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ORIGINAL REPORT

# Monitoring of Minimal Residual Disease in *NPM1*-Mutated Acute Myeloid Leukemia: A Study From the German-Austrian Acute Myeloid Leukemia Study Group

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## ABSTRACT

### Purpose
To evaluate the prognostic value of minimal residual disease (MRD) in patients with acute myeloid leukemia (AML) with *NPM1* mutation (*NPM1*$^{mut}$).

### Patients and Method
RNA-based real-time quantitative polymerase chain reaction (RQ-PCR) specific for the detection of six different *NPM1*$^{mut}$ types was applied to 1,682 samples (bone marrow, n = 1,272; blood, n = 410) serially obtained from 245 intensively treated younger adult patients who were 16 to 60 years old.

### Results
*NPM1*$^{mut}$ transcript levels as a continuous variable were significantly associated with prognosis after each treatment cycle. Achievement of RQ-PCR negativity after double induction therapy identified patients with a low cumulative incidence of relapse (CIR; 6.5% after 4 years) compared with RQ-PCR–positive patients (53.0%; $P < .001$); this translated into significant differences in overall survival (90% *v* 51%, respectively; $P = .001$). After completion of therapy, CIR was 15.7% in RQ-PCR–negative patients compared with 66.5% in RQ-PCR–positive patients ($P < .001$). Multivariable analyses after double induction and after completion of consolidation therapy revealed higher *NPM1*$^{mut}$ transcript levels as a significant factor for a higher risk of relapse and death. Serial post-treatment assessment of MRD allowed early detection of relapse in patients exceeding more than 200 *NPM1*$^{mut}$/$10^4$ *ABL* copies.

### Conclusion
We defined clinically relevant time points for *NPM1*$^{mut}$ MRD assessment that allow for the identification of patients with AML who are at high risk of relapse. Monitoring of *NPM1*$^{mut}$ transcript levels should be incorporated in future clinical trials to guide therapeutic decisions.

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## INTRODUCTION

Monitoring of minimal residual disease (MRD) using real-time quantitative polymerase chain reaction (RQ-PCR) has become an important diagnostic tool for assessment of response to therapy and for monitoring of postremission therapy and the post-treatment period to detect early relapse and to guide pre-emptive therapy.[1,2] Although MRD monitoring is now routinely performed in chronic myeloid leukemia[3] and acute lymphoblastic leukemia,[4] its clinical utility in acute myeloid leukemia (AML) is still restricted to distinct AML subtypes such as acute promyelocytic leukemia with t(15;17)(q22;q12) *PML-RARA* and AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22) *CBFB-MYH11*. In acute promyelocytic leukemia, early therapeutic intervention after molecular relapse is of clinical benefit.[5,6] In a recent study[7] on a large cohort of patients with AML positive for inv(16)/t(16;16), we defined three MRD checkpoints that allow for the identification of patients who are at high risk of relapse. At present, there is still a paucity of prospective trials determining the value of MRD in AML exhibiting other molecular markers such as t(8;21)(q22;q22) and t(9;11)(p21~p22;q23).[1,8,9]

Mutations in the nucleophosmin (*NPM1*) gene represent some of the most common gene mutations (25% to 30%) in AML.[10] *NPM1* mutations (*NPM1*$^{mut}$) predominantly occur in AML with

normal cytogenetics, where the incidence varies between 45% and 60%. *NPM1*$^{mut}$ are of prognostic value, in particular within the context of *fms-like tyrosine kinase receptor 3* (*FLT3*) internal tandem duplications (ITDs).[11-14] In the majority of patients (approximately 95%), three different *NPM1* mutation types (A, B, and D) are found.[15] Because of their homogeneous mutation pattern, *NPM1*$^{mut}$ represent an attractive target for MRD monitoring, in particular in AML with normal cytogenetics.

The applicability of an RNA- or DNA-based RQ-PCR assay for *NPM1*$^{mut}$ has been shown by several groups.[16-21] However, its clinical impact was investigated in only a few studies. These studies were hampered by small patient numbers and lack of availability of samples at defined time points. In a recent study of 252 patients with *NPM1*$^{mut}$ AML by Schnittger et al,[19] different time intervals were identified where the increase of *NPM1*$^{mut}$ levels was predictive for event-free survival (EFS); threshold values were defined that allowed discrimination of two patient groups with different EFS. There was no impact of *NPM1*$^{mut}$ MRD on risk of relapse and overall survival (OS).

Within the German-Austrian AML Study Group (AMLSG), we retrospectively and, since 2008, prospectively performed MRD monitoring in 245 younger adult patients (16 to 60 years old) with *NPM1*$^{mut}$ AML. We report here on the identification of clinically relevant checkpoints that allow for the identification of patients at high and low risk of relapse.

## PATIENTS AND METHODS

### Patients

The criteria used to include a patient in this study were presence of *NPM1*$^{mut}$ as detected by locked nucleic acid–based PCR[22] at diagnosis and availability of at least one bone marrow (BM) follow-up sample. Two hundred forty-five patients were eligible (Table 1). All patients were enrolled onto one of two AMLSG treatment protocols for younger adult patients (age 16 to 60 years), AMLHD98A[23] (ClinicalTrials.gov identifier, NCT00146120; n = 46) or AMLSG 07-04 (ClinicalTrials.gov identifier, NCT00151242; n = 199). Collection of BM and peripheral blood (PB) samples was recommended after each treatment cycle and every 3 months after completion of therapy. All patients gave informed consent for both treatment and genetic analysis according to the Declaration of Helsinki. Approval was obtained from the institutional review boards of the participating AMLSG institutions.

### Quantification of NPM1$^{mut}$ Transcript Levels

Primers and probes for *NPM1*$^{mut}$-specific RQ-PCR were used according to the study by Gorello et al,[16] with minor modifications to increase signal intensity and specificity (Data Supplement). For normalization, *ABL* expression was quantified as the control gene.[24] MRD levels were reported as the normalized values of *NPM1*$^{mut}$ transcripts per $10^4$ *ABL* transcripts (*NPM1*$^{mut}$/$10^4$ *ABL*), hereafter designated as *NPM1*$^{mut}$ transcript levels. Maximum sensitivity was $10^{-6}$ for mutation types A, B, D, and Ba and $10^{-5}$ for mutation types Qm and Km, as determined by serial dilution, combined with a high specificity because no wild-type *NPM1* was detected. Further details on sample preparation and RQ-PCR analyses are provided in the Data Supplement.

### Statistical Analysis

The definitions of complete response (CR) and survival end points were based on recommended criteria.[2,25] Cumulative incidence of relapse (CIR) was calculated according to Gray.[26] Survival time was calculated from the date the MRD sample was obtained. The median follow-up time for survival was calculated according to the method of Korn.[27]

Cox proportional hazards models were used to identify prognostic variables for OS and additionally for the event relapse referring to the survival end point remission duration.[25] For *NPM1*$^{mut}$ transcript levels, serum lactate dehydrogenase levels, and WBC counts, a $\log_{10}$ transformation of the data was done (hazard ratio [HR] for 10-fold increase). In addition, $\log_{10}$ transformation necessitated substitution of 0 by the first *NPM1*$^{mut}$ transcript level defined as negative, 0.33. Additional covariables in multivariable analysis were BM blast counts and age as continuous variables and *FLT3*-ITD status and *FLT3* tyrosine kinase domain status as dichotomous variables. Multiple imputations were applied in multivariable analysis for missing covariables.[28]

Comparisons of the distribution of variables were performed using Fisher's exact test and the Mann-Whitney *U* or Kruskal-Wallis test for qualitative and quantitative variables, respectively. Correlations between continuous variables were calculated using the Spearman rank test. Survival distributions were calculated using the Kaplan-Meier method, and differences between two groups were analyzed using the two-sided log-rank test. An effect was considered significant if $P \leq .05$. Statistical analyses were performed using the software environment R, version 2.4.1, with the R package design, version 2.0-12 (http://www.r-project.org).[29]

**Table 1.** Patient Demographics and Clinical Characteristics at Diagnosis

| Characteristic | No. of Patients (N = 245) | % |
|---|---|---|
| Age, years | | |
| Median | 49 | |
| Range | 19-61 | |
| Sex | | |
| Female | 131 | 53 |
| Male | 114 | 47 |
| AML history | | |
| De novo AML | 232 | 95 |
| Secondary AML | 6 | 2 |
| Therapy-related AML | 7 | 3 |
| Cytogenetics | | |
| Normal karyotype | 204 | 83 |
| Trisomy 8 | 7 | 3 |
| Deletion 9q | 6 | 2 |
| Other | 11 | 4 |
| Not available | 17 | 7 |
| *FLT3* status | | |
| Wild type | 124 | 51 |
| ITD mutated | 94 | 38 |
| TKD mutated | 27 | 11 |
| *NPM1* mutation type | | |
| A | 200 | 82 |
| B | 19 | 8 |
| D | 17 | 7 |
| Other | 9 | 4 |
| Bone marrow blasts, % | | |
| Median | 80 | |
| Range | 10-100 | |
| WBC count, $\times 10^9$/L | | |
| Median | 23 | |
| Range | 1-394 | |
| Serum LDH, U/L | | |
| Median | 479 | |
| Range | 123-5,438 | |

Abbreviations: AML, acute myeloid leukemia; ITD, internal tandem duplication; LDH, lactate dehydrogenase; TKD, tyrosine kinase domain.

## RESULTS

### Patient Characteristics

The whole cohort comprised 245 patients with AML with *NPM1*$^{mut}$. Distribution of mutation types was similar to previous

  

studies (Table 1). After induction therapy, 223 patients (91%) achieved CR, 21 patients (9%) had refractory disease (RD), and there was one early death. Allogeneic hematopoietic stem-cell transplantation (HSCT) was performed in 65 patients in first CR and in 15 patients with RD. In the remaining 165 patients, consolidation therapy consisted of a two- to three-cycle, high-dose, cytarabine-based chemotherapy (n = 146, 60%) or autologous HSCT (n = 19, 8%). The median follow-up time for survival was 37.7 months. After 48 months, 50.3% patients had experienced relapse, and 45.6% had died.

### NPM1$^{mut}$ Transcript Levels at Diagnosis

At diagnosis, BM samples from 220 patients were available. *NPM1*$^{mut}$ transcript levels varied between $1.8 \times 10^4$ and $1.0 \times 10^7$ *NPM1*$^{mut}$/$10^4$ *ABL* copies (median, $7.2 \times 10^5$ *NPM1*$^{mut}$/$10^4$ *ABL* copies). Pretreatment *NPM1*$^{mut}$ transcript levels did not correlate with age ($P = .28$), sex ($P = .58$), WBC counts ($P = .55$), BM blast counts ($P = .62$), serum lactate dehydrogenase levels ($P = .09$), *NPM1*$^{mut}$ type ($P = .76$), *FLT3*-ITD status ($P = .26$), and *FLT3* tyrosine kinase domain status ($P = .54$). Pretreatment *NPM1*$^{mut}$ transcript levels as a $\log_{10}$ transformed continuous variable did not impact relapse-free survival ($P = .69$), EFS ($P = .60$), or OS ($P = .94$).

### Prognostic Impact of NPM1$^{mut}$ Transcript Levels During Therapy

We performed Cox regression analyses to determine the prognostic impact of *NPM1*$^{mut}$ transcript levels in BM obtained at the following time points: at day 15 to 20 in aplasia (n = 58), after single induction (n = 175), after double induction (n = 153), after first consolidation (n = 109), after second consolidation (n = 88), and after completion of therapy (n = 129), which was defined as the first sample obtained within 3 months after the last cycle of consolidation treatment or HSCT. All patients were included, regardless of response to induction and treatment. At all time points, higher *NPM1*$^{mut}$ transcript levels were associated with higher risk of relapse and shorter remission duration and OS, with the exception of the time point of day 15 to 20 in aplasia, which was only associated with shorter remission duration (Data Supplement). This effect remained significant when the analysis was stratified for *FLT3*-ITD status and treatment trial. In subsequent analyses, we focused on the two clinically important time points of after double induction therapy and after completion of therapy.

### Prognostic Impact of NPM1$^{mut}$ Transcript Levels After Double Induction Therapy

After double induction therapy, BM samples were available from 153 patients. *NPM1*$^{mut}$ transcript levels were significantly lower in patients who achieved CR (n = 137; median, 61 *NPM1*$^{mut}$/$10^4$ *ABL* copies) compared with patients with RD (n = 16; median, 1,542 *NPM1*$^{mut}$/$10^4$ *ABL* copies; $P = .014$).

We next focused on patients achieving CR to evaluate whether *NPM1*$^{mut}$ transcript levels provide further prognostic information for this patient group. Consolidation therapy consisted of intensive chemotherapy (n = 84), autologous HSCT (n = 8), and allogeneic HSCT (n = 45). Multivariable analysis revealed *NPM1*$^{mut}$ transcript level to be an important significant prognostic marker for remission duration (HR for relapse, 1.96 for 10-fold increase; $P < .001$) and OS (HR for death, 1.70; $P < .001$; Table 2). *FLT3*-ITD was the only other variable that impacted remission duration (HR, 2.75; $P = .0024$) and OS (HR, 5.28; $P < .001$). The influence of *NPM1*$^{mut}$ transcript levels remained highly significant when patients were censored for allogeneic HSCT in first CR (Data Supplement).

We then investigated whether achievement of RQ-PCR negativity, defined as no detectable *NPM1*$^{mut}$ transcript, could discriminate prognostic subgroups. After induction therapy, CIR at 48 months was 6.4% (SE, 0.4%) for RQ-PCR–negative patients compared with 53.0% (SE, 0.3%; $P < .001$) for patients with any positive value. The lower

**Table 2.** Multivariable Analysis

| Variable | Relapse | | | Death | | |
|---|---|---|---|---|---|---|
| | HR | 95% CI | *P* | HR | 95% CI | *P* |
| After double induction in patients in CR (n = 137) | | | | | | |
| *NPM1*$^{mut}$ level | 1.96 | 1.55 to 2.47 | < .001 | 1.70 | 1.36 to 2.14 | < .001 |
| *FLT3*-ITD | 2.75 | 1.43 to 5.28 | .002 | 5.28 | 2.62 to 10.61 | < .001 |
| *FLT3*-TKD | 0.58 | 0.22 to 1.52 | .27 | 0.52 | 0.17 to 1.56 | .24 |
| Age | 1.27 | 0.85 to 1.90 | .24 | 0.96 | 0.63 to 1.46 | .85 |
| BM blasts | 1.00 | 0.99 to 1.02 | .67 | 1.01 | 0.99 to 1.03 | .37 |
| LDH | 0.98 | 0.33 to 2.91 | .97 | 0.72 | 0.22 to 2.41 | .60 |
| WBC | 1.25 | 0.64 to 2.43 | .51 | 0.89 | 0.43 to 1.81 | .74 |
| After completion of therapy (n = 129) | | | | | | |
| *NPM1*$^{mut}$ level | 2.24 | 1.83 to 2.74 | < .001 | 1.67 | 1.43 to 1.95 | < .001 |
| *FLT3*-ITD | 1.04 | 0.47 to 2.26 | .92 | 3.23 | 1.57 to 6.67 | .001 |
| *FLT3*-TKD | 0.74 | 0.30 to 1.84 | .52 | 0.62 | 0.22 to 1.72 | .36 |
| Age | 1.40 | 0.85 to 2.30 | .32 | 1.44 | 0.93 to 2.24 | .10 |
| BM blasts | 1.02 | 1.00 to 1.04 | .03 | 1.03 | 1.01 to 1.04 | .008 |
| LDH | 0.56 | 0.15 to 2.04 | .38 | 0.42 | 0.09 to 1.91 | .26 |
| WBC | 1.34 | 0.62 to 2.89 | .46 | 0.83 | 0.38 to 1.77 | .62 |

NOTE. *NPM1*$^{mut}$ transcript levels were determined at the respective time point; HR is per 10-fold increase. *FLT3*-ITD and *FLT3*-TKD mutation status, BM blasts, age, serum LDH, and WBC count were determined at diagnosis.

Abbreviations: BM, bone marrow; CR, complete remission; HR, hazard ratio; ITD, internal tandem duplication; LDH, lactate dehydrogenase; TKD, tyrosine kinase domain.

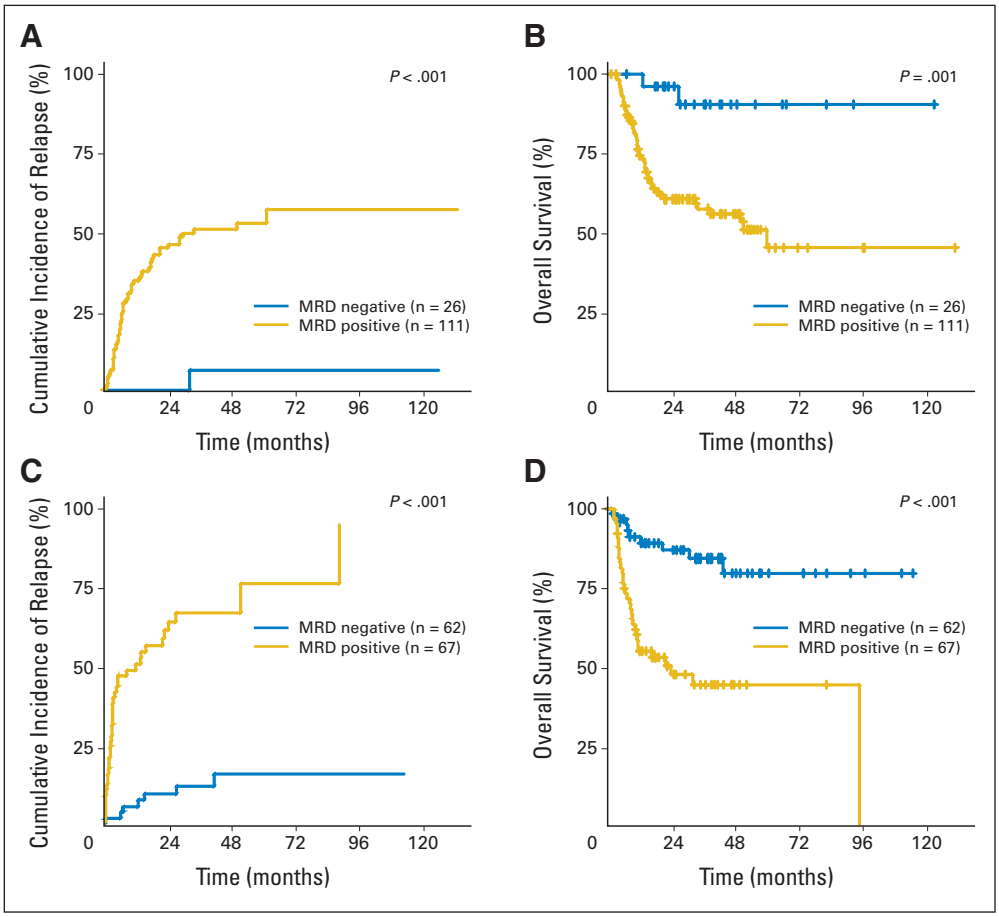


**Fig 1.** (A) Cumulative incidence of relapse (CIR) and (B) overall survival (OS) for patients in complete remission according to minimal residual disease (MRD) status after induction therapy in bone marrow (negative *v* any positive *NPM1* mutation transcript level value). (C) CIR and (D) OS according to MRD status after completion of therapy in bone marrow.

CIR in RQ-PCR–negative patients translated into a significantly better OS ($P = .001$; Figs 1A and 1B). The two deaths in the RQ-PCR–negative group were related to complications of allogeneic HSCT.

### *Prognostic Impact of* NPM1$^{mut}$ *Transcript Levels After Completion of Therapy*

BM samples from 129 patients were available after completion of therapy. Consolidation therapy consisted of intensive chemotherapy (n = 93), autologous HSCT (n = 11), and allogeneic HSCT (n = 25). In multivariable Cox regression analysis, higher *NPM1*$^{mut}$ transcript levels were associated with shorter remission duration (HR, 2.24; $P < .001$) and inferior OS (HR, 1.67; $P < .001$; Table 2).

Again, we compared RQ-PCR–negative patients (n = 62) with patients with any positive RQ-PCR value (n = 67). At 48 months, CIR was 15.7% (SE, 0.3%) versus 66.5% (SE, 0.5%; $P < .001$) and OS was 80% (SE, 7%) versus 44% (SE, 7%; $P < .001$) in RQ-PCR–negative and –positive patients, respectively (Figs 1C and 1D). Remarkably, all 19 of 67 positive patients that were still in CR had low *NPM1*$^{mut}$ transcript levels (maximum, 138 *NPM1*$^{mut}$/$10^4$ *ABL* copies). Of the seven negative patients who experienced relapse, four patients had increasing *NPM1*$^{mut}$ transcript levels at later time points, one patient had no detectable *NPM1*$^{mut}$ at relapse, and two patients had no available samples at later time points.

### *Impact of* NPM1$^{mut}$ *Transcript Levels During Follow-Up*

To further assess the risk of relapse after completion of therapy, we performed serial measurements in 392 samples obtained from 136 patients in the post-treatment period. In 69 (51%) of 136 patients, at least one BM sample was RQ-PCR positive. Of these patients, 43 patients experienced relapse, whereas the remaining 26 patients were still in CR (median follow-up after positive sample, 24.8 months; range, 1.8 to 80.5 months). *NPM1*$^{mut}$ transcript levels were less than 145 *NPM1*$^{mut}$/$10^4$ *ABL* copies in these 26 patients at all time points (Data supplement), and 15 of the 26 patients became negative in a consecutive sample. On the basis of these data, we set an arbitrary cutoff at more than 200 *NPM1*$^{mut}$/$10^4$ *ABL* copies for the definition of a molecular relapse. All 36 patients with a value greater than 200 *NPM1*$^{mut}$/$10^4$ *ABL* experienced relapse. The median time to relapse was 2.6 months, with a broad range from 0.4 to 23.6 months, determined from the first sample exceeding the cutoff value (Fig 2).

Of note, in five patients (patients 103, 123, 351, 172, and 524), *NPM1*$^{mut}$ transcript levels were not detectable or only minimally detectable at the time of relapse (9% of all evaluable relapses), which occurred after 2.8, 6.8, 29.5, 34.2, and 97.7 months. In single nucleotide polymorphism array analysis, no genomic aberrations were identified at diagnosis and at relapse in the two patients with early relapse, whereas aberrations differed at times of diagnosis and relapse in the remaining three patients (Table 3).

### NPM1$^{mut}$ *Transcript Levels and* FLT3-*ITD Mutation Status*

We compared *NPM1*$^{mut}$ transcript levels in BM samples in patients with and without concurrent *FLT3*-ITD. BM samples obtained

  

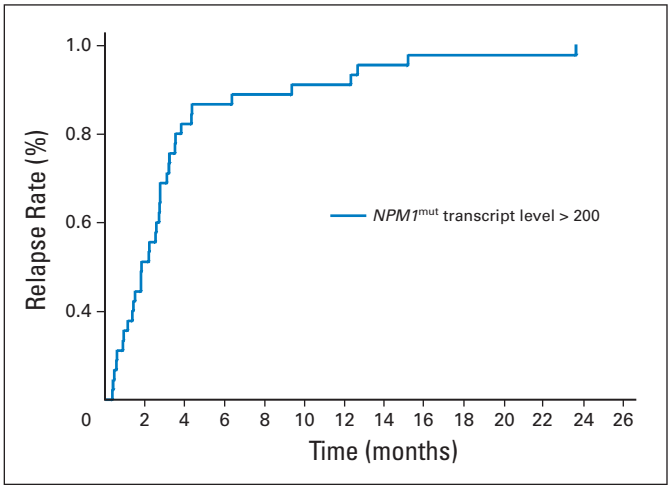


**Fig 2.** Cumulative incidence of relapse during follow-up in 36 patients exceeding *NPM1* mutation (*NPM1*$^{mut}$) transcript levels of 200 *NPM1*$^{mut}$/10$^4$ *ABL* copies in at least one follow-up sample obtained in the post-treatment period. Time to relapse is calculated from the first sample with an *NPM1*$^{mut}$ transcript level of more than 200 *NPM1*$^{mut}$/10$^4$ *ABL* copies to relapse.

after salvage therapy in case of RD and after allogeneic and autologous HSCT were excluded. After the second induction cycle, the median *NPM1*$^{mut}$ transcript levels were significantly lower in the *FLT3*-ITD–negative group (34.7 *NPM1*$^{mut}$/10$^4$ *ABL* copies) compared with the *FLT3*-ITD–positive group (126.4 *NPM1*$^{mut}$/10$^4$ *ABL* copies; $P = .002$), and this effect was observed throughout subsequent treatment cycles (Fig 3).

### *Paired BM and PB Analysis*

To determine whether PB could provide similar prognostic information as BM, we compared 410 paired samples at the following time points: diagnosis (n = 50), induction (n = 103), consolidation (n = 101), and after treatment (n = 156). At diagnosis, median *NPM1*$^{mut}$ transcript levels tended to be lower in PB ($5.5 \times 10^5$ *NPM1*$^{mut}$/10$^4$ *ABL* copies) than in BM ($9.1 \times 10^5$ *NPM1*$^{mut}$/10$^4$ *ABL* copies; $P = .08$; Data Supplement). This was also the case at later time points, where *NPM1*$^{mut}$ transcript levels were higher (median, 8.73-fold) in BM than in PB. *NPM1*$^{mut}$ transcript levels in BM and PB correlated well ($r = 0.89$; $P < .001$; Data Supplement). However, 10.7%, 25.7%, and 15.6% of all pairs were discrepant (BM positive/PB negative) in the induction, consolidation, and post-treatment periods, respectively. Moreover, of 80 negative PB samples obtained in the induction and consolidation periods, 37 samples (46%) still showed *NPM1*$^{mut}$ transcript levels in BM. In the post-treatment period, this fraction decreased to 18% (18 BM-positive/98 PB-negative pairs; Data Supplement). Of note, *NPM1*$^{mut}$ transcript levels in all but one of the 18 positive BM samples were less than the cutoff of 200 *NPM1*$^{mut}$/10$^4$ *ABL* copies.

## DISCUSSION

Here, we report on the results of MRD monitoring in a large cohort of 245 younger adult patients with *NPM1*$^{mut}$ AML treated on two AMLSG treatment trials. Using a sensitive RNA-based RQ-PCR assay, we defined clinically relevant MRD time points during and after therapy that allowed for the identification of patients with high risk of relapse. First, after two induction cycles, *NPM1*$^{mut}$ transcript levels were a highly significant prognostic factor for remission duration and OS. Achievement of RQ-PCR negativity at this time point identified patients with a low CIR and, therefore, allowed a refined risk assessment for patients in CR after induction therapy (Fig 1). In fact, 24 of 26 patients achieving RQ-PCR negativity after induction therapy were alive, and the remaining two patients died from transplantation-related complications. Second, after completion of therapy, *NPM1*$^{mut}$ transcript levels again were an independent prognostic factor for remission duration and OS. Finally, during follow-up, serial RQ-PCR analyses allowed prediction of relapse in all patients exceeding a cutoff value of greater than 200 *NPM1*$^{mut}$/10$^4$ *ABL* copies.

The reduction of *NPM1*$^{mut}$ transcript levels correlated with the *FLT3*-ITD status, with *FLT3*-ITD–negative patients achieving a significantly better molecular response (Fig 3). Importantly, in multivariable analysis, besides *NPM1*$^{mut}$ transcript levels, *FLT3*-ITD remained a significant factor for inferior survival.

Recently, Schnittger et al[19] investigated MRD in a similarly sized cohort of patients with *NPM1*$^{mut}$ AML. In univariable and multivariable analysis, they showed that *NPM1*$^{mut}$ transcript levels evaluated at different time points during therapy and during follow-up independently affected EFS but not the risk of relapse or OS. In contrast to their study, we focused our analysis on patients achieving CR and did not analyze EFS, which also includes patients with RD and early and hypoplastic death for whom MRD-based risk assessment does not provide clinically meaningful information. In addition, the patient population in the study by Schnittger et al[19] was more heterogeneous with respect to age (20 to 79 years) and possibly treatment (not further characterized).

Our observation that detectable *NPM1*$^{mut}$ transcript levels after completion of therapy are of prognostic relevance is in accordance with MRD studies investigating different targets.[4,6,7,30] During follow-up, our cohort of 136 patients comprised 26 MRD-positive patients in CR, and 15 of these patients converted to negativity in a consecutive sample without further treatment. Using a cutoff value of greater than 200 *NPM1*$^{mut}$/10$^4$ *ABL* copies, we increased the specificity for relapse prediction to 100%, demonstrating the importance of serial MRD assessment in the follow-up period. The median time of 2.6 months from exceeding the cutoff value to relapse may not be reliable because this figure depends on the sampling interval, which was heterogeneous in our follow-up cohort. For a more accurate calculation of the time interval from molecular to morphologic relapse, a cohort with a consistent sampling in the follow-up period (eg, every 3 months) is needed. Recently, Ommen et al[31] developed a mathematical model to determine the time to relapse that is not dependent on sampling interval. On the basis of a larger set of patients with *NPM1*$^{mut}$ AML, they calculated a somewhat longer median time interval of 3.5 and 6.5 months for *FLT3*-ITD–positive and *FLT3*-ITD–negative patients, respectively. However, in their analysis the authors only included patients achieving molecular remission after completion of therapy, who may have slower relapse kinetics than patients with persisting MRD.

One reason for failure of relapse prediction is because of the fact that *NPM1*$^{mut}$ may not be a stable marker in all patients. In fact, in our cohort, *NPM1*$^{mut}$ was not sufficiently detectable in five patients (9% of all investigated relapse samples) at the time of relapse. Although some groups made similar observations,[18,32] *NPM1*$^{mut}$ was stable in all 81

**Table 3.** Cytogenetic and Molecular Genetic Findings in Cases That Lost *NPM1* Mutation at The Time of Relapse (n = 5)

| | | Diagnosis | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Study | Patient No. | BM Blasts (%) | Karyotype | SNP Array Analysis | *NPM1* Diagnostic PCR* | *NPM1* Type | *NPM1*$^{mut}$ Level (*NPM1*$^{mut}$/10$^4$ *ABL* copies) | *MLL* PTD | *FLT3* ITD | *FLT3* TKD | *IDH* | *TET2* | No. of Days to Relapse |
| 07-04 | 103 | 90 | 46,XY | UPD1pter-p32.3 | Mut | B | 10,126,291.7 | WT | Mut† | WT | WT | WT | 886 |
| 07-04 | 123 | 90 | 46,XX | — | Mut | A | 441,045.2 | WT | WT | WT | WT | WT | 205 |
| 07-04 | 351 | 65 | 46,XX | — | Mut | B | 326,798.9 | WT | WT | Mut | Mut | WT | 84 |
| 98A | 172 | 31 | 47,XY,+Y | +Y, del9q21.32-q21.33 | Mut | A | 1,710,801.3 | WT | WT | WT | WT | WT | 2,931 |
| 98A | 524 | 90 | 46,XY, del9q22q34[2]/ 46, XY[13] | del9q21.1-q31.1 | Mut | A | 1,106,853.7 | WT | WT | Mut | WT | WT | 1,028 |

Abbreviations: BM, bone marrow; Mut, mutated; ITD, internal tandem duplication; *NPM1*$^{mut}$, *NPM1* mutation; PCR, polymerase chain reaction; PTD, partial tandem duplication; SNP, single nucleotide polymorphism; TKD, tyrosine kinase domain; UPD, uniparental disomy; WT, wild type.
*PCR was DNA based.
†Different *FLT3*-ITD clones.
‡Genetic change in relapse compared with diagnosis.

paired diagnosis/relapse samples in the cohort of Schnittger et al.[19] Three of the five patients who experienced relapse more than 2 years after diagnosis displayed novel genomic aberrations, whereas primary alterations were absent. Of note, in high-resolution single nucleotide polymorphism array analysis, all three patients had an intragenic gain in *MLL* (11q23.3) corresponding to an *MLL* partial tandem duplication that was confirmed by PCR (Table 3). These findings suggest that at least in these three patients, there was secondary AML rather than relapse.

An important practical issue addressed in our study was whether PB samples could be used for MRD monitoring, in particular in the follow-up period. During therapy, in almost half (46%) of the negative PB samples, the corresponding BM samples were positive, whereas during follow-up, the majority (88%) of the paired samples were concordant. Comparable observations were made in two previous studies comparing BM and PB.[20,21] Given these results, we recommend BM samples to be used for MRD monitoring during therapy, whereas during follow-up, PB samples are clinically meaningful for MRD monitoring, particularly in patients with a preceding RQ-PCR–negative BM sample.

In summary, *NPM1*$^{mut}$-based MRD monitoring during and after therapy allows for the discrimination of patients at high and low risk of relapse. We propose to incorporate *NPM1*$^{mut}$ MRD assessment in future clinical trials to evaluate whether early intervention (eg, using an investigational agent or allogeneic HSCT) will improve outcome of high-risk patients.

## AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

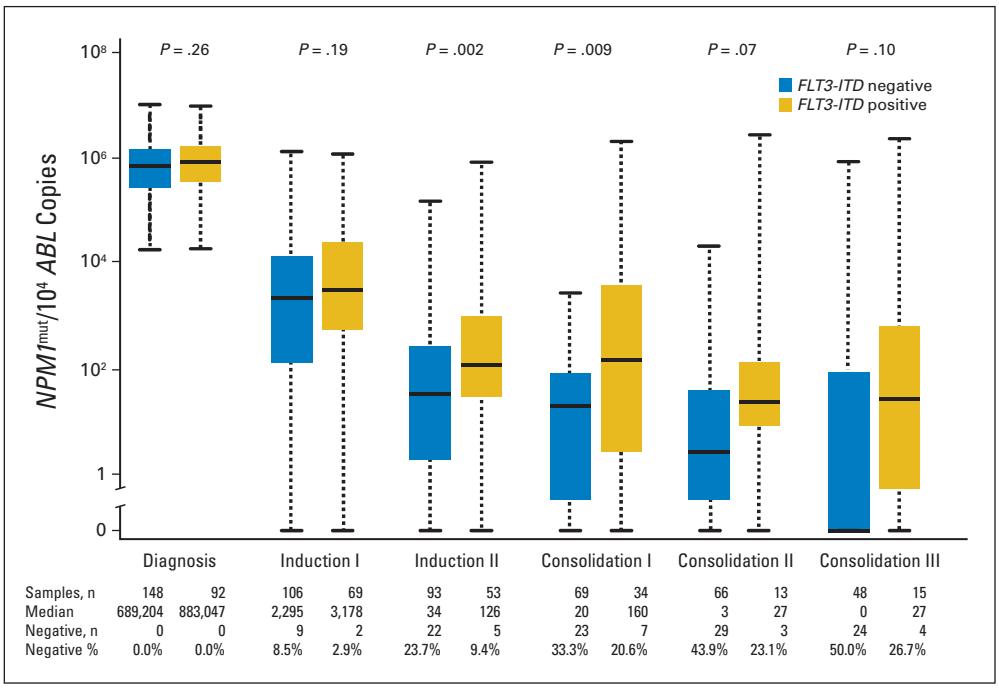


**Fig 3.** *NPM1* mutation (*NPM1*$^{mut}$) transcript levels during therapy according to the *FLT3* internal tandem duplication (ITD) status. Transcript levels are shown for both groups after each treatment cycle. Only samples obtained after standard chemotherapy were included; samples obtained after salvage therapy or autologous and allogeneic transplantation were excluded.

  

**Table 3.** (continued)

| | | Relapse | | | | | | |
|---|---|---|---|---|---|---|---|---|
| BM Blasts (%) | SNP Array Analysis | *NPM1* Diagnostic PCR* | $NPM1^{mut}$ Level ($NPM1^{mut}/10^4$ *ABL* copies) | *MLL* PTD | *FLT3* ITD | *FLT3* TKD | *IDH* | *TET2* |
| 80 | +11q23.3, del12p13.2‡ | WT‡ | 0.0 | Mut‡ | Mut†‡ | WT | WT | WT |
| 10 | — | WT‡ | 0.0 | WT | WT | WT | WT | WT |
| 36 | — | WT‡ | 0.8 | WT | WT | WT‡ | WT‡ | WT |
| 30 | +8, +11q23.3‡ | WT‡ | 0.0 | Mut‡ | WT | WT | WT | WT |
| 50 | +11q23.3‡ | WT‡ | 0.0 | Mut‡ | WT | WT‡ | Mut‡ | WT |

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**Manuscript writing:** All authors
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