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ORIGINAL ARTICLE

Pre-transplant MRD predicts outcome following reduced-intensity and myeloablative allogeneic hemopoietic SCT in AML

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The presence of minimal residual disease (MRD) by multiparametric flow cytometry (MFC) has been associated with adverse outcomes in AML patients treated with chemotherapy alone, but its impact in the setting of allogeneic hematopoietic SCT (HSCT) is less clear. We studied 88 patients who underwent myeloablative (MA) or reduced-intensity conditioned allogeneic HSCT for AML in first or subsequent remission at our center. MRD status was determined using three-color MFC on pre-HSCT BM aspirates, and patients were stratified by MRD status into MRD-negative, low-level MRD-positive (<1%) or high-level MRD-positive groups (1–4.9%). Two-year survival estimates in these groups were 66.8%, 51% and 30%, respectively ($P=0.012$), and 2-year estimates of relapse were 7.6, 37 and 70% ($P<0.001$). Pre-HSCT MRD was related to disease characteristics including secondary AML ($P=0.002$) and primary induction failure ($P=0.005$), but, despite these strong correlations, MRD remained independently associated with poorer survival in multivariate analysis (hazard ratio, 1.92; $P=0.014$). Pre-HSCT MRD is associated with adverse clinical outcomes in AML patients undergoing reduced-intensity or MA HSCT in first or subsequent remission and should be integrated into transplant strategies for patients with AML.

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INTRODUCTION

Current induction chemotherapy achieves CR rates of >80% in patients with AML. The OS of these patients, however, remains poor with a substantial proportion subsequently relapsing. Prognostic stratification by cytogenetic risk[1–4] and the presence of FLT3/ITD[5–7] can define a population of patients whose outcome is improved by allogeneic hematopoietic SCT (HSCT), but relapse post-HSCT remains the major cause of treatment failure.

Options for treatment of post-HSCT relapse in AML are extremely limited, and since interventions such as DLI or rapid withdrawal of immunosuppression are most effective when the leukemic burden is low, it is critical to identify patients with the highest risk of relapse at the time of allograft and/or to detect relapse post-HSCT at a very early stage.

Although the presence of minimal residual disease (MRD) has consistently been shown to be associated with an adverse outcome in patients treated with chemotherapy alone,[8–13] the impact in patients undergoing HSCT is less well characterized. The techniques most commonly used to evaluate MRD are RQ–PCR and multiparametric flow cytometry (MFC). Of these, RQ–PCR for abnormal fusion transcripts (PML-RARA, CBFB-MYH11, RUNX1-RUNX1T1, MLL rearrangements) offers the highest level of sensitivity at 1×10^{-6}.[12] Owing to the low incidence of these reciprocal translocations, this technique can only be applied to approximately 25% of patients with AML.[14] Furthermore, as the most common of these are associated with a favorable outcome (where allograft is not generally recommended in first remission), they become less relevant as a marker for MRD in the setting of allogeneic transplantation. Other PCR targets include mutations of the *NPM1* gene or WT1 overexpression, but, again, these can only be applied to a fraction of patients with AML and measurement of WT1 can be complicated by physiological background expression.[15–17]

MRD monitoring by MFC relies on the detection of a patient-specific leukemia-associated immunophenotype at diagnosis by the co-expression of aberrant markers, which can be identified in >90% of the patients with AML if a comprehensive panel of MoAbs is employed.[18,19] Current techniques offer sensitivities between 1×10^{-4} and 1×10^{-5} [16] and also offer the advantage of rapid analysis.

Walter *et al.*[20,21] have reported an association between pre-transplant MRD measured by MFC and an increased risk of relapse and death in recipients of myeloablative (MA) HSCT for AML, but the clinical utility in patients undergoing reduced-intensity conditioned (RIC) allogeneic HSCT, is less clear. A recently published study by Ustun *et al.*[22] indicates that this association may be extended to the recipients of RIC allografts, but with very few patients in their MRD-positive group, these results warrant verification.

Smaller studies have suggested an association between the presence of MRD post-HSCT and relapse,[23,24] but currently there is insufficient evidence to suggest modification of post transplant management on the basis of post transplant MRD status.

In this study we retrospectively assessed the impact of pre-transplant and post transplant MRD by MFC on transplant outcome in adults with AML. We explicitly investigated the quantitative effect of pre-transplant MRD and the relationship between MRD and other prognostic variables.

PATIENTS AND METHODS

Patients were identified using our hospital-specific electronic database and were included in our study if they were ⩾17 years of age with (1) a

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diagnosis of AML as defined by 2008 WHO criteria;[25] (2) expression of an aberrant leukemia-associated phenotype tested in our laboratory at diagnosis; (3) achievement of morphological and cytogenetic CR prior to allograft; (4) RIC or MA allograft at The Royal Marsden Hospital (London, UK) between January 2006 and December 2011. Cytogenetic analysis was performed at diagnosis by the G banding method, and cytogenetic risk was assessed using the revised MRC prognostic classification.[26]

A total of 88 patients meeting our criteria were identified and data pertaining to these patients were collected retrospectively from electronic patient records with follow-up complete as of 27th August 2012. Approval for this study was obtained from our institutional Audit Committee (Haem066), and all patients signed informed consent prior to HSCT.

Detection of MRD

Immunophenotypic analysis was performed at diagnosis and follow-up on whole BM specimens after stain-lyse-wash standard techniques was carried out. Three-color immunostaining with fluorochrome directly conjugated with MoAbs was performed, with a panel consisting of membrane CD45 CD2 CD7 CD10 CD19 CD20 CD22 CD13 CD14 CD15 CD33 CD34 CD64 CD117 HLA DR and intracytoplasmic TdT MPO Lysozyme IgM CD3 CD79a CD22. FACSCalibur (Becton-Dickinson, Franklin Lakes, NJ, USA) and Cantoll (Becton-Dickinson) instruments were used with CellQuest (Becton-Dickinson) and FACSDiva (Becton-Dickinson) analysis software, respectively. A CD45 gating strategy was used for analysis and 10^5 mononuclear cells were acquired from follow-up samples, sensitivity of the technique: 4×10^{-3}.

Based on immunophenotype at diagnosis, leukemia-associated immunophenotypes were defined and used to assess follow-up samples for the presence of MRD. BM samples were taken for assessment of MRD pre-HSCT at a median of 22 days prior to the transplant date, and 3 months post allograft. We considered any level of residual disease as MRD-positive, and sub-stratified patients into high-level MRD-positive if they had $\geqslant 1\%$ (but less than 5%) MRD, or low-level MRD-positive if they had $<1\%$ MRD.

Statistical analyses

Statistical analyses were performed using SPSS version 20 and 'R' software (2011-12-22 Copyright 2011, The R Foundation for Statistical Computing, Vienna, Austria), with competing risks calculated using the package 'cmprsk' (RJ Gray, 2010; http://CRAN.R-project.org/package=cmprsk).

Relationships between categorical variables were analyzed using χ^2-test or Fisher's exact test. OS defined as the time from HSCT to final follow-up or death was calculated by Kaplan–Meier analysis. Univariate analysis of OS was performed using the log-rank test, and the Cox proportional hazard method was used for multivariate analysis. Cumulative incidence of relapse and non-relapse mortality (NRM) were calculated by competing risks analysis; NRM being the competing risk for relapse, while relapse was the competing risk for NRM.

Univariate analysis was performed to determine the factors associated with shorter post-HSCT survival, and variables were then included in multivariate analysis if significantly associated with survival at $P<0.2$. This included positive MRD pre-allograft, patient age at transplant, poor cytogenetic risk at diagnosis, requiring greater than one cycle of chemotherapy to achieve remission and recipient CMV-positivity.

RESULTS

Patient characteristics

The median age at the date of transplant was 45 years (range: 18.2–70.1). All 88 patients studied were in CR, defined as $<5\%$ blasts by morphology in pre-transplant BM aspirates, and no patients had measurable disease by cytogenetic or molecular analyses. Overall, 64 patients were in CR1, 22 patients were in their second CR and 2 patients were in third CR. Three patients had favorable risk cytogenetics at diagnosis, 58 patients had intermediate risk disease, 26 had adverse risk abnormalities and in 6 patients cytogenetic studies failed.

Forty six patients received MA conditioning (CY and TBI in 40/46) with *in vivo* alemtuzumab where unrelated donors were used; 42 patients had RIC (36/42 receiving fludarabine, melphalan and alemtuzumab). Thirty patients had identical sibling donors, 51 patients had unrelated donors and 7 patients underwent cord allografts. Seven patients received BM and 74 received PBSC.

All patients in this study had their MRD status assessed on BM samples prior to allograft, 35 of whom were shown to be MRD-positive by MFC. Of these patients, 16 were low-level MRD-positive ($<1\%$), 9 were high-level MRD-positive (1–4.9%) and in 7 patients MRD was positive but quantification had not been reported (these 7 patients were excluded from the stratified analyses).

As shown in Table 1, clinical characteristics differed between MRD-positive and negative patients. Notably, 8 of the 10 patients with secondary AML remained MRD-positive pre-HSCT ($P=0.012$), and of 17 patients who had failed induction chemotherapy, only 4 attained MRD-negative status at the time of allograft ($P=0.001$). Patients with positive MRD therefore tended to have had more cycles of induction chemotherapy ($P=0.061$) and were more likely to have received salvage chemotherapy with a FLAG (fludarabine, cytarabine and GCSF)-based regimen ($P=0.007$).

Interestingly, we found no association between adverse (vs intermediate or favorable) cytogenetic risk and the presence of MRD pre-allograft ($P=0.62$), and patients in CR>1 were equally likely to achieve MRD-negative status as those in CR1 ($P=0.95$). Patients in the MRD-positive group tended to be older but this finding was not statistically significant ($P=0.061$).

Relationship between MRD status, survival, relapse and NRM

The median follow-up after transplant date amongst survivors was 3.32 years (range 0.57–7.68) with no patients lost to follow-up. The 2-year estimate of OS in the entire cohort was 57%, with a significant difference between patients who were MRD-negative, low-level MRD-positive and high-level MRD-positive pre-transplant at 66.8%, 51% and 30%, respectively ($P=0.012$; Figure 1).

The results of univariate survival analyses are shown in Table 2; age at transplant, poor cytogenetic risk at diagnosis, recipient CMV status and requiring greater than one cycle to attain CR were

Table 1. Clinical characteristics of patients studied

Variables	*MRD-negative pre-HSCT*	*MRD-positive Pre-HSCT*	P-*value*
Number of patients	53	35	
Reduced intensity			
Myeloablative	21	21	0.061
	32	14	
Median age (range)	44 (18–70)	52 (21–70)	0.061
Cytogenetic risk			
Favorable	2	1	0.62
Standard	33	22	
Poor	16	9	
CR 1	39	25	0.95
CR 2	13	9	
CR 3	1	1	
Secondary AML	2	8	0.012
1 induction cycle	6	2	
2 induction cycles	41	25	
3 induction cycles	6	6	
4 induction cycles	0	2	
Induction Failure	4	13	0.001
Received salvage regimen	33	31	0.007
No salvage regimen	20	4	
CMV-positive recipient	25	22	0.149
Sibling donor	19	11	0.67
Unrelated donor	30	21	
Cord	4	3	

Abbreviations: HSCT = hemopoietic stem cell transplant; MRD = minimal residual disease.

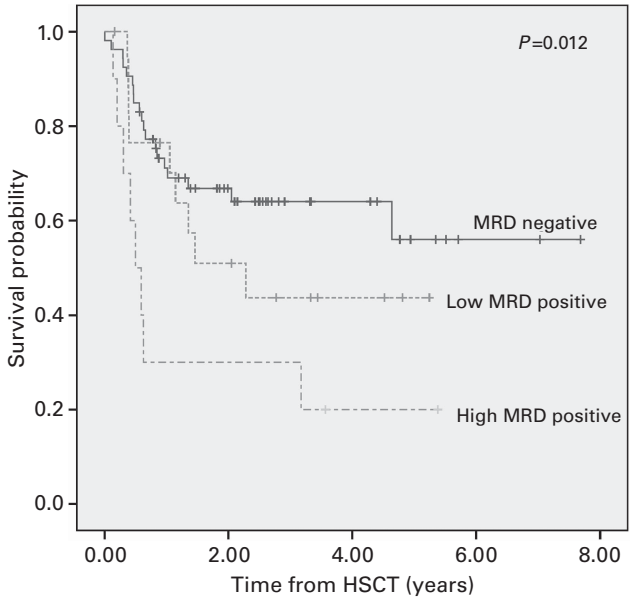

Figure 1. Probability of OS for 81 patients with AML stratified by high-level positive, low-level positive or negative minimal residual disease (MRD) status by multiparametric flow cytometry on BM aspirate samples pre-allograft.

Table 2. Univariate analysis of factors affecting OS

Variable	*HR*	*95% CI*	P-*value*
MRD positivity	2.03	1.10–3.75	0.02
Greater than 1 cycle to CR	1.67	0.89–3.15	0.11
Age	1.02	0.99–1.04	0.07
Adverse cytogenetic risk	1.69	0.89–3.22	0.11
Failure of induction	1.27	0.62–2.60	0.51
Recipient CMV	1.66	0.89–3.12	0.11
Donor type	1.45	0.74–2.86	0.27
Secondary AML	1.36	0.57–3.24	0.48
MA vs RIC	1.24	0.67–2.80	0.50
CR1 vs >1	1.05	0.53–2.09	0.89

Abbreviations: MA = myeloablative; MRD = minimal residual disease; RIC = reduced-intensity conditioning.

found to be significantly associated with death. These factors, in addition to the remission number (1 vs >1) and type of conditioning (MA vs RIC) were included in multivariate analysis (Table 3), MRD status pre-transplant remained significantly associated with increased mortality; hazard ratio, 1.92 (95% confidence interval, 1.15–3.22), $P = 0.014$.

The risk of relapse at 2 years was significantly increased in the high-level MRD-positive group compared to the low-level positive group and the MRD-negative patients at 70%, 37% and 7.6%, respectively ($P < 0.001$; Figure 2). NRM did not differ significantly between MRD-positive and -negative groups ($P = 0.38$).

We examined the impact of pre-HSCT MRD on survival in the subgroup receiving RIC-HSCT and again found significant differences in 2-year OS between MRD-negative, low-level MRD-positive and high-level MRD-positive groups at 65%, 50.5% and 25%, respectively ($P = 0.015$). Repeating this analysis exclusively in recipients of MA-HSCT, we found a trend toward poorer survival in MRD-positive patients.

Seventy patients had BM samples evaluated by MFC at 3 months post-transplant, 10 of whom were found to be MRD-positive. Seven of these 10 patients had also been MRD-positive pre-allograft and, of the patients who were MRD-positive at both

Table 3. Multivariate analysis of factors affecting OS

Variable	*HR*	*95% CI*	P-*value*
MRD positivity	1.92	1.14–3.22	0.014
Poor cytogenetic risk	2.07	0.95–4.48	0.065
Recipient CMV-positivity	1.53	0.72–3.26	0.271
Patient age	1.04	1.0–1.08	0.41
Greater than 1 cycle to CR	1.32	0.55–3.16	0.538
2nd or subsequent remission	1.54	0.6–3.98	0.369
MAC	2.90	0.91–9.20	0.07

Abbreviations: MAC = myeloablative conditioned; MRD = minimal residual disease.

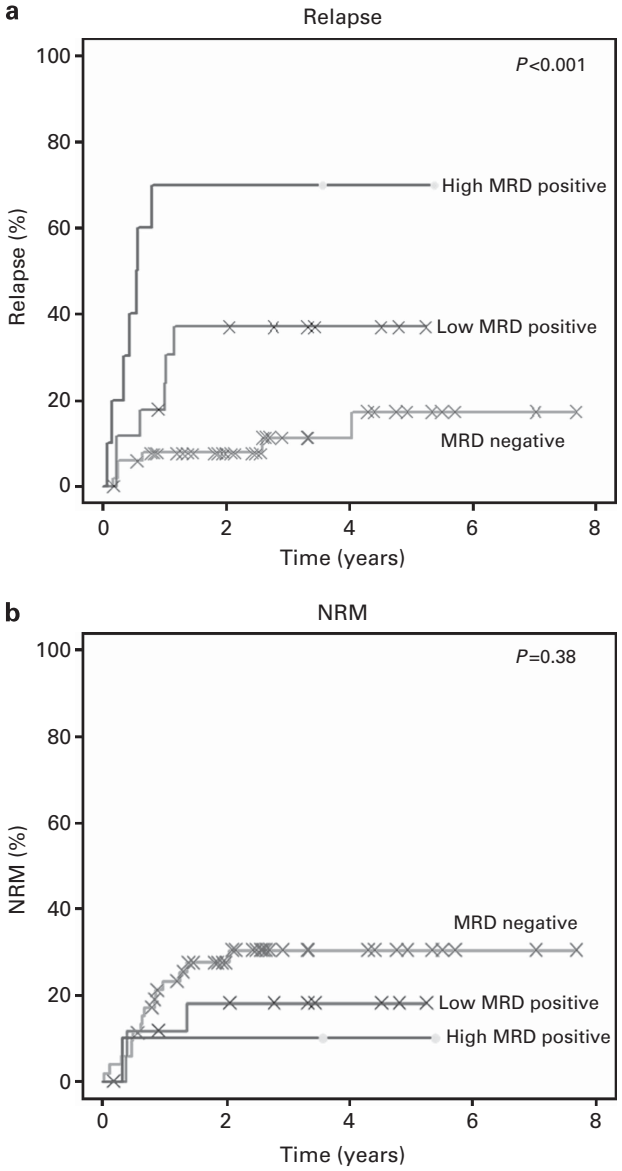

Figure 2. Probability of relapse (**a**) and non-relapse mortality (**b**) for patients with negative vs low-level positive and high-level positive minimal residual disease (MRD) status by multiparametric flow cytometry on pre-allograft BM aspirate samples.

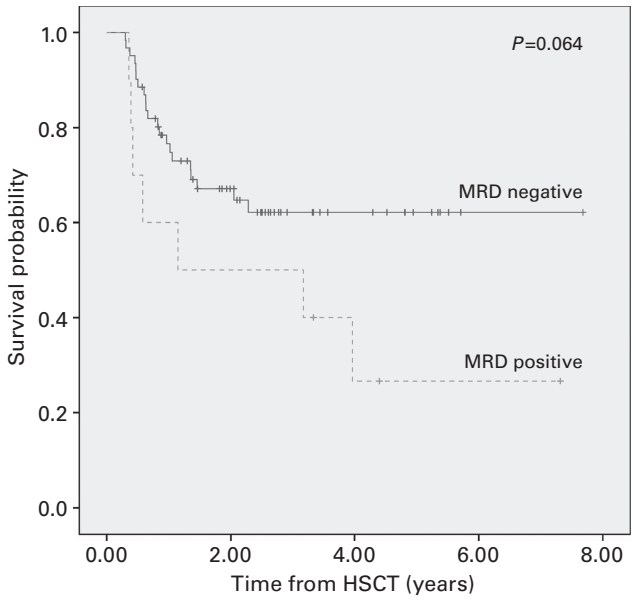

Figure 3. Probability of OS for 71 patients with AML stratified by positive or negative MRD status at 3 months post-transplant.

time points, only 1 survived at last follow-up ($P = 0.01$). As shown in Figure 3, there was a trend toward improved 2-year survival in patients who were MRD-negative at 3 months post-HSCT, with 65% OS in the MRD-negative in comparison to 50% in the MRD-positive group, but this finding did not reach statistical significance ($P = 0.064$).

DISCUSSION

Our findings add to the previous data, confirming that MRD detected by MFC pre-transplant is associated with an increased risk of death and relapse in recipients of MA and RIC-HSCT in first and second CR.[20–22] In our study, the adverse OS in the MRD-positive patients was solely due to a higher risk of relapse in this group, with no demonstrable difference in NRM. Unlike other studies we only included patients in a CR by all other methodology in the analysis.

We showed that a higher level of MRD predicts a worse outcome than lower level MRD, although we did not have a large enough study group to ascertain the comparative significance of levels of MRD $<1\%$. There remains a need for standardization of MFC techniques and to establish the level at which MRD becomes clinically relevant.[8,10,11,13,20,23,24,27]

As expected, we identified a difference in clinical characteristics between patients who are MRD-positive and -negative pre-HSCT; 80% of patients with secondary AML remained MRD-positive and patients in the MRD-positive group were more likely to have failed induction therapy. MRD-positive patients were more heavily treated pre-transplant, having received a salvage regimen in 36/41 cases, suggesting that this group had a higher risk to develop disease than the MRD-negative cohort. Despite this, multivariate analysis showed the presence of pre-transplant MRD to be an independent prognostic factor.

Previous studies have indicated that post-remission chemotherapy does not improve outcomes following allogeneic transplantation in AML; however, this question has not been specifically addressed in MRD-positive patients.[28,29] Our results argue against giving further chemotherapy in an attempt to achieve MRD-negative status, but the optimal treatment of patients who remain MRD-positive pre-allograft is not yet known; it will be important to establish whether modification of conditioning or of post-HSCT management will benefit these patients.

In accordance with Diez-Campelo *et al.*,[23] our data suggest a worse prognosis for patients who are MRD-positive by MFC at 3 months post-transplant, compared with those who are MRD-negative. With only 10 patients demonstrating residual disease at this time point in our study, these results warrant confirmation, and additional evaluation is required to determine the prognostic potential of MRD monitoring at other time points post-HSCT.

Accepting the limitations of retrospective analyses, and that our patient cohort was relatively small, we believe there is sufficient evidence to support the incorporation of pre-HSCT MRD into transplant strategies for patients with AML. Prospective studies with larger patient cohorts will be necessary to assess the clinical utility of tailoring treatment on the basis of MRD results, and to establish the relative benefits of interventions such as modification of conditioning, or enhancing the GVL effect by early withdrawal of immunosuppression as well as, or in addition to, intervention with planned or prophylactic DLI post-HSCT.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

CA designed the research, collected and analyzed the data and wrote the paper. FLD collected data and wrote the paper, RM and AM performed research and wrote the paper. MEE and MNP wrote the paper, BES designed the research, analyzed and interpreted the data, and wrote the paper.

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