2.4.2 Deintensification of Treatment

Despite achieving a CR without *FLT3*-ITD MRD, the relapse risk of AML patients after autologous transplantation remains high in our study (2-year risk of relapse; 42.1% autologous transplant vs. 22.7% allogeneic transplant). However, the overall survival of AML patients without *FLT3*-ITD MRD is similar regardless the type of transplant. This implies that allogeneic transplantation is not necessarily better. However, the numbers are not sufficient to investigate the impact of other prognostic factors in this group such as hyperleukocytosis, the number of chemotherapy cycles to attain CR and transplant related factors. Therefore, at this time, it seems to be premature to retain AML patients without *FLT3*-ITD MRD from allogeneic transplantation. Perhaps this group may benefit from additional treatment with FLT3 inhibitors instead of allogeneic transplantation and therefore the results from the HOVON 156 trial are eagerly awaited.

2.4.3 Alternative Treatment Options

Finally, unfortunately, there are AML patients in whom current standard treatment fails. One notorious example involves AML patients with mutated *TP53* that we studied in Chapter 2. In this study, all patients received induction chemotherapy and an allogeneic transplant when feasible. With the exception of a subset of mutant *TP53* AML patients without CK, the vast majority develop relapsed AML after allogeneic transplantation resulting in very poor overall survival (2-year overall survival; 63.6% non-CK, 16.7% CK). It is clear that mutant TP53 non-CK AML/MDS-EB patients should remain eligible for allogeneic transplant. However, these results show that conventional allogeneic transplant strategies for mutant *TP53* AML/MDS-EB patients with CK warrants further improvement. Trials investigating the

intensity of conditioning regimens had no impact on relapse or survival in mutant *TP53* AML and MDS-EB undergoing allogeneic transplantation.[57,58] There is no data available on enhancing the graft-versus-leukemia effect by early tapering of immunosuppression or DLI specifically in mutant *TP53* myeloid malignancies. Therefore, there is an urgent need for novel therapies that with higher efficacy. One example of such therapy is Eprenetapopt (APR-246), a small molecule that restores TP53 wild-type protein conformation and function in *TP53* mutated leukemic cells. Upfront or maintenance treatment post-allogeneic transplant with Eprenetapopt in recent phase 1 and 2 trials resulted in encouraging CR and survival rates.[59-61] Immunotherapy with bispecific antibodies such as the CD123 x CD3 dual-affinity retargeting antibody Flotetuzumab and the anti-CD47 antibody Magrolimab that inhibits the anti-phagocytic signaling of CD47 resulting in destruction of leukemic cells by the immune system are emerging.[62-64] Mutant *TP53* AML patients with CK should upfront be included in clinical trials with those new agents instead of standard intensive induction chemotherapy and conventional allogeneic transplantation strategies. This can be accomplished by joint efforts of large international cooperative AML trial platforms that pursuit rapid inclusion of mutant *TP53* AML patients.

2.5 Paving the Way for Implementation

Although we are on the verge of implementing molecular MRD by NGS in current AML treatment protocols, several steps remain to be taken for the application in routine clinical practice. First, this will be a technological standardization of error-corrected approaches for deep sequencing at multiple time points for longitudinal monitoring. Mutational thresholds have to be established, in particular for late mutations in CR such as *FLT3*-ITD, since those residual mutations appear to be of most important prognostic relevance. International expert panels such as the ELN AML MRD working party are generating global consensus criteria for universal standardization of molecular MRD by NGS in AML.[28] On short term, I expect that the implementation of molecular MRD will result in personalized molecular MRD tailored treatment and improve outcome for our patients with AML.

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ADDENDUM

NEDERLANDSE SAMENVATTING

Acute myeloïde leukemie (AML) is een heterogene ziekte met een variabele respons op behandeling. Hoewel de meerderheid (>80%) van de AML patiënten met een leeftijd <60 jaar een complete morfologische remissie (CR) bereikt na inductiechemotherapie, blijft het recidief de belangrijkste doodsoorzaak in AML. Evaluatie van het recidief risico en de keuze voor post-remissietherapie is afhankelijk van een genetisch gestuurde classificatie bij diagnose en de detectie van meetbare restziekte (MRD) door multiparameter flowcytometrie (MFC) in CR. In de klinische praktijk is moleculaire MRD monitoring geëvalueerd en geïmplementeerd voor mutaties in individuele genen door real-time kwantitatieve polymerasekettingreactie (RQ-PCR). Dankzij vooruitgang in next-generation sequencing (NGS) technologie kunnen mutaties in meerdere genen in één enkele test worden gedetecteerd. In de afgelopen jaren is NGS op grote schaal toegepast om de aanwezigheid van klinisch relevante genetische markers bij diagnose te bepalen, maar de toepassing van op NGS gebaseerde MRD detectie in CR wordt nog steeds onderzocht. Het doel van dit proefschrift was om de risicoclassificatie bij diagnose verder te verfijnen en de waarde van moleculaire MRD detectie middels NGS tijdens de behandeling te onderzoeken, met als uiteindelijk doel om de voorspelling van het recidief AML te verbeteren.

Patiënten met AML en het myelodysplastisch syndroom met een overmaat aan blasten (MDS-EB) met gemuteerd *TP53* hebben een zeer slechte uitkomst. Substantiële heterogeniteit binnen deze genetisch gedefinieerde subtypen van AML en MDS-EB verhinderde de exacte beoordeling van de prognostische impact voor individuele patiënten. Daarom hebben we een gedetailleerde moleculaire karakterisering uitgevoerd in een groot cohort van patiënten met mutant *TP53* AML (>20% blasten) en hoog-risico MDS-EB (5-19% blasten) (**Hoofdstuk 2**). We toonden aan dat de moleculaire karakteristieken (zoals *TP53* mutant allelische status (mono- of bi-allelisch), het aantal *TP53* mutaties, de mutant *TP53* kloongrootte, co-mutaties en cytogenetica) niet verschilden tussen mutant *TP53* AML en MDS-EB. Bovendien identificeerden de moleculaire karakteristieken van mutant *TP53* AML/MDS-EB, en in het bijzonder de allelische status van mutant *TP53* (mono- of bi-allelic), geen patiënten met gunstigere responsen. **Deze bevindingen geven aan dat mutant *TP53* AML/MDS-EB een homogene groep vormt die als één ziekte entiteit moet worden beschouwd.**

In een zeer groot AML cohort toonden we aan dat persisterende mutaties in CR kunnen worden gedetecteerd met NGS en dat deze methode toepasbaar is op de overgrote meerderheid (>95%) van nieuw gediagnosticeerde volwassenen met AML (**Hoofdstuk 3**). We toonden aan dat mutaties in genen die geassocieerd zijn met klonale hematopoëse, d.w.z. in *DNMT3A*, *TET2* of *ASXL1*, afgekort *DTA*, vaak persisteren in CR met hoge variant allel frequenties (VAF) en dat persisterende *DTA* mutaties in CR niet gerelateerd zijn aan een verhoogd recidiefrisico. **Daarentegen is de aanwezigheid van moleculaire restleukemie, gedefinieerd door persisterende niet-*DTA* mutaties in CR, een krachtige en onafhankelijke**

voorspeller voor een recidief en overleving. Een vergelijking tussen moleculaire en MFC-gebaseerde MRD detectie afgezet tegen het risico op een recidief, toonde additieve prognostische waarde voor moleculaire restziektedetectie met NGS.

Na het onderzoeken van moleculaire MRD met een breed panel van genen die frequent gemuteerd zijn in myeloïde maligniteiten, evalueerden we moleculaire MRD bepalingen in specifieke AML subgroepen, te weten AML met *FLT3*-ITD afwijkingen of gemuteerd *TP53*. In **Hoofdstuk 2** toonden mutant *TP53* deep sequencing experimenten in het beenmerg van AML/MDS-EB in CR, detecteerbare *TP53* mutaties aan in de meerderheid van de patiënten zonder associatie met overleving. In **Hoofdstuk 4** onderzochten we de impact van detectie van *FLT3*-ITD restziekte op de behandeluitkomst in *FLT3*-ITD AML. **We lieten zien dat detectie van *FLT3*-ITD restziekte AML patiënten identificeert met een hoog risico op een recidief en overlijden en dat dit een betere voorspeller is dan de momenteel gebruikte prognostische factoren bij diagnose en NGS-gebaseerde detectie van mutant *NPM1* en MFC MRD in CR.** Tot slot leverden we bewijs dat intensivering van conditioneringsschema's voor allogene stamceltransplantatie bij AML patiënten met *FLT3*-ITD restziekte het risico op een recidief kan verminderen en de overleving kan verbeteren.

CURRICULUM VITAE

Tim Grob was born on November 25th 1988 in Hilversum, The Netherlands. In 2007 Tim started Medical School at the VU University in Amsterdam. During his studies he got funded by a student grant from the Dutch Heart Foundation and performed one year research at the department of Pharmacology at the University of Illinois at Chicago (Illinois, United States of America) focusing on endothelial cell permeability and G-protein-coupled receptor inhibition. After Tim obtained his medical degree in 2014 he started working as a physician at the department of Hematology at the Erasmus University Medical Center in Rotterdam. Under the supervision of dr. M. Jongen-Lavrencic (co-promotor), prof. dr. B. Löwenberg, dr. P.J.M. Valk (co-promotor) and prof. dr. H. R. Delwel (promotor), Tim was awarded with a personal grant of the Queen Wilhelmina Fund Foundation of the Dutch Cancer Society. In the next years, he performed research to refine the molecular diagnostic classification of myeloid malignancies and pioneered the use of molecular residual disease detection by next-generation sequencing in acute myeloid leukemia. His work has been selected for presentation in the presidential late-breaking abstract session at the annual meeting of the American Society of Hematology in 2017 and published in internationally renowned medical journals resulting in this thesis. Subsequently, Tim became a resident at the department of internal medicine in 2020 and started his fellowship Hematology in 2023 at the Erasmus University Medical Center. Tim pursues a career in academia to combine high quality clinical care and translational research with the ultimate goal to improve treatment strategies and outcome for patients with acute myeloid leukemia.

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PORTFOLIO

Courses and workshops	**EC**
Systematic literature retrieval in PubMed (2015)	0.4
EndNote (2015)	0.2
Microsoft Access 2010: Basic (2015)	0.3
Microsoft Excel 2010: Basic (2015)	0.3
Scientific Integrity (2015)	0.3
Survival Analysis (2015)	0.6
Photoshop and Illustrator (2016)	0.3
Course on Laboratory Animal Science (2016)	3.0
Molecular Medicine (2017)	0.7
Bayesians statistics and JASP (2018)	0.6
Molecular Aspects of Hematological Disorders (2018)	0.7
Scientific meetings Department of Hematology	
Work discussion (2019)	8.0
Friday floor meeting (2019)	8.0
Journal club (2019)	6.0
Erasmus Hematology Lectures (2019)	2.0
National and international conferences	
Workshop Molecular Aspects of Hematological Disorders (2015)	0.7
International conference on Acute Myeloid Leukemia (2015)	1.0
Workshop Molecular Aspects of Hematological Disorders (2016)	0.7
SOHO annual meeting - Presidential oral and poster presentation (2016)	1.5
Dutch Hematology Congress - Oral presentation (2017)	1.5
International conference on Acute Myeloid Leukemia (2017)	1.0
Annual conference American Society of Hematology - Late breaking abstract presentation (2017)	2.0
Dutch Hematology Congress - Oral presentation (2018)	1.5
Dutch Hematology Congress - TOP abstract presentation (2019)	1.5
Annual conference European Hematology Association (2019)	1.0
Annual conference European Hematology Association – Poster presentation (2022)	1.5
Other activities	
Writing grant (2015)	1.0
Supervision master student (2017)	2.0
Supervision 3 bachelor students (2018)	1.0

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