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Pre-transplant Quantitative Determination of *NPM1* Mutation Significantly Predicts Outcome of AIlogeneic Hematopoietic Stem Cell Transplantation in Patients with Normal Karyotype AML in Complete Remission

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Abstract. *Background/Aim: Minimal residual disease (MRD) in patients with acute myeloid leukemia (AML) before allogeneic hematopoietic stem cell transplantation (alloHSCT) can influence the results of therapy. With the aim of evaluating the potential role of pre-transplant MRD, we studied the impact of pre-transplant MRD level on the outcome of alloHSCT in patients with AML in complete remission (CR). Patients and Methods: From 2/2005 to 9/2014, 60 patients with a median age of 54 years (range=30-66 years) with normal karyotype-AML harboring nucleophosmin 1 (NPM1) mutation [53% Fms-related tyrosine kinase receptor 3 internal tandem duplication (FLT3/ITD)-positive] in first (n=45) or second (n=15) CR underwent myeloablative (n=16) or reduced-intensity (n=44) alloHSCT (27% related, 73% unrelated). The MRD level was determined from bone marrow samples using real-time polymerase chain reaction for detection of NPM1 mutations before starting the conditioning regimen. Results: The estimated probabilities of 3-year relapse, event-free survival (EFS) and overall survival (OS) for the whole cohort were 28%, 54%, and 59%, respectively. Statistical analysis showed that only age over 63 years and high MRD level affected alloHSCT outcome. Pre-transplant MRD level of 10 mutant copies of NPM1 per 10,000 Abelson murine leukemia viral oncogene homolog 1 (ABL) copies had the strongest statistical significance, and detection of higher MRD level (>10 NPM1-mutant copies) before alloHSCT was associated with increased overall mortality (hazard ratio=3.71; 95% confidence interval=1.55-9.06; p=0.004). The estimated probabilities of 3-year relapse, EFS, and OS were 6%, 72%, and 75% for patients with a low level of MRD and 48%, 35%, and 40% for patients with a higher level. Conclusion: Our data showed that the pre-transplant level of MRD in patients with normal karyotype AML harboring NPM1 mutation in CR provides important prognostic information, which as an independent prognostic factor predicts transplant results.*

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Acute myeloid leukemia (AML) is currently curatively treated using an intensive induction chemotherapy treatment, which generally achieves complete remission (CR) in about 60-80% of cases. However, without further consolidation treatment, relapse would occur in most patients. Consolidation treatment options essentially consist of either further chemotherapy or allogenic hematopoietic stem cell transplantation (alloHSCT), and a range of prognostic factors are important for the choice of consolidation therapy (1-5). While the strategies and methods of curative treatment of AML have undergone no major changes over the last 20 years, the past decade has seen an improvement in treatment results due to more detailed insight into AML biology and improvements in supportive treatment, which make it possible to manage once-fatal complications of intensive chemotherapy or alloHSCT. AlloHSCT is currently the most effective treatment for AML. Mainly due to improvements in supportive treatment and transplant procedures, mortality from transplant (TRM) has fallen significantly, and a wider spectrum of patients can now undergo alloHSCT (6, 7). At

the same time, a number of studies have investigated the role of alloHSCT in AML treatment (8-12). With TRM decreasing, AML relapse after alloHSCT remains the principal limitation to transplant outcomes, especially because the prognosis of AML relapse after alloHSCT is highly unfavorable (13). For this reason, efforts are underway to identify additional prognostic factors that may help determine which patients are at increased risk of AML relapse after alloHSCT.

In recent years, the importance of the detection of minimal residual disease (MRD) for the prediction of AML treatment outcomes has been rising. With regard to chemotherapy of AML, the published data seems to indicate that treatment response evaluation based on MRD detection is among the independent prognostic factors relevant to risk of relapse and therefore to AML treatment results (14-26). In alloHSCT, where the efficacy of treatment is significantly influenced by the graft-*versus*-host disease (GVHD) effect and which has proven effective even in the case of chemoresistant AML, with a reported long-term survival of 20-30%, it is important to ask whether a finding of MRD before alloHSCT has an impact on transplant outcome (27, 28). Moreover, most patients with AML undergo alloHSCT in CR, *i.e.* at a time when any potential residual disease is generally lower than in resistant AML. Considering the importance of the GvL effect, it is a key question whether determining MRD in AML in CR before alloHSCT can offer prognostic information about the treatment outcome, as it does with intensive chemotherapy. The importance of determining MRD before alloHSCT is thus the subject of intensive research; findings published so far, which mostly used multiparametric flow cytometry to detect MRD, indicate that determining MRD before alloHSCT may carry prognostic information about the transplant outcome. However, the existing research is frequently limited by a small number of studied patients, inclusion of heterogeneous AML types, different diagnostic methods for MRD determination, as well as the fact that support for MRD importance is not universal among these studies (29-35).

With the aim of evaluating the importance of determining the level of MRD levels before alloHSCT in patients with AML in CR, we analyzed alloHSCT outcomes in patients in first or second CR of normal karyotype AML (NK-AML) with *NPM1* gene mutation. AML with normal karyotype represents the largest group of patients with AML and the most common molecular lesion in this group is a mutation in the gene encoding nucleophosmin (NPM1). *NPM1* mutation was also previously found to be a suitable and stable marker of MRD in a number of published studies (16-19). We used the relative expression of the mutated *NPM1* gene, evaluated using real-time polymerase chain reaction (RT-PCR), as a marker for MRD monitoring immediately prior to the start of the conditioning regimen.

Patients and Methods

Study group. Our study group was made up of all patients aged 18 and above diagnosed with NK-AML with an *NPM1* gene mutation (types A, B, and D) at the Department of Hematology and Oncology of the University Hospital in Pilsen and who underwent alloHSCT in first or second CR between January 2005 and September 2014. AML was diagnosed according to the World Health Organization (WHO) classification (36).

Cytogenetic examination by G-banding was performed on all AML samples at the time of diagnosis. *NPM1* gene mutation presence and type were determined by DNA sequencing and quantitative examination of initial relative expression of mutated NPM1 was also performed. As part of the molecular genetic marker assay, presence of Fms-related tyrosine kinase *receptor* 3 internal tandem duplication (*FLT3/ITD*) was also determined for all patients at the time of diagnosis. Complete remission of AML was evaluated according to standard recommendations (37).

Human leukocyte antigen (HLA) typing of donors and recipients was performed according the European Federation for Immunogenetics/European Society for Blood and Marrow Transplantation (EFI/EBMT) recommendations (accessible at www.efiweb.eu/efi-committees/standards-committee.htlm) in an EFI-accredited laboratory. Acute and chronic GVHD was diagnosed according to published criteria (38, 39).

All patients were evaluated post-transplant for overall survival (OS), event-free survival (EFS), TRM, and incidence of relapse. All patients were treated according to protocols approved by the Quality Control Boards of a Joint Accreditation Committee-ISCT and EBMT accredited facility and all patients provided consent for monitoring and data processing in accordance with the Declaration of Helsinki.

Detection of MRD. Bone marrow sample collection for MRD detection was performed no later than 1 week before the start of the pre-transplant conditioning regimen. RNA was isolated from bone marrow samples using the commercial QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized from 500 ng of RNA using SuperScript III First Strand Synthesis SuperMix commercial kit (Invitrogen, Carlsbad, CA, USA).

Quantitative real-time PCR was performed on the samples using the NPM1Quant kit (Ipsogen SA, Marseille, France). The calibration curves for the quantitative assessment of the number of copies of the mutated *NPM1* gene and the control gene *ABL* were determined based on the standards supplied with the kit for all analyses. All samples were analyzed in duplicate, using the average of the two analyses in further calculations. If the threshold cycle (Ct) discrepancy between the two samples exceeded 0.6 cycles, the analysis was repeated. The minimum required expression for the *ABL* gene control was set at 3,000 copies. The results of the expression analysis of the mutated *NPM1* gene are given as the number of copies of mutated *NPM1* per 10,000 copies of *ABL*. Amplification and data analysis were performed on Light Cycler version 1.5 or version 2.0 devices (Roche Applied Science, Mannheim, Germany). The assays were performed at the accredited Molecular Genetics Laboratory of Department of Hematology and Oncology, Faculty Hospital, Plzen, Czech Repblic and the method of relative quantification of mutated *NPM1* was regularly verified, validated and monitored as part of external inter-laboratory quality control processes.

Table I. *Characteristics of the study group of patients with acute myeloid leukemia (AML).*

Characteristic	Value
Median of age (range), years	54 (30-66)
Gender, n (%)	
Male	32 (53%)
Female	28 (47%)
AML status, n (%)	
CR1	45 (75%)
CR2	15 (25%)
FLT3/ITD positivity, n (%)	32 (53%)
Conditioning protocol, n (%)	
Myeloablative	16 (27%)
Reduced-intensity	44 (73%)
Type of donor, n (%)	
Related	16 (27%)
Unrelated	44 (73%)
Gender recipient/donor, n (%)	
Male/female	12 (20%)
Other	48 (80%)
CMV status recipient/donor,n (%)	
Negative/negative	6 (10%)
Other	54 (90%)
Source of stem cells, n (%)	
Bone marrow	12 (20%)
PBSC	48 (80%)
WHO status, n (%)	
0-1	60 (100%)
≥2	0 (0%)

CR: First complete remission; CR2: second complete remission; *FLT3/ITD*: Fms-related tyrosine kinase receptor 3 internal tandem duplication; CMV: cytomegalovirus; PBSC: peripheral blood stem cells.

Statistical analysis. Patient characteristics were summarized using frequency tables and standard descriptive statistics, Pearson chi-square test, Fisher's exact test, and *t*-test.

OS was calculated from the date of alloHSCT until death from any cause, and surviving patients were censored at the last follow-up. The EFS was calculated from the date of alloHSCT until death or relapse, and patients who were alive and disease-free were censored at the last follow-up. Probabilities of OS and EFS were estimated using the Kaplan–Meier method. TRM was defined as death due to any cause unrelated to disease. Probabilities of TRM and relapse were summarized using cumulative incidence estimates. Cumulative incidence of TRM and relapse were adjusting for competing risk. TRM was a competing risk for relapse, while relapse was a competing risk for TRM. Univariate analyses to evaluate differences in survival between groups of patients were performed using the log-rank and Wilcoxon tests. The Cox proportional hazards model was considered for the survival modeling to specify the role of individual prognostic factors in assessing the OS and EFS. The multivariable Cox proportional hazards model (stepwise regression) was used for identification of the significant prognostic factors in OS and EFS. The level of statistical significance of α=0.05 was used in all analyses. All computations were performed using SAS software (SAS Institute Inc., Cary, NC, USA) and STATISTICA software (StatSoft, Inc., Tulsa, OK, USA).

Table II. *Transplant results of the entire study group.*

Characteristic	n (%)
Acute GVHD	36 (60%)
Acute GVHD III-IV	8 (13%)
Chronic GVHD	24 (40%)
Mild chronic GVHD	12 (20%)
Moderate chronic GVHD	8 (13%)
Severe chronic GVHD	4 (7%)
3-Year cumulative relapse	28%
3-Year cumulative TRM, %	21%
3-Year EFS	54%
3-Year OS	59%

GVHD: Graft-*versus*-host disease; TRM: transplant-related mortality; EFS: event-free survival; OS: overall survival.

Results

Patient characteristics. The group of patients consisted of 60 individuals (32 women, 28 men), with a median age of 54 years (range: 30-66) with *NK-AML* and NPM1 mutation. *FLT3/ITD* was found in 32 patients (53%) at the time of diagnosis. All patients underwent alloHSCT, most (73%) from an unrelated donor. More patients underwent alloHSCT after reduced-intensity conditioning (RIC) (73%) than after myeloablative conditioning (MAC). The RIC consisted of a combination of fludarabine (30 mg/m^2/day for 4 days) and melphalan (140 mg/m^2/day for 1 day); the MAC consisted of a combination of busulfan (3.2 mg/kg/day *i.v.* for 4 days) and cyclophosphamide (60 mg/kg/day for 2 days). In unrelated alloHSCT, ATG Fresenius S (15 mg/kg dose) was used as part of the conditioning regimen. The source of the hematopoietic stem cells was mainly peripheral blood stem cells (80%).

At the time of transplant, the WHO performance status of patients was between 0 and 1. The median follow-up of surviving patients was 55 months (range=6-101 months). Cyclosporine A and methotrexate were administered to all patients as GVHD prophylaxis. Patient characteristics are summarized in Table I.

Transplant outcomes of the entire study group. All patients underwent transplant and were in CR at day 30 post-transplant. Although many patients developed acute GVHD (60%), only eight developed acute GVHD grade III-IV.

With a median follow-up of 55 months (range=6-101 months), 36 patients (60%) remained alive. Out of the entire

Table III. *Univariate analysis of factor affecting event-free survival (EFS) and overall survival (OS).*

	EFS			OS		
Variable	HR	95% CI	*p*-Value	HR	95% CI	*p*-Value
Age >63 years	3.40	1.24-9.62	0.0341	5.40	1.82-16.02	0.0071
High *vs*. low MRD*	3.69	1.60-8.51	0.0021	3.50	1.40-8.47	0.0034
FLT3/ITD positivity	1.29	0.60-2.78	0.51	1.05	0.47-2.34	0.90
CR2 *vs*. CR1	1.72	0.77-3.87	0.19	1.92	0.82-4.49	0.13
RIT *vs*. MAT	1.14	0.48-2.71	0.76	1.71	0.63-4.58	0.29
Unrelated *vs*. related donor	0.98	0.42- 2.32	0.97	1.22	0.45- 2.83	0.81
BM *vs*. PBSCs	1.18	0.50-2.81	0.70	1.09	0.43-2.74	0.86
Recipent M/donor F *vs*. other	1.00	0.38-2.66	0.99	1.77	0.53-5.95	0.35
CMV donor/recipient positive *vs*. other	2.16	0.97-4.83	0.06	1.75	0.76-3.99	0.18

HR: Hazard ratio; CI: confidence interval; CR1: first complete remission; CR2: second complete remission; RIT: reduced-intesity transplantation; MAT: myeloablative transplantation; BM: bone marrow; PBSC: peripheral blood stem cells; M: male; F: female. *High level of minimal residual disease (MRD): nucleophosmin 1 (*NPM1*) >10 copies/10,000 copies Abelson murine leukemia viral oncogene homolog 1 (*ABL*).

Table IV. *Multivariate analysis of factors affecting event-free survival (EFS) and overall survival (OS).*

	EFS			OS		
Variable	HR	95% CI	*p*-Value	HR	95% CI	*p*-Value
Age >63 years	3.40	1.24-9.62	0.0341	6.23	1.99-19.48	0.0017
High *vs*. low MRD*	3.69	1.60-8.51	0.0021	3.71	1.52-9.06	0.0040

*High level of minimal residual disease (MRD): nucleophosmin 1 (*NPM1*) >10 copies/10,000 copies Abelson murine leukemia viral oncogene homolog 1 (*ABL*).

group, 16 patients experienced relapsed. The median time from transplant to relapse was 4 months (range=3-13 months). Thirteen patients died as a result of relapse, two patients achieved a subsequent CR lasting 51 and 61 months, respectively, and one patient was alive in relapse.

Eleven patients (18%) had died due to TRM. The most common cause of death (64% of TRM cases) was infectious complications related to acute or chronic GVHD. One-year TRM was 13%. Estimated 3-year EFS, OS, cumulative incidence of TRM, and cumulative incidence of relapse were, for the whole group, 54%, 59%, 18%, and 28%, respectively. Transplant outcomes for the entire study group are summarized in Table II and Figure 1.

Importance of pre-transplant prognostic markers for transplant outcome. In univariate analysis of the listed pre-transplant prognostic factors, only age over 63 years and the pre-transplant MRD level (most significant for those above 10 mutated *NPM1* copies per 10,000 *ABL* copies) had a statistically significant negative impact on EFS and OS. A negative trend in EFS and OS was found for alloHSCT having been performed in the second (as opposed to first) CR and for positive serological cytomegalovirus (CMV) status of donor and recipient, but these trends were not statistically significant. Univariate analysis of factors affecting EFS and OS is summarized in Table III.

Multivariate analysis of the listed pre-transplant factors confirmed a statistically significant negative prognostic impact on EFS and OS for age over 63 years and the level of pre-transplant MRD (most significant for levels above 10 mutated *NPM1* copies per 10,000 *ABL* copies). Multivariate analysis results are summarized in Table IV.

Importance of pre-transplant residual disease level for transplant outcome. As the relative expression of mutated *NPM1* was known at the time of *AML* diagnosis [median=38,245 (range=14,350-144,707) mutated *NPM1* copies per 10,000 *ABL* copies], it was possible to evaluate pre-transplant MRD with two different methods: (i) as solely the pre-transplant relative expression of mutated *NPM1*, and (ii) as the decrease in relative expression of mutated *NPM1* between initial AML diagnosis and immediately before the

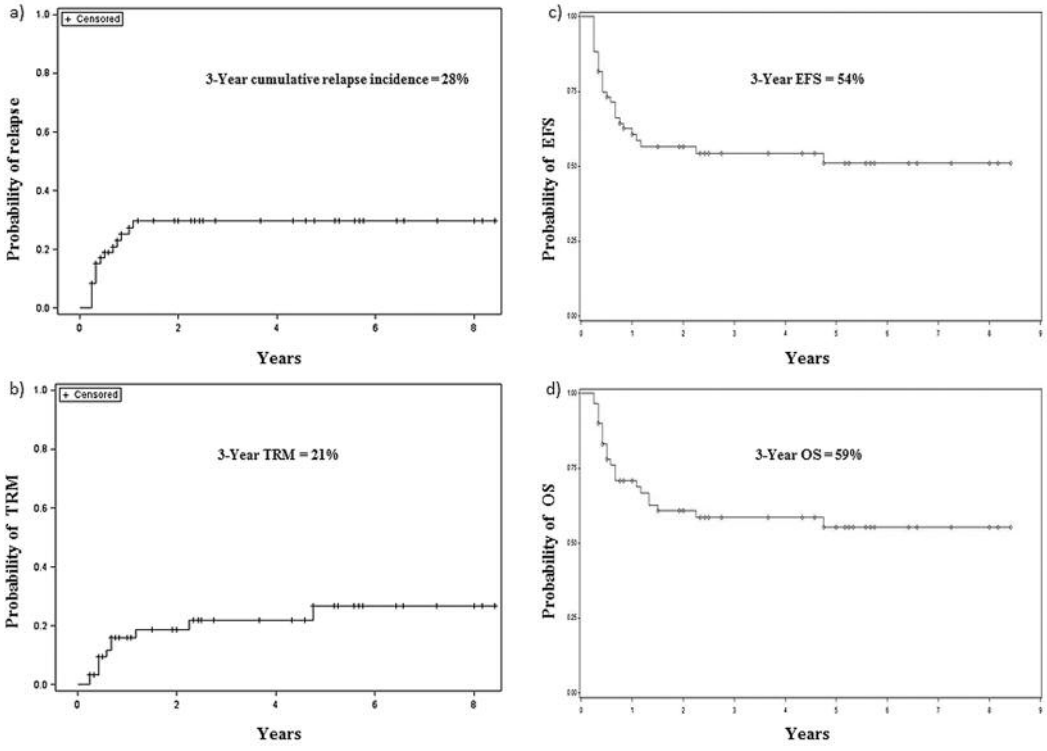

Figure 1. *Probability of relapse (a), transplant-related mortality (b), event-free survival (EFS) (c) and overall survival (OS) (d) for the entire group of patients.*

transplant procedure. Higher levels of statistical significance were achieved using the first method, *i.e.* measuring only the pre-transplant level of relative expression of mutated *NPM1*. We set the cut-off point at 10 mutated *NPM1* copies per 10,000 ABL copies, as this division of the patient group produced the most statistically significant difference in EFS and OS, although statistically significant differences were also found for several other cut-off values (see Figure 2).

The division of the patient cohort by pre-transplant MRD level (more or less than 10 mutated *NPM1* copies per 10,000 *ABL* copies) reveals a marked difference in the results of the alloHSCT. Considering the entire patient group, 16 patients (28%) experienced relapse. However, in the low-MRD group there were only two patients with relapse (6% of the low-MRD group), while in the high-MRD group there were 14 (48% of the high-MRD group). A total of 11 patients (18%) died due to TRM, five (17%) in the high-MRD group and six (19%) in the low-MRD group. Estimated 3-year EFS and OS for the entire study group were 54% and 59%, respectively. Risk of relapse or death was 3.69 times higher in the high-MRD group than in the low-MRD group [hazard ratio (HR)=3.69; 95% confidence interval (CI)=1.60-8.51, p=0.0021). Estimated 3-year EFS was 35% in the high-MRD group – significantly lower than the 72% found in the low-MRD group (p=0.0021). This is presented in Figure 3a.

Table V. *Outcome probalities stratified by minimal residual disease (MRD) status whereby a high level of MRD was defined as nucleophosmin 1 (NPM1) >10 copies/10,000 copies Abelson murine leukemia viral oncogene homolog 1 (ABL).*

3-Year endpoint	Low MRD	High MRD
CIR	6%	48%
EFS	72%	35%
OS	75%	40%

CIR: Cumulative incidence of relapse; EFS: event-free survival; OS: overall survival.

Risk of death was 3.71-times higher in the high-MRD group than in the low-MRD group (HR=3.71; 95% CI=1.52-9.06, p=0.0040). The estimated 3-year OS rate was 40% in the high-MRD group – significantly lower than the 75% found in the low-MRD group (p=0.004). This may be seen in Figure 3b.

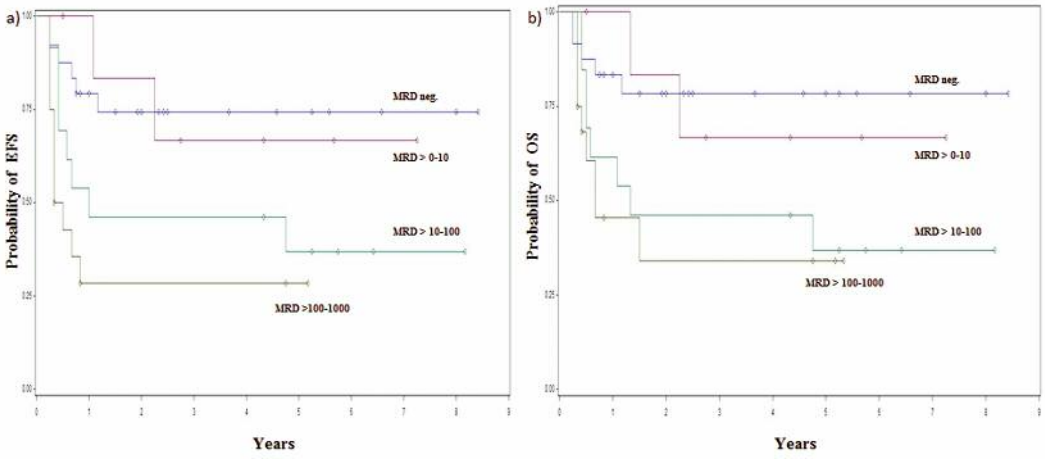

Figure 2. *Probability of event-free (EFS) (a) and overall (OS) (b) survival for transplanted patients stratified by pre-transplant minimal residual disease (MRD) level [mutated nucleophosmin 1 (NPM1) copies/10,000 copies Abelson murine leukemia viral oncogene homolog 1 (ABL)].*

The transplant outcomes for the patients according to pre-transplant MRD level are summarized in Table V.

Discussion

We investigated the importance of pre-transplant MRD level for alloHSCT outcome in patients with NK-AML with a mutation of the *NPM1* gene in first or second CR of AML. While our sample size is somewhat small, it has the virtue of being homogeneous in both diagnosis and treatment. We determined MRD according to the relative expression of mutated *NPM1*, evaluated using standardized RT-PCR. *NPM1* mutation was previously found to be a suitable and stable marker of MRD in a number of published studies (16-19, 40-42). Our sample is among the largest of studies of patients with AML examining the impact of pre-transplant MRD quantified by the expression of mutated *NPM1* on transplant outcome. Thanks to a distinct molecular genetic marker, the use of RT-PCR eliminates certain limitations of multiparametric flow cytometry – in particular, its lower sensitivity and the risk of antigenic shifts in the leukemia cells (33, 43-45). MRD was determined immediately (less than 1 week) prior to the start of conditioning regimen. This approach reduces the risk of error in MRD measurement arising from potentially fast changes in MRD in patients with AML (46, 47). Other published studies either do not disclose the time interval between MRD assessment and the transplant procedure, or this interval was longer than in our study (34, 48).

The results of our analysis show that in our sample population, the pre-transplant MRD level in patients with NK-AML and *NPM1* gene mutation in CR was an independent prognostic factor for alloHSCT outcome. In our study group, determining the pre-alloHSCT relative expression of mutated *NPM1* proved to be a superior method of predicting alloHSCT outcome compared to the evaluation of the decrease of mutated *NPM1* expression between diagnosis and the transplant procedure. We assume this is due to a large range of expression levels of mutated *NPM1* in the AML diagnostic samples. In our study group, these results were statistically significant for several levels of pre-transplant mutated *NPM1* expression (negative *vs*. positive; more *vs*. less than 1; more *vs*. less than 10; more *vs*. less than 100 mutated *NPM1* copies per 10,000 *ABL* copies), which suggests that alloHSCT outcomes (EFS, OS) deteriorate with increasing pre-transplant MRD. This finding is not completely in line with some prior published studies, where a difference in alloHSCT outcomes had been documented only between MRD-positive and MRD-negative patients, and the impact on transplant outcome of different levels of positivity among MRD-positive patients was not further documented (29, 31). This might be explained by the fact that these studies used a less sensitive method of MRD detection (multiparametric flow cytometry), as well as by their lower number of enrolled MRD-positive patients which might have caused further sample divisions to fall short of statistical significance.

In our study, we chose a cut-off point of 10 mutated NPM1 copies per 10,000 ABL copies (0.1% mutated NPM1 to ABL ratio); this cut-off value divided the entire patient cohort into two groups between which the statistical significance of the difference in EFS and OS was highest, in univariate as well as multivariate analysis. Patients with an *NPM1* mutation in CR and higher pre-transplant MRD level (>10 mutated *NPM1* copies per 10,000 *ABL* copies) exhibited higher incidence of relapse and lower EFS and

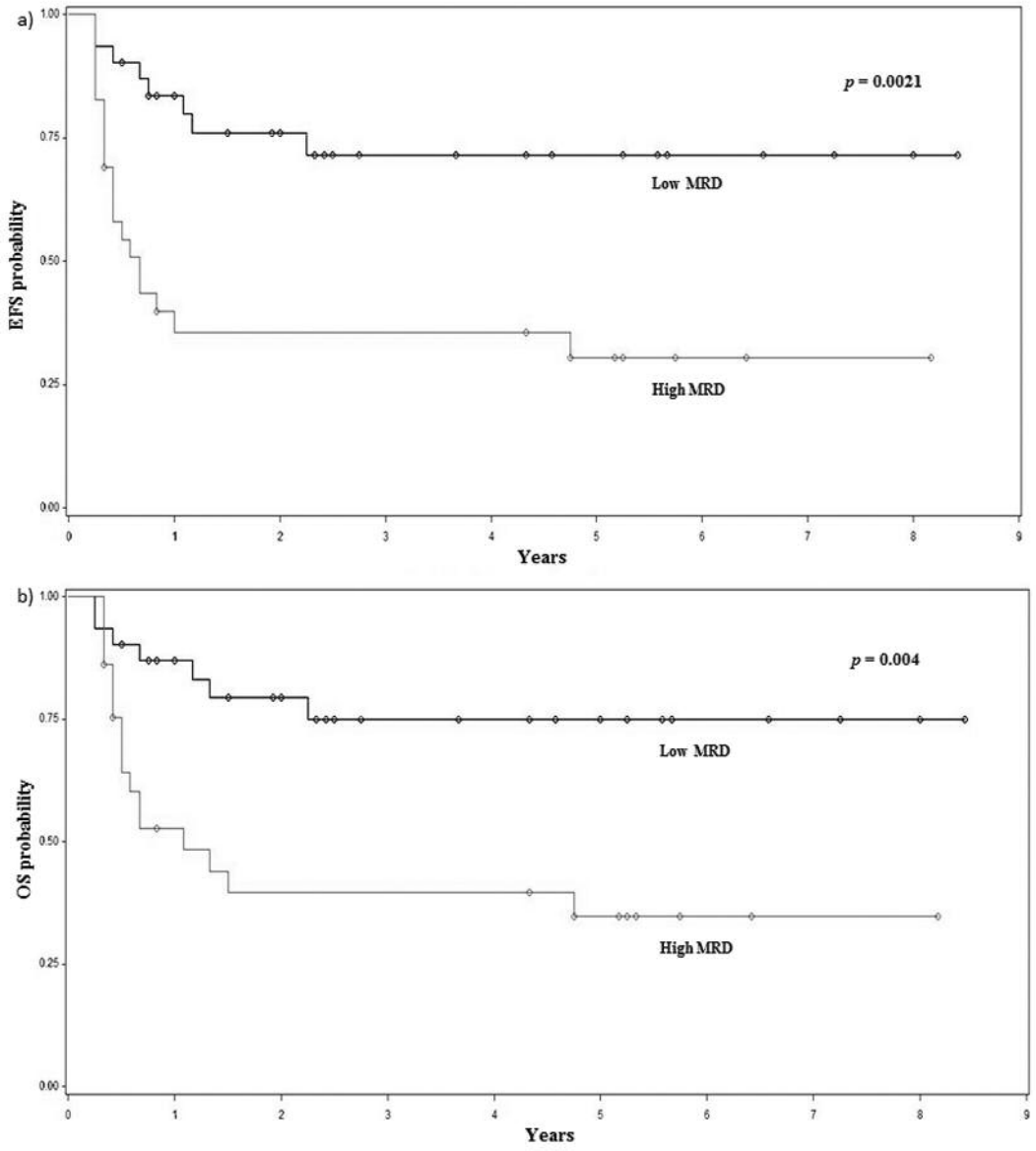

Figure 3. *Probability of event-free (EFS) (a) and overall (OS) (b) survival for patients with acute myeloid leukemia (AML) with negative/low level vs. high level pre-transplant minimal residual disease (MRD) status [defined by cut-off of 10 mutated nucleophosmin 1 (NPM1) copies/10,000 copies Abelson murine leukemia viral oncogene homolog 1 (ABL)].*

overall survival, while TRM was not significantly different between the two groups. Our results thus provide further support for the importance of MRD determination in alloHSCT prognosis among patients with AML, in line with the majority of previously published research (29-34, 48, 49).

An interesting finding is that *FLT3/ITD* positivity had no adverse effect on the transplant outcome in our group of patients with NK-AML with an *NPM1* mutation in CR. In other published research, diagnostic *FLT3/ITD* positivity had a negative impact on the prognosis of such patients (19, 46, 50, 51). From our results it would seem that as long as the MRD level is taken into account, diagnostic *FLT3/ITD* positivity or negativity among patients with AML harboring *NPM1* mutations in CR has only minimal impact on alloHSCT outcome. Certain other researchers' results also support this conclusion (42). The status of CR of AML also had no impact on transplant outcomes in our population when pre-transplant MRD levels were taken into account, outcomes were not significantly different between patients

in first and second CR. Similar results are also reported by other researchers who took pre-transplant MRD levels into account for transplant outcome evaluation (29, 31, 33). However, we cannot rule-out the influence of sample size on our results. We noticed a trend towards poorer transplant outcomes in patients in second CR, but this trend was not statistically significant. This ambiguity is also in line with the results of a recent study of alloHSCT outcomes in patients with AML with mutated *NPM1* which also found worse outcomes in patients in second CR compared with those in the first; however, it did not evaluate pre-transplant MRD level (52).

It is also important to mention the role of the intensity of pre-transplant conditioning regimen in alloHSCT outcomes – especially since, unlike other potential pre-transplant factors (donor type and gender, *etc.*), we are able to influence this. In our study group, we did not find a statistically significant difference between patients transplanted after MAC and those after RIC. This is supported by the results of several other published studies, where alloHSCT outcomes in AML in CR were also not influenced by the conditioning regimen, but rather only influenced by pre-transplant MRD positivity (29-32). In general, however, the published data suggests that reduced-intensity pre-transplant conditioning is associated with a higher risk of post-transplant AML relapse when compared to a MIC; this includes several studies which also evaluated pre-transplant MRD (35, 53-55). In our study group, other potentially prognostic factors (donor type and gender, graft type, donor CMV status) did not significantly influence alloHSCT outcomes, with the exception of age over 63 years, where the 3-year EFS and OS were 38%. However, this last result was impacted by higher TRM (38%) among these older patients.

From a practical standpoint, our sample includes a group of patients with low MRD whose 3-year OS was 75%, especially due to low incidence of relapse (only 6%). Similar results may be found in other published studies – pre-transplant MRD-negative patients were reported across several studies to have 3-year OS of 62-77% and relapse incidence rates of 0-21% (29-31, 34). Thus, this patient group has an overall low risk of AML relapse, leaving TRM, morbidity and quality of life as key factors for alloHSCT outcome. Therefore, for these patients we can preferentially choose an RIC regimen to reduce transplant toxicity. We can also use more potent GVHD prophylaxis and slower tapering of immunosuppression to reduce the risk of GVHD, which is the principal cause of morbidity and mortality after alloHSCT. However, such approaches should be verified in further research. A bolder question is whether alloHSCT is necessary at all in the treatment of these patients, with regard to some recently published data (42).

On the other hand, our study group also included the high pre-transplant MRD group, whose prognosis was notably worse. In our study, these patients had a 3-year OS of 40% and a relapse incidence rate of 48%. Similar results are again found in other published studies, where 3-year OS in pre-transplant MRD-positive patients were in the range of 18-47% and relapse incidence rates were 41-70% (29-31, 34). The outcomes for pre-transplant MRD-positive patients are thus worse than for MRD-negative patients at a statistically significant level. MRD positivity has been shown to be an independent negative prognostic factor for AML treatment outcomes of patients treated with only standard chemotherapy in a fairly large body of research (15-18, 20-22, 26, 42, 56). Therefore, MRD positivity can be considered an independent negative biological characteristic of the disease in the context of standard chemotherapy of AML. Outcomes in MRD-positive patients where chemotherapy treatment is used alone are highly unfavorable. One of the most recent larger published studies on patients with AML with *NPM1* mutation gives 3-year OS of only 24% for patients with persistent positive NPM1 expression after two treatment cycles (42). Combining our results with other published research, pre-transplant MRD-positive patients with AML in CR achieve 3-year OS of around 40%; we can thus infer that alloHSCT partially improves their unfavorable prognosis compared to chemotherapy alone. At present, it remains unclear whether the outcome of alloHSCT in patients with MRD-positive AML in CR can be improved. In this potentially high-risk group, several ways to influence alloHSCT outcomes can be contemplated. In cases where MRD positivity persists during chemotherapy, there is the possibility of attempting to achieve MRD negativity through further cytostatic treatment; however, based on published data, the efficacy of this approach is debatable (26, 57). Another option is to try and influence MRD positivity with a more intensive pre-alloHSCT regimen, but based on our results as well as the results of several other published studies, this approach also does not guarantee an improvement in transplant outcomes (21, 35, 53-55). Furthermore, more intensive pre-transplant conditioning can increase the risk of TRM; this may offset any potential decrease in the risk of relapse, so that OS among alloHSCT patients with an MAC regimen might not change significantly (58). Other options for influencing alloHSCT outcomes in patients with MRD-positive AML in CR include an attempt to increase the graft *versus* leukemia effect by early post-transplant tapering of immunosuppression or by a pre-emptive infusion of donor lymphocytes (59, 60). In recent years, it has also become attractive to combine the abovementioned graft *versus* leukemia potentiation with other treatments, either standard cytostatics or newer targeted therapy (hypomethylating agents, antibodies, tyrosine kinase inhibitors, *etc.*). Certain recent publications have shown azacytidine and deoxyazacytidine to be effective in post-transplant pre-emptive relapse treatment (61-63). However,

the studies published so far are not large and no standard post-transplant pre-emptive treatment is currently being generally recommended for AML.

The results of our study show that any potential post-transplant therapeutic intervention should be initiated in a timely manner, as our patients with AML in CR and high MRD levels experienced relapse after a fairly short median period of 4 months. In general, we can say that the inferior alloHSCT outcomes found in patients with MRD-positive AML in CR open the field for further research that would identify additional negative prognostic factors for this patient group, as well as for prospective intervention studies that would further investigate the benefits of the discussed therapeutic options with regard to improving the prognosis of these patients.

Conflicts of Interest

The Authors declare no conflict of interest in regard to this study.

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