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Molecular response assessment by quantitative RT-PCR after induction therapy in NPM1 mutated patients identifies patients at high risk for relapse

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Running title: MRD in NPM1 mutated AML patients

Abstract

Monitoring of minimal residual disease represents an important diagnostic tool to identify patients with acute myeloid leukemia at high risk for relapse. In this study the prognostic potential of minimal residual disease monitoring by quantitative real-time PCR of NPM1 mutations of patients treated in the AMLCG trials 1999, 2004 and 2008 was investigated.

Minimal residual disease monitoring was performed in 588 samples of 158 NPM1 mutation A, B and D positive patients at diagnosis, in aplasia, after induction therapy, after consolidation therapy, and during follow-up with a sensitivity of 10^{-6}.

127 patients (80.4%) achieved complete remission after induction therapy and of these 56 patients (44.1%) relapsed. At each checkpoints, minimal residual disease cut-offs were calculated. After induction therapy a cut-off NPM1 mutation ratio of 0.01 revealed 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 cut-off positive patients versus 26.4% for cut-off negative patients, respectively. In the favorable subgroup according to European LeukemiaNet, the cut-off after induction therapy also separates 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 individual minimal residual disease course is an important prognostic factor and could be included into clinical trials for the guidance of postremission therapy.

Trials were registered at 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 their cytogenetic and molecular genetic characteristics. At present, therapy decisions are guided by pretherapeutic risk assessments (1). Nevertheless, there are still patients suffering from relapse despite belonging to the favorable risk group (2, 3) and more individualized therapy regimens are needed to prevent relapses.

To identify these patients at high risk for relapse, monitoring of minimal residual disease (MRD) could become an important diagnostic tool (4). In acute lymphoblastic leukemia (ALL) and in chronic myeloid leukemia (CML) patients, the MRD status routinely guides therapy decisions at different check points (5, 6). ALL patients, 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). Also, preemptive arsenic trioxide therapy in acute promyelocytic leukemia (APL) with t(15;17)(q22;q12) PML-RARA initiated by increasing MRD levels has prevented relapse in the MRC 15 trial (7).

AML has a wide spectrum of molecular markers (1). 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 a higher sensitivity by RT-PCR as compared to other methods such as immunophenotyping (4, 8).

MRD assessments after induction and consolidation therapy showed a significant impact on relapse-free and overall survival (RFS, OS) and serial MRD assessments throughout the further follow-up could predict impending relapse (9-12). One of the established MRD markers in AML is the mutated Nucleophosmin gene 1 (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(13, 14). Depending on an additional fms-like tyrosine kinase receptor 3 internal tandem duplication (FLT3-ITD) the general prognosis of patients with NPM1 mutation is favorable (1, 15). The NPM1 mutations are reliable markers to monitor MRD after conventional chemotherapy, allogeneic HSCT, or during the further follow-up (10, 11, 16, 17). Stability of this MRD marker in relapse has been discussed controversial, but NPM1 mutations could be detected in at least 91% of all relapsed patients (10, 18).

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

Methods

Patients

Since 2005 all AML patients were routinely retrospectively and prospectively screened for NPM1 mutations by melting curve-based LightCycler assay (19) at the Laboratory for Leukemia Diagnostics of the Department of Internal Medicine III, University of Munich, Grosshadern. Patients, screened positive for NPM1 mutations (NPM1mut) and with at least one RT-PCR result after the initial diagnosis were included into this retrospective study. All patients were enrolled into one of the following AMLCG trials: AMLCG 1999 (n= 91)(20), AMLCG 2004 (n= 29)(21), and AMLCG 2008 (n= 38, NCT01382147) and gave written informed consent to treatment and genetic analyses according to the Declaration of Helsinki. All trials received approval of the responsible institutional review board and the ethics committees of the participating institutions.

Treatment, Sampling and MRD Monitoring

Younger patients under the age of 60 received either sequential high dose cytarabine with mitoxantrone (sHAM, (21)), or double induction with standard dose cytarabine, daunorubicine and thioguanine followed by high dose cytarabine with mitoxantrone (TAD-HAM), or 2 courses of high dose cytarabine with mitoxantrone (HAM-HAM, (20)). In older patients over the age of 60, induction therapy consisted of either reduced dose sHAM (21) or 1-2 courses of intermediate dose cytarabine with mitoxantrone (HAM-HAM, (20, 21)). Post-induction therapy consisted of TAD consolidation (with subsequent maintenance therapy according to the AMLCG standard (20, 22)) and/or HSCT. MRD monitoring via RT-PCR were performed in 588 bone marrow (BM) samples of 158 patients. Collection of BM was recommended and MRD analyzed at diagnosis, during aplasia within induction therapy, after induction therapy, after consolidation therapy, and every 3 months within the maintenance therapy or after the completion of the therapy, respectively (Table 1, Supplement Figure 1).

Quantitative assessment of NPM1mut A, B, and D

Sample preparation and the conditions of the relative RT-PCR assay of NPM1mut A were performed as previously described by Papadaki(18). Primer and probes for NPM1mut B and D were used according to Gorello et al.(16). The assay conditions and analyses of the relative RQ-PCR of NPM1mut B and D were performed in analogy to Papadaki et al. with a maximum sensitivity of 10^{-6} in a serial dilution of a NPM1mut negative cell line. MRD levels of the samples were expressed as a ratio of the NPM1mut normalized to the housekeeping gene ABL1 and divided by the NPM1mut/ABL1 ratio of an internal calibrator (OCI/AML3 cell line).

Clinical endpoints and statistical analyses

The clinical endpoint definitions (eg, RFS and OS) and remission criteria follow IWG guidelines (23). Survival data was censored for patients in which HSCT in first complete remission (CR) was performed at the time of HSCT.

Cut-off values in aplasia, after induction therapy and after consolidation therapy of the absolute NPM1mut ratios and of its kinetics (Log difference to NPM1mut ratio at diagnosis) were determined by Cox's proportional hazards regression models with respect to the highest HRs and the lowest p values. For the prediction of relapse within an observation time of 100 days during the follow-up period, we considered the absolute values before relapse or the peak value of measurements for patients without relapse during the follow-up, respectively. With the help of receiver-operating characteristics (ROC) 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 (CIR) was calculated according to Gray (24).

All analyses were performed by SPSS 21 Windows software package (IBM, Armonk, NY, USA), or the software environment package R, version 3.0.1 (see also supplement material).

Results

Patients characteristics

The whole study population consisted of 158 AML patients (median age 57, range 18 – 80 years) with NPM1mut A, B or D. 127 patients achieved CR (80.4%) and 16 CRi (10.1%) after induction therapy. Eight patients (5.1%) had refractory disease, 2 patients (1.3%) died during induction therapy, and in 5 patients (3.2%) no remission status was available. HSCT in first CR was performed in 30 patients (19%). After median follow-up time of 18.6 months (range 0.8 – 108.9 months), 56 patients in CR developed relapse (44.1%) and 48 patients (30.4%) died. Further characteristics are summarized in Table 2.

NPM1 mutation ratios at different time points

<u>NPM1mut ratios at diagnosis</u>

At diagnosis, in 138 BM samples NPM1mut ratios were determined by RT-PCR. NPM1mut ratios (median ratio 73.2, range 0.3 – 947.9, Supplement Figure 2) did not impact CIR (p= 0.59), OS (p= 0.69), or CR rate after induction therapy (p= 0.78). NPM1mut ratios at diagnosis showed no correlation or association to FLT3-ITD mutation status (p= 0.54), ELN risk stratification (1) (p= 0.86), blast count (p=0.82), WBC (p= 0.76), or LDH (p= 0.64).

<u>NPM1mut ratios at aplasia during induction therapy</u>

Regardless to the response of induction therapy, BM samples of 64 patients in aplasia (day 16 – 18 after initiation of induction therapy) were available for analysis. The median BM blast count in aplasia of all patients was 0% (range 0 – 96%). Patients with refractory disease after induction therapy had a higher blast count in aplasia in contrast to patients in CR after induction therapy (median 7.5% vs 0%, p= 0.03). BM blast count did not show any impact on CIR or OS in CR patients after induction therapy. Interestingly, neither the absolute NPM1mut ratios nor the NPM1mut kinetics were significantly different between patients who achieved CR and patients who did not. For subsequent analyses only patients who achieved CR 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 towards lower NPM1mut ratios and higher NPM1mut Log reduction kinetics in contrast to patients who developed relapse (median NPM1mut ratio 1.00 vs. 2.4, p= 0.077, and median NPM1mut ratio reduction of - 1.6 Log versus - 1.4 Log, p= 0.074, respectively;

Figure 1). To determine the prognostic value of MRD monitoring in CR patients, we performed Cox regression models. The NPM1mut kinetics but not the absolute levels were significant prognostic indicators for the occurrence of relapse. Clinical cut-offs were determined by Cox's proportional hazards regression. A cut-off ratio of 10 for the absolute NPM1mut 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 (HR 2.81, p=0.034, and HR 4.55, p=0.002, respectively) and on cumulative incidence of relapse (CIR, Supplement Figure 3A, 3B), respectively. OS was not significantly influenced by MRD levels at this checkpoint (Table 4, Supplement Figure 3C, 3D). In addition, multivariate analyses revealed MRD cut-offs at aplasia to be significant independent predictors for relapse if combined with the ELN risk stratification (Table 4).

NPM1mut ratios after induction therapy

BM samples of 91 patients in CR after induction therapy were available for analysis. Absolute NPM1mut ratios and its 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 ratio and higher Log reductions than patients with relapse during follow-up (median NPM1mut ratio of 0.004 vs. 0.11, p= 0.001 and median Log reduction of - 4.4 vs. - 3.0 Log, p= 0.001, respectively; Figure 1). We determined a cut-off ratio of 0.01 for absolute NPM1mut ratios and a Log 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 with high risk for relapse (Table 3). Both cut-off levels identified patients at risk for relapse (HR 4.26, p< 0.0001 for absolute levels, and HR 5.09, p< 0.0001 for kinetics, Table 3) and CIR after two years was 77.8% for MRD cut-off positive patients and thus significantly higher than for MRD cut-off negative patients (26.4%, p< 0.0001, Figure 2 A, B). This prognostic impact on the detection on relapse persisted in the multivariate model if added to established risk stratification factors, which are age, the ELN risk stratification, WBC, and LDH (Table 4). Our cut-off level for absolute NPM1mut ratios showed a trend to impact on OS (Figure 2 C, Table 4) and the cut-off level for kinetics significantly separated two prognostic groups in the Kaplan-Meier plot, but did not reach significance in multivariate model (Figure 2 D, Table 4).

NPM1mut ratios after consolidation therapy

After consolidation therapy 58 patients in CR were available for the analysis on survival data. The absolute NPM1mut ratio and its 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 showed significant lower NPM1mut ratio and higher Log reductions than patients with relapse during follow-up (median NPM1mut ratio of 0.00001 vs. 0.005, p=0.004 and median Log reduction of - 6.99 vs. - 4.39 Log, p= 0.027, respectively; Figure 1).
Cut-off levels of NPM1mut ratio of 0.01 and of - 3 Log were established, displaying 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 (Supplement Figure 4 A, B) with HRs of 2.72 and 7.58. In the multivariate model with age and ELN risk stratification, the NPM1mut ratios and kinetics exceeding the established cut-off levels were the only prognostic variables for CIR and OS (Table 4).

NPM1mut ratios during follow-up

During the follow-up period, MRD was monitored in 191 BM samples (including the samples after consolidation therapy) of 81 patients in CR. In accordance to previously published data on relapse kinetics (25) and as a result of individual different MRD monitoring intervals, an evaluation period of 100 days after sampling were chosen for a prediction analysis of relapse. Seventy-three patients in CR were available for this prediction analysis. Using ROC analysis a cut-off level of NPM1mut 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). All patients below this cut-off (specificity of 100%) did not relapse in the 100 days observation period (p< 0.0001, Table 3).

ELN favorable risk group

Seventy-eight patients (51%) with normal karyotype and no FLT3-ITD were categorized to the ELN favorable risk group (1). 80.8% (63 patients) achieved CR after induction therapy and 11.5 % achieved CRi. Within this prognostic favorable group, 45% of patients developed relapse during the follow-up. In patients in CR, there were no difference in relapse rate, remission duration, and OS between the prognostic favorable and intermediate I (with FLT3-

ITD) group.
All estimated cut-offs for the whole cohort were confirmed in the ELN favorable subgroup (data not shown). Especially the NPM1mut absolute levels after induction therapy seemed to be the most clinically relevant. At this checkpoint, the NPM1mut ratios above the cut-off levels define patients with in an increased risk of relapse as compared to patients with MRD levels below this cut-off (HR 8.59, p= 0.005). In accordance, this is also reflected by a significantly lower CIR in the MRD cut-off negative group (Supplement Figure 6).

Stability of NPM1 mutations at relapse

MRD assessment at relapse could be performed 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 3 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 at diagnosis was also detected in the relapse sample. The second patients relapsed after HSCT and lost the initial leukemia-associated immunophenotype (LAIP) at relapse. The third patient relapsed after 5.6 months and gained a JAK2 mutation at relapse (Supplement Table 2).

Impact of Consolidation Therapy on MRD courses

We analyzed the impact of consolidation therapy on the individual MRD courses of patients in CR after induction and consolidation therapy. Fourty-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 NPM1mut ratio cut-off) after the end of consolidation therapy and 5 patients relapsed after a median time of 4 months (range 1 – 11 months). The remaining double MRD positive patient had slightly 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, 9 of these patients relapsed and 4 stayed in CR. Three of these 4 patients underwent HSCT in first CR.
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 CR and in 14 double MRD cut-off negative patients no relapse did occur after a median follow-up time of 26 months (range 1-105 months). Four double MRD cut-off negative

patients relapsed after a median time of 15 months (range 5-32 months). In one of these patients, MRD sampling 90 days prior to relapse was possible and resulted in NPM1mut 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 slightly positive MRD levels after the consolidation therapy (NPM1mut ratio 0.02). The further follow-up sample was RT-PCR negative and the patient is still in remission after 21 months.
In this paired samples analysis, the MRD status after consolidation therapy in comparison to the MRD status after induction therapy has changed in 14 patients (32%), and in only 4 patients (29%) this MRD change has indicated the correct outcome.

Discussion

In this study, we report on MRD assessments at different checkpoints throughout conventional chemotherapy of 158 NPM1 mutated AML patients. All patients received an intensive high-dosed cytarabine induction therapy within one of three AMLCG trials. It was one of the major goals of this study to identify (I) relevant assessment checkpoints for MRD and (II) establish cut-off levels for the prediction of relapse.

NPM1mut ratio at diagnosis did not show any impact on survival, or on relapse occurrence. The MRD levels after induction therapy seemed to be the most appropriate checkpoint to identify CR patients at high risk for relapse. The cut-off values of the NPM1mut ratios and its kinetics after induction therapy revealed high HRs in multivariable analysis and yielded high values for sensitivity and specificity. Interestingly, the same cut-off levels after consolidation therapy had less impact on CIR 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 (25), a quick and deeper molecular response after induction therapy seems to be more prognostic 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 for relapse.

Krönke et al. (10), and Shayegi et al. (11) have analyzed clinically relevant MRD checkpoints in NPM1 mutated patients. They also identified the MRD assessment after induction therapy, or after the achievement of CR, as one of the most important MRD checkpoints. They demonstrated that positive MRD levels at this checkpoint identify patients at high risk for relapse and shorter OS. In contrast to Krönke et. al and in accordance to 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 to identify patients at high risk for relapse (see supplemental 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 CIR, we only observed a trend for OS (Figure 2). This might be caused by a smaller patient cohort, study design, the censored survival data at HSCT, or a short follow-up after relapse.

NPM1mut MRD monitoring and interpretation is still restricted to centralized laboratories. As a result of different RT-PCR assays, conditions, treatment protocols and clinical situations the estimated cut-offs at the different MRD checkpoints are almost not comparable. Shayegi et al. established clinical cut-offs by ROC analyses and cox regression models. After the achievement of CR they estimated a cut-off level of 1% and after HSCT a higher cut-off level of 10%(11). 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. Corresponding to our data, Schnittger et al. (17) also identified a 3 Log reduction to identify patients at high risk of relapse. Joint efforts such as the standardizations of Wilms tumor gen 1 expression (9) or the BCR-ABL (5) are needed. Unless there is no harmonization of the NPM1mut RT-PCR assays with subsequent interlaboratory comparison, a centralized MRD assessment and interpretation at one laboratory of each study group is obligatory.

The ELN has published a risk stratification considering the cytogenetics and molecular genetic analyses at diagnosis (1, 3). The ELN favorable risk group of our study cohort is the most clinical interesting subgroup. In contrast to the intermediate – I group (with an additional FLT3-ITD) HSCT is usually not recommended in these patients (26). However, in our patient cohort 45% of the NPM1mut patients without FLT3-ITD relapsed and we found no significant differences in relapse rate, remission duration, and OS between the prognostic ELN favorable and intermediate I group. This might be due to the heterogeneous age within the AMLCG trials without age restriction. Depending on the quantitative FLT3-ITD mRNA level (27), FLT3-ITDs might have a prognostic impact in NPM1mut AML and require a more individualized treatment. In other hematological malignancies like ALL and CML MRD guided therapies are already established. Within the MRC AML15 trial, a pre-emptive therapy based on MRD monitoring in APL patients has improved the outcome in a historical comparison to previously trials (7). However, in non-APL AML only a few prospective studies with MRD guided therapies are available (28-30).

Our results and those of others demonstrate the feasibility and reliability of serial NPM1mut MRD monitoring to identify patients at high risk for relapse (10, 11, 19). Since HSCT in NPM1mut patients does not impair the RFS (26) and as shown in our analyses, the MRD

cut-off positive patients are at high risk for relapse, these patients should be considered for a more intensified postremission therapy to improve relapse rate and OS and undergo HSCT, if eligible.

The instability of the MRD markers has been discussed as a problem of MRD guided therapy, i.e. FLT3-ITD can be unstable during follow-up (31, 32). The stability of NPM1mut has been discussed controversially. In the analyzed relapse samples of Schnittger et al.(17), all relapsed patients showed the initial NPM1mut at relapse. This is in contrast to our results and those of Krönke at al. (10), where mutated NPM1 was undetectable at relapse in 6.7%, and 9%. In our analyses, 1 of the 3 relapsed patients with undetectable NPM1mut at relapse developed relapse after more than 2 years with different chromosomal aberrations and a different LAIP, but with a stable DNMT3A mutation (33). The other two patients relapsed within 6 months after CR. One patient also showed the initial leukemia-associated immunophenotype with an additional JAK2 mutation at relapse (Supplement Table 2). Thus, in these patients a clonal evolution or a relapse of a subclone of the initial leukemia is very likely. Considering this aspect, the monitoring of a second stable molecular marker, e.g. DNMT3A (33), or the MRD monitoring 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 for relapse, especially in the clinical relevant subgroup of the ELN favorable risk patients. Particularly high MRD levels above our estimated cut-off after the induction therapy were strongly associated with a high CIR. This and previously published data of others demonstrate that in addition to the pre-therapeutic prognostic factors, the individual MRD course 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

MH, KS, MS, MF, WH designed the study; EZ, SS, AD, SKB performed molecular genetic analysis; MH, KS, EH, TK analyzed data and compiled statistics; MH, TK, MCS collected and documented clinical data; MCS, JB, WEB, TB, BW, and WH coordinated the AMLCG trials; KS, WH supervised the project; MH and KS wrote the manuscript.

The authors declare no conflict of interest.

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Tables

Table 1: Sampling and median NPM1mut 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
All patients						
Median NPM1mut ratio (range)	73.2 0.3 -947	1.4 0.000001 – 1010.0	0.008 0.000001 – 55.3	0.0003 0.000001 – 126.0	0.000001 0.000001 – 115.0	58.8 0.000001 – 326.0
Median NPM1mut kinetics* (range)	--	- 1.7 - 8.1 – 0.2	- 3.7 - 8.4 – 0.4	- 4.8 - 8.4 – 0.5	--	--
Patients in CR after induction therapy						
Median NPM1mut ratio (range)	71.6 0.3 -947	1.6 0.02 - 1010.0	0.009 0.000001 – 55.3	0.000001 0.000001 – 58.5	0.000001 0.000001 – 115.0	--
Median NPM1mut kinetics* (range)	--	- 1.6 - 4.2 – 0.2	- 3.7 - 8.4 – 0.4	- 5.5 - 8.4 – 0.2	--	--

Abbreviations: pts – patients; NPM1mut – NPM1 mutation; CR – complete remission;

* Log reduction in relation to the sample at diagnosis;

Table 2: Demographics and Clinical Characteristics of 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
Trisomie 4	2/154	1
Complex karyotype	3/154	2
Other	7/154	5
NPM1 mutation type		
A	136	86
B	11	7
D	11	7
FLT3-ITD		
Mutated	68/157	43
FLT3-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 CR1	30	19
Median Follow-up in months (range)	19 (1-109)	
Median WBC x 10^9 /L at diagnosis (range)	22 (1-406)	

Median LDH in U/L at diagnosis (range)	487 (108 – 14332)

Abbreviations: ITD – internal tandem duplication; TKD – tyrosine kinase domain; ELN – European LeukemiaNet; HSCT – allogeneic hematopoietic stem cell transplantation; CR1 – 1st complete remission; WBC – white blood cell count; LDH – lactate dehydrogenase;
* according to Döhner et al.(1)

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

Abbreviations: HR – hazard ratio; CI – Confidence Interval; PPV – positive predictive value; NPV – negative predictive value; N PM1mut – NPM1 mutation; NA – not analyzed;

* Cox regression model of all NPM1mut measurements for the occurence of relapse

** Cut-offs determined by Cox regression models

*** Cox regression model of NPM1mut Cut-off values for the occurence of relapse

**** Chi square test of 2 × 2 Contingency Tables

‡ Relapse within an observation period of 100 days during follow-up; Cut-off determined by Receiver-Operating Curve analysis;

Table 4: Multivariate Analyses

	CIR			OS		
Variable	**N=**	**HR (95% CI)**	**P**	**N=**	**HR (95% CI)**	**P**
In Aplasia	**42**			32		
MRD cut-off (NPM1mut ratio)		2.82 (1.08 – 7.31)	0.033		0.93 (0.11 – 7.83)	0.946
ELN (favorable vs 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 vs 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 (NPM1mut ratio)		6.16 (2.49 – 15.24)	0.0001		2.40 (0.84 -6.87)	0.103
ELN (favorable vs 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 10fold 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 vs 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 10fold 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 (NPM1mut ratio)		2.91 (1.16 – 7.31)	0.023		5.06 (1.46 – 17.49)	0.010
ELN (favorable vs 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 vs 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	

Abbreviatios: CIR – cumulative incidence of relapse; OS – overall survival; HR – hazard ratio; CI – confidence interval; NPM1mut – NPM1 Mutation; ELN – Risk stratifiation according to European LeukemiaNet (1); WBC – white blood cell count at diagnosis; LDH – lactate dehydrogenase at diagnosis; NA – not analyzed;

Legends to Figures

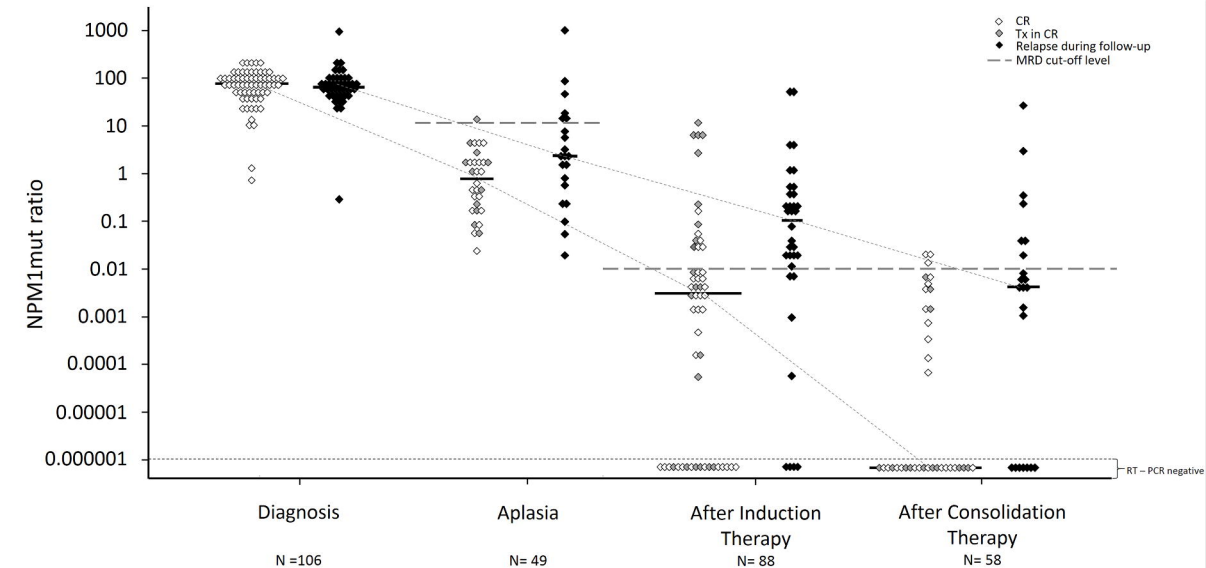
Figure 1: NPM1mut ratios of CR patients at different checkpoints
Black symbols indicate NPM1mut ratios at different checkpoints of patients developed relapse during follow-up, white symbols indicate NPM1mut ratios at different checkpoints of patients with ongoing remission. Grey symbols indicate NPM1mut ratios of patients with subsequent allogeneic stem cell transplantation in first complete remission. Bars indicate median NPM1mut ratios at the specific checkpoint.
Abbreviations: 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 with a subsequent allogeneic stem cell transplantation in first complete remission

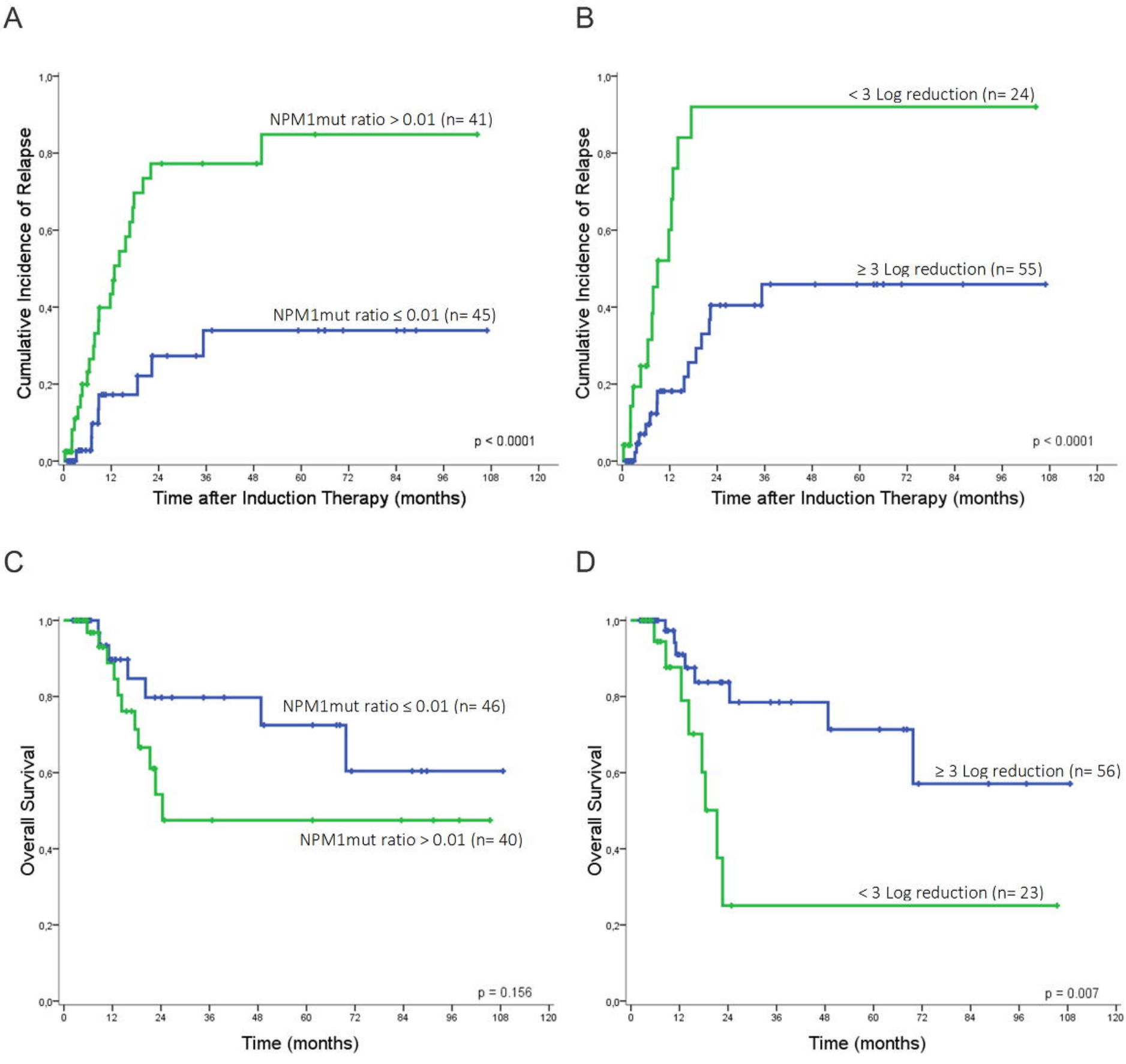
Figure 2: Cumulative incidence of relapse (Figure 2 A + B) and overall survival (Figure 2 C + D) after induction therapy according to MRD status of NPM1mut ratios and NPM1mut kinetics.
(A) + (C) NPM1mut ratios after induction therapy with NPM1mut cut-off ratio of 0.01; (B) + (D) NPM1mut kinetics after induction therapy with a cut-off of – 3 Log

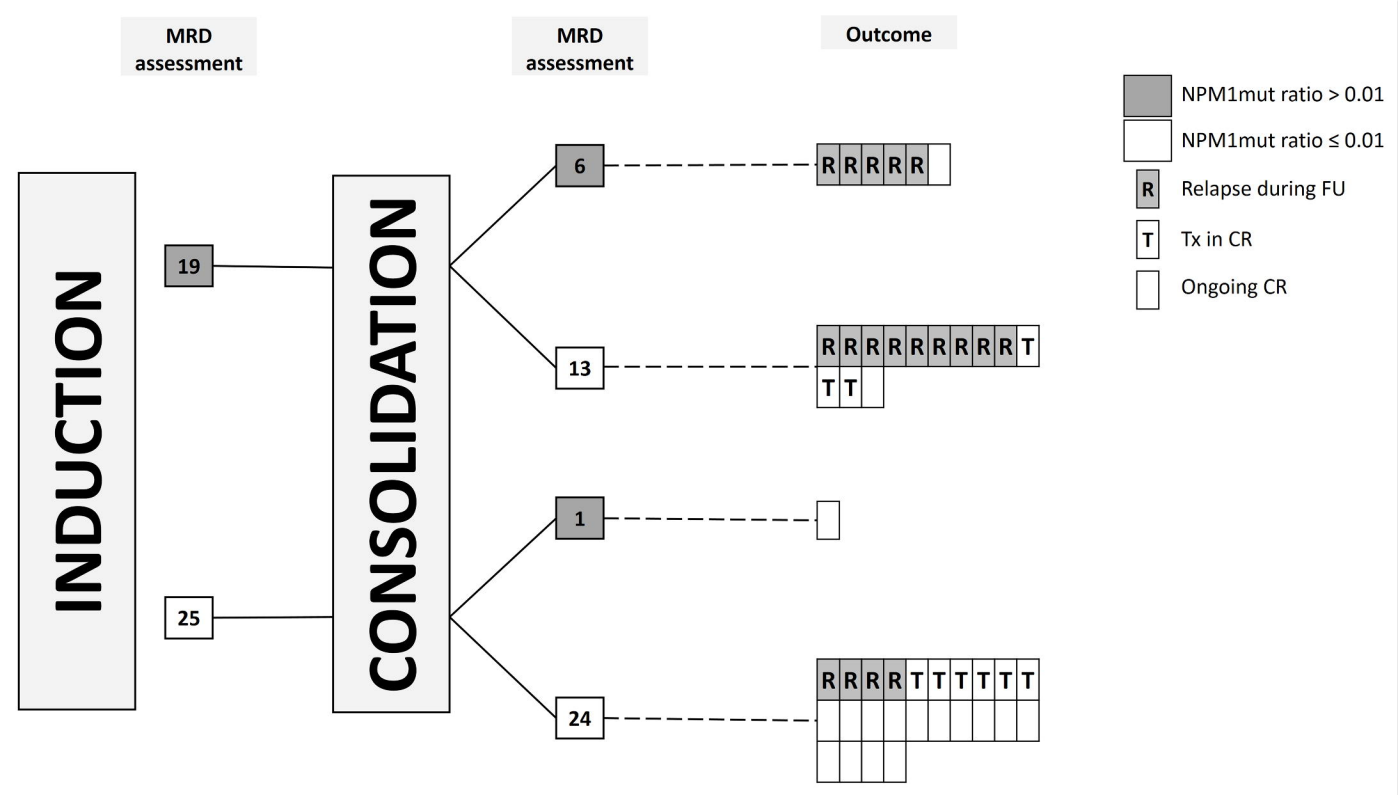
Abbreviations: NPM1mut – NPM1 mutation

Figure 3: MRD assessment after induction and consolidation therapy in paired samples
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 CR 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 time of 31.1 months after consolidation therapy.

Abbreviations: NPM1mut – NPM1 mutation; FU – follow-up period; CR – first complete remission; Tx – allogeneic stem cell transplantation in first complete remission


NPM1mut ratio
1000
100
10
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
CR
Tx in CR
Relapse during follow-up
MRD cut-off level
RT – PCR negative
Diagnosis
N =106
Aplasia
N= 49
After Induction Therapy
N= 88
After Consolidation Therapy
N= 58


A
Cumulative Incidence of Relapse
NPM1mut ratio > 0.01 (n= 41)
NPM1mut ratio ≤ 0.01 (n= 45)
p < 0.0001
Time after Induction Therapy (months)
B
Cumulative Incidence of Relapse
< 3 Log reduction (n= 24)
≥ 3 Log reduction (n= 55)
p < 0.0001
Time after Induction Therapy (months)
C
Overall Survival
NPM1mut ratio ≤ 0.01 (n= 46)
NPM1mut ratio > 0.01 (n= 40)
p = 0.156
Time (months)
D
Overall Survival
≥ 3 Log reduction (n= 56)
< 3 Log reduction (n= 23)
p = 0.007
Time (months)


MRD assessment
MRD assessment
Outcome
NPM1mut ratio > 0.01
NPM1mut ratio ≤ 0.01
R Relapse during FU
T Tx in CR
Ongoing CR
INDUCTION
19
25
CONSOLIDATION
6
13
1
24
R R R R R
R R R R R R R R T
T T
R R R R T T T T T T

Supplemental materials

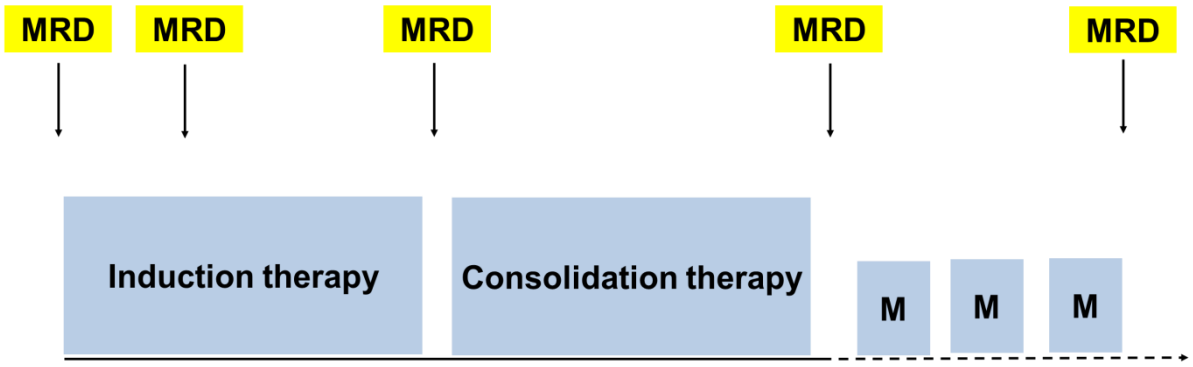
Additional statistical information

Analyses of differences were calculated by the Mann–Whitney U-test, the Kruskal-Wallis-test, or Student's t-test for unpaired data and with Wilcoxon's signed rank test or paired Student's t-test for paired data. Spearman's rank correlation was used to determine the coefficient of correlation as well as the corresponding p value.
To analyze the diagnostic power of the investigated different MRD cut-off values at the different MRD checkpoints, we used Cox's proportional hazards regression and calculate univariate as well as multivariate analyses to analyze the influence of additional baseline factors on the end points: (1) relapse and (2) overall survival. For the prediction of relapse within an observation time of 100 days during the follow-up period, we considered the absolute values before relapse or the peak value of measurements for patients without relapse during the follow-up, respectively. With the help of ROC we selected a cut-off for the prediction of relapse within 100 days in the follow-up period. Characteristics of all selected cut-offs were determined by the analysis of corresponding 2x2 contingency tables of test-positive and -negative cases (with relapse) and controls (without relapse).

RT-PCR negativity versus MRD cut-off ratio

We compared the results of the analyses on relapse of our estimated MRD cut-off after induction and consolidation therapy with the results of RT-PCR negativity at the specific time points. After induction therapy and after consolidation therapy MRD negativity showed inferior results with lower hazard ratios (Supplement Table 1). Likewise, the estimated cut-off of NPM1mut ratio of 0.01 showed a better separation of the cohort in CIR analysis (Supplement Figure 5).

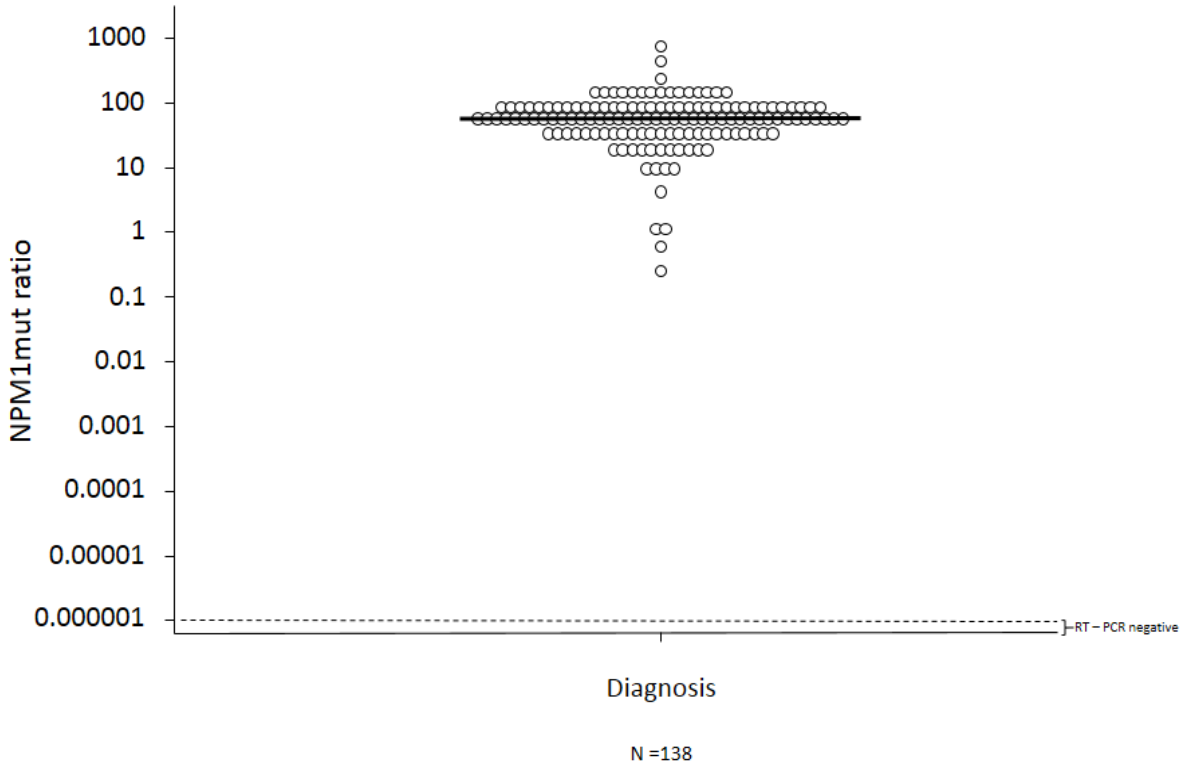
Supplement Figure 1: MRD sampling intervals



Recommended MRD sampling intervals within the AMLCG trials.

Abbreviations: MRD – minimal residual disease; M – three years maintenance therapy of monthly alternating chemotherapy regimens

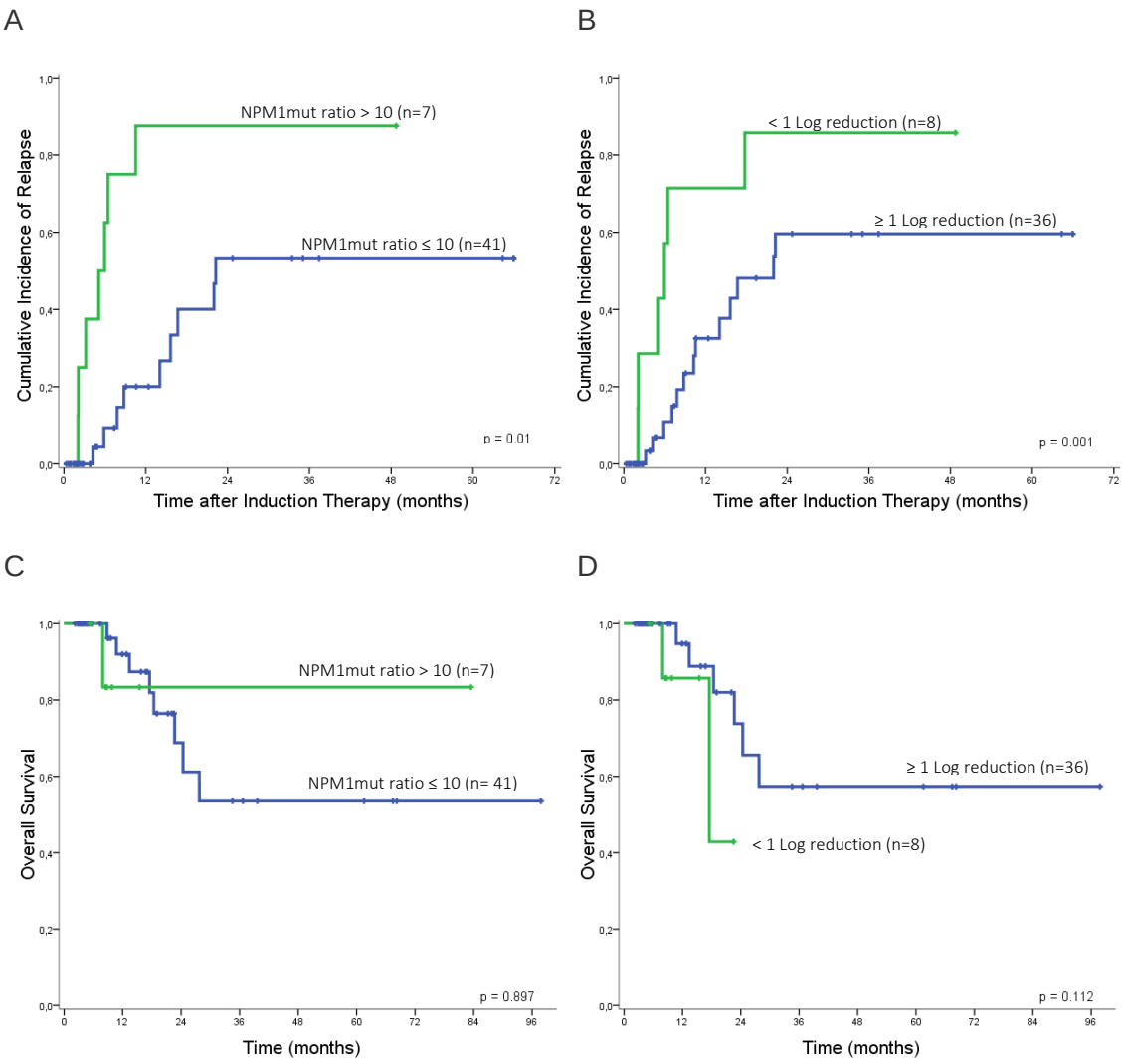
Supplement Figure 2: NPM1mut ratios of all patients at diagnosis



NPM1mut ratios of all patients at diagnosis.

Abbreviations: NPM1mut – NPM1 mutation; RT - PCR – quantitative real-time polymerase chain reaction;

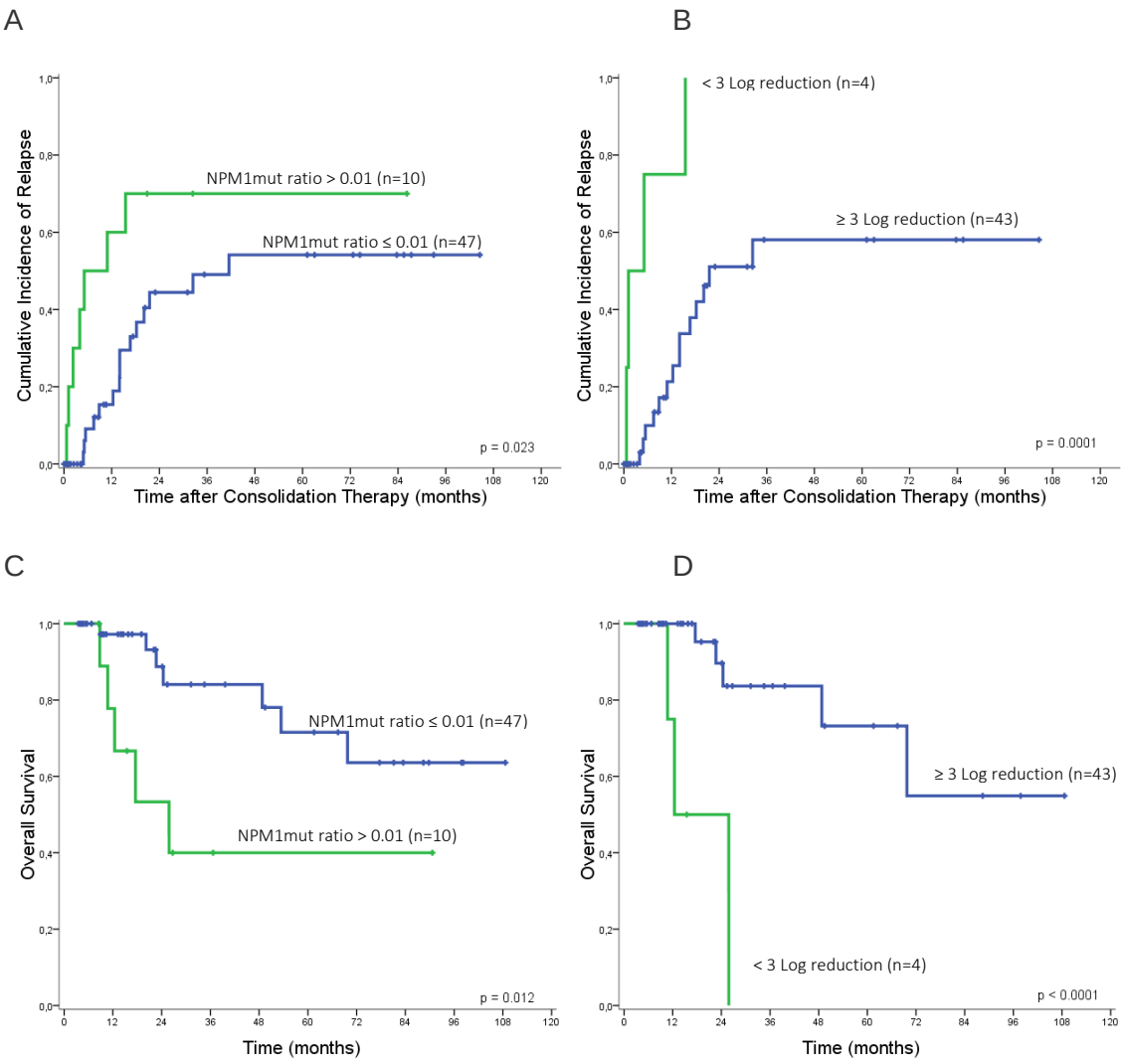
Supplement Figure 3: CIR and OS of patients according to the MRD status in aplasia during induction therapy



(A) + (C) NPM1mut ratios in aplasia with NPM1mut cut-off ratio of 10; (B) + (D) NPM1mut kinetics in aplasia with a cut-off of – 1 Log.

Abbreviations: NPM1mut – NPM1 mutation

Supplement Figure 4: CIR and OS of patients according to the MRD status after consolidation therapy

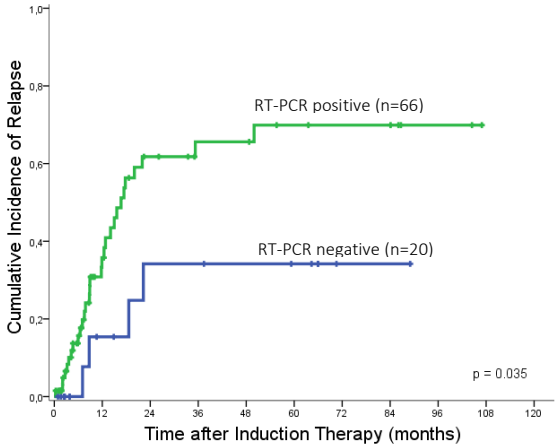


(A) + (C) NPM1mut ratios after consolidation therapy with NPM1mut cut-off ratio of 0.01; (B) + (D) NPM1mut kinetics after consolidation therapy with a cut-off of – 3 Log.

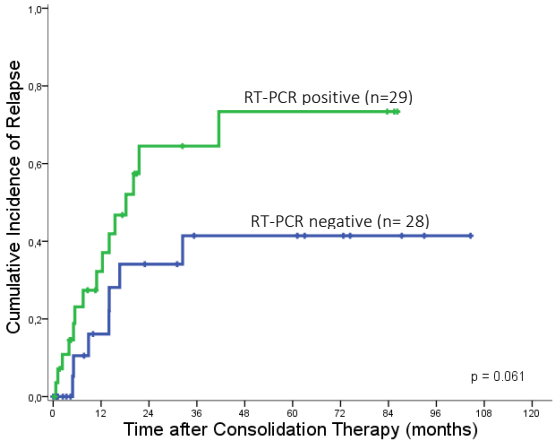
Abbreviations: NPM1mut – NPM1 mutation

Supplement Figure 5: CIR of patients according to RT-PCR negativity after induction (A) and consolidation therapy (B)

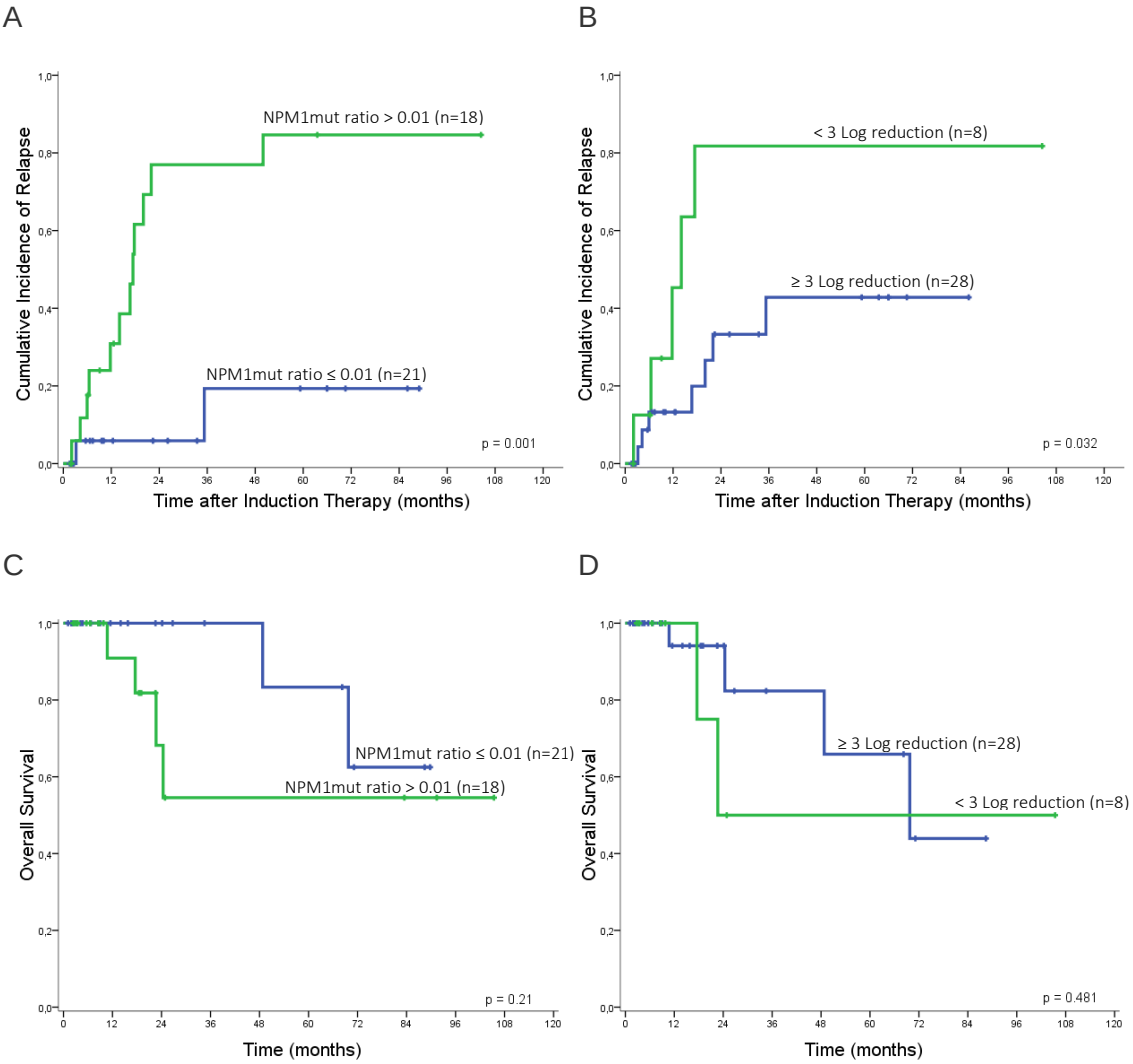
A



B



Supplement Figure 6: CIR and OS of patients within the ELN favorable risk group according to the MRD status after induction therapy.



(A) + (C) NPM1mut ratios after induction therapy with NPM1mut cut-off ratio of 0.01; (B) + (D) NPM1mut kinetics after induction therapy with a cut-off of – 3 Log.

Abbreviations: NPM1mut – NPM1 mutation

Supplement Table 1: Comparison of results of relapse analyses of estimated MRD cut-off with RT-PCR negativity

MRD checkpoint	Cut-off*	HR Cut-off (95% CI)**	P **	Sensitivity	Specificity	PPV	NPV	P ***
After Induction Therapy	0.01	4.26 (1.93 – 9.45)	<0.0001	76% (26/34)	74% (40/54)	65% (26/40)	83% (40/48)	<0.0001
	RT-PCR negative	2.93 (1.03 – 8.35)	0.045	88% (30/34)	33% (18/54)	45% (30/66)	82% (18/22)	0.041
After Consolidation Therapy	0.01	2.72 (1.10 – 6.69)	0.03	32% (7/22)	92% (33/36)	70% (7/10)	69% (33/48)	0.03
	RT-PCR negative	2.31 (0.94 – 5.70)	0.07	68% (15/22)	61% (22/36)	52% (15/29)	76% (22/29)	0.057

Abbreviations: HR – hazard ratio; CI – Confidence Interval; PPV – positive predictive value; NPV – negative predictive value;

* Cut-offs determined by Cox regression models

** Cox regression model of NPM1mut Cut-off values for the occurrence of relapse

*** Chi square test of 2 × 2 Contingency Tables

Supplement Table 2. Patients characteristics and laboratory findings of patients who lost NPM1 mutation at relapse (n=3)

Patient-ID	99185	99074	45019*
Study	**AMLCG99**	**AMLCG99**	**AMLCG99**
Age in years	**42**	**64**	**60**
Time to relapse in months	**5**	**6**	**38**
NPM1mut	**A**	**A**	**A**
Karyotype at diagnosis	**NK**	**NK**	**NK**
Karyotype at relapse	**NK**	**NK**	**Translocation (1;7)**
BM blast at diagnosis in %	**79**	**82**	**95**
BM blast at relapse in %	**30**	**18**	**unknown**
LAIP at diagnosis	**HLA-DR/CD33/CD34**	**CD65/CD87/CD34**	**CD34/CD56/CD33**
LAIP at relapse	**initial LAIP undectable**	**CD65/CD87/CD34**	**CD15/CD13/CD33**
Additional molecular findings at diagnosis	**--**	**--**	**FLT3-ITD, DNMT3A mutation**
Additional molecular findings at relapse	**--**	**JAK2 mutation**	**DNMT3A mutation**
NPM1mut ratio at diagnosis	**40.9**	**47.5**	**5.1**
NPM1mut ratio at relapse	**0.000001**	**0.000001**	**0.000001**

Abbreviations: NPM1mut – NPM1 mutation; NK – normal karyotype; BM – bone marrow; LAIP – leukemia-associated immunophenotype;

* this patient was already published by Papadaki et al.[18]