REGULAR ARTICLE

blood advances

Advantages of a genomic DNA-based next-generation sequencing assay for detection of mutant *NPM1* measurable residual disease in AML

Christian M. Vonk,[1] Tim Grob,[1] Melissa Rijken,[1] François G. Kavelaars,[1] Jolinda M. L. Konijnenburg,[1] Gert J. Ossenkoppele,[2] Markus G. Manz,[3] Violaine Havelange,[4] Yngvar Fløisand,[5,6] Bob Löwenberg,[1] and Peter J. M. Valk[1]

[1]Department of Hematology, Erasmus Medical Center Cancer Institute, University Medical Center Rotterdam, Rotterdam, The Netherlands; [2]Department of Hematology, Cancer Center Amsterdam, Amsterdam University Medical Center, Amsterdam, The Netherlands; [3]Department of Medical Oncology and Hematology, Comprehensive Cancer Center Zurich, University and University Hospital Zurich, Zurich, Switzerland; [4]Department of Hematology, Cliniques universitaires Saint-Luc, Brussels, Belgium; [5]Centre for Cancer Cell Reprogramming, Institute of Clinical Medicine, Faculty of Medicine, University of Oslo, Oslo, Norway; and [6]Department of Hematology, Oslo University Hospital, Oslo, Norway

Key Points

- Detection of m*NPM1* MRD by NGS using DNA overcomes several limitations of RT-qPCR and is therefore an attractive alternative assay.
- The prognostic significance of m*NPM1* MRD appears greatest in patients with AML with a concomitant *FLT3*-internal tandem duplications.

Mutations in the nucleophosmin-1 (*NPM1*) gene are among the most common molecular aberrations in acute myeloid leukemia (AML). Various studies have established mutant *NPM1* (m*NPM1*) as a faithful molecular measurable residual disease (MRD) marker with prognostic significance. Assessment of prognostic m*NPM1* is included in the European LeukemiaNet recommendations on MRD detection in AML. Because of recent advancements of promising drugs targeting m*NPM1* AML, monitoring of m*NPM1* MRD has gained interest, and is generally done by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). However, these RT-qPCR assays use complementary DNA (cDNA) as input, are based on gene expression levels of m*NPM1*, and are generally limited to specific m*NPM1* gene variants. The main advantages of next-generation sequencing (NGS) using genomic DNA as input are stability, independence of gene expression levels, and the ability to detect any *NPM1* variant in a single assay. Here, we comprehensively investigated the applicability of NGS on DNA to detect m*NPM1* MRD in a cohort of 119 (cDNA) and 310 (DNA) patients with m*NPM1* AML in complete remission after 2 cycles of induction chemotherapy. We demonstrate high correlations in levels and prognostic value between RT-qPCR/cDNA and NGS/DNA approaches, postulating NGS/DNA as an attractive alternative to RT-qPCR. We report that the 2% m*NPM1*/*ABL1* threshold by RT-qPCR/cDNA corresponds to an NGS/DNA variant allele frequency of 0.01%. The NGS/DNA threshold of >0.01% after 2 cycles of induction chemotherapy identifies significantly more patients with AML with an increased relapse risk than current RT-qPCR/cDNA assays. The prognostic significance of m*NPM1* MRD appears greatest in patients with AML with *FLT3*-internal tandem duplications.

Introduction

Acute myeloid leukemia (AML) is a heterogeneous hematological malignancy characterized by an increased proliferation of undifferentiated myeloid cells that arises from the sequential acquisition of

Submitted 12 August 2024; accepted 2 November 2024; prepublished online on *Blood Advances* First Edition 5 December 2024. https://doi.org/10.1182/bloodadvances.2024014490.

The data sets generated and/or analyzed during this study are available on reasonable request from the corresponding author, Peter J. M. Valk (p.valk@erasmusmc.nl).

The full-text version of this article contains a data supplement.

gene mutations in a hematopoietic stem cell and its daughter cells. Of patients with AML, ~30% carry mutations in the nucleophosmin-1 (*NPM1*) gene in their leukemic cells. *NPM1* encodes a protein critical for various important cellular processes, including ribosome biogenesis and genomic stability.[1] Mutations in *NPM1* are typically 4 base-pair insertions in exon 12 and result in the aberrant cytoplasmic localization of the NPM1 protein. During AML development, mutant *NPM1* cells frequently harbor various additional other gene mutations, in particular and most commonly *FLT3* internal tandem duplications (ITDs). Patients with mutant *NPM1* AML are classified with a favorable (*FLT3* wild type) or intermediate (*FLT3*-ITD) prognosis according to the European LeukemiaNet (ELN) 2022 risk classification, and are therefore of particular interest for monitoring treatment response with the possibility of intensifying treatment.[2] Mutant *NPM1*–targeted treatment is currently in active development in AML. Various therapeutic compounds specifically targeting *NPM1*-mutant disease show promising activity and registration of menin inhibitors is likely to be expected in the near future.[3]

Monitoring of measurable residual disease (MRD) in AML offers important insights into disease response and therapeutic decision-making.[2,4-6] Because of the stability of the mutation during the course of disease from diagnosis to relapse, and high expression of mutant transcripts, mutant *NPM1* residual cells can be detected with high sensitivity by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR), that is, 1 in 100 000 cells (0.001%), and is generally established as the standard method of measurement.[7] The recent update on MRD recommendations in AML by the ELN expert panel accepts mutant *NPM1* as a robust and clinically valuable molecular target for MRD testing.[8] Mutant *NPM1* MRD is generally assessed in the bone marrow (BM) or peripheral blood (PB) at the end of remission induction or after consolidation therapy. In follow-up, mutant *NPM1* predicts an increased relapse probability when mutant *NPM1/ABL1* levels exceed 2%, whereas low-level MRD (mutant *NPM1/ABL1* of <2%) is associated with a more favorable response.[8] Recommendations focusing on mutant *NPM1* MRD after 2 cycles of chemotherapy have not been firmly established in the ELN guidelines. Two landmark studies showed that patients with AML with either 200 copies of mutant *NPM1* of $>10^4$ *ABL1* copies[9] or with mutant *NPM1* at any level in the PB after regeneration after the second cycle of chemotherapy[10] were shown to have the highest risk of relapse. Multiple other studies have explored the prognostic value of mutant *NPM1* MRD, but substantial variations in treatment protocols, MRD detection time points, and prognostically relevant cutoffs do not permit a critical direct intercomparison of these results.[11-18]

Although the majority of investigational treatment protocols incorporate mutant *NPM1* MRD detection by RT-qPCR, several issues remain unresolved. First, the optimal prognostic threshold to distinguish patients in complete hematological remission (CR) after remission induction chemotherapy with high and low risk of recurrence has remained subject of debate. Second, differences in mutant *NPM1* gene expression levels among patients with AML together with the instability of RNA molecules before molecular testing makes standardization challenging.[19] In fact, compared with DNA assays, RNA/complementary DNA (cDNA) approaches are merely an estimate of the actual level of expression of mutant *NPM1*. Finally, RT-qPCR assays are generally designed to detect only the most frequent *NPM1* mutations (designated type A, B, and D), excluding 10% of all mutant *NPM1* AML cases from effective MRD testing.

Next-generation sequencing (NGS) usually requires genomic DNA as input source, which is more stable compared with RNA/cDNA-based approaches. Because mutant *NPM1* MRD levels measured by NGS/DNA are independent of gene expression levels, results are likely more reproducible, interpretable, and comparable among patients with AML. Another potential advantage of NGS is the ability to comprehensively detect any *NPM1* variants in a single assay such that the broad variety of patients with mutant *NPM1* AML can be monitored. Finally, the 4–base-pair insertion, constituting an *NPM1* mutation, can be easily distinguished from background noise, enabling NGS-based mutant *NPM1* detection at relatively high sensitivity, that is, 1 in 100 000 cells (0.001%). NGS for mutant *NPM1* MRD detection has not been widely implemented yet[20,21] and associations between variant allele frequency (VAF) thresholds and relapse risk have not been explored.

In this study, we comprehensively compared mutant *NPM1* MRD detection by RT-qPCR/cDNA and NGS/DNA assays in a large cohort of patients with newly diagnosed AML.

Methods

Patients and samples

Adult patients with newly diagnosed AML were treated with remission induction chemotherapy in the Dutch-Belgian Hemato-Oncology Cooperative Group and Swiss Group for Clinical Cancer Research clinical trials HO42A (NL193/NTR230), HO102 (NL2070/NTR2187), or HO132 (NL4231/NTR4376).[22-24] The treatment protocols and patient eligibility criteria are available in supplemental Figure 1. Trial participants provided written informed consent in accordance with the Declaration of Helsinki. To be included in the study, patients with mutant *NPM1* AML had to be in either complete remission or complete remission with incomplete hematologic recovery (defined according to the ELN recommendation; hereafter collectively referred to as complete remission [CR]) after 2 cycles of chemotherapy. Patients with AML enrolled in the HO132 trial of whom RNA and DNA CR material was available were included (n = 119 [110 BM and 9 PB]). The NGS/DNA cohort was expanded with patients with AML from HO42A (n = 54), HO102 (n = 90), and HO132 (n = 47) to add up to a final cohort of 310 patients with AML for NGS/DNA mutant *NPM1* MRD analyses in CR (Figure 1). Gene mutations at diagnosis of 54 commonly mutated genes in AML were assessed using the TruSight myeloid sequencing panel (Illumina, San Diego, CA) according to the manufacturer's protocol. *FLT3*-ITDs at diagnosis were assessed using capillary fragment length analysis.[9]

Mutant *NPM1* MRD detection by RT-qPCR

Detection of mutant *NPM1* by RT-qPCR was carried out according to the recommendations set by the ELN for MRD detection in 2021.[8] Mutant *NPM1* copies were normalized to the number of copies of the reference gene *ABL1*. *NPM1* and *ABL1* primer and probe sequences are summarized in supplemental Table 1A. MRD was defined as mutant *NPM1* detectable in duplicate, and assay criteria included a minimum of 10 000 copies of *ABL1* and a Cycle threshold (Ct)-value difference between duplicates of no >1. Analysis was performed using QuantStudio design and analysis

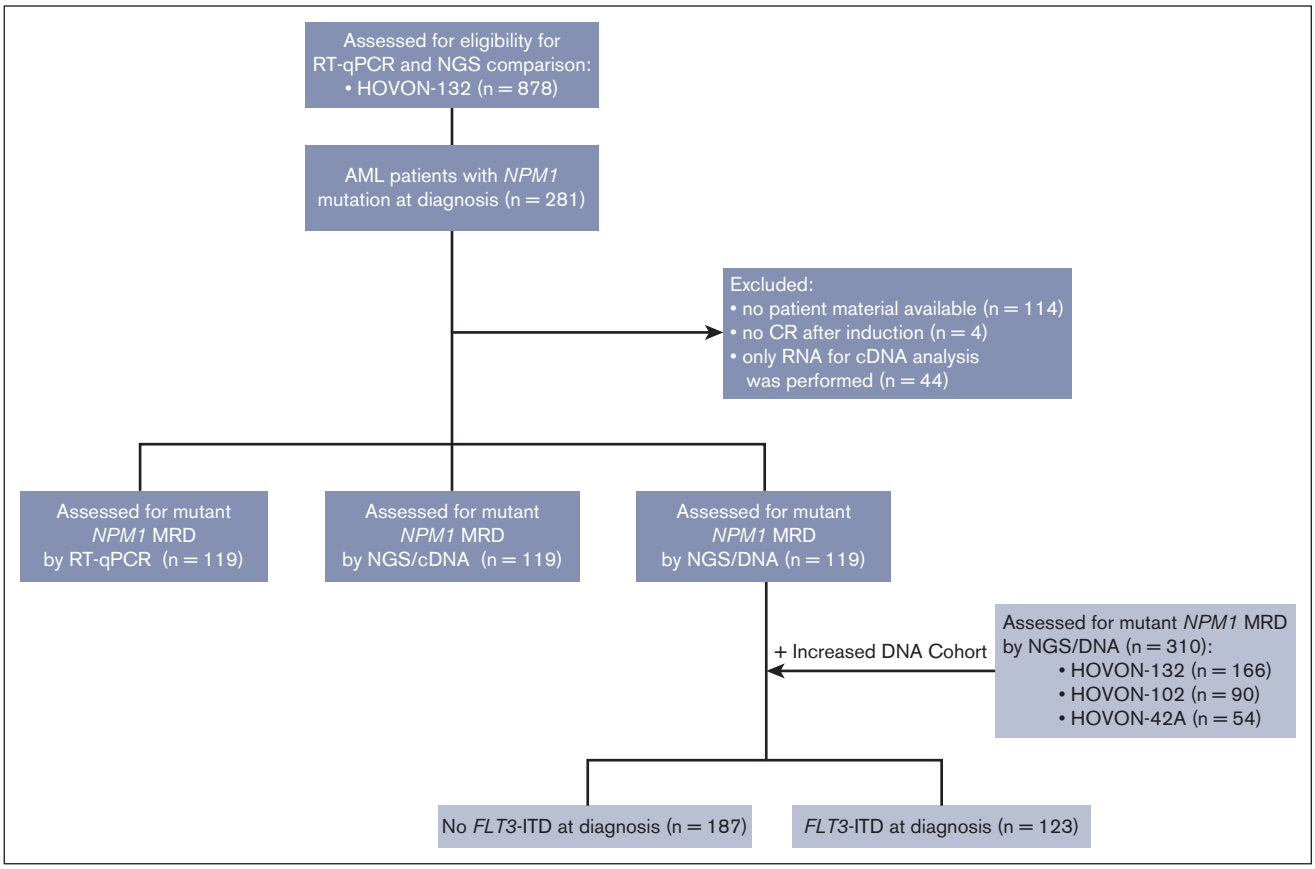

Figure 1. Flow diagram of selection of cases of patients with AML included in mutant *NPM1* cohort. HOVON, Dutch–Belgian Cooperative Trial Group for Hematology–Oncology.

software version 1.4.1 with threshold set at 0.1 (ThermoFisher, Waltham, MA). Detailed RT-qPCR protocol is described in the supplemental Methods.

Mutant *NPM1* MRD detection by NGS

NPM1 mutations were determined at diagnosis on genomic DNA by NGS using the TruSight myeloid sequencing panel (Illumina, San Diego, CA). For mutant *NPM1* MRD detection, a targeted single-amplicon deep NGS approach was used, covering exon 12 of the *NPM1* gene. For MRD analysis by NGS/DNA, 100 ng high–molecular weight genomic DNA was used as input. The library preparation protocols for NGS/DNA and NGS/cDNA are described in the supplemental Methods, and only differed in the primers used for the first target PCR (supplemental Table 1B). The average read coverage of the NGS/DNA approach was 1 264 927 (range, 85 277-2 333 905).

FLT3-ITD MRD detection by NGS

FLT3-ITD MRD was assessed using a targeted deep sequencing approach as described before.[25]

Statistical analysis

The primary end point of the MRD analysis was cumulative incidence of relapse (CIR). Time to relapse and survival were estimated from date of sampling in CR until the date of the event of interest. Survival analyses were performed using Kaplan-Meier estimates, and significance was assessed using the log-rank test. Multivariable analysis was performed using Cox proportional hazards model. Differences in patient characteristics were tested with the Fisher exact test (categorical) or Mann-Whitney *U* test (continuous). *P* values of <.05 were considered statistically significant, and all tests were 2-sided. Stata statistics and data science software, Release 17.0 (Stata, College Station, TX) was used for all statistical analyses.

Results

Mutant *NPM1* MRD detection by RT-qPCR on cDNA

Mutant *NPM1* detection by RT-qPCR/cDNA in CR after 2 cycles of induction chemotherapy was performed on patients with mutant *NPM1* AML. Mutant *NPM1* MRD was detected in 94 cases (79%) with a limit of detection (LOD) of 10^{-6} (Figure 2A). Stratification of patients with AML following various mutant *NPM1/ABL1* thresholds demonstrate that the group with >2% mutant *NPM1/ABL1* after cycle 2 was most significantly associated with increased relapse risk ($P = .016$; Figure 2B) and inferior overall survival (OS; $P < .001$; supplemental Figure 3A-B) compared with other thresholds. In fact, the groups of patients with AML with mutant *NPM1/ABL1* MRD of <2% did not differ statistically significantly in terms of treatment outcome from the patient group without mutant *NPM1* MRD. However, relapse incidence was slightly increased for patients with AML with low-level MRD, suggesting that these

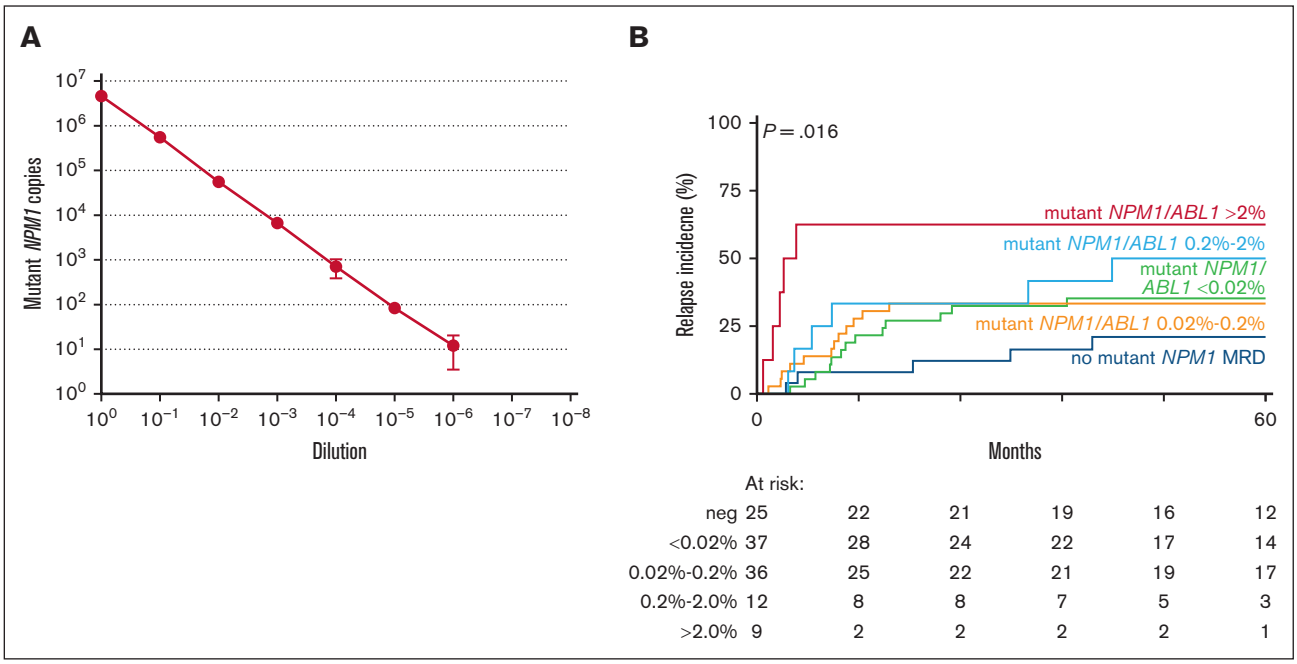

Figure 2. Mutant *NPM1* MRD detection by RT-qPCR/cDNA. (A) LOD of mutant *NPM1* by RT-qPCR/cDNA. (B) CIR of patients with AML with different mutant *NPM1/ABL1* thresholds by RT-qPCR/cDNA. Neg, LOD, Limit of detection.

groups contain a mix of patients with or without a higher risk of relapse that cannot be distinguished based on mutant *NPM1* levels.

Mutant *NPM1* MRD detection by NGS on genomic DNA

Next, we explored NGS-based mutant *NPM1* MRD detection on DNA. The LOD of mutant *NPM1* by NGS/DNA was 10^{-4} (VAF, 2.1×10^{-3}; Figure 3A). Among 119 AML cases in CR, 29 (24%) had measurable mutant *NPM1* by NGS/DNA, of which 28 (97%) were also detectable by RT-qPCR/cDNA. In addition, there were 67 discordant cases in which mutant *NPM1* was detectable by RT-qPCR/cDNA but undetectable with NGS/DNA (supplemental Table 2). All these discordant cases carried mutant *NPM1/ABL1* ratios at low levels (<0.56% mutant *NPM1/ABL1* by RT-qPCR). The correlation between VAF values by NGS/DNA and mutant *NPM1/ABL1* by RT-qPCR was high ($r = 0.98$; Figure 3B). In fact, a similar NGS approach was carried out on cDNA(LOD, 10^{-5}; supplemental Figure 4A). Mutant *NPM1* MRD was detected in 48 (40%) patients, and a strong correlation between mutant *NPM1* VAFs by NGS/cDNA and mutant *NPM1/ABL1* by RT-qPCR/cDNA was found, with 0.1% VAF approximately corresponding to 2% mutant *NPM1/ABL1* ($r = 0.99$; supplemental Figure 4B). CIR and OS were similar for NGS/cDNA compared with RT-qPCR, with MRD defined as VAF of >0.1% (supplemental Figure 4C-D).

All samples with mutant *NPM1/ABL1* of >2% by RT-qPCR were detected with NGS/DNA with VAFs of >0.01% (Figure 3B). This illustrates that the 2% mutant *NPM1/ABL1* by RT-qPCR threshold corresponds to ~1 mutant *NPM1* cell in 10 000 *NPM1* wild-type cells. Relapse rates in patients with AML with mutant *NPM1* VAF of >0.01% by NGS/DNA were significantly increased (4-year CIR: 57.1% MRD^+ vs 32.2% MRD^-; hazard ratio [HR], 2.82; 95% confidence interval [CI], 1.13-7.03; $P = .026$; supplemental Figure 5A). Notably, the NGS/DNA VAF threshold of >0.01% identified patients with AML with a significantly increased risk of relapse, including cases that were missed by RT-qPCR/cDNA with mutant *NPM1/ABL1* of >2% ($P = .025$; Figure 3C). Although this is only a small number of patients with AML in our cohort (n = 6), it increases the number of cases at risk considerably (7.5% to 13%).

We confirmed the prognostic value of mutant *NPM1* MRD by NGS/DNA with the >0.01% VAF threshold in an independent cohort of 191 AML cases with available DNA samples (4-year CIR: 50.0% MRD^+ vs 25.6% MRD^-; HR, 2.55; 95% CI, 1.38-4.71; $P = .003$; supplemental Figure 5B).

Next, we combined all mutant *NPM1* MRD analyses by NGS/DNA to a cohort of 310 AML cases in total (BM, n = 301 and PB, n = 9). Eighty-three (27%) of 310 AML samples had measurable mutant *NPM1* by NGS/DNA at any level. These cases showed a significant increase in relapse incidence compared with patients without detectable mutant *NPM1* (4-year CIR: 42.7% MRD^+ vs 26.7% MRD^-; HR, 1.76; 95% CI, 1.17-2.65; $P = .007$; supplemental Figure 5C). Applying the previously established MRD threshold of VAF >0.01% by NGS/DNA in the enlarged cohort, resulted in 37 patients (12%) with relatively high-level mutant *NPM1* disease and a highly significant increased risk of relapse (4-year CIR: 52.8% MRD^+ vs 28.1% MRD^-; HR, 2.56; 95% CI, 1.54-4.25; $P < .001$; Figure 3D), and inferior OS ($P > .001$; supplemental Figure 5D).

Multiparameter flow cytometry (MFC) MRD results were available in 245 of 310 patients with AML. No clear correlation was apparent between MFC-MRD and mutant *NPM1* MRD by NGS/DNA with or without a VAF threshold of 0.01% (supplemental Table 3A-B). Furthermore, MFC-MRD was not found to be prognostically relevant in this cohort of patients with AML (supplemental Figure 6).

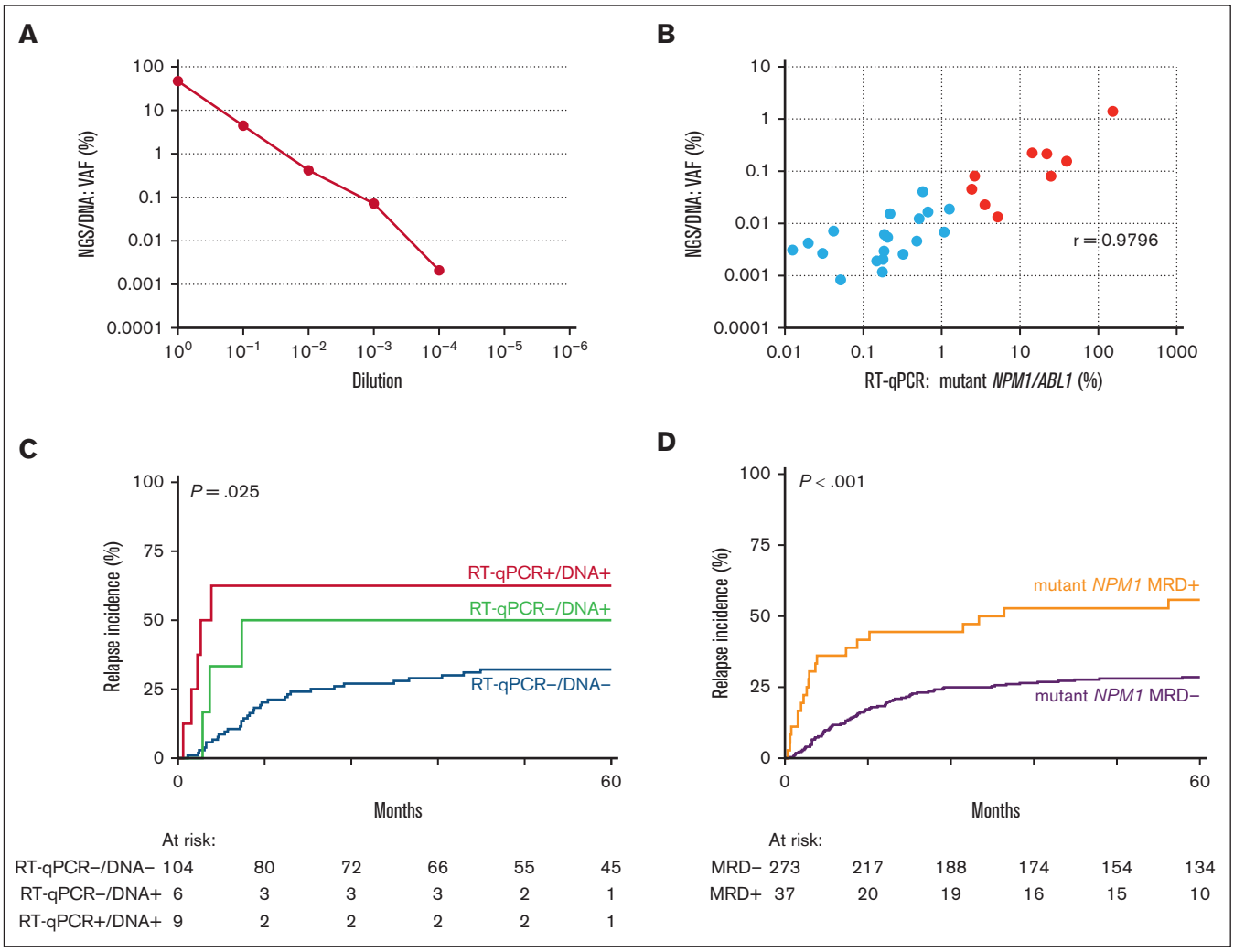

Figure 3. Mutant *NPM1* MRD detection by NGS/DNA. (A) LOD of mutant *NPM1* by NGS/DNA. (B) Correlation of mutant *NPM1* detection by RT-qPCR/cDNA and NGS/DNA, with cases of >2% mutant *NPM1/ABL1* indicated in red. (C) CIR of mutant *NPM1* MRD as detected by RT-qPCR/cDNA (MRD with >2% mutant *NPM1/ABL1*) and/or NGS/DNA (MRD at >0.01%) in a cohort of 119 patients with mutant *NPM1* AML. (D) CIR of mutant *NPM1* MRD as detected by NGS/DNA in a cohort of 310 patients with mutant *NPM1* AML (MRD of >0.01% VAF). Patients with detectable mutant *NPM1* MRD indicated in yellow, patients without detectable mutant *NPM1* MRD indicated in purple.

Influence of concomitant mutations on mutant *NPM1* MRD outcome

Next, we addressed the influence of concomitant mutations at diagnosis on the prognostic value of mutant *NPM1* MRD by NGS/DNA (VAF > 0.01%). Mutations in *DNMT3A* (n = 172, 55%) and *FLT3*-ITDs (n = 123, 40%) were the most common coexisting aberrations (supplemental Figure 7). Patients with AML with *FLT3*-ITD had significantly higher white blood cell count (WBC) at diagnosis ($P = .013$), were more often consolidated with allogeneic hematopoietic stem cell transplantation ($P < .001$), and less frequently had a complex karyotype ($P = .015$; Table 1).

Next, we explored the effect of mutant *NPM1* MRD (VAF > 0.01%) by NGS/DNA in patients with AML with a *FLT3*-ITD (n = 123) and patients without a *FLT3*-ITD (n = 187) at diagnosis. In the subgroup without a *FLT3*-ITD (n = 187), mutant *NPM1* MRD was detected in 23 patients with AML (12%). Persistence of mutant *NPM1* in CR only shows a trend of a higher relapse rate after 5 years compared with patients with AML without mutant *NPM1* MRD ($P = .067$; Figure 4A). In contrast, in patients with a concomitant *FLT3*-ITD the presence of mutant *NPM1* MRD (n = 14) was associated with a significantly higher relapse incidence ($P < .001$; Figure 4B), with 75% of the patients facing a relapse after 5 years compared to ~30% of patients with AML with mutant *NPM1* MRD with VAF of <0.01%. Similarly, in terms of OS, persistence of mutant *NPM1* in CR was inferior in patients with a co-occurring *FLT3*-ITD than patients without (supplemental Figure 8). In multivariable analysis including age, number of cycles to attain CR, and white blood cell count at diagnosis, mutant *NPM1* MRD with VAF of >0.01% was not prognostic in patients with wild-type *FLT3*-ITD ($P = .102$; supplemental Table 4) but remained a strong independent predictor of relapse in patients with *FLT3*-ITD AML ($P = .001$; Table 2).

FLT3-ITD MRD has recently been shown to be a strong predictor of relapse in *FLT3*-ITD AML.[25-27] Of 14 patients with AML with

Table 1. Patient characteristics of patients with AML with mutated *NPM1*

	No *FLT3*-ITD (n = 187)	*FLT3*-ITD (n = 123)	Total (n = 310)	*P*-value
Age, y				$P = .204$
Median	52	53	52	
Range	19-65	26-66	19-66	
Sex, n (%)				$P = .561$
M	91 (49)	55 (45)	146 (47)	
F	96 (51)	68 (55)	164 (53)	
ELN risk classification (%)				$P < .001$
Favorable	182 (97)	1 (1)	183 (59)	
Intermediate	0 (0)	121 (98)	121 (39)	
Adverse	5 (3)	1 (1)	6 (2)	
Disease type (%)				$P = .565$
De novo AML	186 (99.5)	121 (98)	307 (99)	
Therapy-related AML	1 (0.5)	2 (2)	3 (1)	
WBC at diagnosis, n (%)				$P = .013$
$<100 \times 10^9$/L	171 (91)	100 (81)	271 (87)	
$>100 \times 10^9$/L	16 (9)	23 (19)	39 (13)	
Last treatment before first CR, n (%)				$P = .243$
Cycle 1	172 (92)	108 (88)	280 (90)	
Cycle 2	15 (8)	15 (12)	30 (10)	
Consolidation therapy, n (%)				$P < .001$
None	16 (9)	15 (12)	31 (10)	
Chemotherapy	55 (29)	17 (14)	72 (23)	
Autologous HSCT	88 (47)	23 (19)	111 (36)	
Allogeneic HSCT	28 (15)	68 (55)	96 (31)	
Cytogenetics, n (%)*				$P = .015$
Normal karyotype	158 (87)	113 (96)	271 (91)	
Aberrant karyotype	23 (13)	5 (4)	28 (9)	
Mutations at diagnosis, n (%)				$P = .035$
DNMT3A				
Wild-type	92 (49)	45 (37)	137 (44)	
Mutant	95 (51)	78 (63)	173 (56)	

F, female; HSCT, hematopoietic stem cell transplant; M, male; WBC, white blood cell.
*Cytogenetics failed in 11 patients.

mutant *NPM1* MRD with VAF of >0.01% in CR, 11 cases also had detectable *FLT3*-ITD MRD with VAF of >0.01%. The 3 patients with mutant *NPM1* MRD without *FLT3*-ITD MRD in CR did not relapse within 5 years (Figure 4C). In contrast, the 5 patients without mutant *NPM1* MRD but with detectable *FLT3*-ITD MRD showed an increased CIR (Figure 4C).

Discussion

MRD assessment of mutant *NPM1* in patients with AML is currently performed primarily by using RT-qPCR assays on cDNA. For various reasons this assay has limitations and particular drawbacks. The most prominent disadvantages relate to (1) the instability of the RNA input material, (2) the MRD levels that are based on expression of the target genes that vary among patients with AML, and (3) the MRD analyses that are generally limited to the most frequent *NPM1* mutation types. There is an intense clinical interest in a robust mutant *NPM1* MRD detection assay, and the establishment of reliable prognostically relevant thresholds that would resolve these issues and enable better comparison, verification, and harmonization of assays across molecular diagnostic laboratories.

Here, we show that NGS can reliably assess mutant *NPM1* MRD with a very high concordance between RT-qPCR/cDNA (mutant *NPM1/ABL1*) and NGS/DNA (mutant *NPM1*/wild-type *NPM1*). Although NGS/DNA is ~10-fold less sensitive compared with cDNA-based assays, a high correlation between mutant *NPM1* VAFs by NGS/DNA and mutant *NPM1/ABL1* by RT-qPCR is apparent. The difference in sensitivity does not preclude the association with relapse risk, because patients with AML with higher levels of mutant *NPM1/ABL1* expression (>2% by RT-qPCR) were easily detected by NGS/DNA (VAF > 0.01%). In

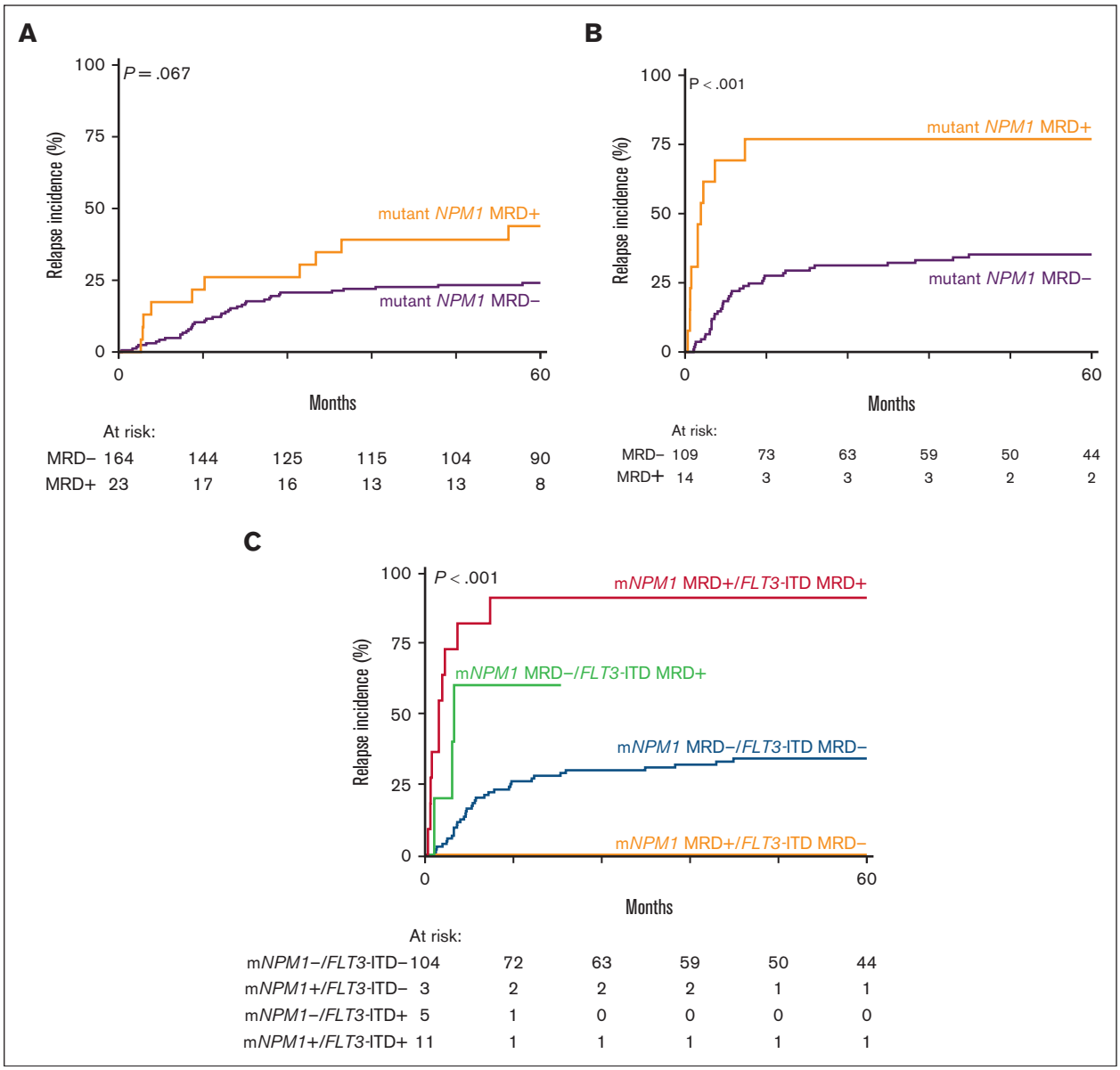

Figure 4. Mutant *NPM1* MRD detection in patients with AML with a concomitant *FLT3*-ITD. (A) Relapse incidence of mutant *NPM1* (m*NPM1*) MRD (NGS/DNA, >0.01% VAF) in patients with AML without *FLT3*-ITD at diagnosis. Patients with detectable m*NPM1* MRD indicated in yellow, patients without detectable *NPM1* MRD indicated in purple. (B) Relapse incidence of m*NPM1* MRD (NGS/DNA, >0.01% VAF) in patients with AML with a *FLT3*-ITD at diagnosis. Patients with detectable m*NPM1* MRD indicated in yellow, patients without detectable *NPM1* MRD indicated in purple. (C) Relapse incidence of patients with AML with and without m*NPM1* MRD in patients with AML with a *FLT3*-ITD at diagnosis in the context of *FLT3*-ITD MRD.

fact, a substantial number of additional patients with AML at higher risk of relapse were identified with >0.01% VAF by NGS/DNA, compared with >2% mutant *NPM1/ABL1* by RT-qPCR/cDNA. Moreover, we show that MRD assessment for mutant *NPM1* after 2 cycles of chemotherapy is prognostic for relapse with a mutant *NPM1/ABL1* threshold of 2% in the BM. Whereas the current guidelines state that PB is preferred for post–cycle 2 assessment, we show here that cDNA/DNA derived from the BM is equally proficient. However, because of the unavailability of paired PB/BM samples, a comparison could not be performed. In addition, although MFC-MRD is established for MRD assessment in AML, it did not correlate with mutant *NPM1* MRD by NGS/DNA, and MFC-MRD has limited prognostic value in our mutant *NPM1* AML patient cohort. Altogether, our data indicate that NGS can be used as a reliable MRD detection method that identifies patients with mutant *NPM1* AML with prognostic significance. Importantly, NGS-based assays with DNA as input will allow an easier way of harmonizing mutant *NPM1* MRD detection among users.

Models of AML progression support the acquisition of *NPM1* mutations as an intermediate event that is frequently preceded by mutations in *DNMT3A* and followed by *FLT3*-ITDs or other mutations in activated signaling genes, such as *KRAS*, *NRAS*, *PTPN11*, or *FLT3*-TKDs.[28] Previous work has shown that *FLT3*-ITD

Table 2. Multivariable analysis of mutant *NPM1* MRD in patients with AML with a *FLT3*-ITD at diagnosis

	Relapse incidence	
Prognostic factor	**HR (95% CI)**	***P* value**
Mutant *NPM1* MRD status (detectable vs not detectable)	4.79 (1.84-12.44)	.001
Age (per year)	1.00 (0.97-1.04)	.86
No. of cycles to attain CR (2 vs 1 cycle)	0.70 (0.26-1.85)	.47
WBC counts at diagnosis (>100 × 10^9 vs ≤100 × 10^9)	1.59 (0.78-3.22)	.20

WBC, white blood cell.

persistence in AML with *FLT3*-ITD in CR after induction treatment is a strong and independent prognostic marker for inferior OS and increased relapse incidence.[25-27] The threshold of *FLT3*-ITD MRD showing the best correlation with disease outcomes in independent studies corresponded with a value of >0.01%.[18,25,26] The optimal prognostically relevant threshold of mutant *NPM1* VAF of >0.01% reported here is as expected because the VAFs of concomitant mutations, in this case mutant *NPM1* and *FLT3*-ITD, are expected to be similar.

We demonstrated that mutant *NPM1* MRD detection is particularly prognostic in *FLT3*-ITD AML. Mutant *NPM1* will act as a surrogate MRD marker for *FLT3*-ITD MRD in mutant *NPM1*/*FLT3*-ITD AML. A similar but less profound association of mutant *NPM1* MRD with a higher relapse risk in *FLT3*-ITD AML has been reported before,[12] however, in the majority of studies this association was not addressed[9,11,18] or absent.[10] Although the number of patients with mutant *NPM1*/*FLT3*-ITD AML in our study was small, cases with *FLT3*-ITD MRD had a higher CIR and none of the patients with only mutant *NPM1* MRD experienced relapse within 5 years. Altogether, this may indicate that combinatorial MRD analyses of mutant *NPM1* and *FLT3*-ITD MRD analyses are preferred in mutant *NPM1*/*FLT3*-ITD AML, however additional molecular MRD analyses in this subtype of AML are warranted.

In conclusion, we showed that mutant *NPM1* MRD detection with VAF of >0.01% by NGS/DNA-based assays reliably identifies patients with AML with an increased CIR after 2 cycles of chemotherapy, most profoundly in mutant *NPM1*/*FLT3*-ITD AML. Harmonization of this alternative NGS/DNA-based assay is expected to be relatively easy and may be useful in resolving many of the current issues with respect to mutant *NPM1* MRD detection in clinical practice.

Acknowledgments

The authors thank all the patients, clinicians, research nurses, data managers, and laboratory scientists who provided samples, conducted cytogenetic analyses, and performed analyses at the participating centers of the Dutch-Belgian Cooperative Trial Group for Hematology-Oncology and the Swiss Group for Clinical Cancer Research, including centers in The Netherlands, Belgium, Switzerland, Norway, Sweden, Lithuania, Germany, and Denmark. The authors thank the Erasmus MC Hematology department, the Molecular Diagnostic unit of the Hemato-oncology laboratory, and Eric Bindels for performing the sequencing; as well as Mathijs Sanders and Remco Hoogenboezem for assisting with bioinformatics.

This study was supported by grants from the Queen Wilhelmina Fund Foundation of the Dutch Cancer Society (EMCR 2019-12507 and 2021-13650 infra).

Authorship

Contribution: C.M.V., T.G., B.L., and P.J.M.V. designed the study; G.J.O., M.G.M., V.H., Y.F., and B.L. provided study materials or patients; C.M.V., M.R., and F.G.K. performed experiments; C.M.V., T.G., M.R., F.G.K., and J.M.L.K. analyzed data; C.M.V. and T.G. prepared the figures; C.M.V., T.G., B.L. and P.J.M.V. drafted the manuscript with input from the remaining authors; and all authors approved the final version of the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

ORCID profiles: C.M.V., 0009-0001-4321-9104; T.G., 0000-0002-2520-4603; M.G.M., 0000-0002-4676-7931; B.L., 0000-0001-8982-5217.

Correspondence: Peter J. M. Valk, Department of Hematology, Erasmus University Medical Center Rotterdam, Nc806, Wytemaweg 80, 3015 CN Rotterdam Z-H, The Netherlands; email: p.valk@erasmusmc.nl.

References

1. Heath EM, Chan SM, Minden MD, Murphy T, Shlush LI, Schimmer AD. Biological and clinical consequences of NPM1 mutations in AML. *Leukemia*. 2017;31(4):798-807.
2. Dohner H, Wei AH, Appelbaum FR, et al. Diagnosis and management of AML in adults: 2022 recommendations from an international expert panel on behalf of the ELN. *Blood*. 2022;140(12):1345-1377.
3. Ranieri R, Pianigiani G, Sciabolacci S, et al. Current status and future perspectives in targeted therapy of NPM1-mutated AML. *Leukemia*. 2022;36(10):2351-2367.
4. Short NJ, Zhou S, Fu C, et al. Association of measurable residual disease with survival outcomes in patients with acute myeloid leukemia: a systematic review and meta-analysis. *JAMA Oncol*. 2020;6(12):1890-1899.
5. Walter RB, Ofran Y, Wierzbowska A, et al. Measurable residual disease as a biomarker in acute myeloid leukemia: theoretical and practical considerations. *Leukemia*. 2021;35(6):1529-1538.
6. Vonk CM, Al Hinai ASA, Hanekamp D, Valk PJM. Molecular minimal residual disease detection in acute myeloid leukemia. *Cancers 2021*. 2021;13(21):5431.

7. Voso MT, Ottone T, Lavorgna S, et al. MRD in AML: the role of new techniques. *Front Oncol*. 2019;9:655.
8. Heuser M, Freeman SD, Ossenkoppele GJ, et al. 2021 Update on MRD in acute myeloid leukemia: a consensus document from the European LeukemiaNet MRD Working Party. *Blood*. 2021;138(26):2753-2767.
9. Krönke J, Schlenk RF, Jensen KO, et al. Monitoring of minimal residual disease in NPM1-mutated acute myeloid leukemia: a study from the German-Austrian Acute Myeloid Leukemia Study Group. *J Clin Oncol*. 2011;29(19):2709-2716.
10. Ivey A, Hills RK, Simpson MA, et al. Assessment of minimal residual disease in standard-risk AML. *N Engl J Med*. 2016;374(5):422-433.
11. Shayegi N, Kramer M, Bornhäuser M, et al. The level of residual disease based on mutant NPM1 is an independent prognostic factor for relapse and survival in AML. *Blood*. 2013;122(1):83-92.
12. Dillon R, Hills R, Freeman S, et al. Molecular MRD status and outcome after transplantation in NPM1-mutated AML. *Blood*. 2020;135(9):680-688.
13. Balsat M, Renneville A, Thomas X, et al. Postinduction minimal residual disease predicts outcome and benefit from allogeneic stem cell transplantation in acute myeloid leukemia with NPM1 mutation: a study by the Acute Leukemia French Association Group. *J Clin Oncol*. 2017;35(2):185-193.
14. Schwind S, Bischof L, Bill M, et al. Quantifying *NPM1* MRD in AML patients prior to allogeneic stem cell transplantation: where to draw the line? *HemaSphere*. 2024;8(3):e55.
15. Kapp-Schwoerer S, Weber D, Corbacioglu A, et al. Impact of gemtuzumab ozogamicin on MRD and relapse risk in patients with NPM1-mutated AML: results from the AMLSG 09-09 trial. *Blood*. 2020;136(26):3041-3050.
16. Hubmann M, Köhnke T, Hoster E, et al. Molecular response assessment by quantitative real-time polymerase chain reaction after induction therapy in NPM1-mutated patients identifies those at high risk of relapse. *Haematologica*. 2014;99(8):1317-1325.
17. Tiong IS, Dillon R, Ivey A, et al. Clinical impact of NPM1-mutant molecular persistence after chemotherapy for acute myeloid leukemia. *Blood Adv*. 2021;14(5):5107-5111.
18. Othman J, Potter N, Ivey A, et al. Postinduction molecular MRD identifies patients with NPM1 AML who benefit from allogeneic transplant in first remission. *Blood*. 2024;143(19):1931-1936.
19. Handschuh L, Kaźmierczak M, Milewski MC, et al. Gene expression profiling of acute myeloid leukemia samples from adult patients with AML-M1 and -M2 through boutique microarrays, real-time PCR and droplet digital PCR. *Int J Oncol*. 2018;52(3):656-678.
20. Pettersson L, Johansson Alm S, Almstedt A, et al. Comparison of RNA- and DNA-based methods for measurable residual disease analysis in NPM1-mutated acute myeloid leukemia. *Int J Lab Hematol*. 2021;43(4):664-674.
21. Thol F, Gabdoulline R, Liebich A, et al. Measurable residual disease monitoring by ngs before allogeneic hematopoietic cell transplantation in AML. *Blood*. 2018;132(16):1703-1713.
22. Pabst T, Vellenga E, Van Putten W, et al. Favorable effect of priming with granulocyte colony-stimulating factor in remission induction of acute myeloid leukemia restricted to dose escalation of cytarabine. *Blood*. 2012;119(23):5367-5373.
23. Lowenberg BL̈, Pabst T, Maertens J, et al. Therapeutic value of clofarabine in younger and middle-aged (18-65 years) adults with newly diagnosed AML. *Blood*. 2017;129(12):1636-1645.
24. Löwenberg B, Pabst T, Maertens J, et al. Addition of lenalidomide to intensive treatment in younger and middle-aged adults with newly diagnosed AML: the HOVON-SAKK-132 trial. *Blood Adv*. 2021;5(4):1110-1121.
25. Grob T, Sanders MA, Vonk CM, et al. Prognostic value of *FLT3*-internal tandem duplication residual disease in acute myeloid leukemia. *J Clin Oncol*. 2023;41(4):756-765.
26. Loo S, Dillon R, Ivey A, et al. Pretransplant FLT3-ITD MRD assessed by high-sensitivity PCR-NGS determines posttransplant clinical outcome. *Blood*. 2022;140(22):2407-2411.
27. Dillon LW, Gui G, Ravindra N, et al. Measurable residual FLT3 internal tandem duplication before allogeneic transplant for acute myeloid leukemia. *JAMA Oncol*. 2024;10(8):1104-1110.
28. Patel JL, Schumacher JA, Frizzell K, et al. Coexisting and cooperating mutations in NPM1-mutated acute myeloid leukemia. *Leuk Res*. 2017;56:7-12.