

# Molecular response assessment by quantitative real-time polymerase chain reaction after induction therapy in *NPM1*-mutated patients identifies those at high risk of relapse

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## ABSTRACT

Monitoring minimal residual disease is an important way to identify patients with acute myeloid leukemia at high risk of relapse. In this study we investigated the prognostic potential of minimal residual disease monitoring by quantitative real-time polymerase chain reaction analysis of *NPM1* mutations in patients treated in the AMLCG 1999, 2004 and 2008 trials. Minimal residual disease was monitored - in aplasia, after induction therapy, after consolidation therapy, and during follow-up - in 588 samples from 158 patients positive for *NPM1* mutations A, B and D (with a sensitivity of  $10^{-6}$ ). One hundred and twenty-seven patients (80.4%) achieved complete remission after induction therapy and, of these, 56 patients (44.1%) relapsed. At each checkpoint, minimal residual disease cut-offs were calculated. After induction therapy a cut-off *NPM1* mutation ratio of 0.01 was associated with a high hazard ratio of 4.26 and the highest sensitivity of 76% for the prediction of relapse. This was reflected in a cumulative incidence of relapse after 2 years of 77.8% for patients with ratios above the cut-off versus 26.4% for those with ratios below the cut-off. In the favorable subgroup according to European LeukemiaNet, the cut-off after induction therapy also separated the cohort into two prognostic groups with a cumulative incidence of relapse of 76% versus 6% after 2 years. Our data demonstrate that in addition to pre-therapeutic factors, the course of minimal residual disease in an individual is an important prognostic factor and could be included in clinical trials for the guidance of post-remission therapy. The trials from which data were obtained were registered at [www.clinicaltrials.gov](http://www.clinicaltrials.gov) (#NCT01382147, #NCT00266136) and at the European Leukemia Trial Registry (#LN\_AMLINT2004\_230).

## Introduction

Molecular analyses have led to an improvement of the prognostic evaluation of patients with acute myeloid leukemia (AML). The European LeukemiaNet (ELN) has published a classification that identifies prognostic subgroups based on the patients' cytogenetic and molecular genetic characteristics. At present, therapy decisions are guided by pre-therapeutic risk assessments.<sup>1</sup> Nevertheless, there are still patients who suffer from relapse despite belonging to the favorable risk group<sup>2,3</sup> and more individualized therapy regimens are needed to prevent relapses.

Monitoring minimal residual disease (MRD) could become an important way to identify patients at high risk of relapse.<sup>4</sup> In patients with acute lymphoblastic leukemia or chronic myeloid leukemia, the MRD status routinely guides therapy decisions at different check points.<sup>5,6</sup> Patients with acute lym-

phoblastic leukemia in complete remission after treatment within the GMALL protocol and with a MRD level of  $>10^{-4}$  at week 16 should undergo hematopoietic allogeneic stem cell transplantation (HSCT). Pre-emptive arsenic trioxide therapy in patients with acute promyelocytic leukemia with t(15;17)(q22;q12) *PML-RARA*, initiated on the basis of increasing MRD levels, was seen to prevent relapses in the MRC 15 trial.<sup>7</sup>

AML has a wide spectrum of molecular markers.<sup>1</sup> Some of these markers can be assessed by quantitative real-time polymerase chain reaction (RT-PCR), e.g. leukemia-specific fusion transcripts, mutated genes, or aberrantly expressed genes. These molecular markers can be monitored with higher sensitivity by RT-PCR than by other methods such as immunophenotyping.<sup>4,8</sup>

MRD assessments after induction and consolidation therapy showed a significant impact on relapse-free and overall

survival and serial MRD assessments throughout the further follow-up could predict impending relapse.<sup>9-12</sup> One of the established MRD markers in AML is the mutated *Nucleophosmin 1* gene (*NPM1*). In approximately 30% of all AML patients *NPM1* is mutated with the main point mutations A, B, and D which occur in 95% of all *NPM1*-mutated patients.<sup>13,14</sup> Depending on an additional *fms*-like tyrosine kinase receptor 3 internal tandem duplication (*FLT3*-ITD) the general prognosis of patients with *NPM1* mutations is favorable.<sup>1,15</sup> *NPM1* mutations are reliable markers for monitoring MRD after conventional chemotherapy, allogeneic HSCT, or during the subsequent follow-up.<sup>10,11,16,17</sup> There has been some discussion on the stability of this MRD marker in relapse, but *NPM1* mutations could be detected in at least 91% of all relapsed patients.<sup>10,18</sup>

In this study, we analyzed MRD monitoring by RT-PCR in 158 adult patients with *NPM1* A, B and D mutations treated with high-dose cytarabine induction therapies within the AMLCG 1999, 2004 and 2008 trials. We established clinically useful cut-offs at different time points and showed the impact of MRD levels, in terms of *NPM1* mutation ratios, on clinical outcome.

## Methods

### Patients

Since 2005 all AML patients have been routinely retrospectively and prospectively screened for *NPM1* mutations by melting curve-based LightCycler assay<sup>19</sup> at the Laboratory for Leukemia Diagnostics of the Department of Internal Medicine III, University of Munich, Grosshadern. Patients screened positive for *NPM1* mutations (*NPM1*mut) and with at least one RT-PCR result after the initial diagnosis were included in this retrospective study. All patients were enrolled into one of the following AMLCG trials: AMLCG 1999 (n=91),<sup>20</sup> AMLCG 2004 (n=29),<sup>21</sup> and AMLCG 2008 (n=38, NCT01382147) and gave written informed consent to treatment and genetic analyses according to the Declaration of Helsinki. All trials were approved by the relevant institutional review boards and ethics committees of the participating institutions.

### Treatment, sampling and minimal residual disease monitoring

Younger patients, i.e. those under the age of 60, received either sequential high-dose cytarabine with mitoxantrone (sHAM<sup>21</sup>), or double induction with standard-dose cytarabine, daunorubicin and thioguanine followed by high-dose cytarabine with mitoxantrone (TAD-HAM), or two courses of high-dose cytarabine with mitoxantrone (HAM-HAM<sup>20</sup>). In older patients, over the age of 60, induction therapy consisted of either reduced dose sHAM<sup>21</sup> or one to two courses of intermediate-dose cytarabine with mitoxantrone (HAM-HAM<sup>20,21</sup>). Post-induction therapy consisted of TAD consolidation (with subsequent maintenance therapy according to the AMLCG standard<sup>20,22</sup>) and/or HSCT. MRD was monitored by RT-PCR in 588 bone marrow samples from 158 patients. It was recommended that bone marrow was collected and MRD analyzed at diagnosis, during aplasia within induction therapy, after induction therapy, after consolidation therapy, and every 3 months during the maintenance therapy or after completion of the therapy (Table 1, *Online Supplementary Figure S1*).

### Quantitative assessment of *NPM1* mutations A, B, and D

Sample preparation and the conditions of the RT-PCR assay of *NPM1*mut A were as described by Papadaki *et al.*<sup>18</sup> The primers and probes used for *NPM1*mut B and D were those published by Gorello *et al.*,<sup>16</sup> while the RT-PCR assay conditions and analyses for these mutations were analogous to those described by Papadaki *et al.* with a maximum sensitivity of 10<sup>-6</sup> in a serial dilution of a *NPM1*mut-negative cell line. MRD levels of the samples were expressed as a ratio of the *NPM1*mut normalized to the housekeeping gene *ABL1* and divided by the *NPM1*mut/*ABL1* ratio of an internal calibrator (the OCI/AML3 cell line).

### Clinical endpoints and statistical analyses

The definitions of the clinical endpoints (relapse-free survival and overall survival) and remission criteria follow the International Working Group guidelines.<sup>23</sup> Survival data were censored at the time of HSCT for those patients in whom HSCT was performed in first complete remission. Cut-off values in aplasia, after induction therapy and after consolidation therapy of the absolute *NPM1*mut ratios and of their kinetics (log difference to *NPM1*mut ratio at diagnosis) were determined by Cox

**Table 1.** Sampling and median *NPM1*mut ratios of 158 patients at different MRD checkpoints.

|                                                               | At diagnosis | In aplasia        | After induction therapy | After consolidation therapy | During maintenance/follow-up | At relapse       |
|---------------------------------------------------------------|--------------|-------------------|-------------------------|-----------------------------|------------------------------|------------------|
| Samples (n)                                                   | 138          | 64                | 106                     | 71                          | 164 (of 49 pts)              | 45               |
| <b>All patients</b>                                           |              |                   |                         |                             |                              |                  |
| Median <i>NPM1</i> mut ratio                                  | 73.2         | 1.4               | 0.008                   | 0.0003                      | 0.000001                     | 58.8             |
| (range)                                                       | 0.3 - 947    | 0.000001 – 1010.0 | 0.000001 – 55.3         | 0.000001 – 126.0            | 0.000001 – 115.0             | 0.000001 – 326.0 |
| Median <i>NPM1</i> mut kinetics*                              | --           | - 1.7             | - 3.7                   | - 4.8                       | --                           | --               |
| (range)                                                       | --           | - 8.1 – 0.2       | - 8.4 – 0.4             | - 8.4 – 0.5                 | --                           | --               |
| <b>Patients in complete remission after induction therapy</b> |              |                   |                         |                             |                              |                  |
| Median <i>NPM1</i> mut ratio                                  | 71.6         | 1.6               | 0.009                   | 0.000001                    | 0.000001                     | --               |
| (range)                                                       | 0.3 - 947    | 0.02 - 1010.0     | 0.000001 – 55.3         | 0.000001 – 58.5             | 0.000001 – 115.0             | --               |
| Median <i>NPM1</i> mut kinetics*                              | --           | - 1.6             | - 3.7                   | - 5.5                       | --                           | --               |
| (range)                                                       | --           | - 4.2 – 0.2       | - 8.4 – 0.4             | - 8.4 – 0.2                 | --                           | --               |

pts: patients; *NPM1*mut: *NPM1* mutation; \*Log reduction in relation to the sample at diagnosis.

proportional hazards regression models with respect to the highest hazard ratios and the lowest *P* values. For the prediction of relapse within an observation period of 100 days during the follow-up, we considered the absolute values before relapse for the patients who did relapse or the peak value of measurements for patients who did not relapse during the follow-up. With the help of receiver-operating characteristics (ROC) curve analysis we selected a cut-off for the prediction of relapse within 100 days in the follow-up period. The Kaplan-Meier estimator and log-rank test were used to calculate survival data. The cumulative incidence of relapse was calculated according to Gray.<sup>24</sup>

All analyses were performed using the SPSS 21 Windows software package (IBM, Armonk, NY, USA), or the software environment package R, version 3.0.1 (see also *Online Supplementary Material*).

## Results

### Patients' characteristics

The whole study population consisted of 158 AML patients (median age 57 years, range 18 – 80) with *NPM1*mut A, B or D. Of these 158 patients, 127 patients achieved a complete remission (80.4%) and 16 an incomplete complete remission (10.1%) after induction therapy. Eight patients (5.1%) had refractory disease, two patients (1.3%) died during induction therapy, and in five patients (3.2%) no remission status was available. HSCT was performed in first complete remission in 30 patients (19%). After a median follow-up of 18.6 months (range, 0.8 – 108.9 months), 56 patients in complete remission had a relapse (44.1%) and 48 patients (30.4%) died. Further characteristics are summarized in Table 2.

### *NPM1* mutation ratios at different time points

#### *NPM1* mutation ratios at diagnosis

*NPM1*mut ratios were determined by RT-PCR at diagnosis in 138 bone marrow samples. *NPM1*mut ratios (median ratio 73.2; range, 0.3 – 947.9, *Online Supplementary Figure S2*) did not affect the cumulative incidence of relapse (*P*=0.59), overall survival (*P*=0.69), or complete remission rate after induction therapy (*P*=0.78). *NPM1*mut ratios at diagnosis showed no correlation or association with *FLT3*-ITD mutation status (*P*=0.54), ELN risk stratification<sup>1</sup> (*P*= 0.86), blast count (*P*=0.82), white blood cell count (*P*=0.76), or lactate dehydrogenase level (*P*= 0.64).

#### *NPM1* mutation ratios at the time of aplasia during induction therapy

Bone marrow samples from 64 patients in aplasia (day 16 – 18 after the initiation of induction therapy), regardless of the response to induction therapy, were available for analysis. The median bone marrow blast count of all patients during aplasia was 0% (range, 0 – 96%). Patients with refractory disease after induction therapy had a higher blast count in aplasia than patients in complete remission after induction therapy (median 7.5% *versus* 0%, *P*=0.03). Bone marrow blast count did not show any impact on cumulative incidence of relapse or overall survival in complete remission patients after induction therapy. Interestingly, neither the absolute *NPM1*mut ratios nor the *NPM1*mut kinetics were significantly different between patients who achieved complete remission and patients who did not.

For subsequent analyses only patients who achieved complete remission after induction therapy were analyzed (*n*=49). At this early stage of treatment all patients still showed detectable RT-PCR signals (Figure 1, Table 1). Patients who stayed in remission showed a trend to having lower *NPM1*mut ratios and greater *NPM1*mut log reduction kinetics than those in patients who had a relapse (median *NPM1*mut ratio 1.00 *versus* 2.4, *P*=0.077, and median *NPM1*mut ratio reduction of -1.6 log *versus* -1.4 log, *P*=0.074; Figure 1). To determine the prognostic value of MRD monitoring in complete remission patients, we analyzed Cox regression models. The *NPM1*mut kinetics but not the absolute levels were significant prognostic indicators for the occurrence of relapse. Clinical cut-offs were determined by Cox proportional hazards regression. A cut-off ratio of 10 for the absolute *NPM1*mut ratios and a cut-off of -1 log for the kinetics resulted in sensitivities of 29% and 39%, respectively, and specificities of 96% (Table 3). Both cut-offs showed a significant prognostic impact on remission duration [hazard ratio (HR) 2.81, *P*=0.034, and HR 4.55, *P*=0.002, respectively] and on cumulative incidence of relapse (*Online Supplementary Figure 3A,B*). Overall survival was not significantly influenced by MRD levels at this checkpoint (Table 4, *Online Supplementary Figure 3C,D*). In addition, multivariate analyses revealed that MRD cut-offs at aplasia were significant independent predictors for relapse if combined with the ELN risk stratification (Table 4).

**Table 2.** Demographics and clinical characteristics of the 158 patients.

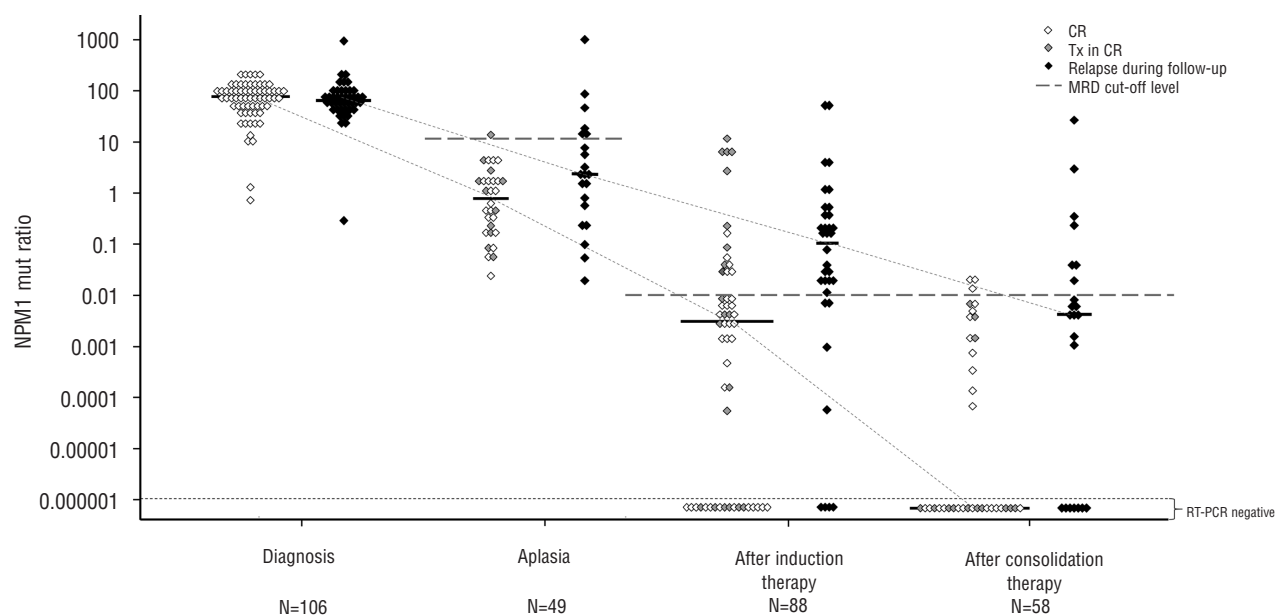
|                                                     | N.              | %  |
|-----------------------------------------------------|-----------------|----|
| Median age in years (range)                         | 57 (18-80)      |    |
| Sex                                                 |                 |    |
| Female                                              | 86              | 54 |
| Male                                                | 72              | 46 |
| Cytogenetics                                        |                 |    |
| Normal karyotype                                    | 139/154         | 90 |
| Deletion 9q                                         | 3/154           | 2  |
| Trisomy 4                                           | 2/154           | 1  |
| Complex karyotype                                   | 3/154           | 2  |
| Other                                               | 7/154           | 5  |
| <i>NPM1</i> mutation type                           |                 |    |
| A                                                   | 136             | 86 |
| B                                                   | 11              | 7  |
| D                                                   | 11              | 7  |
| <i>FLT3</i> -ITD                                    |                 |    |
| Mutated                                             | 68/157          | 43 |
| <i>FLT3</i> -TKD                                    |                 |    |
| Mutated                                             | 19/150          | 13 |
| ELN risk stratification*                            |                 |    |
| Favorable                                           | 78/154          | 51 |
| Intermediate – I                                    | 61/154          | 40 |
| Intermediate – II                                   | 11/154          | 7  |
| Adverse                                             | 4/154           | 3  |
| HSCT in first complete remission                    | 30              | 19 |
| Median follow-up in months (range)                  | 19 (1-109)      |    |
| Median WBC x10 <sup>9</sup> /L at diagnosis (range) | 22 (1-406)      |    |
| Median LDH in U/L at diagnosis (range)              | 487 (108-14332) |    |

ITD: internal tandem duplication; TKD: tyrosine kinase domain; ELN: European LeukemiaNet; HSCT: allogeneic hematopoietic stem cell transplantation; WBC: white blood cell count; LDH: lactate dehydrogenase; \*According to Döhner et al.<sup>1</sup>

## NPM1 mutation ratios after induction therapy

Bone marrow samples from 91 patients in complete remission after induction therapy were available for analysis. Absolute NPM1mut ratios and their kinetics (Table 1) after induction therapy had a significant prognostic impact on the occurrence of relapse (Table 3).

Patients with ongoing remission had significantly lower NPM1mut ratios and greater log reductions than patients with relapse during follow-up (median NPM1mut ratio of 0.004 versus 0.11,  $P=0.001$  and median log reduction of -4.4 versus -3.0 log,  $P=0.001$ ; Figure 1). We determined a cut-off ratio of 0.01 for absolute NPM1mut ratios and a log



**Figure 1.** NPM1mut ratios at different checkpoints in patients who achieved complete remission. Black symbols indicate NPM1mut ratios at different checkpoints of patients who relapsed during follow-up, white symbols indicate NPM1mut ratios at different checkpoints of patients with ongoing remission. Gray symbols indicate NPM1mut ratios of patients who subsequently underwent allogeneic stem cell transplantation in first complete remission. Bars indicate median NPM1mut ratios at the specific checkpoint. NPM1mut: NPM1 mutation; RT-PCR: quantitative real-time polymerase chain reaction; CR: NPM1 mutation ratios of patients with ongoing complete remission; Tx in CR: NPM1 mutation ratios of patients who subsequently underwent allogeneic stem cell transplantation in first complete remission.

**Table 3.** Results of relapse analyses at different MRD checkpoints.

| MRD checkpoint                     | N. | HR (95% CI)*        | P*       | Cut-off** | HR Cut-off (95% CI)*** | P***    | Sensitivity    | Specificity     | PPV            | NPV             | P****   |
|------------------------------------|----|---------------------|----------|-----------|------------------------|---------|----------------|-----------------|----------------|-----------------|---------|
| <b>Aplasia</b>                     |    |                     |          |           |                        |         |                |                 |                |                 |         |
| NPM1mut ratios                     | 49 | 1.43<br>(0.97-2.05) | 0.068    | 10        | 2.81<br>(1.08-7.29)    | 0.034   | 29%<br>(6/21)  | 96%<br>(27/28)  | 86%<br>(6/7)   | 64%<br>(27/42)  | 0.033   |
| NPM1mut kinetics                   | 45 | 2.29<br>(1.22-4.29) | 0.01     | 1 Log     | 4.55<br>(1.73-11.97)   | 0.002   | 39%<br>(7/18)  | 96%<br>(26/27)  | 87%<br>(7/8)   | 70%<br>(26/37)  | 0.004   |
| <b>After induction therapy</b>     |    |                     |          |           |                        |         |                |                 |                |                 |         |
| NPM1mut ratios                     | 88 | 1.47<br>(1.17-1.84) | 0.001    | 0.01      | 4.26<br>(1.93-9.45)    | <0.0001 | 76%<br>(26/34) | 74%<br>(40/54)  | 65%<br>(26/40) | 83%<br>(40/48)  | <0.0001 |
| NPM1mut kinetics                   | 81 | 1.64<br>(1.26-2.14) | <0.0001  | 3 Log     | 5.09<br>(2.39-10.82)   | <0.0001 | 50%<br>(15/30) | 84%<br>(43/51)  | 65%<br>(15/23) | 74%<br>(43/58)  | 0.002   |
| <b>After consolidation therapy</b> |    |                     |          |           |                        |         |                |                 |                |                 |         |
| NPM1mut ratios                     | 58 | 1.39<br>(1.10-1.77) | 0.006    | 0.01      | 2.72<br>(1.10-6.69)    | 0.03    | 32%<br>(7/22)  | 92%<br>(33/36)  | 70%<br>(7/10)  | 69%<br>(33/48)  | 0.03    |
| NPM1mut kinetics                   | 48 | 1.34<br>(1.06-1.70) | 0.016    | 3 Log     | 7.58<br>(2.31-24.86)   | 0.001   | 22%<br>(4/18)  | 100%<br>(30/30) | 100%<br>(4/4)  | 68%<br>(30/44)  | 0.016   |
| <b>Follow-up*</b>                  |    |                     |          |           |                        |         |                |                 |                |                 |         |
| NPM1mut ratios                     | 73 | NA                  | <0.0001† | 1‡        | NA                     | NA      | 100%<br>(7/7)  | 100%<br>(66/66) | 100%<br>(7/7)  | 100%<br>(66/66) | <0.0001 |

HR: hazard ratio; CI: confidence interval; PPV: positive predictive value; NPV: negative predictive value; NPM1mut: NPM1 mutation; NA: not analyzed; \*Cox regression model of all NPM1mut measurements for the occurrence of relapse; \*\*Cut-offs determined by Cox regression models; \*\*\*Cox regression model of NPM1mut cut-off values for the occurrence of relapse; \*\*\*\* $\chi^2$  test of 2 × 2 contingency tables; †Relapse within an observation period of 100 days during follow-up; ‡Cut-off determined by receiver-operating characteristic curve analysis.

reduction of -3 log. With these cut-offs we calculated sensitivities of 76% and 50% and specificities of 74% and 81% for the identification of patients at high risk of relapse (Table 3). Both cut-off levels identified patients at risk of relapse (HR 4.26,  $P<0.0001$  for absolute levels, and HR 5.09,  $P<0.0001$  for kinetics, Table 3). The cumulative incidence of relapse after 2 years was 77.8% for MRD cut-off-positive patients and thus significantly higher than that for MRD cut-off-negative patients (26.4%,  $P<0.0001$ ; Figure 2A,B). This ability to predict relapse persisted in the multivariate model if added to established risk stratification factors: age, ELN risk stratification, white blood cell count, and lactate dehydrogenase level (Table 4). Our cut-off level for absolute *NPM1*mut ratios showed a trend to affect overall survival (Figure 2C, Table 4) and the cut-off level for kinetics separated two prognostic groups in the Kaplan-Meier plot with statistically significantly different overall survival, although the difference did not reach statistical significance in the multivariate model (Figure 2D, Table 4).

#### *NPM1* mutation ratios after consolidation therapy

After consolidation therapy 58 patients in complete remission were available for analysis of survival data. The absolute *NPM1*mut ratios and their kinetics (Table 1) had a significant impact on relapse detection (HR of absolute levels 1.39,  $P=0.006$ , and HR of kinetics 1.34,  $P=0.016$ , Table 3). In accordance, patients with ongoing remission had significantly lower *NPM1*mut ratios and greater log reductions than patients with relapse during follow-up (median *NPM1*mut ratio of 0.00001 *versus* 0.005,  $P=0.004$

and median log reduction of -6.99 *versus* -4.39 log,  $P=0.027$ , respectively; Figure 1).

Cut-off levels of *NPM1*mut ratio of 0.01 and of -3 log were established and displayed low sensitivities of 32% and 22% but high specificities of 92% and 100% (Table 3). The cut-off levels separated the cohort into two prognostic groups (Online Supplementary Figure 4A,B) with hazard ratios of 2.72 and 7.58. In the multivariate model with age and ELN risk stratification, the *NPM1*mut ratios and kinetics exceeding the established cut-off levels were the only prognostic variables for cumulative incidence of relapse and overall survival (Table 4).

#### *NPM1* mutation ratios during follow-up

During the follow-up period, MRD was monitored in 191 bone marrow samples (including the samples after consolidation therapy) from 81 patients in complete remission. In accordance with previously published data on relapse kinetics<sup>25</sup> and as a result of individual different MRD monitoring intervals, an evaluation period of 100 days after sampling was chosen for a prediction analysis of relapse. Seventy-three patients in complete remission were available for this prediction analysis. Using ROC analysis a cut-off *NPM1*mut ratio of 1 was assessed. With this cut-off all patients with an upcoming relapse (sensitivity of 100%) within the next 100 days could be detected prior to relapse with a median time to relapse of 58 days (range, 20 – 98 days). None of the patients with values below this cut-off (specificity of 100%) relapsed in the 100-day observation period ( $P<0.0001$ , Table 3).

**Table 4.** Multivariate analyses.

| Variable                             | Cumulative incidence of relapse |                   |        | N. | Overall survival  |       |
|--------------------------------------|---------------------------------|-------------------|--------|----|-------------------|-------|
|                                      | N.                              | HR (95% CI)       | P      |    | HR (95% CI)       | P     |
| In aplasia                           | 42                              |                   |        | 32 |                   |       |
| MRD cut-off ( <i>NPM1</i> mut ratio) |                                 | 2.82 (1.08-7.31)  | 0.033  |    | 0.93 (0.11-7.83)  | 0.946 |
| ELN (favorable <i>vs.</i> others)    |                                 | 0.83 (0.35-2.00)  | 0.686  |    | 0.50 (0.12-2.08)  | 0.340 |
| In aplasia                           | 38                              |                   |        | 28 |                   |       |
| MRD cut-off (Kinetics)               |                                 | 4.87 (1.80-13.17) | 0.002  |    | 3.01 (0.48-18.95) | 0.240 |
| ELN (favorable <i>vs.</i> others)    |                                 | 0.58 (0.21-1.56)  | 0.279  |    | 0.29 (0.05-1.57)  | 0.152 |
| After induction therapy              | 72                              |                   |        | 52 |                   |       |
| MRD cut-off ( <i>NPM1</i> mut ratio) |                                 | 6.16 (2.49-15.24) | 0.0001 |    | 2.40 (0.84-6.87)  | 0.103 |
| ELN (favorable <i>vs.</i> others)    |                                 | 2.83 (1.19-6.75)  | 0.018  |    | 5.19 (1.59-16.97) | 0.006 |
| Age (per decade)                     |                                 | 1.31 (0.91-1.88)  | 0.145  |    | 2.96 (1.41-6.21)  | 0.004 |
| WBC (per 10 fold increase)           |                                 | 2.52 (1.00-6.31)  | 0.049  |    | 3.86 (1.34-11.10) | 0.012 |
| LDH (per one upper limit of normal)  |                                 | 0.94 (0.73-1.20)  | 0.615  |    | NA                | NA    |
| After induction therapy              | 66                              |                   |        | 46 |                   |       |
| MRD cut-off (Kinetics)               |                                 | 5.33 (2.02-14.05) | 0.001  |    | 3.02 (0.89-10.30) | 0.077 |
| ELN (favorable <i>vs.</i> others)    |                                 | 1.93 (0.85-4.425) | 0.118  |    | 3.23 (0.91-11.48) | 0.070 |
| Age (per decade)                     |                                 | 1.05 (0.72-1.54)  | 0.803  |    | 2.53 (1.12-5.68)  | 0.025 |
| WBC (per 10 fold increase)           |                                 | 2.05 (0.94-4.44)  | 0.07   |    | 3.96 (1.30-12.19) | 0.016 |
| After consolidation therapy          | 55                              |                   |        | 45 |                   |       |
| MRD cut-off ( <i>NPM1</i> mut ratio) |                                 | 2.91 (1.16-7.31)  | 0.023  |    | 5.06 (1.46-17.49) | 0.010 |
| ELN (favorable <i>vs.</i> others)    |                                 | 1.07 (0.45-2.52)  | 0.878  |    | 0.66 (0.19-2.32)  | 0.658 |
| Age (per decade)                     |                                 | 1.21 (0.83-1.78)  | 0.320  |    | 1.44 (0.81-2.58)  | 0.215 |
| After Consolidation Therapy          | 45                              |                   |        | 30 |                   |       |
| MRD cut-off (Kinetics)               |                                 | 7.62 (2.25-25.88) | 0.001  |    | 8.80 (1.73-44.78) | 0.009 |
| ELN (favorable <i>vs.</i> others)    |                                 | 1.04 (0.40-2.73)  | 0.936  |    | 0.16 (0.02-1.38)  | 0.164 |
| Age (per decade)                     |                                 | 1.20 (0.80-1.79)  | 0.369  |    | NA                |       |

HR: hazard ratio; CI: confidence interval; *NPM1*mut: *NPM1* mutation; ELN: risk stratification according to European LeukemiaNet; WBC: white blood cell count at diagnosis; LDH: lactate dehydrogenase level at diagnosis; NA: not analyzed.

**European LeukemiaNet favorable risk group**

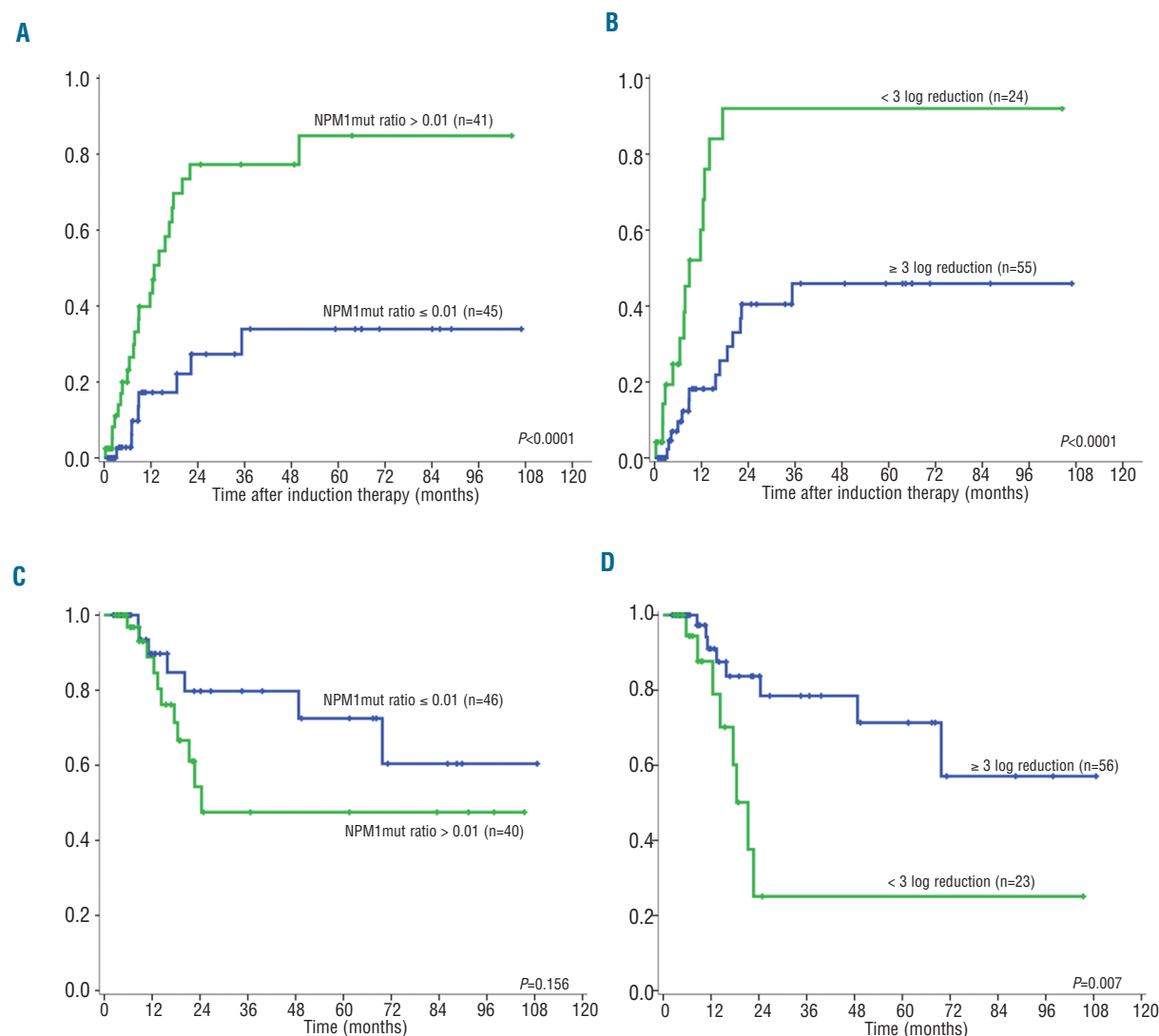
Seventy-eight patients (51%) with normal karyotype and no *FLT3*-ITD were classified as being in the ELN favorable risk group.<sup>1</sup> Of these, 80.8% (63 patients) achieved complete remission after induction therapy and 11.5% achieved an incomplete complete remission. Within this group with a favorable prognosis, 45% of the patients had a relapse during the follow-up. In patients in complete remission, there were no differences in relapse rate, remission duration, and overall survival between the prognostically favorable and intermediate I (with *FLT3*-ITD) groups.

All estimated cut-offs for the whole cohort were confirmed in the ELN favorable subgroup (*data not shown*). The absolute level of NPM1mut after induction therapy seemed to be the most clinically relevant parameter. At this checkpoint, NPM1mut ratios above the cut-off levels defined patients with an increased risk of relapse as compared to patients with MRD levels below this cut-off (HR

8.59,  $P=0.005$ ). In accordance with this, the cumulative incidence of relapse was significantly lower in the MRD cut-off negative group (*Online Supplementary Figure S6*).

**Stability of NPM1 mutations at relapse**

MRD could be assessed at relapse in 45 patients with a median NPM1mut ratio of 58.8 (range, 0.000001 – 326.0). The NPM1mut ratios at relapse did not differ from those at diagnosis ( $P=0.25$ ). In three out of the 45 patients (6.7%) no NPM1mut signal could be detected by RT-PCR. One of these patients also lost *FLT3*-ITD at relapse after 37.6 months and gained a chromosomal aberration t(1;7). In this patient, a *DNMT3A* mutation present at diagnosis was also detected in the relapse sample. The second patient relapsed after HSCT and lost the initial leukemia-associated immunophenotype at relapse. The third patient relapsed after 5.6 months and gained a *JAK2* mutation at relapse (*Online Supplementary Table S2*).



**Figure 2.** Cumulative incidence of relapse (A and B) and overall survival (C and D) after induction therapy according to MRD status of NPM1mut ratios and NPM1mut kinetics. (A) and (C) NPM1mut ratios after induction therapy with a NPM1mut cut-off ratio of 0.01; (B) and (D) NPM1mut kinetics after induction therapy with a cut-off of  $\geq 3$  log. NPM1mut: NPM1 mutation.

### Impact of consolidation therapy on the course of minimal residual disease

We analyzed the impact of consolidation therapy on the course of MRD in individual patients in complete remission after induction and consolidation therapy. Forty-four patients had paired MRD samples at both checkpoints before and after consolidation therapy (Figure 3).

Nineteen patients showed MRD levels above the estimated cut-off after induction therapy. Six out of these 19 patients stayed MRD-positive (according to the estimated *NPM1*mut ratio cut-off) after the end of consolidation therapy and five patients relapsed after a median of 4 months (range, 1 – 11 months). The remaining double MRD-positive patient had weakly positive MRD levels at both checkpoints (0.07 and 0.03, respectively) and became RT-PCR negative throughout the maintenance therapy. In 13 patients MRD levels decreased below the estimated cut-off after consolidation therapy. Nevertheless, nine of these patients relapsed, while four remained in complete remission. Three of these four patients underwent HSCT in first complete remission.

Twenty-five patients were MRD cut-off negative after induction therapy and 24 patients stayed MRD cut-off negative after consolidation therapy. Six patients were transplanted in first complete remission and in 14 double MRD cut-off negative patients no relapse occurred after a median follow-up of 26 months (range, 1-105 months). Four double MRD cut-off-negative patients relapsed after a median of 15 months (range, 5-32 months). In one of these patients, MRD sampling 90 days prior to relapse

was possible and resulted in a *NPM1*mut ratio of 8.6, clearly above the follow-up cut-off ratio of 1.

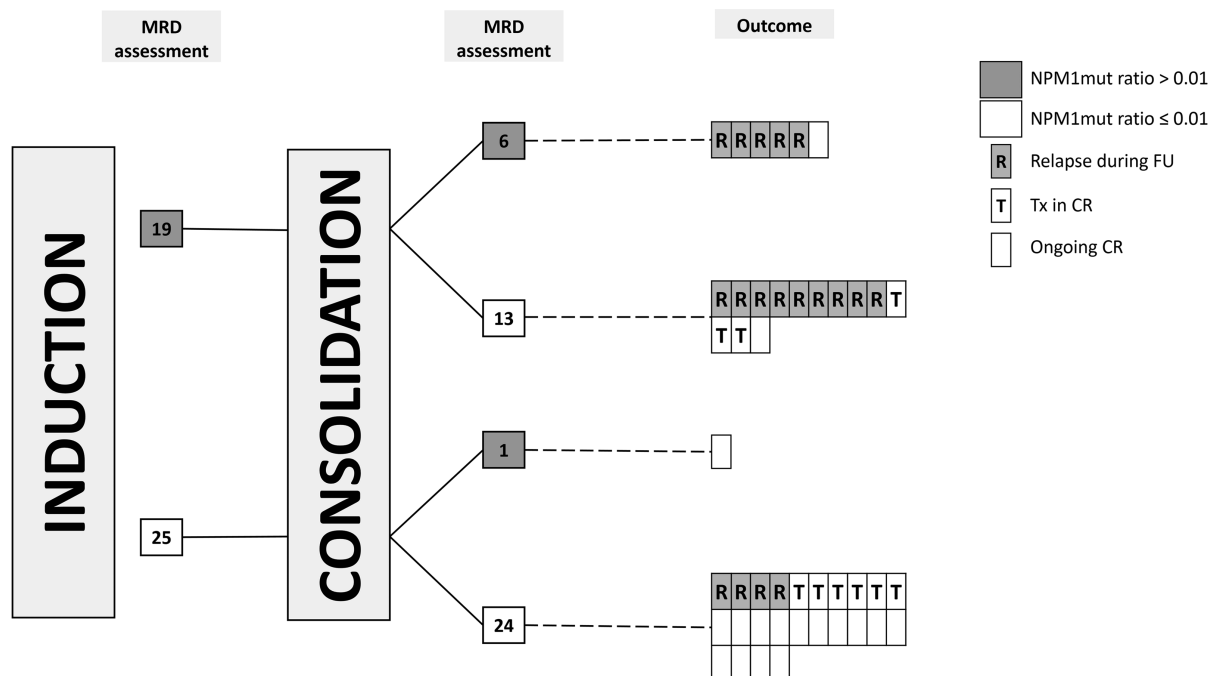
Only one patient was initially MRD cut-off-negative after the induction therapy and showed weakly positive MRD levels after the consolidation therapy (*NPM1*mut ratio 0.02). The further follow-up sample was RT-PCR-negative and the patient is still in remission after 21 months.

In this analysis of paired samples, MRD status after consolidation therapy in comparison to the MRD status after induction therapy has changed in 14 patients (32%), and in only four patients (29%) did this change in MRD indicate the correct outcome.

### Discussion

In this study, we assessed MRD at different checkpoints throughout conventional chemotherapy in 158 AML patients with *NPM1* mutations. All patients received intensive, high-dose cytarabine induction therapy within one of three AMLCG trials. Major goals of this study were: (i) to identify relevant assessment checkpoints for MRD and (ii) to establish cut-off levels for the prediction of relapse.

The *NPM1*mut ratio at diagnosis did not show any impact on survival, or on relapse occurrence. After induction therapy seemed to be the most appropriate checkpoint to assess MRD levels in order to identify patients in complete remission at high risk of relapse. The cut-off



**Figure 3.** MRD assessment after induction and consolidation therapy in patients with paired samples. In total, 18 patients relapsed after consolidation therapy with a median time to relapse of 9.9 months. Nine patients were transplanted in first complete remission with a median time to allogeneic stem cell transplantation of 1.2 months and 17 patients had an ongoing remission with a median follow-up of 31.1 months after consolidation therapy. *NPM1*mut: *NPM1* mutation; FU: follow-up period; CR: first complete remission; Tx: allogeneic stem cell transplantation in first complete remission.

values of the NPM1mut ratios and the kinetics of these ratios after induction therapy had high hazard ratios in multivariable analysis and yielded high sensitivity and specificity values. Interestingly, the same cut-off levels after consolidation therapy had less impact on cumulative incidence of relapse than the MRD cut-off after induction therapy. In addition, consolidation therapy showed no clear MRD reduction in MRD cut-off-positive patients after induction therapy. Hence, in this biological subgroup with its slow relapse kinetics,<sup>25</sup> a quick and deeper molecular response after induction therapy seems to be more prognostically relevant than persistent MRD positivity after consolidation therapy. Thus, we conclude that MRD levels after induction therapy should be used to identify patients at high risk of relapse.

Krönke *et al.*,<sup>10</sup> and Shayegi *et al.*<sup>11</sup> analyzed clinically relevant MRD checkpoints in *NPM1*-mutated patients. They, too, identified MRD assessment after induction therapy, or after the achievement of complete remission, as one of the most important MRD checkpoints. They demonstrated that positive MRD levels at this checkpoint identify patients at high risk of relapse and shorter overall survival. In contrast to Krönke *et al.* and in accordance with Shayegi *et al.* we found that a low level of MRD (cut-off ratio of 0.01) after induction therapy is superior to RT-PCR negativity for identifying patients at high risk of relapse (see *Online Supplementary Material*). During the follow-up period, our estimated cut-off ratio of 1 identified all relapses within the next 100 days after sampling and all patients with lower NPM1mut ratios stayed in remission for the next 100 days. While our analysis confirmed the significant impact of MRD positivity on cumulative incidence of relapse, we observed only a trend for overall survival (Figure 2). This might be the consequence of a smaller cohort of patients, study design, the censored survival data at HSCT, or a short follow-up after relapse.

The monitoring and interpretation of NPM1mut MRD assessments are still restricted to centralized laboratories. As a result of different RT-PCR assays, conditions, treatment protocols and clinical situations, it is very difficult to compare the estimated cut-offs at the different MRD checkpoints. Shayegi *et al.* established clinical cut-offs by ROC analyses and Cox regression models. After the achievement of complete remission they estimated a cut-off level of 1% and after HSCT a higher cut-off level of 10%.<sup>11</sup> We focused our MRD analyses on patients after conventional chemotherapy and identified an NPM1mut ratio of 0.01 as the best cut-off value after induction therapy and a cut-off ratio of 1 during the follow-up period. Only the MRD log reduction can be compared throughout the different MRD studies. Consistent with our data, Schnittger *et al.*<sup>17</sup> also found that a 3-log reduction identified patients at high risk of relapse. Joint efforts such as the standardization of Wilms' tumor 1 (*WT1*) gene expression<sup>9</sup> or *BCR-ABL*<sup>5</sup> are needed. Until there is harmonization of NPM1mut RT-PCR assays, enabling subsequent interlaboratory comparison, centralized MRD assessment and interpretation at one laboratory of each study group is obligatory.

The ELN has published a risk stratification considering cytogenetics and molecular genetic analyses at diagnosis.<sup>1,3</sup> The ELN favorable risk group of our study cohort is the most clinically interesting subgroup. In contrast to the intermediate I group (with an additional *FLT3*-ITD)

HSCT is usually not recommended in these patients.<sup>26</sup> However, in our cohort of patients, 45% of the NPM1mut patients without *FLT3*-ITD relapsed and we found no significant differences in relapse rate, remission duration, and overall survival between the ELN favorable and intermediate I groups. This might be because of the heterogeneous age of patients within the AMLCG trials, which had no age restrictions. Depending on the quantity of *FLT3*-ITD mRNA,<sup>27</sup> *FLT3*-ITD might have a prognostic impact in NPM1mut AML and require a more individualized treatment. In other hematologic malignancies, such as acute lymphoblastic leukemia and chronic myeloid leukemia, MRD-guided therapies are already established. Pre-emptive therapy based on MRD monitoring in patients with acute promyelocytic leukemia improved the outcome of patients in the MRC AML15 trial, in a historical comparison to previous trials.<sup>7</sup> However, only a few prospective studies with MRD-guided therapies are available in non-acute promyelocytic forms of AML.<sup>28-30</sup>

Our results and those of others demonstrate the feasibility and reliability of serial NPM1mut MRD monitoring to identify patients at high risk of relapse.<sup>10,11,19</sup> Since HSCT in NPM1mut patients does not impair the relapse-free survival<sup>26</sup> and, as shown in our analyses, MRD cut-off-positive patients are at high risk of relapse, these patients should be considered for more intensified post-remission therapy, to improve the relapse rate and overall survival, and should undergo HSCT, if eligible.

Instability of MRD markers is a potential problem of MRD-guided therapy and *FLT3*-ITD can be unstable during follow-up.<sup>31,32</sup> The stability of NPM1mut has been debated. In the relapse samples analyzed by Schnittger *et al.*<sup>17</sup> all relapsed patients showed the initial NPM1mut at relapse. This is in contrast to our results and those of Krönke *et al.*,<sup>10</sup> with mutated *NPM1* being undetectable at relapse in 6.7% and 9% of patients, respectively. In our analyses, one of the three relapsed patients with undetectable NPM1mut at relapse did relapse after more than 2 years with different chromosomal aberrations and a different leukemia-associated immunophenotype, but with a stable *DNMT3A* mutation.<sup>33</sup> The other two patients relapsed within 6 months after achieving complete remission. One patient also showed the initial leukemia-associated immunophenotype with an additional *JAK2* mutation at relapse (*Online Supplementary Table S2*). Thus, in these patients a clonal evolution or a relapse of a subclone of the initial leukemia is very likely. Considering this aspect, monitoring a second, stable molecular marker, e.g. *DNMT3A*,<sup>33</sup> or monitoring MRD by an alternative method, e.g. flow cytometry, may improve the relapse prediction rate and lower the rate of false negative MRD results.

In conclusion, our results showed the prognostic impact of *NPM1* MRD monitoring by RT-PCR. MRD monitoring can identify patients at high risk of relapse, especially in the clinically relevant subgroup of the ELN favorable-risk patients. Particularly high MRD levels above our estimated cut-off after induction therapy were strongly associated with a high cumulative incidence of relapse. This and data previously published by others demonstrate that, in addition to pre-therapeutic prognostic factors, the individual course of MRD should be used as new prognostic factor for the guidance of treatment and patients with high or increasing levels of MRD should undergo HSCT, if eligible.

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### Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at [www.haematologica.org](http://www.haematologica.org).

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