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# Multiparametric flow-cytometry minimal residual disease before hematopoietic stem cell transplantation predicts outcome in pediatric acute myeloid leukemia

Barbara Buldini [1,2], Pietro Merli [3,4], Elena Varotto [1], Riccardo Masetti [5], Maria Gabelli [1], Marco Zecca [6], Davide Leardini [5], Pamela Scarparo [1], Maddalena Benetton [1], Luisa Strocchio [3], Franca Fagioli [7], Alessandra Biffi [1,2], Adriana Balduzzi [8,9], Martina Pigazzi [1,2], Daria Pagliara [3], Franco Locatelli [3,10].

1. Pediatric Hematology, Oncology and Stem Cell Transplant Division, Maternal and Child Health Department, University of Padova and Padova University Hospital, Padova, Italy.
2. Pediatric Onco-Hematology, Stem Cell Transplant and Gene Therapy Laboratory, Istituto di Ricerca Pediatrica (IRP) Fondazione Città della Speranza, Padova, Italy
3. Department of Pediatric Hematology and Oncology, IRCCS Ospedale Pediatrico Bambino Gesù, Rome, Italy
4. Saint Camillus International University of Health Sciences, Rome, Italy
5. Pediatric Hematology and Oncology, IRCCS Azienda Ospedaliero-Universitaria di Bologna, Bologna. Italy
6. Pediatric Hematology-Oncology, IRCCS Policlinico San Matteo, Pavia, Italy
7. Pediatric Onco-Hematology, Stem Cell Transplantation and Cellular Therapy Division, Regina Margherita Children's Hospital, University of Turin, Turin, Italy
8. Pediatric Department, Fondazione IRCCS San Gerardo, Monza, Italy
9. School of Medicine and Surgery, University of Milano-Bicocca, Monza, Italy
10. Catholic University of the Sacred Heart, Rome, Italy

**Correspondence**
Barbara Buldini,
Pediatric Hematology, Oncology and Stem Cell Transplant Division, Maternal and Child Health Department, University of Padova and Padova University Hospital, Via Giustiniani 3, 35128 Padova, Italy
Email: barbara.buldini@unipd.it

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## Author Contributions

Contribution: B.B., P.M., E.V., and F.L. conceptualized the study; B.B., E.V., M.G. and P.S. performed formal analysis; B.B., P.M., E.V., M.G., R.M., M.Z., D.L., L.S., F.F., A.Bi., A.Ba., M.P., D.P. and F.L. were responsible for patient management and data collection; M.P. was responsible for genetic diagnosis; M.B. performed genetic screening; B.B., P.M., E.V., R.M., M.G., M.P. and D.L were responsible for data curation; P.M. was responsible for statistical analysis; B.B., P.M., E.V., and M.G. were responsible for preparation of the original manuscript draft; B.B., P.M., E.V., M.G., and F.L. were responsible for review and editing of the manuscript; E.V. and P.M. were responsible for figure preparation; B.B., P.M., F.L. supervised the study; F.L., M.P., B.B. and A.Bi. provided access to financial resources for conducting the study; all authors have read and agreed to the published version of the manuscript.

## Competing Interests

B.B. participated in a speaker’s bureau over the last 2 years: Amgen S.r.l (Milano), Beckman Coulter SRL (Milano).

P.M. reports personal fees from Sobi, Miltenyi, and Amgen, outside the submitted work.

A.Ba. participated in a Speaker's bureau fee by Amgen, Novartis; participated in an Advisory board for Sanofi, Orchards, Novartis, Jazz.

F.L. participated in a speaker’s bureau: Miltenyi, Amgen, Novartis, BMS, Medac, GILEAD, Sobi; participated in an Advisory Board for Amgen, Novartis, Sanofi, Vertex.

Other authors declare no conflict of interest.

### Data sharing statement

Data that support this study are available from the corresponding author upon reasonable request.

The prognostic impact of minimal/measurable residual disease by multiparametric flow-cytometry (MFC-MRD) before hematopoietic stem cell transplantation (pre-HSCT) in children with *de novo* acute myeloid leukemia (AML) has yet to be established. In this retrospective, multicenter analysis, we demonstrated that negative pre-HSCT MFC-MRD was associated with better 8-year outcome than any positive values, this finding being confirmed also after multivariable analysis. Notably, patients with a positive pre-HSCT MRD developing acute graft-versus-host disease (aGvHD) (any grade) had a cumulative incidence of relapse (CIR) superimposable to that of patients with pre-HSCT MRD negativity. In conclusion, pre-HSCT MFC-MRD is critical for predicting relapse-risk and optimizing therapy-intervention decisions in pediatric AML.

Outcome of pediatric patients with acute myeloid leukemia (AML) has significantly improved in the last decades, thanks to patient-tailored therapeutic approaches based on refined molecular/cytogenetic risk stratification, improvement in response-to-therapy monitoring and large use of allogeneic hematopoietic stem cell transplant (HSCT)[1,2]. Nowadays, minimal/measurable residual disease detection by multiparametric flow-cytometry (MFC-MRD) after induction treatment of *de novo* pediatric AML (pedAML) is an internationally recognized tool used for final patients' risk-group stratification[3–5]. However, differently from pediatric acute lymphoblastic leukemia (ALL)[6], the prognostic impact of MFC-MRD level before HSCT has not been extensively investigated in pedAML. In particular, while MFC-MRD positivity before HSCT has been associated with worse outcomes in adult AML cohorts[7], its prognostic role has yet to be clarified in children. In 2012, on a cohort of 122 children, the St. Jude group demonstrated a significant impact on survival of pre-HSCT MFC-MRD in ALL, but not in pedAML[8]. A recent systematic review

and meta-analysis including studies published between January 2005 and June 2016 indicated a significant association between positive pre-HSCT MRD and worse outcome in pediatric and adult AML[9].

In addition, a collaborative retrospective study from the iBFM group evaluating 112 pedAML cases demonstrated the prognostic value of molecular MRD positivity before HSCT[10].

We conducted an analysis on the impact of pre-HSCT MFC-MRD level in pedAML patients, evaluating data of the AIEOP (*Associazione Italiana Ematologia Oncologia Pediatrica*) pedAML cohort.

In this retrospective, multicenter analysis, we included pediatric, adolescent and young adult (AYA) patients (aged 0-23 at time of diagnosis) with *de novo* pedAML (patients with secondary or therapy-related AML were excluded) who underwent a first allogeneic HSCT from December 2011 to December 2021 in morphological complete remission (CR) according to therapeutic trials[2,11] with available pre-HSCT MFC-MRD assessment within 60 days before HSCT and no additional treatments between the evaluation and start of the conditioning regimen.

Patients' parents or legal guardians consented to data collection/analysis. The study protocol received approval from local institutional boards at each AIEOP center.

Diagnosis and MFC-MRD analyses were centrally performed at the Diagnostic Laboratory of Pediatric Hematology, Oncology and Stem Cell Transplant Division of Padova University Hospital, on fresh bone marrow (BM) samples. AML diagnosis relied on morphologic, cytochemical, immunophenotypic, and genetic analysis (karyotype, molecular genetic panels) as per the therapeutic protocols[2,11]. MFC-MRD was performed using 5-10 color-combinations according to standardized operating procedures previously described[3,7,12].

Allogeneic HSCT was performed locally following each center policy. Myeloablative conditioning based on busulfan, cyclophosphamide and melphalan (Bu-Cy-Mel) was recommended by national guidelines. Other conditioning regimens (myeloablative in all cases) were adopted according to center/physician preference.

Eight-year overall (OS) and event-free survival (EFS) were estimated using the Kaplan and Meier method and differences between groups were tested using the log-tank test. The 8-year CIR and non-relapse mortality (NRM) were calculated using the Fine&Gray method to consider competitive risks (namely, death in remission for relapse and disease recurrence for NRM).

A total of 218 patients met the inclusion criteria. Patients' characteristics are shown in Table 1. Median age at diagnosis was 7.3 years (range 0-23) with 25/218 (11.4%) of patients aged less than 1 year. Molecular analysis was performed and evaluable in 209/218 (96%) patients. Distribution of genetic lesions is summarized in Table 1. Regarding HSCT, 177/218 (81.2%) patients were transplanted in first CR (CR1), while 41/218 (18.8%) in second CR (CR2).

A chemotherapy-based conditioning regimen was used in 184/218 (84.4%) cases [140/218 (64.2%) Bu-Cy-Mel, 17/218 (7.8%) busulfan-thiotepa-fludarabine, 14/218 (6.4%) treosulfan-based regimens, 13/218 (6.0%) other], while total body irradiation together with cytotoxic agents was employed in the remaining 34 patients (15.6%). The stem cell source was BM in 125/218 patients (57.3%), peripheral blood (PB) in 84/218 (38.5%), and cord blood in 9/218 (4.2%). HSCT was performed from a matched unrelated donor (MUD) in 61/218 (28.0%) patients, a matched family donor (MFD) in 48/218 (22.0%), mismatched family donor (MMFD) in 80/218 (36.7%), and mismatched unrelated donor (MMUD) in 29/218 (13.3%). Information on graft-versus-host disease (GvHD) prophylaxis and acute (aGvHD) or chronic GvHD (cGvHD) occurrence are detailed in Table 1.

Pre-HSCT MFC-MRD was assessed at a median time of 22 days before HSCT (range 10-52). Overall, 180/218 (82.6%) samples were MFC-MRD negative, while 38/218 (17.4%) showed a positive MFC-MRD. Among them, MFC-MRD was <0.1% in 10/218 (4.6%) samples, 0.1-<1.0% in 15/218 (6.9%) and ≥1.0% (range 1-4.9%) in 13/218 samples (5.9%).

Median follow-up from HSCT for patients alive was 57 months (range 12-120). A total of 178 patients were alive at last follow-up, while 45 died (15 of transplant-related causes and 30 because of relapse).

The projected 8-year OS and EFS of the whole cohort were 74.2% (95% CI 63.1-82.5) and 69.1% (95% CI 62.1-75.0), respectively (Figure 1A-B), while the 8-year CIR and NRM were 23.7% (95% CI 18.0-29.9) and 7.1% (95% CI 4.2-11.2), respectively (Figure 1C).

Patients with pre-HSCT negative MRD showed a better 8-year OS compared to those with positive MRD (any positivity) [77.8% (95% CI 63.1-87.2) vs. 54% (95% CI 35.7-69.1), p<0.0001], better EFS [74.4% (95% CI 66.9-80.4) vs. 42.9% (95% CI 25.6-59.1), p<0.0001] and lower 8-year CIR [18.5% (95% CI 13.0-25.0) vs. 49.1% (95% CI 31.1-65.0), p<0.0001] (Figure 1D-E).

Timing of BM assessment (<22 vs. ≥22 days before HSCT) did not affect outcomes (Supplemental Figure 1).

The impact of MFC-MRD on OS, EFS and CIR was confirmed also in subgroup analysis in patients transplanted either in CR1 or in CR2 (Supplemental Figure 2); in patients with *KMT2A*-rearrangements, a negative MFC-MRD correlated with lower CIR, but not with better OS and EFS (Supplemental Figure 2). These findings may be due to the low number of patients per group.

Notably patients with a positive pre-HSCT MRD who experienced aGvHD (any grade) had a CIR superimposable to that of patients with negative pre-HSCT MRD [in details, 4-yr CIR of MFC-MRD+/aGvHD+ 18.1% (95% CI 2.9-44.2) vs 67.1% (95% CI 41.8-83.3) of MFC-

MRD+/aGvHD- patients, with 4-yr CIR of MFC-MRD-/aGvHD- patients 20.7% (95% CI 13.7-28.8) and MFC-MRD-/aGvHD+ 15.1% (95% CI 6.5-27.3), p<0.0001; Supplemental Figure 3]. The effect of cGvHD on CIR was not evaluable, because no patients with MFC-MRD+ patients developed cGVHD.

The 8-year EFS was not significantly different for patients given the allograft in CR1 versus those transplanted in CR2 (p=0.13). Also, year of HSCT, type of donor and stem cell source employed did not correlate with outcome (data not shown).

No significant difference in NRM according to pre-HSCT MRD status was observed (data not shown).

In a multivariable model for OS, EFS and CIR, which included MFC-MRD (pos vs neg), disease phase, donor type, median age at HSCT, aGvHD, cGvHD, and high-risk genetic features as per therapeutic protocols[2,11], MFC-MRD positivity was confirmed to predict poorer outcomes (HR for OS 3.77, p<0.001; HR for EFS 3.39, p<0.001; HR for CIR 3.66, p<0.001) (Table 2). aGvHD occurrence correlated with a lower CIR but did not influence OS and EFS. By contrast, cGvHD was associated with worse OS and EFS, because of an increased risk of NRM (data not shown). High-risk genetic features correlated with OS and EFS, but not with CIR, in multivariate analysis.

Post-induction MRD assessments by MFC have become an important clinical tool in the management of pedAML, influencing treatment intensity stratification in recent trials. The use of MRD before HSCT with the aim of identifying patients at higher risk of relapse is widely adopted; however, robust data supporting this use in pedAML are lacking. Buckley et al. in a meta-analysis demonstrated a significant association between pre-HSCT MRD positivity (either by MFC or molecular techniques) and dismal outcomes also in pedAML[9]. However, the meta-analysis included only 5 pediatric studies, with lower number of patients (72 in the larger cohort) as compared to our study[9]. Moreover, only 3 studies used MFC assays to detect MRD and with a limited number of fluorochromes.

By contrast, in this study, we report a large cohort of pedAML patients with a very long follow-up, homogeneously managed according to national guidelines and all evaluated with at least 5-color MFC in a central laboratory.

With the limits of a retrospective analysis, our findings indicate that pre-HSCT MFC-MRD has an important prognostic role in pedAML patients transplanted both in CR1 and in CR2 using a fully myeloablative conditioning regimen, with negative MFC-MRD patients having a significantly lower risk of relapse which led to better OS and EFS.

The clinical dilemma of how to manage pre-HSCT MFC-MRD positivity is not easily solvable. The advantage of obtaining negative MRD before HSCT should be weighed on one side against the risk of toxicities associated with further chemotherapy courses in already heavily treated patients. On the other hand, immune modulation approaches, including early tapering/withdrawal of immunosuppression after HSCT to optimize the graft-versus-leukemia (GvL) effect, carry the risk of increased GvHD-associated NRM.

Noteworthy, in our cohort, patients with positive pre-HSCT MRD and aGvHD had lower CIR, as compared to those without, this finding suggesting the relevance of a GvHD-associated GvL effect for preventing leukemia recurrence.

Additionally, whenever possible, the post-HSCT use of targeted treatments to maintain remission based on pre-transplant MRD assessment can be beneficial for patients. Indeed, the need for a precision-medicine treatment based on genetic characterization has been worldwide recognized as a critical tool for optimizing patient outcome[13]. This is the case for patients with FMS-like tyrosine kinase 3 internal tandem duplication (*FLT3-ITD*$^{+}$)-AML who could benefit from FLT3-inhibitors[14] or patients with *KMT2A*-rearrangements, *UBTF-TD* or *NUP98*-rearrangements given menin-inhibitors[15]. Also, patients harboring *CBFA2T3-GLIS2* fusions could benefit from post-HSCT FOLR1-targeted immunotherapy for preventing relapses.

In conclusion, with the limits of a retrospective study including patients transplanted in both

CR1 and CR2, pre-HSCT MFC-MRD was a critical tool for predicting the risk of relapse in our cohort of pedAML. Further studies are necessary to evaluate its potential role in optimizing therapy-intervention decisions before or after HSCT and its correlation with post-HSCT MFC-MRD.

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## Table 1: Study cohort main features

| Variable | N° | % |
|---|---|---|
| **Total pts** | 218 | |
| **Age at diagnosis (year)** | | |
| Median (range) | 7.3 (0-23) | |
| <1 year | 25 | 11.4 |
| **Age at HSCT (year)** | | |
| Median (range) | 8.3 (0.4-23.3) | |
| < 1 year | 12 | 5.5 |
| **Sex** | | |
| Male | 114 | 52.3 |
| Female | 104 | 47.7 |
| **Genetics** | | |
| Negative | 91 | 43.6 |
| *KMT2A-rearr* | 40 | 19.1 |
| *CBF-rearr* | 27 | 12.9 |
| *NUP98-rearr* | 10 | 4.8 |
| *FLT3-ITD*$^{+}$ | 9 | 4.3 |
| *CBFA2T3::GLIS2* | 8 | 3.8 |
| *NPM1-mutation* | 5 | 2.4 |
| *MOZ::CBP* | 4 | 1.9 |
| *FLT3-ITD* $^{+}$*+NPM1*$^{+}$ | 2 | 1.0 |
| Others | 13 | 6.2 |
| NA | 9 | |
| **Remission status** | | |
| CR1 | 177 | 81.2 |
| CR2 | 41 | 18.8 |
| **MFC-MRD pre-HSCT** | | |
| Negative | 180 | 82.6 |
| <0.1% | 10 | 4.6 |
| 0.1-<1.0% | 15 | 6.9 |
| ≥1.0% | 13 | 5.9 |
| **Donor** | | |
| MFD | 48 | 22.0 |
| MUD | 61 | 28.0 |
| MMUD | 29 | 13.3 |
| MMFD | 80 | 36.7 |
| **Stem cell source** | | |
| BM | 125 | 57.3 |
| PBSC | 84 | 38.5 |
| CB | 9 | 4.2 |
| **Conditioning regimen** | | |
| Bu-Cy-Mel | 140 | 64.2 |
| Bu-Thio-Fluda | 17 | 7.8 |
| Treo-based | 14 | 6.4 |
| TBI-based | 34 | 15.6 |
| Other | 13 | 6.0 |
| **GvHD prophylaxis** | | |
| CNI | 20 | 9.2 |
| CNI+MTX | 109 | 50.0 |
| CNI+MMF | 8 | 3.7 |
| Ex-vivo TCD | 58 | 26.6 |
| Other | 23 | 10.5 |
| **aGvHD grade** | | |
| I | 21 | 9.6 |
| II | 29 | 13.3 |
| III | 7 | 3.2 |
| IV | 7 | 3.2 |
| **cGvHD*** | 12/190 | 6.3 |
| **ATG** | | |
| Yes | 138 | 63.3 |
| No | 80 | 36.7 |
| **Follow-up (day)** | | |
| Median (range) | 1712 (365-3606) | |

**Abbreviations:** N°: number; pts: patients; HSCT: hematopoietic stem cell transplantation; NA: not available; CR: complete remission; MFC: multiparametric flow cytometry; MRD: minimal/measurable residual disease; MFD: matched family donor; MUD: matched unrelated donor; MMUD: mismatched unrelated donor; MMFD: mismatched family donor; BM: bone marrow; PBSC: peripheral blood stem cell; CB: cord blood; Bu: busulfan; Cy: cyclophosphamide; Mel:melphalan Thio:thiotepa; Fluda: fludarabine; Treo: treosulfan; TBI: Total Body Irradiation; aGvHD: acute graft-versus-host disease; cGvHD: chronic graft-versus-host disease; CNI: calcineurin inhibitor; MTX; methotrexate; MMF: mycophenolate mofetil; TCD: T cell depletion; ATG: anti-thymocyte globulin.
cGVHD*: data on cGVHD was available on 190 patients

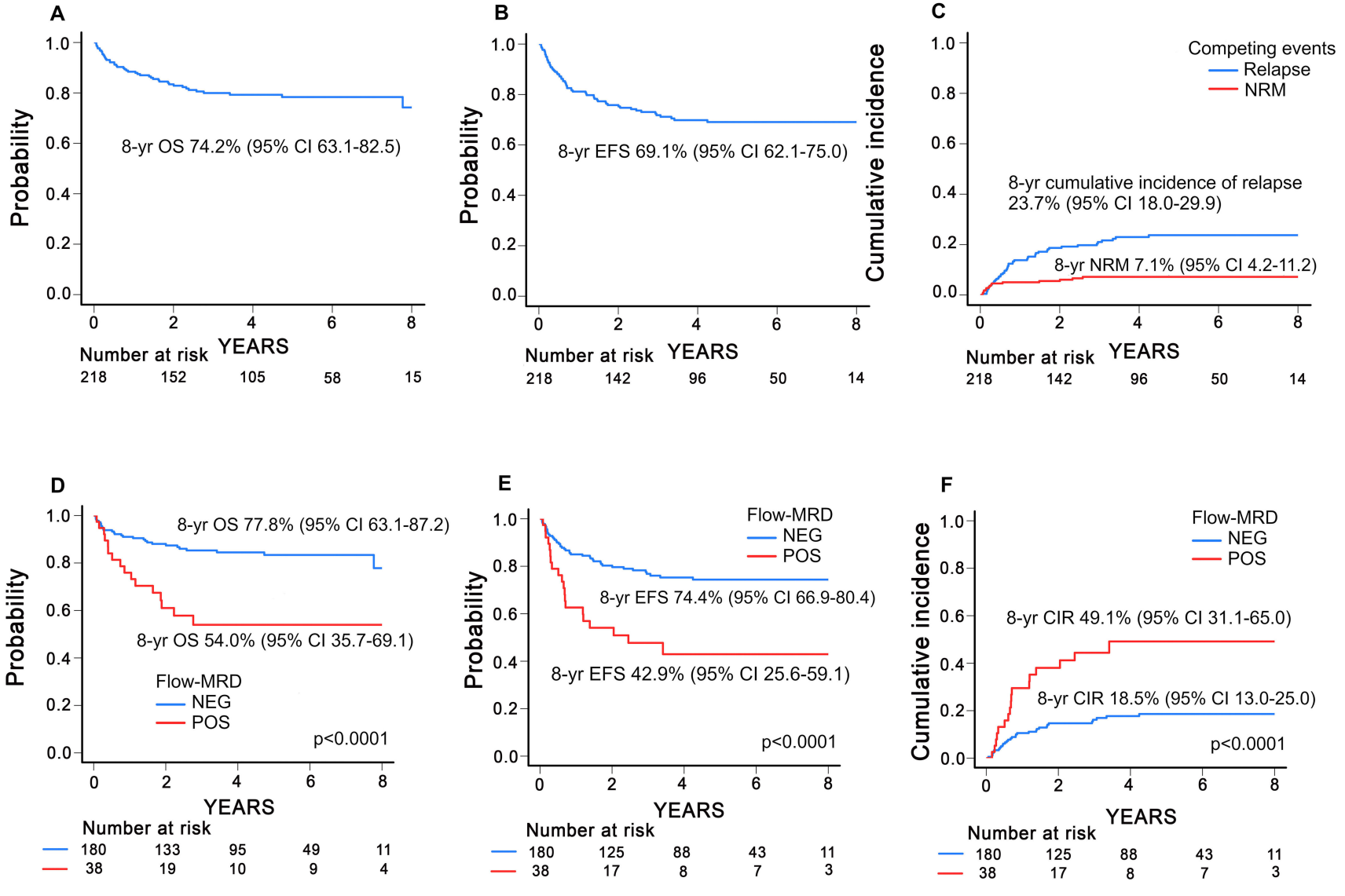
## Table 2: Multivariable analysis

| Variable | Hazard ratio | Lower 95%CI | Upper 95%CI | p-value |
|---|---|---|---|---|
| **EFS** | | | | |
| MFC-MRD pre-HSCT | 3.399 | 1.928 | 5.992 | **<0.001** |
| (NEG reference) | | | | |
| CR2 vs CR1 | 1.229 | 0.667 | 2.266 | 0.509 |
| Donor | 1.084 | 0.872 | 1.347 | 0.469 |
| Age at HSCT (median) | 1.087 | 0.649 | 1.819 | 0.751 |
| aGVHD* | 0.688 | 0.390 | 1.212 | 0.195 |
| cGVHD | 2.621 | 1.105 | 6.213 | **0.029** |
| High-risk genetics | 1.820 | 1.083 | 3.056 | **0.024** |
| **OS** | | | | |
| MFC-MRD pre-HSCT | 3.772 | 1.928 | 7.377 | **<0.001** |
| CR2 vs CR1 | 1.362 | 0.659 | 2.816 | 0.404 |
| Donor | 1.210 | 0.921 | 1.591 | 0.172 |
| Age at HSCT (median) | 1.327 | 0.704 | 2.499 | 0.382 |
| aGVHD* | 0.874 | 0.456 | 1.678 | 0.687 |
| cGVHD | 3.546 | 1.331 | 9.450 | **0.011** |
| High-risk genetics | 2.178 | 1.151 | 4.119 | **0.017** |
| **CIR** | | | | |
| MFC-MRD pre-HSCT | 3.669 | 2.013 | 6.686 | **<0.001** |
| CR2 vs CR1 | 1.657 | 0.875 | 3.136 | 0.120 |
| Donor | 1.204 | 0.941 | 1.539 | 0.140 |
| Age at HSCT (median) | 0.884 | 0.491 | 1.593 | 0.680 |
| aGvHD* | 0.416 | 0.197 | 0.879 | **0.022** |
| cGvHD | 1.717 | 0.480 | 6.149 | 0.410 |
| High-risk genetics | 1.831 | 0.994 | 3.374 | 0.052 |

**Abbreviations:** CI: confidence interval; EFS: event free survival; MFC: multiparametric flow cytometry; MRD: minimal/measurable residual disease; HSCT: hematopoietic stem cell transplantation; OS: overall survival
aGVHD*: any grade of acute graft-versus-host disease

**Figure 1. Clinical outcomes of the whole study cohort and according to pre-transplant MFC-MRD**

8-year OS (Panel A), 8-year EFS (Panel B) and 8-year CIR and NRM (Panel C) of the whole cohort of patients. 8-year OS (Panel D), 8-year EFS (Panel E) and 8-year CIR (Panel F) of patients according to MFC-MRD (pos or neg) before HSCT.

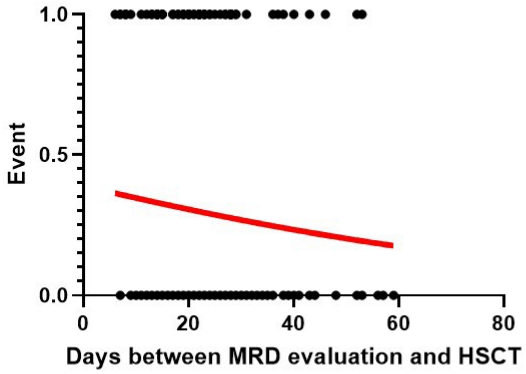

A
Probability
1.0
0.8
0.6
0.4
0.2
0.0
8-yr OS 74.2% (95% CI 63.1-82.5)
0
2
4
6
8
YEARS
Number at risk
218
152
105
58
15
B
Probability
8-yr EFS 69.1% (95% CI 62.1-75.0)
Cumulative incidence
Number at risk
YEARS
218
142
96
50
14
C
Competing events
Relapse
NRM
8-yr cumulative incidence of relapse
23.7% (95% CI 18.0-29.9)
8-yr NRM 7.1% (95% CI 4.2-11.2)
Number at risk
YEARS
218
142
96
50
14
D
Probability
8-yr OS 77.8% (95% CI 63.1-87.2)
8-yr OS 54.0% (95% CI 35.7-69.1)
Flow-MRD
NEG
POS
p<0.0001
YEARS
Number at risk
180
133
95
49
11
38
19
10
9
4
E
Probability
Flow-MRD
NEG
POS
8-yr EFS 74.4% (95% CI 66.9-80.4)
8-yr EFS 42.9% (95% CI 25.6-59.1)
p<0.0001
YEARS
Number at risk
180
125
88
43
11
38
17
8
7
3
F
Cumulative incidence
Flow-MRD
NEG
POS
8-yr CIR 49.1% (95% CI 31.1-65.0)
8-yr CIR 18.5% (95% CI 13.0-25.0)
p<0.0001
YEARS
Number at risk
180
125
88
43
11
38
17
8
7
3

# Multiparametric flow-cytometry minimal residual disease before hematopoietic stem cell transplantation predicts outcome in pediatric acute myeloid leukemia

Barbara Buldini [1,2], Pietro Merli [3,4], Elena Varotto [1], Riccardo Masetti [5], Maria Gabelli [1], Marco Zecca [6], Davide Leardini [5], Pamela Scarparo [1], Maddalena Benetton [1], Luisa Strocchio [3], Franca Fagioli [7], Alessandra Biffi [1,2], Adriana Balduzzi [8,9], Martina Pigazzi [1,2], Daria Pagliara [3], Franco Locatelli [3,10].

1. Pediatric Hematology, Oncology and Stem Cell Transplant Division, Maternal and Child Health Department, University of Padova and Padova University Hospital, Padova, Italy.
2. Pediatric Onco-Hematology, Stem Cell Transplant and Gene Therapy Laboratory, Istituto di Ricerca Pediatrica (IRP) Fondazione Città della Speranza, Padova, Italy
3. Department of Pediatric Hematology and Oncology, IRCCS Ospedale Pediatrico Bambino Gesù, Rome, Italy
4. Saint Camillus International University of Health Sciences, Rome, Italy
5. Pediatric Hematology and Oncology, IRCCS Azienda Ospedaliero-Universitaria di Bologna, Bologna,Italy
6. Pediatric Hematology-Oncology, IRCCS Policlinico San Matteo, Pavia, Italy
7. Pediatric Onco-Hematology, Stem Cell Transplantation and Cellular Therapy Division, Regina Margherita Children's Hospital, University of Turin, Turin, Italy
8. Pediatric Department, Fondazione IRCCS San Gerardo, Monza, Italy
9. School of Medicine and Surgery, University of Milano-Bicocca, Monza, Italy
10. Catholic University of the Sacred Heart, Rome, Italy

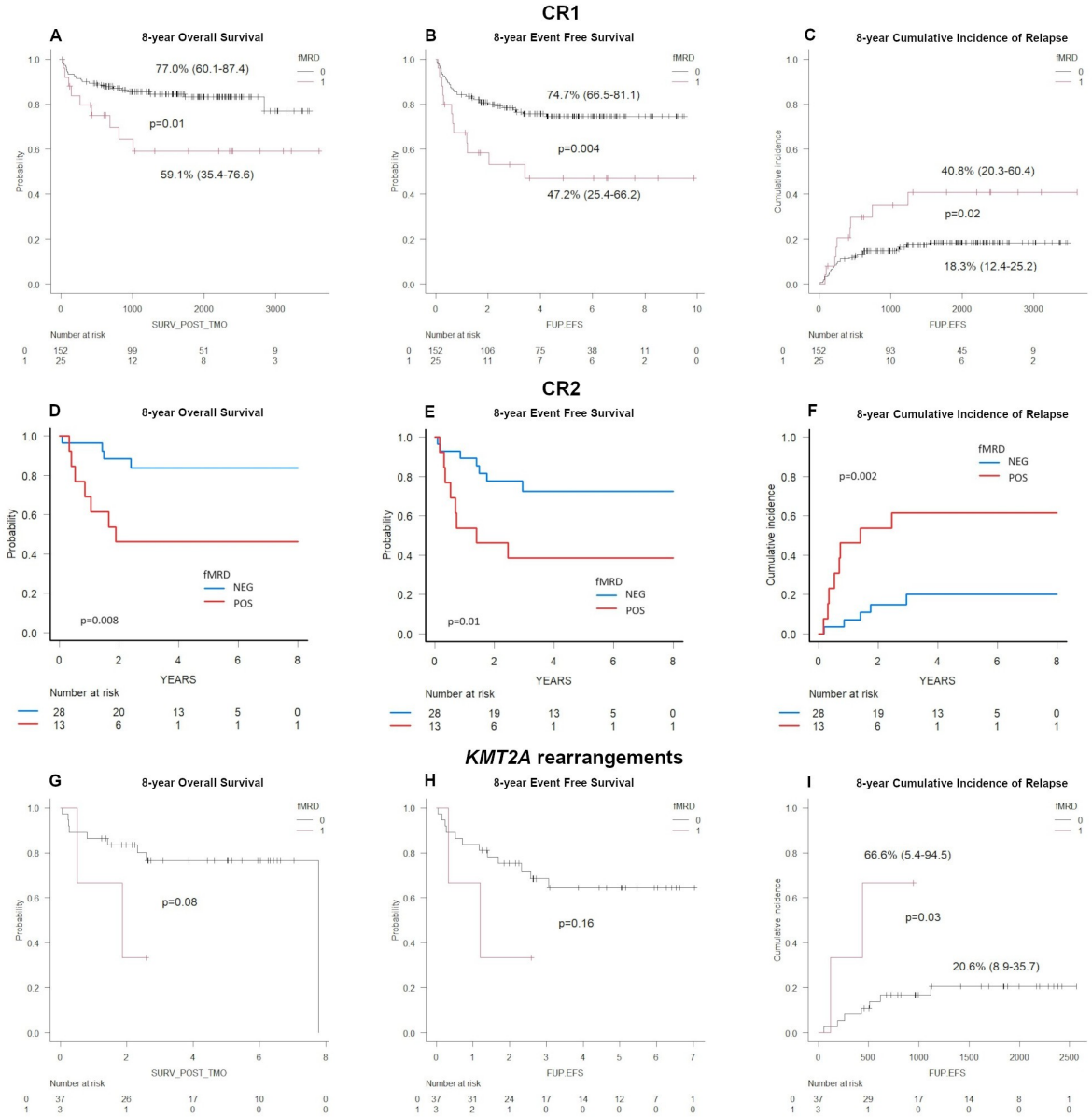
**Supplemental Figure 1:** Timing of MFC-MRD assessment before HSCT. After stratifying patients according to median time of BM evaluation before HSCT (<22 vs. ≥ 22 days before HSCT), both survival analysis (A) and logistic regression analysis (B) did not show a detrimental effect of time of pre-HSCT MFC-MRD evaluation on outcome.




Equation log odds = -0,4481-0,01846*X

| | |
|---|---|
| Best-fit values | |
| β0 | -0,4481 |
| β1 | -0,01846 |
| X at 50% | -24,27 |
| Std. Error | |
| β0 | 0,3665 |
| β1 | 0,01466 |
| X at 50% | 38,26 |
| 95% CI (profile likelihood) | |
| β0 | -1,167 to 0,2744 |
| β1 | -0,04828 to 0,009468 |
| X at 50% | ??? to 6,118 |
| Odds ratios | |
| β0 | 0,6388 |
| β1 | 0,9817 |
| 95% CI (profile likelihood) for odds ratios | |
| β0 | 0,3111 to 1,316 |
| β1 | 0,9529 to 1,010 |
| Is slope significantly non-zero? | |
| Likelihood ratio | 1,650 |
| P value | 0,1989 |
| Deviation from zero? | Not Significant |
| Likelihood ratio test | |
| Log-likelihood ratio (G squared) | 1,650 |
| P value | 0,1989 |
| Reject Null Hypothesis? | No |
| P value summary | ns |
| Area under the ROC curve | |
| Area | 0,5539 |
| Std. Error | 0,04273 |
| 95% confidence interval | 0,4701 to 0,6376 |
| P value | 0,2106 |
| Goodness of Fit | |
| Tjur's R squared | 0,007527 |
| Cox-Snell's R squared | 0,007542 |
| Model deviance, G squared | 262,3 |

**Supplemental Figure 2: Pre-transplant MFC-MRD prognostic impact in subgroups**. Pre-transplant MFC-MRD prognostic role in patients in CR1 (A, B, C) and CR2 (D, E, F): a negative pre-transplant MFC-MRD value was associated with better 8-year OS (A, D), EFS (B, E), and CIR (C, F) in patients undergoing HSCT in both CR1 and CR2. Pre-transplant MFC-MRD prognostic role in patients with a *KMT2A* rearrangement (G, H, I): a negative pre-transplant MFC-MRD value was associated with a better CIR (I) but did not impact on OS (G), and EFS (H).



**Supplemental Figure 3: Prognostic impact of pre-transaplant MFC-MRD in patients who experienced acute GVHD after HSCT.** Patients with a positive pre-HSCT MFC-MRD who experience acute GVHD of any grade after HSCT showed a better OS (A) and EFS (B) in comparison to patients with positive pre-HSCT MFC-MRD without acute GVHD after HSCT. Notably, patients with acute GVHD of any grade after HSCT and positive pre-HSCT MFC-MRD showed a superimposable CIR (C) to patients with negative pre-HSCT MFC-MRD