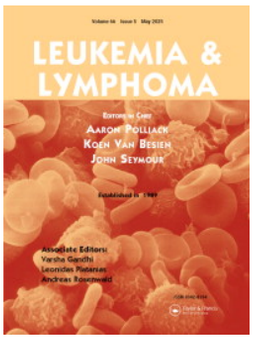

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ORIGINAL ARTICLE



# Prognostic significance of deep sequencing for analysis of measurable residual disease in acute myeloid leukemia with *NPM1* mutation

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**ABSTRACT**

In acute myeloid leukemia with *NPM1* mutation, analysis of measurable residual disease (MRD) with reverse transcription quantitative polymerase chain reaction (RT-qPCR) is recommended for response assessment and monitoring after treatment. For rare mutations in *NPM1*, this is not readily available. Therefore, we evaluated the prognostic value of deep sequencing covering all *NPM1* exon 11 variants, using retrospectively analyzed bone marrow samples from 97 patients in remission during treatment. MRD positivity was defined as *NPM1* mutation at ≥0.05% variant allele frequency based on a previous comparison with RT-qPCR. Deep sequencing MRD positivity at any time during consolidation predicted relapse-free survival (at 3 years: 23.1±11.7% vs. 70.8±6.1%, $p<0.001$) and overall survival (at 3 years: 30.8±12.8% vs. 63.8±6.6%, $p=0.014$). In multivariable analysis, MRD status during consolidation was the sole predictor for relapse. In conclusion, deep sequencing of *NPM1* has high prognostic value and extends MRD monitoring to patients with rare mutations in *NPM1*.

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**KEYWORDS**
Acute myeloid leukemia; measurable residual disease; NPM1; deep sequencing

## Introduction

Mutations in the gene nucleophosmin 1 (*NPM1*), located on chromosome 5q35, is the most common genetic aberration in adult acute myeloid leukemia (AML), present in around one-third of all patients and half of patients with normal karyotypes [1]. Given its unique features, AML with *NPM1* mutation forms a specific diagnostic entity according to both the World Health Organization Classification of Haematolymphoid Tumours and the International Consensus Classification [2,3]. Mutations in *NPM1* are found in exon 11 (previously identified as exon 12) and over 90 different types have been described. The so called type A mutation (c.860_863dupTCTG, p.(Trp288CysfsTer12)) is the most frequent mutation, accounting for 70-85%, depending on the studied cohort. Types B (c.863_864insCATG, p.(Trp288Cysfs*12)) and D (c.863_864insCCTG, p.(Trp288Cysfs*12)) each account for 2–7% of *NPM1* mutated cases, and the remaining 10–20% are individually rare [4–6].

To improve the prognosis for AML patients, it is crucial to early identify patients who are at risk of relapse. This risk is determined mainly by the genetics at diagnosis and level of measurable residual disease (MRD) in morphologic remission. According to the 2022 European LeukemiaNet (ELN) risk classification, AML with mutated *NPM1* is classified as favorable risk unless there is a concurrent *FLT3*-mutation or adverse risk cytogenetics [7]. For patients with *NPM1* mutation, the response to chemotherapy treatment, assessed by molecular MRD analysis, has shown prognostic value in a number of previous studies and can accordingly modify genetic risk assessment [7–11].

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Although strict guidelines are lacking, MRD is thus becoming increasingly important for the choice and timing of allogeneic hematopoietic stem cell transplantation (alloSCT) [12].

For MRD analysis in *NPM1*-mutated AML, ELN recommends reverse transcription quantitative polymerase chain reaction (RT-qPCR) performed on RNA [13]. Due to the high number of reported mutations in *NPM1*, a clinical laboratory needs to set up multiple RT-qPCR assays to enable MRD analysis for all patients with *NPM1*-mutated AML. With the requirement for stringent quality control, most clinical laboratories therefore restrict monitoring to the most common mutation(s), resulting in inequality in the care of patients with *NPM1*-mutated AML. In order to provide molecular MRD analysis for more patients, we and others have developed DNA-based deep sequencing methods covering all different variants in exon 11 of *NPM1* without the need for mutation-specific primers or probes [14,15]. We have previously shown the potential of deep sequencing as a highly sensitive tool for patient-tailored MRD analysis in AML [15], and demonstrated that deep sequencing of *NPM1* can provide prognostic information regarding relapse and survival after alloSCT [14]. By comparison with RT-qPCR, we recently proposed a clinically relevant cutoff of 0.05% variant allele frequency (VAF), corresponding to 0.1% leukemic cells [16].

In order to enable guidance for clinical use of deep sequencing for molecular MRD analysis in *NPM1*-mutated AML, we here aimed to verify its applicability for relapse prediction. This was performed through a population-based retrospective study of patients with AML with mutation in *NPM1* treated before the era of molecular MRD monitoring.

## Materials and methods

### *Patients*

Eligible patients were adults diagnosed with *NPM1*-mutated AML that had been treated with curative intent and achieved complete remission (CR) or CR with incomplete peripheral blood count recovery (CRi) between 2006 and 2016 in Region Västra Götaland in Sweden. CR was defined as a bone marrow (BM) blast count <5%, no circulating blasts with Auer rods, absence of extramedullary disease, an absolute neutrophil count $\geq 1.0 \times 10^9$/L, and a platelet count $\geq 100 \times 10^9$/L, while CRi was defined as meeting all CR criteria except for residual neutropenia ($<1.0 \times 10^9$/L) or thrombocytopenia ($<100 \times 10^9$/L) [17]. Using the national AML registry, 757 patients diagnosed with AML in the region during the chosen time period were identified. Of these, 469 were treated with curative intent: 354 achieved CR/CRi and of those, 90 patients had a confirmed *NPM1* mutation as determined by PCR with fragment analysis. After additional screening of samples from patients not screened for *NPM1* at diagnosis, the resulting study cohort consisted of 97 patients (Supplementary Figure 1). BM aspirate slides for isolation and deep sequencing analysis of DNA from at least one time point during treatment were available for all patients. In total 257 samples were analyzed from different time points when patients were in CR/CRi: after the first cycle of chemotherapy ($n = 96$), during consolidation (after two [$n = 56$], or three [$n = 48$] cycles), and at the end of treatment (completion of four [$n = 47$], or five [$n = 8$] cycles, or before alloSCT [$n = 2$]).

Patients were treated at four centers: Sahlgrenska University Hospital (Gothenburg, Sweden), Uddevalla Hospital (Uddevalla, Sweden), Southern Älvsborg Hospital (Borås, Sweden) or Skaraborg Hospital (Skövde, Sweden). Patients were treated by a cytarabine- and daunorubicin-based induction and consolidation regimen [18], and received a median of 4 cycles (range, 2–5 cycles) of chemotherapy, where 32 patients received 2–3 cycles and 63 patients received 4–5 cycles. Two patients received treatment with azacitidine, where one was treated with azacitidine solely and the other received standard chemotherapy as induction and azacitidine as consolidation, both patients received 7 cycles each in total. For the purpose of statistical analyses, patients were divided into groups of full intensity treatment (FIT; $n = 67$) or reduced intensity treatment (RIT; $n = 30$), based on the number of cycles and dosing of chemotherapy (Supplementary Table 1). Thirty-one patients underwent alloSCT; 22 following intensive regimen and 9 following reduced regimens. The decision for alloSCT was made by the treating physician based on the fitness of the patient and the indication for alloSCT according to the Swedish national care program for AML, in which indications for transplantation were *FLT3*-ITD mutation and intermediate-/high-risk cytogenetics. The median follow-up time of patients was 59 months (range, 2–172 months). Patient characteristics, treatment information, relapse and survival status were obtained from patient medical records. The study was approved by the Regional Ethics Review Board (Gothenburg, Sweden) and conducted according to the Declaration of Helsinki. All patients gave their consent for their samples to be stored in a local biobank and be used for research purposes.

### Deep sequencing and definition of MRD cutoffs

Assessment of MRD using deep sequencing of mutated *NPM1* was performed as previously described [16]. DNA was isolated from biobanked BM aspirate slides, stored in room temperature, using the QIAamp DNA Micro Kit (Qiagen, Hilden, Germany). Briefly, after addition of tissue lysis buffer ATL to the slides, cells were scraped off and collected, whereafter slides were rinsed twice with the same buffer to collect remaining cells. DNA was then extracted according to the manufacturer's protocol, with an output of 180 ng – 20 µg DNA/slide. The DNA output was equally distributed over time, indicating no decay in quality despite several years of biobanking. Library preparation of the mutation hotspot region *NPM1* exon 11 with PCR of 100 ng DNA followed by paired-end sequencing on MiSeq (Illumina, San Diego, CA) was performed as previously described [16]. Calling of mutated and wild-type reads was performed with the in-house developed script 'NPM1_DeepSeq,' freely available at https://github.com/ClinicalGenomicsGBG/NPM1_DeepSeq. Coverage was $>5\times10^{\wedge5}$ in all samples except one with $2.6\times10^{\wedge5}$ reads. *NPM1* mutation VAF was calculated as the number of reads with mutated *NPM1* divided by the sum of mutated *NPM1* and corresponding wild-type sequence, expressed as percentage. The limit of detection (LOD) was adjusted for the material used in this study, i.e. BM aspirate slides, and defined as the mean + 3 standard deviations of the 44 negative control analyses; VAF ≥0.005% and ≥50 mutated reads. This LOD was the basis for the definitions MRD detectable ($MRD^{det}$) and MRD undetectable ($MRD^{undet}$). For the definition of MRD positive ($MRD^{pos}$) the cutoff ≥0.05% VAF was used, and MRD negative ($MRD^{neg}$) was defined as <0.05% VAF, based on our previous comparison with RT-qPCR of mutated *NPM1* [16]. In the analyses, MRD status during consolidation was defined from results after two cycles of treatment, or after three cycles if no BM examination was performed after two cycles, and at the end of treatment, from MRD results after the last cycle of treatment.

### Multiparameter flow cytometry

Assessment of MRD with multiparameter flow cytometry (MFC) was performed as previously described with a standardized eight color MRD panel consisting of five tubes [19] in 17 patients during consolidation as part of clinical care. Briefly, 1–2 mL of the BM aspirate was used for MRD analysis, using bulk lysis and stained with monoclonal antibodies. MRD MFC positivity ($MRD\ MFC^{pos}$) was defined as ≥0.1% cells with leukemia associated immunophenotype.

### Diagnostic genetic analyses

Karyotype was obtained through G-banding at the time of diagnosis, and *FLT3*-ITD and *NPM1* mutation with PCR followed by fragment analysis [20,21]. The specific DNA sequences were amplified with two gene-specific primer sequences, where one was labeled with FAM-fluorochrome, and PCR products were detected by capillary electrophoresis. Results were analyzed in GeneMapper (ThermoFisher Scientific, Waltham, MA). For assessment of *FLT3*-ITD allelic ratio (AR), biobanked samples were reanalyzed with PCR [20], and *FLT3*-ITD AR was calculated by dividing the area under the curve (AUC) of the mutant peak with the AUC of the wild-type *FLT3*-ITD. Mutations in *DNMT3A* exons 2–23 were detected with Sanger sequencing at Eurofins Medigenomix GmbH (Ebersberg, Germany). *NPM1* mutation status at relapse was assessed in clinical care ($n=14$) or on biobanked samples ($n=18$).

### Statistical analysis

Differences between groups were performed by Mann–Whitney *U* test for continuous variables and the Chi-square test for categorical variables. Association between deep sequencing and MFC was assessed using Cohen's kappa. Receiver operating characteristic (ROC) curves were used to determine the optimal cutoff value for deep sequencing, with the value corresponding to the highest Youden's index selected as the optimal threshold. Survival differences were calculated by log-rank test and survival fractions were calculated using the Kaplan–Meier estimate. For analysis of impact of different variables, Cox proportional hazards regression with univariable and multivariable analysis was performed, and presented as hazard ratios (HR) with 95% confidence intervals (CI). Relapse-free survival (RFS) was defined as the time from diagnosis to molecular or hematological relapse. Overall survival (OS) was defined as the time from diagnosis to death of any cause. Patients that did not experience relapse and patients that were alive were censored at the last date of follow-up. RFS and OS time are presented as percentage ± standard error. Relapse was defined as ≥5% leukemic blasts in BM, extramedullary disease, or in one individual as molecular relapse. Statistical significance was defined as a two-sided significance level <0.05. All statistical analyses were performed using

IBM SPSS Statistics version 29 (IBM Corp., Armonk, NY). Figures were created in GraphPad Prism version 10 (GraphPad Software, San Diego, CA).

## Results

### Patient characteristics

This population-based study included 97 patients aged 19-82 years, with *NPM1*-mutated AML (Table 1 for clinical characteristics). In total, the patients displayed nine different *NPM1* mutations with type A most common, followed by types B and D (Supplementary Table 2). When divided into groups with either type A, B, D, or other mutation in *NPM1*, the patients displayed similar characteristics (Table 2). All *NPM1* mutations were assessed at the MRD level during treatment in BM aspirate slides using deep sequencing. When dividing the patients based on *FLT3*-ITD and *DNMT3A* mutation status at diagnosis, patients with *FLT3*-ITD had slightly higher VAF of the *NPM1* mutation after the first cycle (Figure 1(A)), but there were no significant differences at other time points or depending on *DNMT3A* mutation status (Figure 1(B)). Patients who later relapsed had significantly higher levels of VAF of the *NPM1* mutation after the first cycle of treatment and during consolidation compared with non-relapsing patients, and had similar levels at the end of treatment (Figure 1(C)). At relapse, 88% of patients (assessed in cases with available samples (32/43)) remained *NPM1*-positive.

**Table 1.** Clinical characteristics for all patients, and for patients defined as $MRD^{pos*}$ and $MRD^{neg}$ by deep sequencing of *NPM1* during consolidation (data available for 70 patients).

| Characteristics | All patients ($n=97$) | $MRD^{pos}$ ($n=13$) | $MRD^{neg}$ ($n=57$) |
|---|---|---|---|
| Age at diagnosis (years, median (min-max)) | 64 (19–82) | 63 (30–78) | 61 (19–80) |
| Sex | | | |
| Male | 37 (38.1) | 5 (38.5) | 18 (31.6) |
| Female | 60 (61.9) | 8 (61.5) | 39 (68.4) |
| Classification according to WHO 5th Edition | | | |
| AML with *NPM1* mutation | 93 (95.9) | 13 (100.0) | 55 (96.5) |
| AML, myelodysplasia-related | 4 (4.1) | – | 2 (3.5) |
| AlloSCT | | | |
| No | 66 (68.0) | 6 (46.2) | 38 (66.7) |
| Yes | 31 (32.0) | 7 (53.8) | 19 (33.3) |
| Cytogenetic risk | | | |
| Intermediate | 93 (95.9) | 13 (100.0) | 57 (100.0) |
| High | 3 (3.1) | – | – |
| Missing data | 1 (1.0) | – | – |
| *FLT3*-ITD mutation at diagnosis | | | |
| No | 53 (54.6) | 6 (46.2) | 29 (50.9) |
| Yes | 44 (45.4) | 7 (53.8) | 28 (49.1) |
| AR > 0.5 | 24 (24.7) | 4 (30.8) | 16 (28.1) |
| *DNMT3A* mutation at diagnosis | | | |
| No | 53 (54.6) | 5 (38.5) | 31 (54.4) |
| Yes | 44 (45.4) | 8 (61.5) | 26 (45.6) |
| p.R882 | 34 (35.1) | 6 (46.2) | 22 (38.6) |
| Relapse | | | |
| No | 54 (55.7) | 2 (15.4) | 37 (64.9) |
| Yes | 43 (44.3) | 11 (84.6) | 20 (35.1) |
| Death | | | |
| No | 48 (49.5) | 2 (15.4) | 32 (56.1) |
| Yes | 49 (50.5) | 11 (84.6) | 25 (43.9) |
| Cause of death | | | |
| Relapse | 39 (40.2) | 10 (76.9) | 18 (31.6) |
| Non-relapse mortality | 10 (10.3) | 1 (7.7) | 7 (12.3) |

Unless otherwise stated, data are presented as number of patients (%).
*$MRD^{pos}$ defined as variant allele frequency ≥0.05% mutated *NPM1* measured by deep sequencing after two cycles of treatment, or after three cycles if no bone marrow examination was performed after two cycles.
MRD: measurable residual disease; WHO: World Health Organization; alloSCT; allogeneic hematopoietic stem cell transplantation; AR; allelic ratio; p.R882: mutation affecting arginine at position 882.

### High prognostic value of MRD analysis with deep sequencing during consolidation

In our previous work comparing deep sequencing and RT-qPCR [16], we proposed a cutoff of 0.05% VAF as a clinically relevant level of MRD positivity using deep sequencing. To verify this, we performed deep sequencing on DNA from BM aspirate slides obtained during consolidation. $MRD^{pos}$ using this cutoff was associated with significantly shorter RFS (3-year RFS for $MRD^{pos}$ 23.1 ± 11.7% vs. 70.8.±6.1% for $MRD^{neg}$, $p<0.001$) and OS (3-year OS for $MRD^{pos}$ 30.8 ± 12.8% vs. 71.9 ± 6.0% for $MRD^{neg}$, $p=0.014$) (Figure 2(A,B)). In total, 20/57 patients were $MRD^{neg}$ during consolidation and later relapsed, and of these, 13 had *FLT3*-ITD mutation at diagnosis and three had both *FLT3*-ITD and *DNMT3A* mutations. Since analysis of MRD with MFC for intensively treated patients was introduced in the Swedish national guidelines in 2012 [18], a subgroup of our patients had been assessed with MFC enabling an evaluation of the concordance between methods. Results from both MFC and deep sequencing of mutated *NPM1* were available from 20 BM samples from 17 patients during consolidation ($n=11$ after cycle two, $n=9$ after cycle three), and showed a moderate agreement (Cohen's kappa = 0.459, $p=0.015$). Of the 20 samples, 17 were negative with both methods, one was positive with both methods, and two were positive with deep sequencing but negative with MFC.

### Worse prognosis for $MRD^{pos}$ patients in the reduced intensity treatment group

Based on the population-based nature of this study, treatment regimens varied in the study cohort. We therefore stratified the cohort into a group with reduced intensity treatment (RIT) with a median age of 69.5 years (range, 30–81 years), and a group with full intensity treatment (FIT) with a median age of 60 years (range, 19–82 years). The effect of MRD status was most obvious in patients treated with RIT where a

**Table 2.** Clinical characteristics for patients with *NPM1* type A, B, D, and other types.

| Characteristics | Type A ($n=78$) | Type B ($n=5$) | Type D ($n=5$) | Other types ($n=9$) |
|---|---|---|---|---|
| Age at diagnosis (years, median (min-max)) | 63.5 (23–81) | 60 (19–82) | 64 (48–72) | 65 (44–81) |
| Sex | | | | |
| Male | 30 (38.5) | – | 1 (20.0) | 6 (66.7) |
| Female | 48 (61.5) | 5 (100.0) | 4 (80.0) | 3 (33.3) |
| Classification according to WHO 5th Edition | | | | |
| AML with *NPM1* mutation | 74 (94.9) | 5 (100.0) | 5 (100.0) | 9 (100.0) |
| AML, myelodysplasia-related | 4 (5.1) | – | – | – |
| AlloSCT | | | | |
| No | 52 (66.7) | 4 (80.0) | 4 (80.0) | 6 (66.7) |
| Yes | 26 (33.3) | 1 (20.0) | 1 (20.0) | 3 (33.3) |
| Cytogenetic risk | | | | |
| Intermediate | 74 (94.9) | 5 (100.0) | 5 (100.0) | 9 (100.0) |
| High | 3 (3.8) | – | – | – |
| Missing data | 1 (1.3) | – | – | – |
| *FLT3*-ITD mutation at diagnosis | | | | |
| No | 41 (52.6) | 2 (40.0) | 2 (40.0) | 8 (88.9) |
| Yes | 37 (47.4) | 3 (60.0) | 3 (60.0) | 1 (11.1) |
| AR > 0.5 | 20 (25.6) | 1 (20.0) | 3 (60.0) | – |
| *DNMT3A* mutation at diagnosis | | | | |
| No | 42 (53.8) | 1 (20.0) | – | 6 (66.7) |
| Yes | 36 (46.2) | 4 (80.0) | 5 (100.0) | 3 (33.3) |
| p.R882 | 27 (34.6) | – | 4 (80.0) | 3 (33.3) |
| MRD$^{pos*}$ | | | | |
| After first cycle | 52/77 (67.5) | 4/5 (80.0) | 5/5 (100.0) | 4/9 (44.4) |
| During consolidation | 11/55 (20.0) | 0/3 (0.0) | 0/5 (0.0) | 2/7 (28.6) |
| End of treatment | 9/42 (21.4) | 0/2 (0.0) | 0/3 (0.0) | 1/4 (25.0) |
| Relapse | | | | |
| No | 44 (56.4) | 3 (60.0) | 3 (60.0) | 4 (44.4) |
| Yes | 34 (43.6) | 2 (40.0) | 2 (40.0) | 5 (55.6) |
| Death | | | | |
| No | 39 (50.0) | 3 (60.0) | 2 (40.0) | 4 (44.4) |
| Yes | 39 (50.0) | 2 (40.0) | 3 (60.0) | 5 (55.6) |
| Cause of death | | | | |
| Relapse | 30 (38.5) | 2 (40.0) | 2 (40.0) | 5 (55.6) |
| Non-relapse mortality | 9 (11.5) | – | 1 (20.0) | – |

Unless otherwise stated, data are presented as number of patients (%). *MRD$^{pos}$ defined as variant allele frequency ≥0.05% mutated *NPM1* measured by deep sequencing after first cycle of treatment, after two cycles of treatment, or after three cycles if no bone marrow examination was performed after two cycles (during consolidation), and after the last cycle of treatment (end of treatment).
MRD: measurable residual disease; WHO: World Health Organization; alloSCT: allogeneic hematopoietic stem cell transplantation; AR: allelic ratio; p.R882: mutation affecting arginine at position 882.

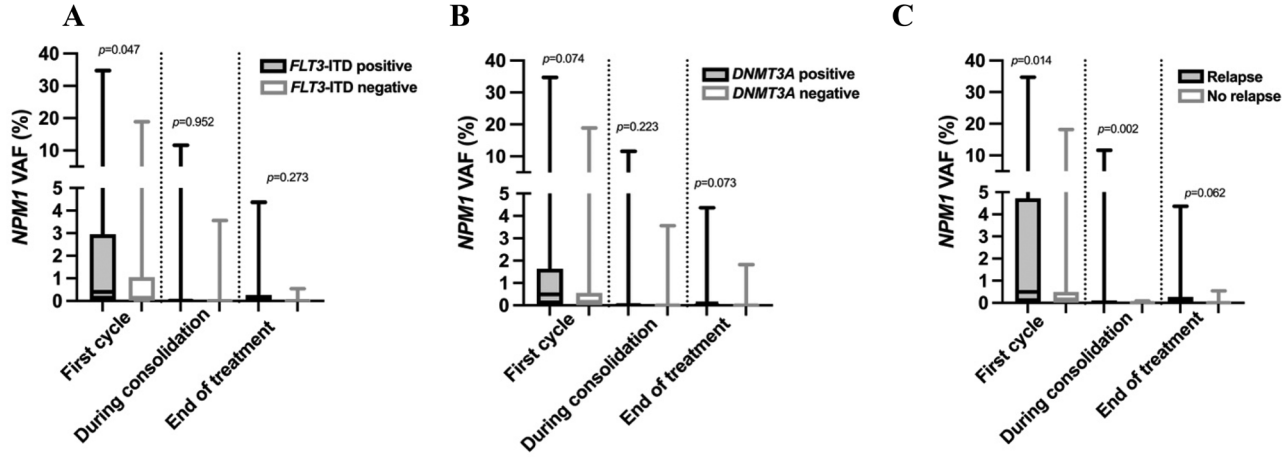


**Figure 1.** Box plots showing the distribution of variant allele frequency (VAF; %) of mutated *NPM1* measured by deep sequencing after first cycle of chemotherapy, during consolidation and at the end of treatment in (A) *FLT3*-ITD positive (black border with light gray fill) and *FLT3*-ITD negative (gray border, no fill) patients, (B) *DNMT3A* positive (black border with light gray fill) and *DNMT3A* negative (gray border, no fill) patients, and (C) relapsing (black border with light gray fill) and non-relapsing (gray border, no fill) patients.

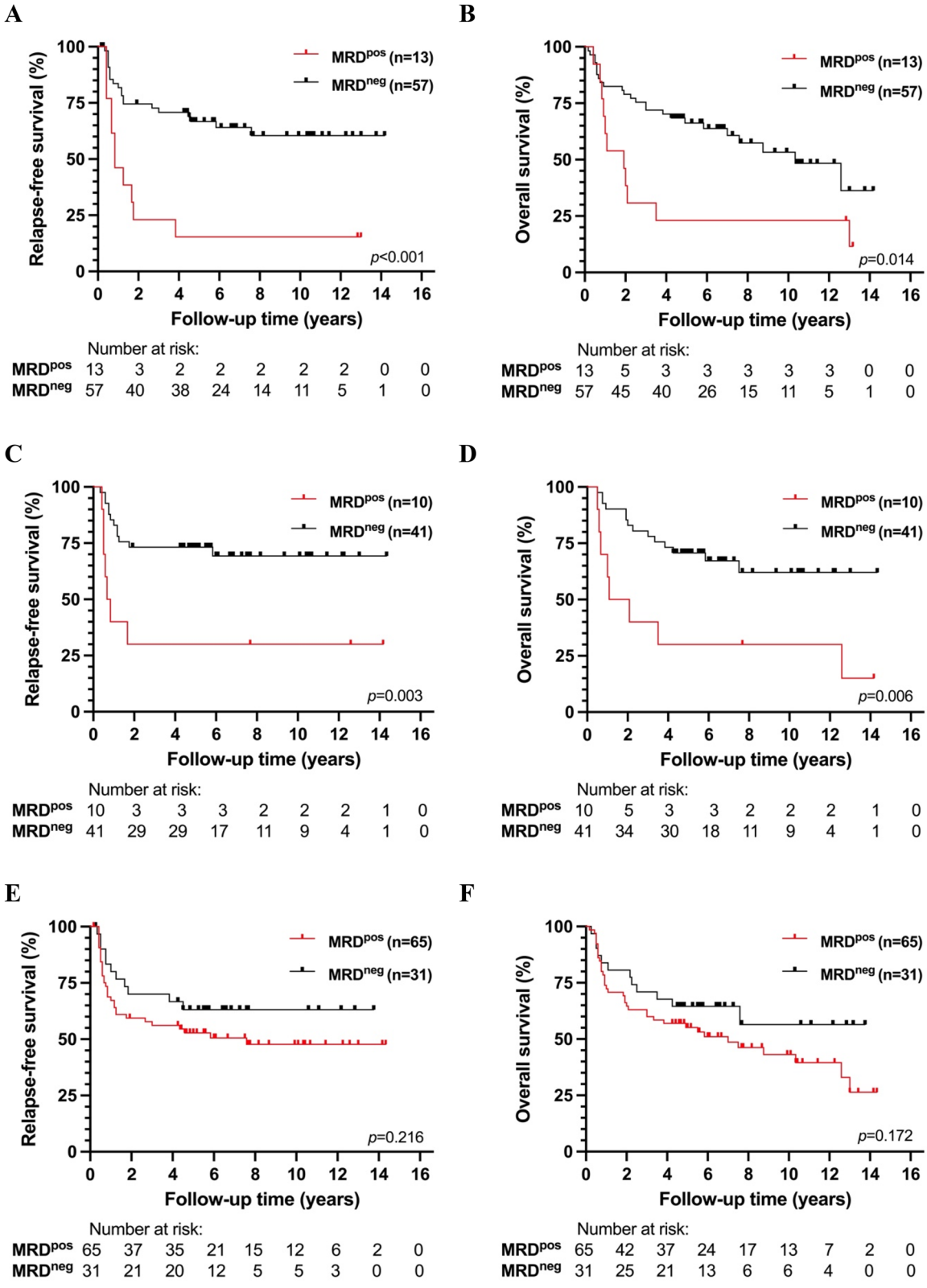


**Figure 2.** Kaplan–Meier curves for probability of relapse-free and overall survival based on measurable residual disease (MRD) status by deep sequencing of *NPM1* (A, B) during consolidation, (C, D) at the end of treatment, and (E, F) after first cycle of treatment. MRD$^{pos}$ was defined as variant allele frequency ≥0.05% mutated *NPM1* after first cycle of treatment, after two cycles of treatment, or after three cycles if no bone marrow examination was performed after two cycles (during consolidation), and after the last cycle of treatment (at the end of treatment).

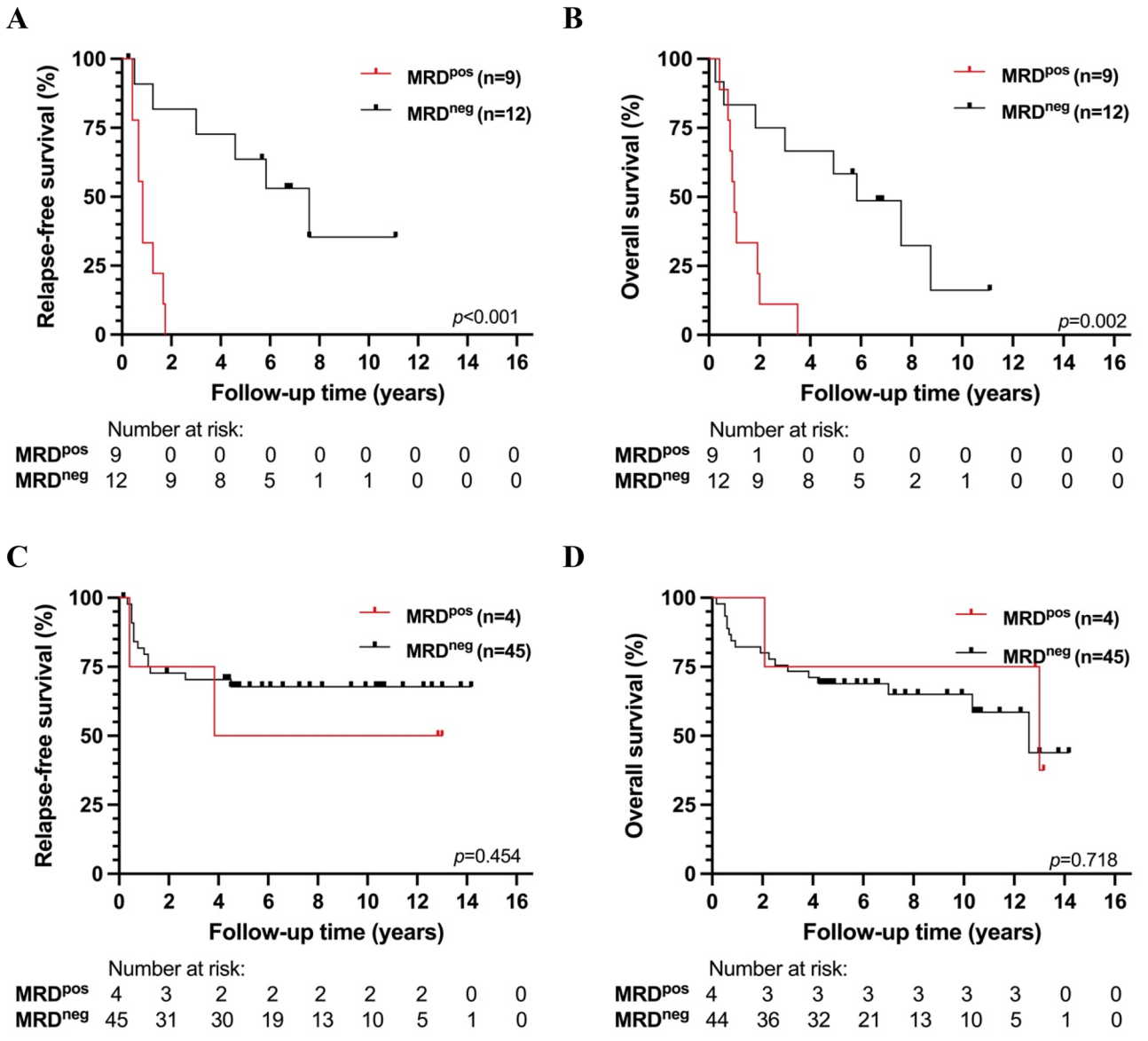


**Figure 3.** Kaplan–Meier curves for probability of relapse-free and overall survival based on measurable residual disease (MRD) status by deep sequencing of *NPM1* during consolidation for patients receiving (A, B) reduced intensity treatment, and (C, D) full intensity treatment. MRD$^{pos}$ was defined as variant allele frequency ≥0.05% mutated *NPM1* after two cycles of treatment, or after three cycles if no bone marrow examination was performed after two cycles.

higher proportion of MRD$^{pos}$ patients relapsed and died, resulting in a shorter 3-year RFS and OS survival for MRD$^{pos}$ patients compared to MRD$^{neg}$ patients (Figure 3(A,B)). In the FIT group, few patients were MRD$^{pos}$, precluding a reliable conclusion on the effect on RFS and OS (Figure 3(C,D)).

### *MRD status during consolidation is the strongest predictor for relapse in multivariable analysis*

In order to assess the impact of MRD during consolidation in relation to other important factors, we performed uni- and multivariable Cox regression analyses. In univariable analysis, MRD$^{pos}$ status was confirmed associated with higher risk of relapse and death. Also, age and treatment intensity, but not alloSCT or *DNMT3A* mutation status, predicted RFS and OS. *FLT3*-ITD at diagnosis predicted OS but not RFS, and there was no effect of *FLT3*-ITD AR on either RFS or OS. Based on these findings, we included MRD status, age, *FLT3*-ITD, and treatment intensity in the multivariable analysis. MRD$^{pos}$ was the only significant predictor for RFS. For OS, *FLT3*-ITD and treatment intensity, but not MRD$^{pos}$, were significant (Table 3). When dividing the cohort based on *FLT3*-ITD status at diagnosis, the groups became rather small with statistically

**Table 3.** Cox proportional hazards regression analysis of relapse-free and overall survival.

| | Univariable | | Multivariable | |
|---|---|---|---|---|
| | Relapse-free survival | Overall survival | Relapse-free survival | Overall survival |
| | HR (95% CI) | HR (95% CI) | HR (95% CI) | HR (95% CI) |
| Variable | *p*-value | *p*-value | *p*-value | *p*-value |
| MRD$^{pos}$* during consolidation | 3.72 (1.76–7.87) <0.001 | 2.42 (1.17–5.02) 0.018 | 3.13 (1.45–6.73) 0.004 | 1.98 (0.94–4.20) 0.074 |
| Age | 1.03 (1.00–1.06) 0.021 | 1.04 (1.02–1.07) 0.001 | 1.01 (0.99–1.04) 0.386 | 1.02 (0.99-1.05) 0.175 |
| *FLT3*-ITD | 1.34 (0.74–2.44) 0.336 | 1.85 (1.05–3.27) 0.033 | 1.94 (0.94–4.01) 0.075 | 2.58 (1.28–5.18) 0.008 |
| *FLT3*-ITD AR (>0.5) | 0.72 (0.30–1.72) 0.462 | 0.91 (0.43–1.91) 0.799 | N/A | N/A |
| *DNMT3A* | 1.10 (0.60–2.0) 0.767 | 1.16 (0.66–2.04) 0.601 | N/A | N/A |
| Treatment intensity (FIT) | 0.41 (0.23–0.75) 0.004 | 0.34 (0.19–0.60) <0.001 | 0.52 (0.24–1.16) 0.109 | 0.40 (0.19–0.87) 0.020 |
| AlloSCT | 0.73 (0.38–1.43) 0.358 | 0.65 (0.35–1.20) 0.167 | N/A | N/A |

*MRD$^{pos}$ defined as variant allele frequency ≥0.05% mutated *NPM1* measured by deep sequencing after two cycles of treatment, or after three cycles if no bone marrow examination was performed after two cycles. HR: hazard ratio; CI: confidence interval; MRD: measurable residual disease; AR: allelic ratio; FIT: full intensity treatment; alloSCT: allogeneic stem cell transplantation.

significant effect of MRD status during consolidation only in the group of patients without *FLT3*-ITD mutation (Supplementary Figure 2). When dividing patients into subgroups based on specific *NPM1* mutation types, the MRD response and outcome was similar, although groups were too small to allow conclusions (Table 2, Supplementary Figure 3).

### *Effect of different cutoffs and time points for deep sequencing MRD*

When assessed at the end of treatment, deep sequencing MRD$^{pos}$ at our previously obtained cutoff was also associated with both shorter RFS (3-year RFS for MRD$^{pos}$ 30.0 ± 14.5% vs. 73.2 ± 6.9% for MRD$^{neg}$, $p = 0.003$), and OS (3-year OS for MRD$^{pos}$ 40.0 ± 15.5% vs. 78.0 ± 6.5% for MRD$^{neg}$, $p = 0.006$) (Figure 2(C,D)). On the other hand, when the same cutoff was used for assessment after the first cycle, MRD$^{pos}$ had no significant effect on either RFS (3-year RFS for MRD$^{pos}$ 56.2 ± 6.2% vs. 70.0 ± 8.4% for MRD$^{neg}$, $p = 0.216$) or OS (3-year OS for MRD$^{pos}$ 60.0 ± 6.1% vs. 71.0 ± 8.2% for MRD$^{neg}$, $p = 0.172$) (Figure 2(E,F)). We then evaluated whether other cutoffs would be more appropriate. First, patients were stratified based on whether MRD was detectable or not. As expected, the effect of MRD detectable status was not statistically significant for RFS or OS when assessed after the first cycle (Supplementary Figure 4(A,B)). During consolidation, the risk of relapse was higher among patients with MRD$^{det}$, but there was no statistically significant effect on OS (Supplementary Figure 4(C,D)). Also at the end of treatment, MRD$^{det}$ was associated with lower RFS but had no effect on OS (Supplementary Figure 4(E,F)). Then, to identify the optimal cutoffs at each time point in this cohort, we performed ROC curve analysis. The resulting optimal cutoffs were 0.72% VAF after first cycle, 0.017% VAF during consolidation, and 0.014% VAF at the end of treatment. Table 4 shows resulting sensitivity and specificity for RFS and OS using these cutoffs. As internal verification, we also applied them to survival analyses, resulting in significant differences in both RFS and OS at all three analysis time points (Table 4).

## Discussion

In *NPM1*-mutated AML, analysis of MRD with RT-qPCR is recommended for the assessment of treatment response and monitoring after treatment. In contrast to RT-qPCR, deep sequencing provides possibility for MRD analysis for all exon 11 *NPM1* mutations, including rare types, reducing inequity. In this retrospective study in a population-based cohort of AML patients, we show

**Table 4.** Performance of the optimal cutoffs for MRD positivity by deep sequencing of *NPM1* obtained from receiver operating characteristic analysis.

| Time point (optimal cutoff, VAF) | After first cycle (0.72%) | During consolidation (0.017%) | End of treatment (0.014%) |
|---|---|---|---|
| Relapse-free survival | | | |
| Sensitivity (%) | 48.8 | 51.6 | 47.4 |
| Specificity (%) | 81.1 | 89.7 | 81.2 |
| Youden's index | 0.300 | 0.414 | 0.286 |
| Resulting 3-year RFS $MRD^{pos}$ | 33.3%±8.6% | 31.6%±10.7% | 40.0%±12.6% |
| Resulting 3-year RFS $MRD^{neg}$ | 73.4%±5.5% | 73.4%±6.3% | 75.0±7.2% |
| *p*-value, Log-rank test RFS | <0.001 | <0.001 | 0.006 |
| Overall survival | | | |
| Sensitivity (%) | 44.9 | 44.4 | 45.5 |
| Specificity (%) | 80.9 | 88.2 | 82.8 |
| Youden's index | 0.257 | 0.327 | 0.282 |
| Resulting 3-year OS $MRD^{pos}$ | 35.5%±8.6% | 35.0%±10.7% | 46.7%±12.9% |
| Resulting 3-year OS $MRD^{neg}$ | 76.9%±5.2% | 76.0%±6.0% | 80.6%±6.6% |
| *p*-value, Log-rank test OS | 0.002 | 0.001 | 0.011 |

During consolidation was defined as after two cycles of treatment, or after three cycles if no bone marrow examination was performed after two cycles, and at the end of treatment was defined as after the last cycle of treatment.
VAF: variant allele frequency; RFS: relapse-free survival; OS: overall survival.

that deep sequencing of mutated *NPM1* during and after treatment can be used as a predictive tool.

We started by confirming the threshold of VAF 0.05% for deep sequencing $MRD^{pos}$ that we previously proposed from a comparison with RT-qPCR. With this cutoff for determination of MRD status during consolidation, deep sequencing predicted relapse, independently of *FLT3*-ITD status, age, and treatment intensity. This is in line with results from the gold standard method RT-qPCR, where multiple earlier studies have demonstrated the prognostic value of MRD-analysis of *NPM1* at various time points during the course of the disease [8–11,22]. It also confirms the clinical relevance of highly sensitive next generation sequencing (NGS)-based MRD analysis of *NPM1*. In a recent study, Vonk et al. showed that MRD-analysis using a targeted single-amplicon deep NGS with a threshold of 0.01% mutated *NPM1* after two cycles of chemotherapy has strong prognostic value, and may even identify more patients at risk of relapse compared to RT-qPCR [23]. While their study cohort of 310 patients was larger than ours, all patients were trial participants, significantly younger (median age 52, range 19–66), and the follow-up period was considerably shorter. The effect of MRD status was significant for patients with *FLT3*-ITD mutation at diagnosis but not for *FLT3*-ITD negative patients. In our cohort on the other hand, the effect of MRD status reached statistical significance only in the group of *FLT3*-ITD negative patients. Further studies may therefore be needed to determine if the prognostic impact of MRD status really varies depending on the genetic profile of the patient. In an earlier study, Patkar et al. used a very similar assay in 83 AML patients in India and showed that a log reduction in the level of mutated *NPM1* between first and second treatment cycle could predict relapse and survival [6]. Also there, the study participants were younger and the follow-up time was only half of our study. These differences in study outline underscore the importance of the findings in our study, confirming the impact of NGS-based MRD analysis in AML with mutated *NPM1*.

In patients treated with reduced intensity regimens, the effect of MRD status was highly significant. We show that the median RFS for $MRD^{neg}$ patients in the RIT group was around seven years, suggesting that $MRD^{neg}$ status also in this patient group indicates prolonged disease-free and overall survival. These findings indicate that for patients treated with reduced intensity regimens, MRD analysis may guide further treatment decisions in another way than in transplantable patients. Although MRD assessment has become standard practice in patients treated with intensive chemotherapy, its utility remains unclear in patients treated with lower-intensity regimens. Molecular MRD measured by RT-qPCR has been shown to be a strong prognostic factor in patients with *NPM1*-mutated AML receiving venetoclax-based non-intensive therapy [24]. As suggested by ELN, establishment of MRD analysis in patients treated with low-dose regimen is clinically relevant and needs to be better defined to improve outcome [25].

When we tested the 0.05% cutoff after the first cycle of treatment, there was no significant difference in either RFS or OS between $MRD^{pos}$ and $MRD^{neg}$ patients when assessed. This could be explained by the aggressive nature of the disease where additional cycles of chemotherapy are needed to eliminate remaining leukemic cells, and is in line with earlier studies showing that presence of MRD during consolidation is associated with a higher risk of poor

outcome [26]. When we performed ROC curve analysis, we found a higher optimal cutoff after the first cycle of treatment, and lower at the end of treatment. The optimal cutoff of 0.017% VAF during consolidation is close to our tested 0.05% cutoff and aligns well with the 0.01% cutoff suggested by Vonk et al., further supporting the validity of our findings. When we applied the 'Vonk cutoff' 0.01% on our cohort, 12 patients were classified as $MRD^{pos}$ instead of $MRD^{neg}$, and among these, half experienced a relapse. In other words, a level of 0.01–0.05% during consolidation seems to be a grey zone, supporting the use of our cutoff of 0.05% for identification of high risk patients.

The main limitations of this study are the retrospective design and limited number of patients. However, we eliminated potential selection bias by including all AML patients in Region Västra Götaland. The population-based nature extends the knowledge about MRD from clinical trials to the real-world situation. Intentionally, we performed the study on a cohort treated in a time when molecular MRD analysis was not introduced in clinical care, to avoid treatment bias based on MRD results. This also means that patients were treated before the introduction of FLT3 inhibitors. Further studies are needed to address deep sequencing-based MRD during treatment with FLT3 inhibitor and azacitidine-venetoclax regimens, as well as in subgroups of patients with specific *NPM1* mutations.

In conclusion, when assessed during or after consolidation, MRD status of *NPM1* by deep sequencing is highly predictive of especially RFS. Since deep sequencing can be used for all *NPM1* mutations, it is widely applicable and extends the use of molecular MRD analysis for risk refinement and monitoring to patients with rare mutations in *NPM1*.

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## Disclosure statement

G.O. has received honoraria from AbbVie for delivering two lectures and has served as an Advisory Board member for Servier. P-O.A. has received honoraria from AbbVie, Astra Zeneca, BeiGene, Johnson & Johnson, KITE/Gilead, Lilly, Roche, Sandoz, SOBI, and Takeda, and has served as an Advisory Board member for AbbVie, Astra Zeneca, BeiGene, Johnson & Johnson, KITE/Gilead, Lilly, Roche, Sandoz, Serb Pharma, and SOBI. All other authors declare no competing interests.

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## Data availability statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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