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Impact of high-sensitivity flow cytometry on peri-transplant minimal residual disease kinetics in acute leukemia

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Minimal residual disease (MRD) detected before hematopoietic cell transplantation (HCT) is associated with adverse outcomes in patients with high-risk acute leukemia. However, the ideal time points for post-transplant MRD assessment and the clinical significance of low levels of residual disease in this context are unclear. We conducted a prospective real-world analysis of high-sensitivity flow cytometry MRD performed before and after transplant (at days 30, 60 and 100) in 77 acute leukemia patients. The aim was to evaluate the kinetics of disease elimination and correlate it with transplant outcomes. Pre-transplant MRD was negative in 42 (MRD-) and positive in 35 patients (MRD+). Post-transplant MRD assessment was feasible at day 30 ($n = 30$, 38.9%), day 60 ($n = 27$, 35.0%) and day 100 ($n = 60$, 77.9%). Relapses occurred in 8 patients in the MRD + group (22.9%) and three in the MRD-negative group (7.1%), $p = 0.02$. Pre-transplant MRD correlated with a decrease in overall survival (OS; 87.9% MRD- vs. 54.0% MRD+) and event-free survival (EFS; 85.3% MRD- vs. 51.1% MRD+), $p = 0.001$. Cumulative incidence of relapse (CIR) was 17.5% in MRD + vs. 2.6% in MRD- ($p = 0.049$). Non-relapse mortality (NRM) was 31.4% in MRD + vs. 12.1% in MRD- ($p = 0.019$). One-year OS was higher in patients with negative MRD at d100 (92.4%, 95% CI: 0.81–0.971) than positive d100 MRD (53.3%, 95% CI: 0.177–0.796), $p < 0.0001$. Disease status and d100 MRD were associated with OS, EFS and CIR. Differences in NRM between leukemia types (ALL: 18.9% MRD- vs. 50% MRD+, and AML 0% MRD- vs. 21.7% MRD+, $p = 0.0158$) were also observed. In conclusion, pre-transplant MRD assessed by highly sensitive flow cytometry accurately identified patients with adverse prognoses. Persistent MRD after HCT could predict relapse with high specificity and clinical sensitivity. These results highlight the importance of incorporating peri-transplant MRD kinetics into the routine treatment of acute leukemia, particularly in low/middle-income countries.

Keywords Minimal residual disease, Measurable residual disease, Acute lymphoblastic leukemia, Acute myeloid leukemia, Allogeneic hematopoietic stem cell transplantation, Flow cytometry, Biomarkers

Minimal or measurable residual disease (MRD) is a useful tool for assessing the quality of response after treatment in pediatric and adult patients diagnosed with acute leukemia[1,2]. High-risk patients typically require allogeneic hematopoietic cell transplantation (HCT) during the first or second complete remission (CR1/CR2) as a unique curative option[3,4]. Unfortunately, relapse and treatment-related mortality limit efficacy and can lead to transplant failure[5–7]. Notably, the presence of residual disease immediately before transplantation is associated with unfavourable outcomes in both lymphoblastic (ALL)[8–11]and myeloblastic (AML)[12–14]acute leukemia. Additionally, post-transplant MRD appears informative for predicting relapses[9,15,16]and has been instrumental in tailoring treatment approaches, such as early reduction of immunosuppression[17–19].

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MRD detection methods, including multiparameter flow cytometry and next-generation multigene sequencing (NGS), are increasingly sensitive[20,21]. Recent advancements in flow cytometry standardisation and validation for high-sensitivity testing have expanded its utility by incorporating new markers and analysing a greater number of cellular events[22–24]. Consequently, the flow cytometry sensitivity can be compared with that of molecular techniques, increasing the accuracy of residual cell detection[25–27]. However, the clinical significance of detecting such low levels of residual disease in the context of hematopoietic stem cell transplantation remains unknown[28,29].

Previous studies have emphasized the critical role of detecting and quantifying residual disease before and after allogeneic hematopoietic stem cell transplantation[8,10,11,14]. Despite this, the role of high-sensitive MRD detection methods, the best time points to assess post-transplant MRD in acute leukemia and the significance of low levels of residual disease in this context are unclear[9,15,30].

In Brazil, acute leukemias represent one of the primary indications for hematopoietic cell transplant across all age groups, with nearly half of the cases transplanted in first remission[31]. While retrospective studies in adult[32]and pediatric[33,34]cohorts have linked MRD negativity after induction therapy with prolonged overall and event-free survival, the importance of peri-transplant MRD and the kinetics of post-transplant MRD in countries with limited resources must be better characterized[35–38]. This prospective study investigates the role of high-sensitivity flow cytometry MRD done before and after transplantation in high-risk acute leukemia in a real-world setting, to evaluate the kinetics of disease elimination and correlate it with transplant outcomes.

Methods

Study design and patient characteristics

A prospective, longitudinal, and observational study was conducted on consecutive patients with high-risk acute leukemia who underwent allogeneic HCT at the Hospital de Clínicas da Universidade Federal do Paraná (CHC-UFPR) in Curitiba, Paraná, Brazil, between June 2019 and June 2023.

Patients who achieved morphological complete remission status according to the international criteria[39] were scheduled for local high-sensitive MRD analysis before transplantation. Based on the results of the MRD test, patients were categorised into two groups: the pre-transplant MRD-positive (MRD+) and negative (MRD-) groups. Patients were assessed for MRD detection at days 30, 60 and 100 after HCT, and at days 180 and 360 according to survival.

Definitions

Complete remission (CR) was characterised by morphological remission with fewer than 5% blasts in the bone marrow (BM) and no evidence of extramedullary disease. Relapse was defined as any evidence of disease above a detectable level at bone marrow or extra-medullary sites, with a threshold of 10^{-4} (< 0.01%) in ALL and 10^{-3} (> 0.1%) in AML patients. Neutrophil engraftment was described as a neutrophil count greater than 0.5×10^9/L for three consecutive days, while platelet engraftment required a platelet count greater than 20×10^9/L for seven days without platelet transfusion. Acute graft-versus-host disease (aGvHD) and chronic graft-versus-host disease (cGvHD) were diagnosed and classified according to established criteria, with grading determined according to the pattern and severity of organ involvement[40]. Donor chimerism was identified by short tandem repeats (STR) analysis using the polymerase chain reaction technique. Complete donor chimerism was defined as the presence of over 95% donor-derived cells, and mixed chimerism was defined as the detection of 5–95% donor-derived cells[44].

Flow cytometry sample preparation

Bone marrow aspiration for MRD testing was routinely performed in the month before transplantation and on days d30, d60, d100, and d360 after HCT, depending on patient survival. Briefly, 2–3 ml of bone marrow sample was collected in ethylenediaminetetraacetic acid (EDTA) anticoagulant and processed within 24 h according to European Leukemia Net guidelines[21]. The staining protocol included a lyse-wash protocol using FACS Lysing Solution (BD Biosciences) and, if necessary, an ammonium chloride-based lysis solution[41,42]. Samples were acquired on a FACSCantoII™ cytometer (BD Biosciences, Erembodegem, Belgium) using FACS-Diva software (BD Biosciences). Instrument settings were generated according to Euroflow guidelines[43] and data analysis was performed using Infinicyt™ software version 2.0 (Cytognos SL, Salamanca, Spain).

The MRD panel consisted of 8-colour tubes designed to detect residual disease in acute leukemia, incorporating core MRD markers according to international guidelines[44,45]. For accurate MRD assignment in cases of B-cell precursor ALL (BCP-ALL), a standard protocol was adopted using the two tubes described by Theunissen et al.[26] in 2017. The panel comprised CD10 APC (clone MEM-78), CD19 Pecy7 (clone J3-119), CD20 V450 (clone L27), CD34 PercpCy5.5 (clone 8G12), CD38 APC-H7 (clone HB7), CD45 V500c (clone 2D1), CD66c PE (clone B62), and CD73 PE (clone AD-2), CD81 FITC (clone JS81), CD123 PE (clone 9F5) and CD304 PE (clone Neuropilin-1). T-cell ALL MRD markers included cytoplasmic CD3 V450 (clone HCHT1), surface CD3 APC-H7 (clone SK7), CD5 Percpcy5.5 (clone L17F12), CD7 APC (clone M-T701), CD45 V500c (clone 2D1), CD99 PE (clone Tü12), and CD117 Pecy7 (clone 104D2). The antibody panel for AML MRD included CD7 FITC (clone 4H9), CD10 APC-H7 (clone H10A), CD11b APC (clone D12), CD13 PE (clone L138), CD14 APC-H7 (clone MφP9), CD16 FITC (clone CLB-Fc-gran/1), CD33 APC (clone P67.6), CD36 FITC (clone CLB-IVC7), CD64 PE (clone 10.1), CD117 Pecy7 (clone 104D2), HL-DR V450 (clone L243), CD34 PercpCy5.5 (clone 8G12), CD38 APC-H7 (clone HB7), CD45 V500c (clone 2D1), CD56 PE (clone N901*), and CD300e APC (clone UP-H2).

The arrangement of the monoclonal antibody panel and fluorescence used are detailed in Supplementary Table S1.

Measurable residual disease detection

MRD was done using a combination of the "different from normal" method (DifN) and leukemia-associated immunophenotype (LAIP), adhering to current literature[21,22].

We routinely acquire more than one to two million cellular events per tube for AML and T-cell ALL and 5,000,000 events per tube in BCP ALL patients[26], ensuring the test's sensitivity. The objective was to obtain a sensitivity of at least 10^{-4}(< 0.01%) for a higher resolution. Thresholds were determined using the limit of detection (LoD) and lower limit of quantification (LLOQ), with a cluster of 20 events required to confirm positive MRD and a cluster of 50 events are necessary for MRD quantification[25,44,45].

The presence of any level of abnormal cells constitutes the **MRD-positive group (MRD+)**, whereas patients with less than 10^{-4} (< 0.01%) blast cells were considered the **MRD-negative (MRD−)** group. Patients with more than 5% of blasts were considered to have **active disease**. The abnormal blast population was quantified as a percentage of total nucleated blood cells and the frequency of normal neutrophils, monocytes, lymphocytes, erythrocytes, and myeloid precursors.

Statistical analysis

Descriptive statistics were used to summarize baseline patient and transplantation characteristics. An independent sample *t*-test assessed data, with rates compared using Pearson's or Fisher´s exact test for categorical variables and Mann-Whitney or Kruskal-Wallis tests for continuous variables. Overall survival (OS) was defined as the time from transplant to death from any cause, and event-free survival (EFS) as the time until disease progression (relapse) or death of any cause, including non-relapse mortality (NRM). Transplant outcomes were calculated for the population as a whole and by the MRD subcategory (pre-transplant MRD + vs. MRD-). The primary endpoint was two-year overall survival, and secondary endpoints included EFS, post-transplant relapse rate, cumulative incidence of relapse (CIR) and NRM. The Kaplan-Meier method was used to generate survival curves, and the Log-Rank test was employed to evaluate statistical differences between the curves. Cumulative incidence (CI) curves and the Gray Test were used to evaluate relapse and NRM. To evaluate the impact of MRD status at day 100 post-transplant we conducted a Kaplan-Meier analysis with a log-rank test, employing a landmark approach at day 100. Correlations between MRD at different time points were analysed using Spearman's test. A contingency table was constructed to analyse MRD positivity before and after transplantation and relapse at two years of follow-up.

A 2-sided $p\text{-}value \leq 0.05$ was considered statistically significant. Statistical analyses were performed in SPSS Statistics v.20.0 software (SPSS Inc., Chicago, IL, USA) and EZR version 1.53 (Saitama Medical Center, Jichi Medical University, Saitama, Japan) was used for competing risk analysis.

Ethics committee

This study was approved by the CHC-UFPR Medical Ethics Committee under protocol number CAAE 84969718.0.000.0096 and was conducted following the principles of the Declaration of Helsinki. The patient or legal guardian consented to use biological material and access to medical records. The authors confirm that NO organs or tissues were harvested from prisoners. All BM or peripheral blood donors, whether related or unrelated, provided informed consent for donation. Medical records were accessed in the hospital's database system.

Results

Patient cohort

During the study period, eighty-six patients with high-risk leukemia underwent transplantation. Nine patients were excluded due to active disease or frank dysplasia. Consequently, the cohort comprised 77 acute leukemia patients who achieved morphological remission and had a local high-sensitivity flow cytometry analysis performed before the procedure. There were 39 ALL patients (29 BCP-ALL, 10 high-risk T-cell ALL), and 38 AML patients. The patients were divided into two groups according to pre-transplant MRD status: 35 MRD+ (23 AML and 12 ALL patients) and 42 MRD- (27 ALL and 15 AML). Post-transplant MRD assessment was possible at d30 ($n = 30$, 38.9%), d60 ($n = 27$, 35.0%) and d100 ($n = 60$, 77.9%).The flowchart in Fig. 1 illustrates the evolution of each subgroup.

Baseline demographic and clinical characteristics of participants

There were 77 patients, a median age of 36.4 (range 2.0 to 62.5 years), 55.8% males. Regarding disease status, 53 (68.8%) patients were in first complete remission (CR1), 22 (28.6%) were in CR2, and two (5.6%) were in CR3 before transplantation. A higher proportion of patients in CR1 were in the MRD negative group (83.3% vs. 51.4%, $p = 0.003$) than other disease status groups. Among acute lymphoblastic leukemia patients the molecular analysis showed *BCR::ABL1* fusion in 14 patients (35.9% of ALL group), one hyperdiploidy karyotype, and two *KMT2A* pro-B ALL.

Considering transplant-related variables, 38 (49.4%) patients received a matched-related donor, 27 (35.1%) received an unrelated donor, and twelve (15.6%) received a haploidentical transplant. Bone marrow was the source of cells in 44 patients, and peripheral blood in 33 patients. All patients received myeloablative conditioning regimens, which included total body irradiation (TBI) and cyclophosphamide 120 mg/kg (Cy/TBI, $n = 33$, 42.9%), Busulfan and cyclophosphamide (Bu/Cy, $n = 37$, 41.8%) ± rabbit anti-thymocyte globulin (ATG), or regimens based on fludarabine (Flu/TBI, $n = 7$, 9.1%). A higher proportion of TBI regimens were used in the MRD- versus MRD + group (66.7% vs. 37.1%, $p = 0.012$). The prophylaxis treatment of graft-versus-host disease (GvHD) was based on short-term methotrexate (MTX) combined with cyclosporine A (CSA) ($n = 60$, 77.9%) or post-transplant cyclophosphamide (PTCy) plus CSA and mycophenolate mofetil ($n = 17$, 22.1%). There were

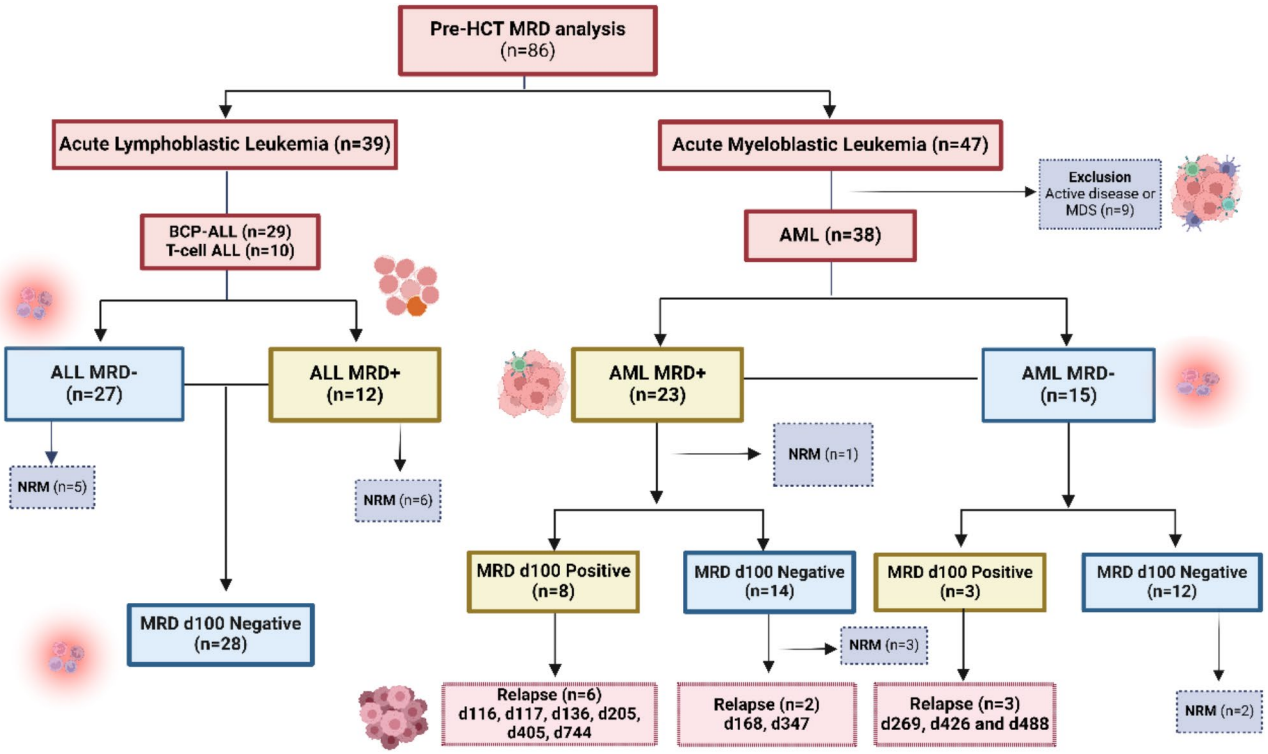

Fig. 1. Flow chart of patients included in the study showing post-transplant MRD kinetic considering the pre-transplant MRD status. Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; BCP-ALL, B-cell precursor acute lymphoblastic leukemia; HCT, hematopoietic stem cell transplantation; days d30; d100; MDS, myelodysplastic disease; MRD, measurable residual disease; NRM, non-relapse mortality.

no significant differences in age, sex, disease subtype and characteristics, cell source, donor type, or GvHD prophylaxis between MRD + and MRD- groups.

Table 1 summarises patient characteristics stratified according to pre-transplant MRD status and univariate analysis.

Hematopoietic engraftment and early post-transplant complications

The median time for neutrophil engraftment was 19 days (range 15–23), and platelet engraftment was 21 days (range 13–28). One patient did not achieve neutrophil engraftment but was successfully rescued with a second transplant, while the other six died without engraftment. Infections were documented within the first 30 days of transplantation in 42 (54.5%) patients, and 17 (22.1%) required intensive care at the time of hospitalisation. Mucositis grade III-IV was observed in 58 (75.3%) with no difference between MRD + and MRD- groups. The cumulative incidence of acute GvHD grades III-IV at d100 was 28.6%, and chronic GvHD was 49.3%, with no difference between MRD + and MRD- groups. Complete chimerism on day 100 was documented in 45 (80.4%) patients and incomplete chimerism in 11 (19.6%) patients, $p = 0.497$.

NRM was higher in pre-transplant MRD + than MRD- groups (11.9% vs. 34.3%, $p = 0.019$). A total of eleven patients relapsed during the study, eight were from the pre-transplant MRD + and three from MRD- (22.9% vs. 7.1%, $p = 0.02$).

Table 2 shows post-transplant events according to pre-transplant MRD status.

Measurable residual disease analysis and sensitivity

A total of 257 bone marrow aspirate samples were analysed, including 77 pre-transplant and 180 post-transplant samples. Most available samples achieved high sensitivity, with 1,000,000 to 10,000,000 events acquired in 255 (99.2%) samples. Considering this data, the median limits of detection and quantification achieved were 0.002% and 0.005% in AML ($< 10^{-5}$), and 0.0002% and 0.0005% in ALL ($< 10^{-6}$), respectively.

Eight patients in the acute lymphoblastic leukemia group had MRD higher than 0.01%, and four had quantifiable MRD at low levels (range: 0.0009–0.009). In the BCP-ALL group, we found 10 patients with bright CD10 and weak/dim CD38/CD34 expression; three cases of CD10 dim with CD34-CD38-; three cases with negative CD10/CD20 expression; three patients were positive for CD304/CD73, and twelve were positive for CD66c/CD123. The high-risk T-cell leukemia patients had strong CD2/CD7, and CD99 and cytoplasmic CD3 positivity with weak or negative surface CD3 in all diagnostic samples, and one patient had gamma-delta TCR expression.

The phenotype of AML patients was known in 33 cases, with 3–4 phenotypic alterations observed in each sample (mean 3). The studied LAIPs included CD34/HLA-DR negativity (15 cases), weak CD38 expression (14

	Total ($n=77$)	MRD-($n=42$, 54.5%)	MRD+ ($n=35$, 45.5%)	p-value
Age (y, median ± sd)	36.4 ± 17.4	34.5 ± 16.2	34.7 ± 17.3	0.785
Children < 14y	10 (14.9%)	5 (14.7%)	5 (15.2%)	0.614
Male (n/%)	43 (55.8%)	24 (57.1%)	19 (54.3%)	0.822
Disease type				
AML ($n=38$)	38 (49.4%)	15 (39.5%)	23 (60.5%)	0.251
ALL ($n=39$)	39 (50.6%)	27 (69.2%)	12 (30.8%)	
BCP-ALL	29 (74.3%)	19 (45.2%)	10 (28.6%)	0.693
T-Cell ALL	10 (13.0%)	8 (19.0%)	2 (5.7%)	--
BCR::ABL positive	14 (35.9%)	9 (37.5%)	5 (41.7%)	0.544
Disease stage				
CR1	53 (68.8%)	35 (83.3%)	18 (51.4%)	0.003
CR2 or more	24 (31.2%)	7 (16.7%)	17 (48.6%)	
Cell source				0.368
Bone marrow	44 (57.1%)	26 (61.9%)	18 (40.9%)	
Peripheral blood	33 (42.9%)	16 (38.1%)	17 (48.6%)	
Number of cells				
CNT ($x10^8$/uL, median ± sd)	6.2 ± 3.2	5.7 ± 3.1	6.3 ± 3.1	0.874
CD34 (x106/uL, median ± sd)	6.2 ± 3.5	5.5 ± 2.85	6.5 ± 3.35	0.125
Donnor				
Matched related donor	38 (49.4%)	19 (45.2%)	19 (54.3%)	0,257
Unrelated donor	27 (35.1%)	18 (42.9%)	9 (25.7%)	
Haploidentical donor	12 (15.6%)	5 (11.9%)	7 (20.0%)	
HLA Incompatibilities				
None	43 (55.8%)	23 (54.8%)	20 (57.1%)	0.860
One	22 (28.6%)	13 (31.0%)	9 (25.7%)	
Two or more	12 (15.6%)	6 (14.3%)	6 (17.1%)	
Preparatory regimen				
Cy + TBI+/-ATG	35 (45.5%)	26 (61.9%)	9 (25.7%)	0.006
BU + Cy	38 (49.4%)	14 (33.6%)	24 (68.6%)	
Other (Fludarabine based)	4 (5.2%)	2 (4.8%)	2 (5.2%)	
TBI (yes)	41 (53.2%)	28 (66.7%)	13 (37.1%)	0.012
GvHD prophylaxis				
MTX + CSA	60 (77.9%)	33 (78.6%)	27 (77.1%)	0.888
PTCy + CSA + MMF	17 (22.1%)	9 (21.4%)	7 (22.9%)	

Table 1. Demographic and clinical characteristics according to pre-transplant MRD status. Legend: Data are described as n (%) or mean ± standard deviation. *p-values* were determined using *t-test*, Pearson's, Fisher´s exact test, chi-square, or Wilcoxon-Mann-Whitney test. ALL: Acute lymphoblastic leukemia; AML: Acute myeloblastic leukemia; ATG, rabbit antithymocyte globulin; MRD: Measurable residual disease; CR: Complete remission; GvHD: graft-versus-host disease; HCT: Hematopoietic cell transplantation; BM: Bone marrow; PB: Peripheral blood; PTCy: Post-transplant cyclophosphamide; TBI: Total body irradiation; Cy: Cyclophosphamide; BU: Busulfan; MTX: Methotrexate; MMF: Mycophenolate mofetil.

cases), asynchronous expression of CD13/CD33 (17 cases), positivity of CD56 in five, and bright expression of CD7 in 10 cases; four cases expressed CD19 and CD56, and six had monoblastic phenotype.

Using DifN and LAIP approaches, we found fourteen AML cases with MRD+. Two cases had blasts detectable above the threshold of 0.1% (0.04% and 0.05%) and were included in the MRD + group. Three patients with low expression or negative CD34 at relapse were considered as MRD- in the pre-transplant analysis. These cases were most likely false negatives, but the flow cytometry analysts did not previously know this LAIP.

The clinical and biological characteristics of the patients, including LAIPs strategy in each case, are shown in Supplementary Table S2.

Pre- and Post-transplant MRD kinetics

Post-transplant minimal residual disease was assessed at the following time points:

(I) MRD d30 tested in 30 patients, with 2 MRD+ (relapses d116 and d117).
(II) MRD d60 tested in 27 patients, with 2 MRD+ (relapses d116 and d168).
(III) MRD d100 tested in 60, with 8 MRD+ (relapses days d116, d117, d205, d289, d405, d744; two cases in follow-up).

	Total ($n=77$)	MRD-($n=42$)	MRD+ ($n=35$)	p-value
Mucositis grade III-IV	58 (75.3%)	30 (71.4%)	28 (80.0%)	0.656
Neutrophil engraftment (days)	19.3 ± 4.3	19.6 ± 4.3	18.5 ± 3.5	0.753
Platelet engraftment (days)	21.2 ± 7.4	24.2 ± 8.3	18.4 ± 5.8	0.257
Infection during HCT	42 (54.5%)	21 (50.0%)	21 (60.0%)	0.491
ICU in first 100 days	17 (22.1%)	6 (14.3%)	11 (31.4%)	0.099
CMV reactivation	26 (33.8%)	15 (37.5%)	11 (31.4%)	0.971
Acute GvHD grades II-IV	22 (28.6%)	9 (21.4%)	13 (37.1%)	0.140
Chronic GvHD	39 (50.6%)	21 (50.0%)	18 (51.4%)	0.901
d100 chimerism				0.497
100% donor	45 (80.4%)	30 (83.3%)	15 (75.0%)	
5–99% donor	11 (19.6%)	6 (16.7%)	5 (25.0%)	

Table 2. Post-transplant events according to pre-transplant MRD status. Legend: Data are described as n (%) or mean +/- standard deviation. *p-value* was determined using a *t-test*, Pearson's and Fisher´s exact test, chi-square or Wilcoxon-Mann-Whitney test. MRD, measurable residual disease; CR, complete remission, HCT, hematopoietic cell transplantation; ICU, intensive care unit; GvHD, graft-versus-host disease; CMV, Cytomegalovirus, NRM, non-relapse mortality.

(IV) MRD d180 tested in 19 patients, with 3 MRD+, (relapses d347 and d405, one case in follow-up).
(V) MRD d360 tested in 18 patients, with 5 MRD+ (relapses d405, d426 and d488, two cases in follow-up).

Relapses occurred in 8 patients from the pre-transplant MRD+ (22.9%) and three from the MRD- (7.1%) groups, $p=0.02$. A significant correlation was observed between pre-transplant MRD + and positivity at d30 ($p=0.02$) and d100 ($p=0.038$), and between positivity at any time point post-transplant with relapse ($p=0.009$).

Considering the 66 patients alive on d100, 39 (59.0%) were from the MRD- group and 27 (41.0%) from MRD+. Among these, 36 (92.3%) from the pre-transplant MRD- group remained negative and alive at d100, while three MRD- patients were assigned as MRD + at d100, and subsequently relapsed (d269, d426 and d488). These three patients had low expression of CD34 at relapse, so they were most probably false negative cases at pre-transplant analysis[54]. Of the MRD + group, 17 out of 27 (62.9%) had MRD negative at d100 and were considered cured. Eight patients (29.6%) had persistent MRD + after transplantation, and six of these relapsed later (d116, d117, d205, d289, d405, d744). Two patients had no detectable MRD on d100 (considered d100 MRD negative) but relapsed later (d347 and d488).

Survival and follow-up

The median follow-up for survivors was 2.96 years (range 2.74–3.2 years), with longer median survival time in the pre-transplant MRD negative than MRD positive groups (3.7 years versus 2.1 years, $p=0.001$). Overall, 27 out of 74 patients died (35.0%), with seven patients (16.6%) from the MRD- group (5 ALL/2 AML), and twenty (57.1%) from the MRD + group (6 ALL/14 AML). The causes of death in the MRD- group included transplant-related toxicity (five patients) and late relapse (two patients). In comparison, in the MRD + group, twelve patients died of transplant-related toxicity (7 bacterial sepsis, two fungal, one adenovirus), two patients had COVID-19 in the first year of follow-up, and eight patients died with relapse. NRM was higher in MRD + than MRD- groups (11.9% vs. 34.3%, $p=0.019$). A total of eleven patients relapsed during the study, eight were from the MRD + and three from the MRD- group (22.9% vs. 7.1%, $p=0.02$).

Prognostic impact according to pre-transplant MRD status

Total cohort OS at 1 year was 74.4% (95% CI: 0.605–0.840) and 63.6% at 2 years (95% CI: 0.360–0.948), with higher OS in pre-transplant MRD negative (87.9%, 95% CI: 0.733–0.948) than the MRD positive group (54.0%, 95% CI: 0.362–0.688), $p=0.0001$. MRD positivity before transplantation was associated with lower EFS (85.3% MRD- vs. 51.1% MRD+, $p=0.0004$), with significant differences in both ALL (81.5% MRD- vs. 50.0% MRD+) and AML (92.9% MRD- vs. 51.4% MRD+) diseases, $p=0.006$ (Fig. 2).

The cumulative incidence of relapse two years after transplantation was 2.6% in MRD- group (95% CI: 0.002–0.118) compared to 17.5% in the MRD + group (95% CI: 0.069–0.321), $p=0.04$. The cumulative incidence of NRM was 31.4% in the MRD + group (95% CI: 16.9–47.1) vs. 12.1% in the MRD- group (95% CI: 4.4–24.1), $p=0.019$, with five deaths in remission in MRD- group and twelve in MRD + group.

Outcome results are presented in Table 3; Fig. 2 curves.

Prognostic impact according to post-transplant MRD status

Patients with persistent detectable post-transplant MRD at d100 had significantly lower one-year OS rates (53.3%, 95% CI: 0.177–0.796) compared to patients with MRD negative at this time point (92.4%, 95% CI: 0.81–0.971), $p<0.0001$ (see Supplementary Figure S5). As expected, patients with positive MRD at d100 exhibited a higher cumulative incidence of relapse (3.9% vs. 55.6%, $p<0.0001$), Fig. 2.

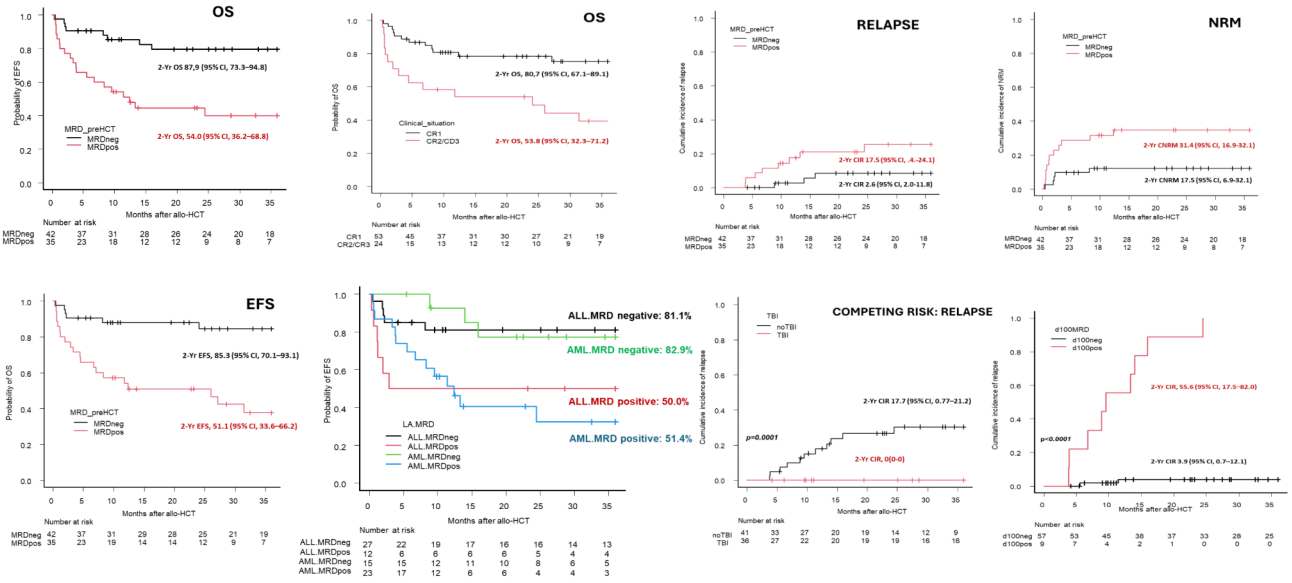

Fig. 2. Overall survival curves (Kaplan-Meier) considering pre-transplant MRD + vs. MRD- groups and clinical situation; cumulative incidence of relapse (CIR) and non-relapse mortality (NRM); event-free survival (EFS) considering pre-transplant MRD + *versus* MRD- groups and the four leukemia disease subgroups (ALL MRD-; ALL MRD+; AML MRD- and AML MRD+), and CIR considering TBI use and MRD + at d100.

Outcomes	MRD-($n=42$)	MRD+ ($n=35$)	*p*-value
OS (2y)	87,9% (73.3–94.8)	54.0% (36.2–68.8)	0.0001
EFS (2y)	85.3% (70.1–93.1)	51.1% (33.6–66.2)	0.0004
CI Relapse	2.6% (2.0–11.8)	17.5% (6.9–32.1)	0.049
CI NRM	12.1% (4.4–24.1)	31.4% (16.9–47.1)	0.197
Relapse (n, %)	3 (7.1%)	8 (22.9%)	0.020
NRM (n, %)	5 (11.9%)	12 (34.3%)	0.019
Median survival time (days)	1345 ± 92	769 ± 125	0.001

Table 3. Outcomes according to pre-transplant MRD status. Legend: Data described as n (%) or mean +/- standard deviation. Kaplan-Meier and Log-Rank test was used to survival rates, with 95% confidence interval and ranges. ALL, acute lymphoblastic leukemia; AML, acute myeloblastic leukemia; CI, cumulative incidence; MRD, mensurable residual disease; NRM, non-relapse mortality.

Competing risks analysis

Competing risk analysis (Gray test) showed that patients transplanted in first complete remission (CR1) had better OS (80.7%, 95% CI: 67.1–89.1) than another clinical situation (53.8%, 95% CI: 32.3–71.2), $p=0.0009$. Other pre-transplant variables such as age, donor type, TBI use and donor chimerism were not significant for OS or EFS in competing risk analysis, as shown in Supplementary Table S3.

The cumulative incidence of relapse was significantly lower in patients transplanted at CR1 ($p=0.02$) and in those who received TBI in the conditioning ($p=0.001$), Fig. 2.

There were no differences in the cumulative incidence of NRM between groups ($p=0.197$). However, considering leukemia subtypes we found that in patients with ALL and negative pre-transplant MRD, the cumulative incidence of NRM was 18.9% (95% CI: 6.7–35.8) compared to 50.0% (95% CI: 19.2–74.8) in MRD+, and no patients with AML and pre-transplant MRD negative died in remission vs. 21.7% (95% CI: 7.6–40.4) of AML with MRD+, $p=0.0158$.

Clinical sensitivity and specificity

A contingency table was constructed considering MRD positivity before HCT, MRD status at d100, and recurrence at two years of follow-up. The presence of detectable pre-transplant MRD had a high negative predictive value (NPV) for predicting relapse (91.4%), but a positive predictive value (PPV) of only 19.1%, with low clinical sensitivity and specificity. However, positive pre-HCT MRD shows 92.0% NPV, with 73.0% sensitivity and 65.0% specificity in predicting positivity at d100. On the other hand, the persistence of post-transplant MRD positivity on d100 showed high specificity and PPV (100%) for predicting relapse, with an NPV of 97.0% and a sensitivity of 71.0% (see Supplementary Table S4).

Discussion

Consistent with prior research our results demonstrated that pre-transplant MRD assessment through flow cytometry accurately identified patients with adverse prognoses, including lower overall and event-free survival rates[46–49], and a higher likelihood of relapse and NRM[10,14]. Furthermore, the persistence of detectable residual disease post-transplantation, especially at day 100, demonstrates high clinical specificity, sensitivity, and predictive values for relapse[9,15,16].

Our analysis confirmed that in a real-world context both MRD positivity and disease status at the time of transplantation were associated with a higher risk of death. Similarly, after adjustment for clinical status, MRD negativity before HCT was significantly associated with improved survival, particularly in patients in first complete remission. These results support the hypothesis that patients with high-risk leukemia in first complete remission who have negative MRD are the ones most likely to benefit from allogeneic transplantation[18,19].

From a methodological point of view, while flow cytometry tests for MRD detection in acute lymphoblastic leukemia have become well-established[26,50], challenges persist in standardizing tests for acute myeloid leukemia[47,51]due to the lack of specific markers and potential clonal evolution[51]. Nevertheless, innovative technologies, including leukemic stem cell quantification[52]and molecular assays, offer promise in reducing the likelihood of false-negative results[53,54], and providing additional insights on clonal architecture[55,56,58]. Although molecular biology was not used as a comparator in this study, identifying residual disease at levels below 10^{-4}allowed the detection of at least four cases with very low disease burden. However, due to the limited number of patients evaluated, no definitive conclusions can be drawn regarding the absolute necessity of such detection levels in this context, as is discussed in literature[29].

In addition to pre-transplant analyses, monitoring the kinetics of minimal residual disease after transplantation can serve as a crucial indicator of relapse risk. Notably, detectable MRD at day 100 post-transplant has emerged as a reliable predictor of subsequent relapses[16,22,27]. In the present study, the persistence of detectable MRD at day 100 was significantly associated with survival and cumulative incidence of relapse compared to patients with negative MRD at this time point. Similarly to our data, a recent multicenter retrospective analysis involving 295 AML patients undergoing hematopoietic cell transplantation indicated that patients who reached d100 and either maintained or developed a new positive MRD had an unfavourable short-term prognosis, regardless of their MRD status before transplantation[59]. Another recent study suggested that MRD identification conducted earlier post-transplant, specifically between days 20 and 40, can aid the possibility of implementing preventive strategies to mitigate the risk of relapse[15,60]. In our cohort, only half of the patients were tested for MRD on day 30 post-transplant, with two cases returning MRD positive. Notably, both patients experienced relapse around d100, suggesting an inferior prognosis. However, the small number of patients who were alive and MRD-positive at post-transplant time points limits our ability to draw definitive conclusions.

Given the heterogeneity of the patient population in this study and the relatively small number of events, careful interpretation of subgroup analyses is essential. Differences in disease biology among leukemia subtypes, treatment-related complications, and patient-specific factors significantly influence relapse rates and relapse-free mortality[38]. For instance, our study found that relapses were more common in myeloid leukemias, a highly heterogeneous group with diverse prognostic factors, including distinct cytogenetic and molecular abnormalities that affect treatment response[55]. Although allogeneic transplant is considered the best option for AML patients with persistent MRD after first-line treatment, tumour burden, even at the MRD level, is one of the variables with the greatest impact on the outcome after treatment, as described in different studies using molecular or flow cytometry techniques[3,12,14]. Importantly, pre-transplant MRD positivity in AML does not always predict imminent relapse, as the graft-versus-leukemia (GVL) effect can effectively target residual leukemic cells[40]. In contrast, MRD positivity at day 100 post-transplant demonstrated high specificity and robust predictive value for relapse[59,60], emphasizing the importance of continuous MRD monitoring during this critical period.

The unexpected association between MRD status and NRM in acute lymphoblastic leukemia in the present study merits further investigation. The potential contributing factors may include treatment-related toxicities, such as the use of high doses of TBI, the presence of aggressive or chemoresistant disease, intensified pre-transplant therapies and delayed immune recovery[38]. Recent comprehensive data from the Brazilian Hematopoietic Stem Cell Transplant Registry (HSCTBR), covering more than 9,800 transplants performed between 2012 and 2022, highlighted infections as the predominant cause of death within the first 100 days across all transplant types[31]. This underlines the urgent need for local and national initiatives not only to improve MRD monitoring but also to adjust treatment plans for this subgroup of patients in our country. Strategies could include earlier referral for transplantation, better infection control measures and adjustments in transplant-related variables[31,32,38]. On the other hand, our findings indicate that, despite the high toxicity associated with total body irradiation in conditioning, it significantly reduced relapse rates. This suggests the need to carefully balance the risks and benefits of TBI use as its enhanced disease control may outweigh potential complications, particularly in resource-limited settings[6,31,35].

Despite several limitations, such as the small number and heterogeneity of the cohort, our study demonstrates the feasibility and usefulness of flow cytometry as a sensitive, suitable, and cost-effective tool for detecting MRD in acute leukemia in the context of hematopoietic cell transplantation, particularly in resource-limited settings where molecular techniques may not be readily available[38]. Furthermore, at a regional level, the implementation of the new generation 8-color flow cytometry technique has proven to be essential both in the peri-transplant context and more generally, as prior to this study, only less sensitive methods, such as 4-color flow cytometry or morphological assessments, were used[50,57].

Overall, our findings underscore the critical role of MRD assessment prior to transplantation in guiding transplant decisions and identifying patients who may require tailored interventions. Furthermore, the persistence of MRD at any point post-transplant signals a high risk of relapse and identifies patients who could benefit from proactive interventions. These interventions might include reducing immunosuppression,

implementing targeted therapies, or introducing novel anti-leukemic agents[16,30,48]. In conclusion, these findings emphasize the importance of incorporating peri-transplant MRD kinetics into the routine management of acute leukemia, particularly in low/middle-income countries, where resource optimization and early intervention can significantly impact patient outcomes.

Data availability

The data supporting the results of this study (e.g. flow cytometry data files, patient consent forms, administrative documents) are available from the authors, but there are restrictions as they were used under license from the Hospital deClínicas da Universidade Federal do Paraná (CHC-UFPR) for this study. However, the data can be obtained on request from the corresponding author: Ana Paula de Azambuja, by e-mail apazamb@gmail.com.

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Author contributions

Author contributions: A.P.A.: wrote the main manuscript text. A.P.A., M.P.B. and M.M.: conceptualization; funding acquisition; methodology; A.P.A., Y.C.S. and J.L.P.J.: flow cytometry analysis. A.C.M.L.: statistical analysis. V.A.M.F., R.P. and C.B.: writing-review & editing. All authors approved the final version of this manuscript.

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Conflict of interest: The authors declare no potential conflict of interest.

Declarations

Compliance with ethical standards

This study was approved by the Institutional Review Boards of the CHC-UFPR Medical Ethics Committee under protocol number CAAE.

Competing interests

The authors declare no competing interests.

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Additional information

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