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**Highlights**:

- full donor chimerism can mitigate negative impact of pre-transplant MRD positivity
- qPCR-MRD is more predictive for post-transplant relapse than MFC-MRD
- mixed chimerism has limited predictive value for post-transplant relapses
- mixed chimerism without post-transplant MRD is not associated with worse outcomes

# Post-transplant day +100 MRD detection rather than mixed chimerism predicts relapses after allo-SCT for intermediate risk AML patients transplanted in CR

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**Short running title: MRD vs post-transplant chimerism studies in intermediate risk AML patients**

**Keywords:** allogeneic hematopoietic stem cell transplantation (allo-SCT), minimal/measurable residual disease (MRD), quantitative real-time PCR (qPCR), mixed chimerism, multiparameter flow cytometry (MFC), acute myeloid leukemia (AML)

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**Abstract**

**Background**: Chimerism and minimal residual disease (MRD) are suggested to be prognostic for post-transplant relapses in AML patients. Nevertheless, the predictive values of both approaches in homogeneous population remain underinvestigated. Here, we suggest that MRD may have a higher predictive value for relapses than mixed chimerism (MC) in intermediate risk AML patients.

**Patients and Methods**: 79 patients with intermediate risk AML (male, n=40, median age, 57 (19-77)) were included. MRD detection on day +100 was performed in bone marrow (multiparameter flow cytometry and quantitative real-time PCR for *NPM1*-mutated patients). Chimerism analysis was measured in peripheral blood. MC was defined as persistence of <99.9% of donor alleles.

**Results**: The area under the ROC curve was highest for qPCR-MRD (0.93) followed by MFC-MRD (0.80) and MC (0.65). The highest relapses at 3 years were observed in day +100 qPCR-MRD positive patients (100%) followed by MFC-MRD positive patients (55%, p<0.001). No patients with MC and without detectable MRD developed relapses. The 3-year OS and LFS for patients with MC without detectable MRD were both 86% (61-96%) compared with day +100 MFC-MRD positive (OS: 61%, 36-84%; LFS: 30%, 11-59%) and with day +100 qPCR-MRD positive patients (OS: 17%, 3-56%, p=0.001; LFS: 0%, p<0.001).

**Conclusions**: In intermediate-risk AML, the qPCR-MRD on day +100 is highly predictive for relapse and long-term survival after allo-SCT, closely followed by MFC-MRD. In contrast, the chimerism status has limited predictive potential. Thus, molecular and flow-cytometric MRD monitoring in the first months post-transplant rather than MC is able to identify patients with an increased relapse risk who may benefit from early post-transplant pre-emptive intervention.

**Introduction**

Acute myeloid leukaemia (AML) is a heterogeneous clonal disease of hematopoietic stem cells with fatal outcomes without treatment. Despite improvements in recent decades resulting in higher remission rates and prolonged survival due to availability of new therapeutic modalities (e.g. targeted treatments or/and improved supportive care), allogeneic stem cell transplantation (allo-SCT) remains the only curative option for the majority of these patients. The post-transplant relapses still remain a main challenge with rates between 20% and 60%, depending on individual risk stratification.(1) Thus, early prediction of relapse is crucial, especially in the context of early post-transplant interventions (e.g. immunotherapeutic approaches or maintenance therapies).(2)

Chimerism represents a dynamic process of coexistence of donor and recipient cells, including blasts. Full donor chimerism (FDC) can correspond to successful engraftment and development of a successful graft-*versus*-leukemia (GvL) effect. Mixed chimerism (MC) may represent a state of dynamic and vulnerable tolerance early post-transplant; this tolerance may be lost resulting in post-transplant relapse or GvHD. Some patients with MC may show a subsequent increase of donor alleles and experience favourable outcomes, whereas others may develop a further decrease of donor alleles and relapse very quickly.(3-5) In this setting, short intervals of chimerism monitoring may be required.(6-8)

Using chimerism as a marker of impending relapse is challenging due to different sensitivity of available tests as well. The classical Short Tandem Repeat polymerase chain reaction (STR-PCR) method has a significant lack of sensitivity; the quantitative real-time PCR (qPCR) technique using short insertion/deletion polymorphisms can be performed on peripheral blood samples and is associated with higher sensitivity.(9-14)

In general, studies using conventional chimerism to predict relapse provided controversial results.(5;7;15-18). More recent studies using qPCR for evaluation of lineage-specific chimerism (e.g. T-cell, CD34+, or CD3-) showed a better predictive potential. However, most of these studies included heterogeneous patients with different risk profiles and remission status at the time of allo-SCT.(19-22)

Disease-related MRD strategies such as multicolor flow cytometry (MFC) and/or detection of molecular markers with qPCR (e.g. *WT1* expression, *NPM1* mutation load) have been expanding in the last decades and clearly demonstrated significant impact on relapse and survival in the pre- and post-transplant settings.(23-26)

Some authors already reported on the use of chimerism together with post-transplant MRD.(16;21;27;28) Most of these studies included patients with overexpression of *WT1* gene despite the lack of clear consensus due to great thresholds' variability.

In this study, we evaluated sensitivity and specificity of post-transplant MRD detection with MFC and/or qPCR (for *NPM1*-mutated patients) and chimerism dynamics in the first 100 days after allo-SCT for relapse prediction in intermediate risk AML patients.

## Patients and Methods

*Study cohort*

Adult (≥18 years old) patients were included in this monocentric retrospective study if they had intermediate risk AML, fulfilled the criteria for CR at allo-SCT, underwent allo-SCT after myeloablative or reduced intensity conditioning, had available MFC-MRD data on day +100 as well as chimerism data in the first 100 days post-transplant. All patients received allo-SCT at the Department for Stem Cell Transplantation of University Cancer Center University of Hamburg in the period 01/2015 to 9/2021. In case of presence of *NPM1*-mutation the qPCR-MRD monitoring was performed including day +100. We used the European Leukemia Net (ELN) criteria (2017) to assign disease-dependent risk.(29) Criteria for response to therapy were used as proposed by an International Working Group.(30) All patients consented in accordance with the Declaration of Helsinki. Follow-up was current as of August 15, 2021.

*Flow-cytometric detection of MRD*

Immunophenotypic analysis was done at day +100 on whole bone marrow specimens after stain-lyse-wash standard techniques.(25) The eight-color based immunostaining analysis was performed according to ELN consensus recommendation.(30) Up to 2,000,000 events per tube (6,000,000 events per sample) were evaluated. All antibodies were obtained from Beckman-Coulter (CA, USA) or Becton Dickinson (BD Biosciences, New Jersey, USA). Analysis of list mode files was performed using Infinicyte™ Flow Cytometry Software (Cytognos, Salamanca, Spain). The assessments were performed using the leukemia-associated phenotype (LAIP) and the "different from normal" strategy. Following ELN guidelines, a threshold of 0.1% or more of aberrant cells in the bone marrow was defining MRD positivity.(31) The sensitivity of our MFC-based approach was $10^{-4}$ – $10^{-5}$.

*Chimerism*

Chimerism assessment in peripheral blood followed our laboratory guidelines.(4) The analysis was performed with qPCR (TaqMan) targeting diverse donor-recipient-specific polymorphisms (short deletions or insertions) according to Alizadeh *et al.*(32) In case of sex mismatched allo-SCT, we used Y-chromosome-specific sequences from the *DFFRY* gene.(33) The chimerism status was exactly quantified with a standard curve

after normalization by use of the hematopoietic kinase gene (*HCK*). Sensitivity was $10^{-4}$. The chimerism assessment was performed at least every week after engraftment. Results were categorized as FDC (= ≥99.9% of donor alleles) and MC (= <99.9% of donor alleles). Patients who achieved FDC but then converted to MC were further subdivided into two categories according to increase or decrease of donor alleles (after the first assessment of MC). Loss of chimerism was defined as decline of donor alleles of 0.5% at least in two separate measurements.

*Molecular mutation MRD monitoring*

Following extraction of genomic DNA from unseparated bone marrow or peripheral blood samples, the *NPM1A, NPM1B* and *NPM1D* mutation status was assessed as described previously.(34) Briefly, the mutation levels were exactly quantified by use of a standard curve following dilution of the OCI/AML1 cell line (carrying an *NPM1A, NPM1B* and *NPM1D* mutations) and were normalized for the DNA content with the *HCK* gene. This assay achieved sensitivity of $10^{-4}$ to $10^{-6}$ depending on the sample's DNA concentration.

*Statistical analysis*

Unadjusted probabilities of overall survival (OS) and leukemia-free survival (LFS) were estimated by using the Kaplan-Meier and Cox regression methods. Probabilities of NRM and relapse were summarized by using cumulative incidence estimates. NRM was defined as death without relapse and was considered a competing risk for relapse, whereas relapse was a competing risk for NRM. The probability of developing acute (grade II-IV) GVHD and chronic GVHD was depicted by calculating the cumulative incidence with death without GVHD as a competing risk.

Categorical characteristics were compared by Pearson's or Fisher's exact test. Continuous variables were compared using non-parametric Mann-Whitney test. To evaluate relapse predictability, sensitivity, specificity, positive- and negative predictive values were calculated.(35) In addition, receiver-operating curves (ROC) were used for assessment of relapse predictability. Statistical analysis was performed with IBM SPSS Version 25 (SPSS, Inc.; Chicago, IL, USA) and R software (Version 3.5.1 R Foundation, Vienna, Austria) with competing risks calculated using the package 'cmprsk' (http://CRAN.R-project.org/package=cmprsk).

## *Results*

### *Patients' characteristics*

The characteristics of the study population are summarized in Table 1. Seventy-nine patients with intermediate-risk AML (male, n=40) with a median age of 57 years (range 19-77) were included. The allografts were performed in majority of the cases from matched unrelated donors after myeloablative conditioning. At day +100, 56 patients (71%) experienced MFC-MRD negativity, while 23 patients (29%) were MFC-MRD positive. The day +100 MFC-MRD positivity was significantly associated with pre-transplant MFC-MRD positivity (p=0.001), post-transplant day +100 qPCR-MRD positivity (p=0.032), and MC (p=0.016).

The qPCR-MRD status referring to *NPM1* mutation (*NPM1A*) at day +100 was available in 25 patients, with six patients (24%) showing a MRD positivity with a median level of 0.02% (0.002-0.52). The remaining patients (n=54) patients were tested for *NPM1* mutations at initial diagnosis and were found not to have any. The *FLT3-ITD*-mutation status was available in all *NPM1*-mutated patients: positive, n=18 (72%); negative, n=7 (28%, Table 1S).

Regarding to the chimerism assessment, 47 patients experienced FDC (60%) and 32 MC (40%). Patients with FDC experienced significantly lower *NPM1*-mutation load (0.007%, 0.002-0.1%) comparing to MC patients (0.2%, 0.02-0.52%, p=0.048). Of patients with MC, four showed subsequent increase of donor alleles, six showed decrease of donor alleles and 22 showed stable MC in the first 100 day post-transplant.

***Post-transplant MRD clearance, conditioning and age***

The MFC-MRD persistence at day +100 was documented in 17 of 36 pre-transplant MRD positive patients and was not associated with conditioning intensity (MAC: 13/26, 50% vs 4/10, 40%, p=0.44). Of those patients, who achieved MFC-MRD clearance at day +100 (n=19) (MAC, n=13; RIC, n=6) only one patient developed relapse, comparing with 11 of 17 patients with post-transplant MFC-MRD persistence (MAC, n=13; RIC, n=4). The median age did not differ between patients who achieved the day +100 MFC-MRD clearance (59 years, 25-75) and those who did not (52 years, 21-71, p=0.20).

Nine of 25 patients (18%) were qPCR-MRD positive at the time of allograft. Of those, 5 patients cleared the NPM1-mutation (MAC, n=2; RIC, n=3) and experienced excellent outcomes without relapses or NRM events. In contrast, all four patients who did not achieve post-transplant qPCR-MRD clearance relapsed (MAC, n=3; RIC, n=1, p=0.36). The median age did not differ between patients who achieved the day +100 qPCR-MRD clearance (66 years, 56-69) and those who did not (54 years, 29-67, p=0.19).

***Correlation between two post-transplant MRD approaches***

Considering the 25 patients who could be evaluated for MRD status by MFC and qPCR in combination, the concordance (=both positive + both negative results measured with two approaches) for MFC-MRD and qPCR-MRD was rather high with 80% (n=20/25).

***Relapses according to post-transplant MRD approaches and chimerism***

An amount of 56 patients (71%) were MFC-MRD negative on day +100, whereas 23 patients (29%) were MFC-MRD positive. The relapse rate at 3 years was 58% (34-79%) in the MRD positive as compared to 7% (2-19%) in the MRD negative patients ($p<0.0001$).

An amount of 19 patients (76%) were qPCR-MRD negative on day +100, whereas six patients (24%) were qPCR-MRD positive. The relapse rate at 3 years was 100% in the MRD positive as compared to 5% (1-24%) in the MRD negative patients ($p<0.0001$). In more details, no patients who were qPCR-MRD negative at day +100 relapsed. All six patients who were qPCR-MRD positive at day +100 relapsed. The time to relapses was shorter in case of NPM1 double positivity (=peripheral blood and bone marrow, Table S1).

An amount of 47 patients (60%) experienced FDC in the first 100 days post-transplant, whereas 32 patients (40%) had MC. The relapse rate at 3 years was 12% (5-25%) in the FDC as compared to 37% (19-59%) in the MRD positive patients ($p=0.03$).

***Relapses in patients with FDC regarding the MRD status***

Of patients with FDC (n=47), 39 (83%) were MFC-MRD negative and eight MFC-MRD positive at day +100. Of the MFC-MRD negative patients, two (5%) developed relapses; of the MFC-MRD positive patients, three (38%) relapsed.

Of 25 patients with available qPCR-MRD data, 16 had FDC. Of them, 12 (75%) were qPCR-MRD negative and four qPCR-MRD positive. There were no relapses within negative patients, whereas three of four qPCR-MRD positive patients (75%) relapsed.

***Relapses in patients with MC regarding the MRD status***

Of patients with MC (n=32), 17 (53%) were MFC-MRD negative and 15 (47%) MFC-MRD positive. In the subgroup of the MFC-MRD negative patients, only one developed relapse (6%), whereas eight (53%) of the MFC-MRD positive patients

developed a relapse. Of 25 patients with available qPCR-MRD data, nine had MC. Of them, seven (78%) were qPCR-MRD negative and two were qPCR-MRD positive. There were no relapses within qPCR-MRD negative patients whereas both qPCR-MRD positive patients developed relapses.

Considering the dynamics of MC, of four patients with subsequent increase of donor alleles, three were MFC-MRD negative and one MFC-MRD positive who developed relapse at day +119 and underwent a second allo-SCT. There were no relapses within MFC-MRD negative patients with increasing donor alleles. Of six patients with MC and subsequently decreasing donor alleles, four were MFC-MRD positive and two MFC-MRD negative. All positive patients developed relapses at 98, 105, 161 and 722 days after allograft. Two of them died and the remaining patients underwent a second allo-SCT. Both MFC-MRD negative patients developed graft failure. One underwent a second allo-SCT and another one died due to severe infection.

***Survival outcomes***

*Overall*

After a median follow up of 25 months (range 3-60), there were 14 deaths, 14 relapses and 6 NRM events. Due to low events a multivariate analysis was not performed. The results of univariate analysis are represented in the Table 2.

The relapse rate at 3 years for all patients was 21% (95% CI 13-32%) with a median of 216 days (range 110-722). The 3-year OS and LFS were 79% (95% CI 68-87%) and 70% (95% CI 58-80%), respectively.

*Outcomes according to chimerism and pre-transplant MRD status*

In 67 patients the pre-transplant MFC-MRD data were available (Table 1). According to post-transplant chimerism dynamics, these patients were subdivided into following four groups: (i) pre-transplant MFC-MRD positive, who achieved FDC (“positive-full”, n=20); (ii) pre-transplant MFC-MRD positive with MC (“positive-mixed”, n=16); (iii) pre-transplant MFC-MRD negative with FDC (“negative-full”, n=19); and (iv) pre-transplant MFC-MRD negative with MC (“negative-mixed”, n=12). The highest relapses at 3 years after allo-SCT were observed in the “positive-mixed” group (79%, 95% CI 29-97%) followed by the “positive-full” (16%, 95% CI 5-40%), the “negative-full” (13%, 95% CI 3-39%) and the “negative-mixed” (8%, 95% CI 1-36%, p=0.003). The NRM at 3 years after allo-SCT was higher in the “negative-mixed” group (26%, 95% CI 9-57%), followed by the “negative-full” (6%, 95% 1-28%), the “positive-full” (6%, 95% 1-28%) and the “positive-mixed” (0%, p=0.11). This resulted in the lowest 3-year LFS in the “positive-mixed” group (17%, 95% CI 3-61%) followed by the “negative-mixed”

(66%, 95% 38-86%), the „positive-full" (78%, 95% CI 55-91%), and the "negative-full" (81%, 95% CI 57-93%, p=0.028). The 3-year OS was not significantly different between four groups ("positive-mixed", 71%, 95% CI 43-89%; "positive-full", 77%, 95% CI 54-91%; "negative-mixed", 64%, 95% CI, 37-85%; "negative-full", 94%, 95% CI 72-99%, p=0.27, Figure 1S).

*Outcomes according to day +100 MRD status*

Regarding the MRD status, the day +100 MFC-MRD negative patients showed significantly higher 3-year OS (92%, 95% CI 81-97% vs 50%, 95% CI 30-70%, p<0.001) and 3-year LFS (88%, 95% CI 75-95% vs 25%, 95% CI 10-50%, p<0.001). The day +100 qPCR-MRD negative patients showed significantly higher 3-year OS (94%, 95% CI 77-99% vs 17%, 95% CI 3-56%, p<0.001) and 3-year LFS (94%, 95% CI 77-99% vs 0%, p<0.001).

*Outcomes for patients with MC*

The patients with MC showed a non-significantly lower 3-year OS (69%, 95% CI 50-83% vs 86%, 95% 72-94%, p=0.15) and significantly lower 3-year LFS (51%, 95% CI 31-71% vs 81%, 95% CI 67-90%, p=0.021) compared to patients with FDC. Of patients with MC, the day +100 MFC-MRD negative patients experienced higher 2-y LFS (81% vs 48%, p=0.017) and lower relapse rates at 2 year (6% vs 67%, p=0.003) compared with MFC-MRD positive ones. There was no significant difference in the 2-year OS (MFC-MRD negative: 78%, 52-92% vs MFC-MRD positive: 63%, 36-84%, p=0.56).

*Outcomes for patients with FDC*

Of patients with FDC, those with MFC-MRD negativity on day +100 (n=39) experienced excellent 2-year OS of 97% (85-100%) and 2-year LFS of 91% (77-97%) compared with MFC-MRD positive patients (2-year OS: 19%, 4-61%, p<0.001; 2-year LFS: 19%, 4-61%, p<0.001) as a result of lower relapse rates (4%, 0-16% vs 42%, 14-76%, p=0.005).

*Outcomes for patients with MC without MRD and for MRD positive patients*

We performed a separate analysis for 41 patients who had either MC (n=16) without detectable MRD with both methods, day +100 MFC-MRD positivity (n=16) or day +100 qPCR-MRD positivity (n=6). We observed the highest relapse rates at 3 years for day +100 qPCR-MRD positive patients (100%) followed by day +100 MFC-MRD positive patients (55%, 95% CI 27-80%, p<0.001). No patients with MC without

detectable MRD relapsed. This resulted into higher 3-year OS and LFS (both 86%, 95% CI 61-96%) for MC patients without detectable MRD comparing with 61% (95% CI 36-84%) and 30% (95% CI 11-59%) for day +100 MFC-MRD positive patients; and with 17% (95% CI 3-56%) and 0% for day +100 qPCR-MRD positive patients, respectively (Figure 1).

**Graft-versus-host disease**

The cumulative incidence of acute severe (grade II-IV) GvHD at 1 year was 16% (95% CI 7-34%) for MC and 26% (95% CI 16-39%, p=0.26) for FDC. The cumulative incidence of acute severe (grade II-IV) GvHD at 1 year was 24% (95% CI 14-37%) for MFC-MRD negative and 18% (95% CI 7-38%, p=0.54) for MFC-MRD positive patients. The cumulative incidence of acute severe (grade II-IV) GvHD at 1 year was 33% (95% CI 16-56%) for qPCR-MRD negative and 33% (95% CI 9-72%, p=0.95) for qPCR-MRD positive patients.

The cumulative incidence of chronic GvHD at 3 years was 54% (95% CI 32-75%) for MC and 51% (95% CI 36-66%, p=0.52) for FDC. The cumulative incidence of chronic GvHD at 3 years was 49% (95% CI 36-62%) for MFC-MRD negative and 55% (95% CI 31-77%, p=0.90) for MFC-MRD positive patients. The cumulative incidence of chronic GvHD at 3 years for qPCR-MRD could not be calculated due to median follow up of 252 days (range 142-820).

***Predictive values for relapse***

The highest sensitivity and specificity were documented for qPCR-MRD (100% and 95%) followed by MFC-MRD (79% and 82%), and MC (64% and 65%). The highest positive-predictive and negative-predicted values were documented for qPCR-MRD (83% and 100%), followed by MFC-MRD (48% and 95%) and MC (28% and 89%; Table 3).

The area under the ROC curve was highest for qPCR-MRD (n=25; 0.93, 77-100%, p=0.001) followed by MFC-MRD (n=79; 0.80, 95% CI 66-94%, p<0.001) and MC (n=79; 0.65, 95% CI 48-81%, p=0.09; Figures 2, 3).

***Discussion***

In this study, we compared predictive potentials of early chimerism dynamics in peripheral blood (qPCR), day +100 MFC- and qPCR-MRD (*NPM1*) for post-transplant relapse in intermediate risk AML patients. Despite that both, MC and MRD positivity (measured by MFC and qPCR) were associated with increased relapses, the highest predictive potential was observed for qPCR-MRD closely following by MFC-MRD. Also,

we showed that patients with FDC and MC in the peripheral blood may represent heterogeneous populations according to the post-transplant MRD status.

In general, different methods of chimerism detection are associated with different sensitivity that may be associated with its' limited prognostic value. Most of the studies in this setting were based on use of STR-PCR and provided controversial results.(5,7,15-17) Although the qPCR-based approach using short insertion/deletion polymorphisms can be performed on peripheral blood with better sensitivity(9,13), the prognostic value of chimerism even in this setting remains controversial.(18,36,37) The lineage specific chimerism was suggested to improve relapse prediction in several retrospective studies.(20,38-40) However, in a phase II randomized study, Devine *et al.* found no significant impact of CD3+ chimerism measured in peripheral blood on post-transplant outcomes in older AML patients after having received RIC-SCT in CR.(22) Recently, the results of the FIGARO study showed, that achievement of CD3+ FDC at 3 months mitigates negative impact of positive MRD in 244 patients with AML and MDS patients, allografted after RIC.(41) In our study, intermediate risk AML patients with MC experienced more relapses and lower LFS compared to those with FDC. This may be due to use of MAC regimens in the majority of our patients. Further, in line with the results of the FIGARO study, we observed that achieving of FDC up to day +100 can mitigate a negative impact of pre-transplant MFC-MRD positivity on relapses resulting in significantly improved 3-year LFS.

Though several studies showed that MAC can improve outcomes in pre-transplant MRD-positive patients(42,43), Dillon et al. did not find any impact of conditioning intensity on survival for pre-transplant MRD positive *NPM1*-mutated AML patients.(44) In our study, we neither observed any impact of conditioning on post-transplant MRD clearance and outcomes, possibly due to low number of patients who received RIC; nevertheless the ELN intermediate risk of included patients may play a role.

The post-transplant MRD persistence in AML patients regardless of the methodology represents a potent risk factor for relapse.(45-48) In this term, the combined use of MRD and chimerism may be able to improve the post-transplant relapse prediction. Previous studies that combined conventional chimerism and *WT1* gene expression analysis demonstrated less efficacy of the former approach.(16,27,49) Recently, Bouvier *et al.* showed a comparable predictivity of CD3-negative cell chimerism and qPCR-MRD detection (*WT1*) for relapses in AML patients. This study included 100 AML patients with all ELN risk groups, 28 of whom were not in remission at the allo-SCT.(21)

In our study, we used the *NPM1*-qPCR-based MRD strategy and correlated this with MFC-MRD monitoring and early chimerism dynamics in 25 patients. The highest

predictive values of qPCR-MRD monitoring can be associated with increased sensitivity of this approach as well with stability of the *NPM1*-mutation during post-transplant period. On the other hand, the MFC-MRD may be associated with relevant amount of false positive or false-negative results due to several factors like the quality of bone marrow aspirate, type of antibodies used, method of sample preparation and phenotype shifting of initial AML clone. In addition, the recognition of clonal hematopoiesis of indeterminate potential (CHIP) with this approach may not be completely excluded.

Further we showed, that patients with FDC and MC represent heterogenic populations. First, FDC in peripheral blood may not exclude presence of malignant clone in the bone marrow at low level and convert to MC in case of morphologic relapse. Second, though it is well known that some patients with non-malignant disease may not require development of FDC after allo-SCT(50), the issue of MC in malignant disorders is challenging. Kinsella *et al.* reported on the importance of hosts' dendritic cells and hosts' and donor's T regulatory cells in maintenance of MC early after allo-SCT. The development of tolerance early after allo-SCT may represent a dynamic and vulnerable process. In case of attenuation of GvL effect due to T regulatory cells, the tolerance may be impaired and relapse occurs. On the other hand, expansion of alloreactive T cells due to downregulation of T regulatory cells may result in GvHD.(51) Moreover, some authors showed that MC may persist long time after allograft and may not be associated with adverse outcomes.(52) Therefore, the use of post-transplant interventions like donor lymphocyte infusions (DLIs) in this setting may not improve outcomes and explain limited predictive value of MC found in our and other studies.

Improved post-transplant relapse prediction in AML patients in terms of risk stratification seems to be crucial for early post-transplant interventions. Though several retrospective studies provided controversial data on chimerism in this setting(5,36,37,53,54), use of post-transplant MRD monitoring was shown to be important. Platzbecker *et al.* evaluated an MRD-guided strategy (qPCR and CD34+ donor chimerism in transplanted patients) in 53 AML/MDS patients who received 6 cycles of a 5'-azacitidine in case of MRD positivity with a response rate of around 60% (60% of ongoing CR).(2)

Thus, in this study on more homogeneous population, even being done frequently with more sensitive molecular methods, chimerism alone seems to have a limited prognostic value for early relapse prediction. Several approaches (e.g. CD3-negative selection) may improve its predictive potential, however, randomizing studies to confirm this are missing. The use of post-transplant MRD detection (especially with

PCR-based methods) may improve relapse prediction and may lead to better stratification of patients who may benefit from early post-transplant interventions.

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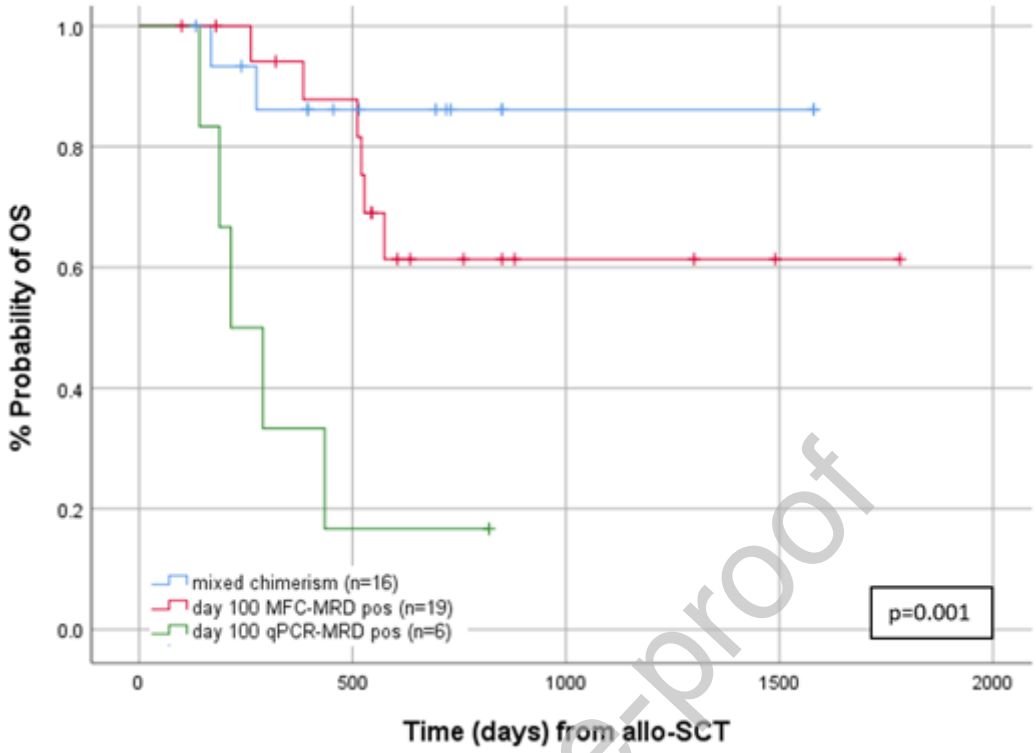
**Figure 1**. Outcomes for patients according early post-transplanr chimerism and day +100 MRD status: a) overall survival (OS); b) leukemia-free survival (LFS); c) relapses and non-relapsed mortality (NRM).

**Figure 2.** ROC curve analysis for chimerism and MFC-MRD (n=79).

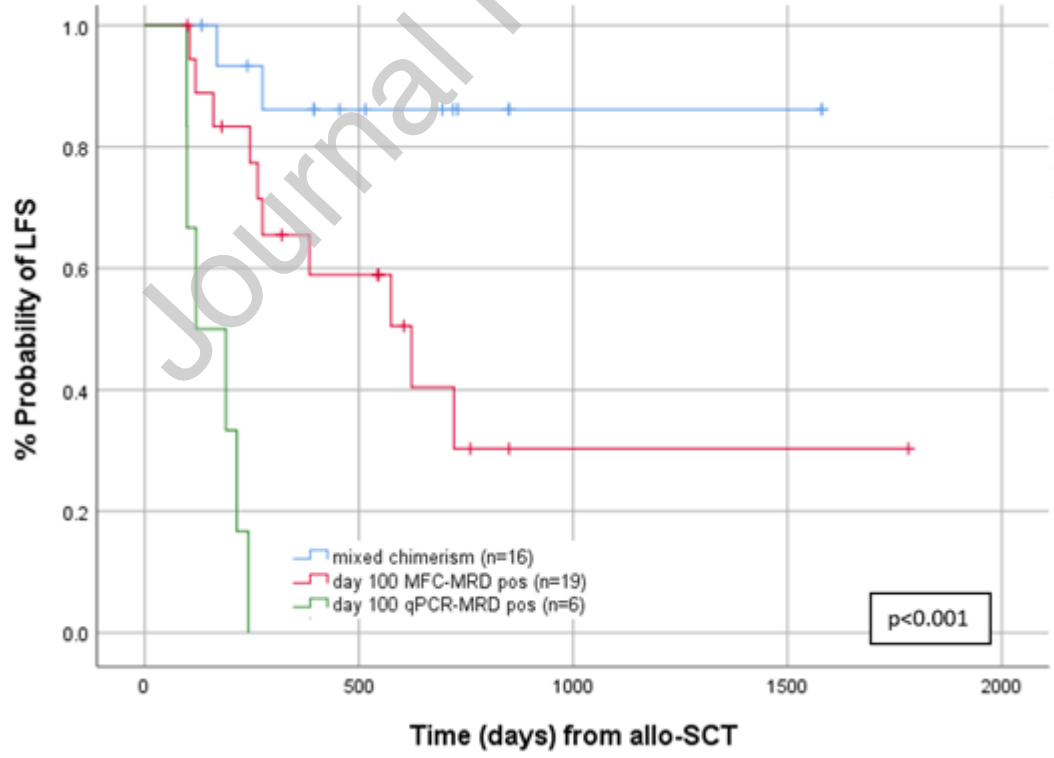
**Figure 3.** ROC curve analysis for chimerism, MFC-MRD and qPCR-MRD (n=25)

**Figure 1.**

**a)**




**b)**



**c)**

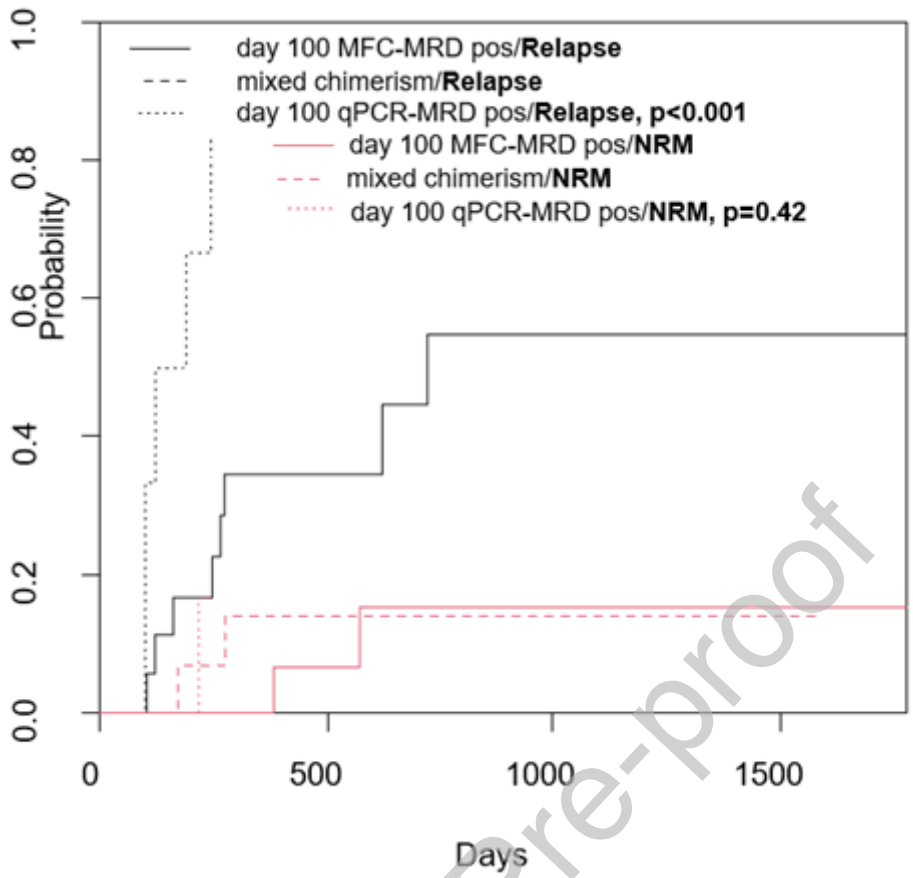


**Figure 2.**

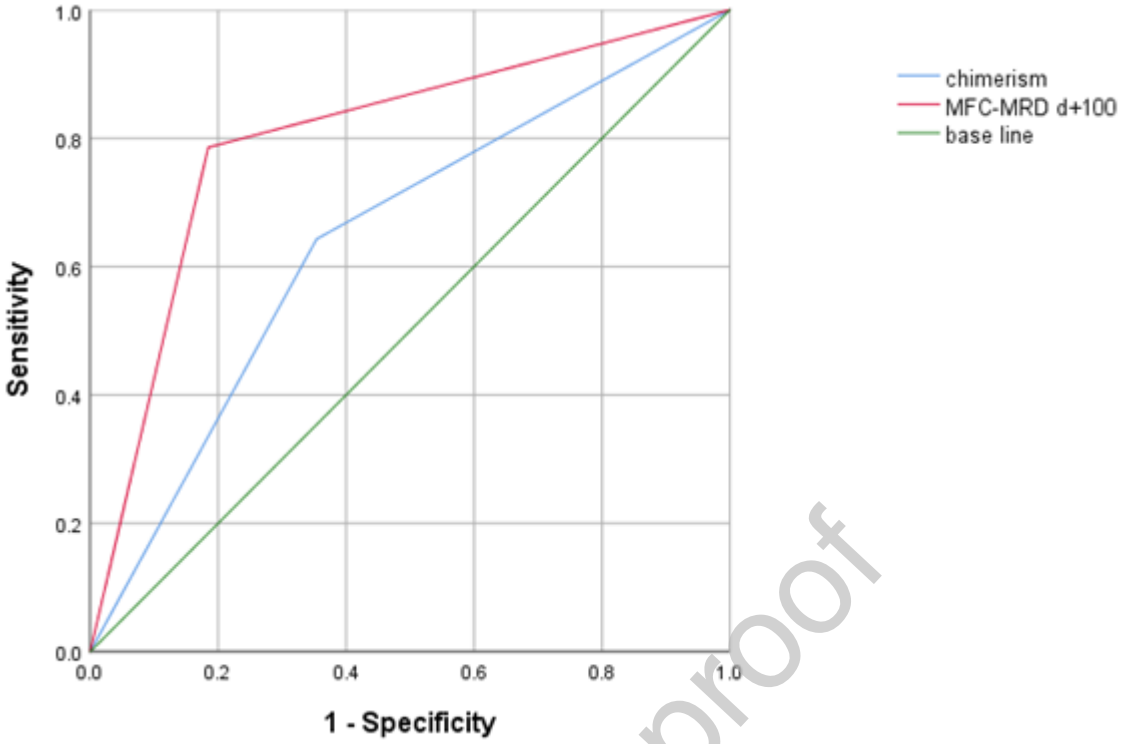
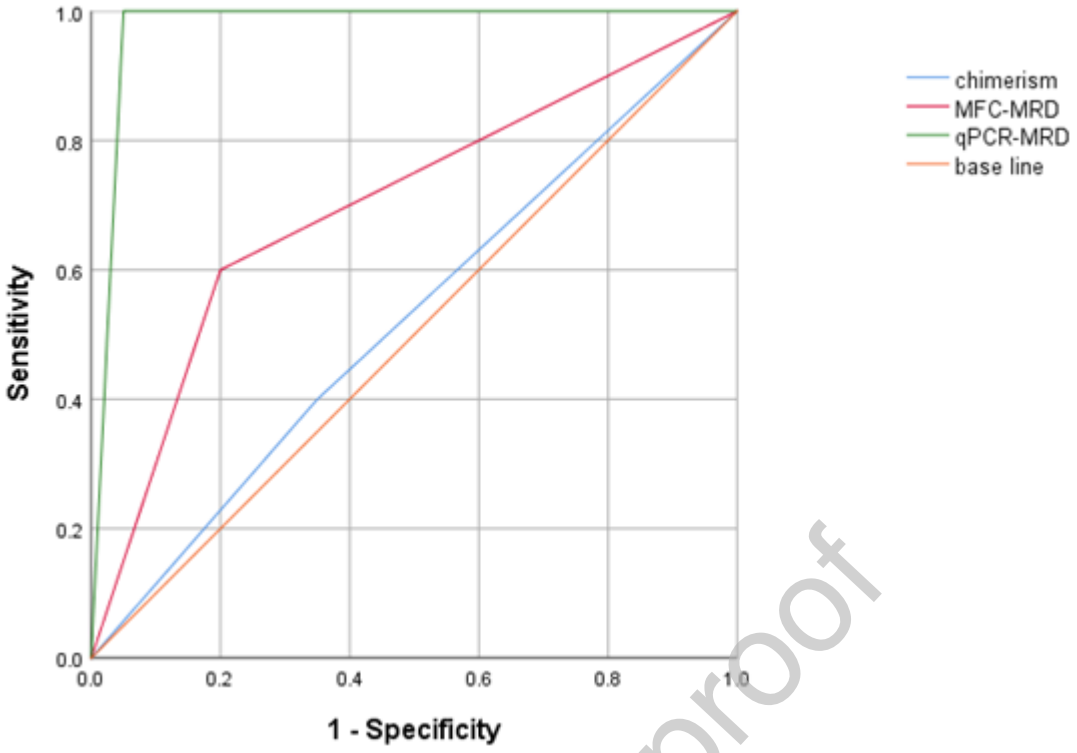


**Figure 3.**



## Tables

**Table 1 Patients' characteristics**(n, number; s/tAML, secondary/therapy-related AML; MSD, matched sibling donor; MUD, matched unrelated donor; MMUD, mismatched unrelated donor; MAC, myeloablative conditioning; RIC, reduced intensity conditioning; ATG, anti-thymocyte globuline; post-Cy, post-transplant cyclophosphamide; MRD, minimal residual disease, PB, peripheral blood)

| Characteristics | Day +100 MFC-MRD negative (n=56) N (%) | Day +100 MFC-MRD positive (n=23) N (%) | Total (n=79) N (%) |
|---|---|---|---|
| **Patients sex** | | | |
| male | 26 (46%) | 14 (61%) | 40 (51%) |
| female | 30 (54%) | 9 (39%) | 39 (49%) |
| **Patient/donor sex match** | | | |
| match | 32 (57%) | 15 (65%) | 47 (60%) |
| mismatch | 24 (43%) | 8 (35%) | 32 (40%) |
| **Patients' age** | | | |
| median (range) | 58 (19-77) | 52 (21-71) | 57 (19-77) |
| **Donors' age** | | | |
| median (range) | 30 (18-69) | 32 (18-66) | 30 (18-69) |
| **Origin of disease** | | | |
| *de novo* | 46 (82%) | 18 (78%) | 64 (81%) |
| s/tAML | 10 (18%) | 5 (22%) | 15 (19%) |
| **Remission status** | | | |
| 1CR | 37 (66%) | 17 (74%) | 54 (68%) |
| 2+CR | 6 (11%) | 4 (17%) | 10 (13%) |
| CRi | 13 (23%) | 2 (9%) | 15 (19%) |
| **Extramedullary involvement** | 4 (7%) | 2 (9%) | 6 (8%) |
| **Cytogenetics at diagnosis** | | | |
| normal | 37 (69%) | 12 (52%) | 49 (64%) |
| abnormal | 17 (31%) | 11 (48%) | 28 (36%) |
| *not available* | 2 | 0 | 2 |
| **Previous therapy** | | | |
| Chemotherapy-based | 50 (90%) | 19 (82%) | 69 (88%) |
| Azacytidine/Decitabine mono | 3 (5%) | 2 (9%) | 5 (6%) |
| Venetoclax in combinations* | 3 (5%) | 2 (9%) | 5 (6%) |
| **Primary induction failure** | 16 (29%) | 3 (13%) | 19 (24%) |
| **Donor type:** | | | |
| MSD | 11 (20%) | 7 (30%) | 18 (23%) |
| MUD | 37 (66%) | 13 (57%) | 50 (63%) |
| MMUD | 4 (7%) | 2 (9%) | 6 (8%) |
| HaploidenticalCord blood | 4 (7%) | 1 (4%) | 5 (6%) |
| **Patients' CMV status** | | | |
| negative | 19 (35%) | 7 (30%) | 26 (33%) |
| positive | 37 (65%) | 16 (70%) | 53 (67%( |
| **Conditioning** | | | |
| MAC | 40 (71%) | 19 (83%) | 59 (75%) |
| RIC | 16 (29%) | 4 (17%) | 20 (25%) |
| **Immunosuppression** | | | |
| ATG | 47 (84%) | 17 (77%) | 64 (82%) |
| post-Cy | 9 (16%) | 5 (23%) | 14 (18%) |
| both | - | 1 | 1 |
| **MRD status at allo-SCT:** | | | |
| *MFC:* | | | |
| negative | 28 (60%) | 3 (15%) | 31 (46%) |
| positive | 19 (40%) | 17 (85%) | 36 (54%) |
| n.a. | 9 | 3 | 12 |
| *qPCR:* | | | |
| $NPM1^{neg}$ | 13 (61%) | 3 (43%) | 16 (76%) |
| $NPM1^{pos}$ | 5 (39%) | 4 (57%) | 9 (24%) |
| ***NPM1* status on day 100** | | | |
| negative | 16 (89%) | 3 (43%) | 19 (76%) |
| positive | 2 (11%) | 4 (57%) | 6 (24%) |
| **Chimerism dynamics (PB)** | | | |
| full donor | 39 (70%) | 8 (35%) | 47 (60%) |
| mixed stable | 12 (21%) | 10 (44%) | 22 (28%) |
| mixed increasing | 3 (5%) | 1 (4%) | 4 (5%) |

| | | | |
|---|---|---|---|
| mixed decreasing | 2 (4%) | 4 (17%) | 6 (8%) |

**Table 2.** Results of univariate analysis (n=79) (s/tAML, secondary/therapy-related AML; MSD, matched sibling donor; MUD, matched unrelated donor; MMUD, mismatched unrelated donor; MAC, myeloablative conditioning; RIC, reduced intensity conditioning; ATG, anti-thymocyte globuline; post-Cy, post-transplant cyclophosphamide; MRD, minimal residual disease, MC, mixed chimerism; FDC, full donor chimerism; HR, hazard ratio; CI, confidence interval, p, p-value)

| Characteristic | OS (HR, 95% CI, p) | LFS (HR, 95% CI, p) | Relapse (HR, 95% CI, p) | NRM (HR, 95% CI, p) |
|---|---|---|---|---|
| **Patients' sex:** | | | | |
| male vs female | 1.8 (0.6-5.5), 0.27 | 2.0 (0.8-5.1), 0.14 | | |
| **Patient/donor sex match** | | | | |
| match vs mismatch | 1.2 (0.4-3.5), 0.76 | 1.3 (0.5-3.3), 0.58 | | |
| **Patients' age** | **1.0 (0.99-1.1), 0.09** | **1.0 (0.97-1.1), 0.77** | **0.96 (0.93-0.99), 0.032** | **1.2 (1.1-1.3), <0.001** |
| **Origin of disease** | | | | |
| *de novo* vs s/tAML | 1.3 (0.3-5.7), 0.76 | 1.3 (0.4-4.6), 0.65 | | |
| **Cytogenetics at diagnosis** | | | | |
| normal vs abnormal | 0.6 (0.2-1.6), 0.26 | 0.8 (0.3-2.0), 0.68 | | |
| **Donor type** | 0.99 | 0.49 | | |
| MUD vs MSD | 0.98 (0.3-3.6), 0.98 | 1.1 (0.3-3.3), 0.91 | | |
| MMUD vs MRD | 0.96 (0.1-9.3), 0.97 | 2.7 (0.6-12), 0.19 | | |
| Haploident vs MRD | 1.3 (0.1-12), 0.84 | 0.9 (0.1-8), 0.92 | | |
| **Patients' CMV status** | | | | |
| negative vs positive | 1.1 (0.4-3.2), 0.93 | 1.0 (0.4-2.5), 0.99 | | |
| **Conditioning** | | | | |
| MAC vs RIC | 0.6 (0.2-1.6), 0.29 | 0.8 (0.3-2.1), p=0.66 | | |
| **Immunosuppression** | | | | |
| ATG vs post-Cy | **0.3 (0.1-1.1), 0.07** | 0.6 (0.2-1.9), p=0.43 | 0.6 (0.2-2.3), 0.45 | |
| **Chimerism dynamics** | | | | |
| MC vs FDC | **2.2 (0.8-6.2), 0.15** | **2.8 (1.1-6.8), 0.027** | **3.2 (1.1-9.5), 0.038** | 1.6 (0.3-7.8), 0.57 |
| **MFC-MRD status on day +100** | | | | |
| negative vs positive | **0.1 (0.04-0.4), 0.001** | **0.1 (0.04-0.3), <0.001** | **0.1 (0.02-0.3), <0.001** | **0.4 (0.1-1.9), 0.24** |
| **qPCR-MRD status on day +100** | | | | |
| negative vs positive | **0.1 (0.01-0.4), 0.004** | **0.05 (0.01-0.3), <0.001** | **0.02 (0.01-0.3), 0.0023** | **0.2 (0.01-3.9), 0.31** |

**Table 3. Predictive values for post-transplant MRD and chimerism dynamics**.(MRD, minimal residual disease; MFC, multicolor flow cytometry; qPCR, quantitative real-time polymerase chain reaction; PPV, positive-predictive value; NPV, negative predictive value)

| | Sensitivity | Specificity | PPV | NPV |
|---|---|---|---|---|
| **Mixed chimerism** | 9/14, 64% | 42/65, 65% | 9/32, 28% | 42/47, 89% |
| **MRD-MFC on day +100** | 11/14, 79% | 53/65, 82% | 11/23, 48% | 53/56, 95% |
| **MRD-qPCR on day +100** | 5/5, 100% | 19/20, 95% | 5/6, 83% | 19/19, 100% |