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Pre- and post-transplant minimal residual disease predicts relapse occurrence in children with acute lymphoblastic leukaemia

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Summary

Relapse remains the leading cause of treatment failure in children with acute lymphoblastic leukaemia (ALL) undergoing allogeneic haematopoietic stem cell transplantation (HSCT). We retrospectively investigated the prognostic role of minimal residual disease (MRD) before and after HSCT in 119 children transplanted in complete remission (CR). MRD was measured by polymerase chain reaction in bone marrow samples collected pre-HSCT and during the first and third trimesters after HSCT (post-HSCT1 and post-HSCT3). The overall event-free survival (EFS) was 50%. The cumulative incidence of relapse and non-relapse mortality was 41% and 9%. Any degree of detectable pre-HSCT MRD was associated with poor outcome: EFS was 39% and 18% in patients with MRD positivity $<1 \times 10^{-3}$ and $\geq 1 \times 10^{-3}$, respectively, *versus* 73% in MRD-negative patients ($P < 0{\cdot}001$). This effect was maintained in different disease remissions, but low-level MRD had a very strong negative impact only in patients transplanted in second or further CR. Also, MRD after HSCT enabled patients to be stratified, with increasing MRD between post-HSCT1 and post-HSCT3 clearly defining cohorts with a different outcome. MRD is an important prognostic factor both before and after transplantation. Given that MRD persistence after HSCT is associated with dismal outcome, these patients could benefit from early discontinuation of immunosuppression, or pre-emptive immuno-therapy.

Keywords: minimal residual disease, acute lymphoblastic leukaemia, haematopoietic stem cell transplantation, children, leukaemia relapse.

Currently, conventional front-line chemotherapy cures a large proportion of children affected by acute lymphoblastic leukaemia (ALL) (Schrappe *et al*, 2011; Pui *et al*, 2012). Furthermore, second-line treatment followed by allogeneic haematopoietic stem cell transplantation (HSCT) can be effective in rescuing 30–50% of relapsed patients (Einsiedel *et al*, 2005; Tallen *et al*, 2010). Nevertheless, relapse remains the most frequent cause of treatment failure for children

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affected by ALL, even after allogeneic HSCT (Balduzzi *et al*, 2005; Locatelli *et al*, 2017).

During the last 2 decades, minimal residual disease (MRD) quantification has progressively acquired a pivotal role in the assessment of early treatment response and defining risk stratification of children with newly diagnosed ALL, (Knechtli *et al*, 1998a,b; Conter *et al*, 2010; Schrappe *et al*, 2011; Vora *et al*, 2013, 2014), as well as of relapsed patients receiving chemotherapy according to second-line protocols (Eckert *et al*, 2001, 2013). Pre-transplant MRD status has also been shown to predict the risk of relapse and final outcome of children affected by ALL and given allogeneic HSCT (Knechtli *et al*, 1998a,b; Bader *et al*, 2009; Leung *et al*, 2012; Umeda *et al*, 2016).

In light of these considerations, MRD has been recently proposed as a tool to guide the extent of pre-transplant chemotherapy administration or post-transplant pre-emptive immunomodulation or immunotherapy, in order to prevent a new disease relapse (Campana & Leung, 2013; Balduzzi *et al*, 2014; Mo *et al*, 2017; Rettinger *et al*, 2017).

The aim of this study was to quantify MRD by real time quantitative polymerase chain reaction (RQ-PCR) immediately before allogeneic HSCT, in order to assess its clinical significance and impact on the risk of relapse and transplant outcome in a cohort of paediatric ALL patients transplanted in first, second or subsequent complete remission (CR). Furthermore, we analysed MRD in the same patients during the first and third trimester after transplantation, to address the question of whether MRD evaluation could provide further information to predict the risk of post-transplant leukaemia relapse.

Patients and methods

Patients

This study included 119 consecutive patients aged between 1 and 18 years, affected by ALL in first, second or subsequent morphological CR (CR1, CR2 or other CR) given allogeneic HSCT in one of the Italian Association for Paediatric Haematology/Oncology (*Associazione Italiana di Ematologia e Oncologia Pediatrica; AIEOP*) transplant centres in Padua, Pavia and Turin. Inclusion criteria were: morphological CR at time of HSCT, defined as less than 5% blasts by morphological examination, allogeneic HSCT from a matched family donor (MFD), an unrelated donor (UD) or a partially matched (haploidentical) family donor (PMFD) and the availability of bone marrow (BM) aspirates for MRD assessment within 30 days before HSCT. In 98 of the 119 patients MRD was also assessed within the first 3 months after HSCT (post-HSCT1), in 59 between the 7th and the 9th month after HSCT (post-HSCT3), and at both these time points in 48 patients. All parents or guardians signed the appropriate informed consent, approved by the local ethics committee or Institutional Review Board. Details on clinical characteristics of patients enrolled in the study are reported in Table I.

Treatment protocols

All patients had been enrolled in one of the following first-line treatment protocols: AIEOP ALL 2000 (Moricke *et al*, 2016), AIEOP ALL R2006, AIEOP-Berlin-Frankfürt-Münster (BFM) ALL 2009 or EsPhALL (Safety and Efficacy of Imatinib Added to Chemotherapy in Treatment of Ph+ Acute Lymphoblastic Leukaemia in Children) (Biondi *et al*, 2012). Eligibility criteria for transplantation in CR1 have been reported elsewhere (Fagioli *et al*, 2013). Patients with first leukaemia relapse were stratified according to the BFM relapse risk stratification (Henze & von Stackelberg, 2002; Tallen *et al*, 2010), and treated according to the AIEOP ALL REC 2003 protocol. Re-induction treatment for patients who presented a second relapse before HSCT varied between centres. Transplants were performed between January 2001 and June 2014. In all donor-recipient pairs, histocompatibility was determined by high-resolution molecular typing of *HLA-A*, *-B*, *-C*, *DRB1* and *DQB1* loci. Forty-five patients (38%) received HSCT from a MFD, 59 (49%) from an UD and 15 (13%), lacking a compatible donor, were transplanted from a PMFD. Forty-three patients (36%) were transplanted in CR1, 65 (55%) in CR2 and 11 (9%) in other CR. Conditioning regimen included total body irradiation (TBI) in 113 cases (95%) and chemotherapy alone in the remaining 6 cases (5%). Details on the transplant procedure and graft-versus-host disease (GVHD) prophylaxis are reported in Table I. In absence of GVHD, ciclosporin A tapering was started within 3 months after HSCT and the drug was discontinued within 6 months after HSCT.

Because of the retrospective nature of the study, clinicians were not informed of the results of MRD before or after HSCT and no decision concerning immunosuppressive treatment tapering and discontinuation was based on MRD results. No patient received additional post-transplant consolidation treatment, including donor lymphocyte infusion (DLI) and tyrosine-kinase inhibitors.

MRD analysis

A total of 276 BM aspirates, collected before and after HSCT, and previously stored in the biological bank "*BioBanca Oncologica Pediatrica BBOP*" were retrospectively analysed for MRD.

DNA samples from BM mononuclear cells were obtained as previously reported (Paganin *et al*, 2014). Clonal immune gene rearrangements identified at diagnosis/relapse were used for MRD assessment by RQ-PCR, and the results were interpreted according to the EuroMRD guidelines, as previously published (van der Velden *et al*, 2007a; Flohr *et al*, 2008). Briefly, a set of PCR reactions were performed on diagnosis/relapse DNA to identify *IGH*, *IGK*, *TRG*, *TRD*, and *TRB*

Table I. Patient characteristics and transplant procedures.

Number of patients	119 (100%)
Gender:	
Male	74 (62%)
Female	45 (38%)
Median age at transplantation (years, range)	7 (1–18)
Immunophenotype:	
B-cell precursor ALL	105 (88%)
T-cell ALL	14 (12%)
Cytogenetics:	
t(9;22)	17 (14%)
t(4;11)	3 (2%)
t(12;21)	4 (3%)
First-line chemotherapy protocol:	
AIEOP ALL 95	4 (3%)
AIEOP ALL 2000	97 (82%)
AIEOP ALL 2009	7 (6%)
EsPhALL	9 (8%)
Other	2 (2%)
Year of transplantation:	
2001–2005	15 (13%)
2006–2010	81 (68%)
2011–2014	23 (19%)
Disease phase at transplantation:	
CR1	43 (36%)
CR2 BFM S1–S2	39 (33%)
CR2 BFM S3–S4	26 (22%)
≥CR3	11 (9%)
Donor:	
Matched family donor	45 (38%)
Unrelated donor	59 (49%)
Partially matched family donor	15 (13%)
Stem cell source:	
Bone marrow	77 (65%)
Peripheral blood	34 (28%)
Cord blood	8 (7%)
Conditioning regimen:	
TBI-based	113 (95%)
Busulfan-based	6 (5%)
Graft-versus-host disease prophylaxis:	
CsA	28 (23%)
CsA + MTX	18 (15%)
CsA + MTX + ATLG	49 (41%)
CsA + Steroids + ATLG	9 (8%)
Ex vivo T-cell depletion	15 (13%)

AIEOP, Associazione Italiana di Ematologia e Oncologia Pediatrica; ALL, acute lymphoblastic leukaemia; BFM S1–S4, Berlin-Frankfürt-Münster standard risk groups; CR1, first complete remission; CR2, second complete remission; CR3, third complete remission; CsA, ciclosporin A; EsPhALL, Safety and Efficacy of Imatinib Added to Chemotherapy in Treatment of Ph+ Acute Lymphoblastic Leukaemia in Children; MTX, short-term methotrexate; ATBI, total body irradiation; TLG, anti-T lymphocyte globulin.

rearrangements. Clonal gene rearrangements, confirmed by homo/heteroduplex analysis, were sequenced and patient-specific primers were designed complementary to the junctional regions of each target. Specific and sensitive RQ-PCR assays were developed and the 2 best performing targets were selected for MRD quantification. As for relapsed patients, we used at least one molecular marker confirmed at the time of relapse. MRD positivity was defined according to the one Ct below background rule (van der Velden *et al*, 2007b). Patients were categorized into 3 groups according to their MRD results: (i) MRD-high: patients with positive quantifiable MRD $\geq 1 \times 10^{-3}$; (ii) MRD-low: patients with positive quantifiable or not-quantifiable MRD $< 1 \times 10^{-3}$; (iii) MRD-negative: patients with a negative MRD result (Paganin *et al*, 2008).

Statistical analysis

The reference date used for analysis was 31 January 2016. Quantitative variables were reported as median value and range, while categorical variables were expressed as absolute value and percentage. Demographic and clinical characteristics of patients were compared using the Chi-square test or Fisher's exact test for categorical variables, while the Mann–Whitney rank sum test or the Student's *t*-test were used for continuous variables as appropriate. Overall survival (OS) and EFS were calculated according to the Kaplan–Meier method (Kaplan & Meier, 1958), while the risk of relapse (REL) and death in remission, defined as non-relapse mortality (NRM) were calculated as cumulative incidences in order to adjust the analysis for the 2 competing risks (Gooley *et al*, 1999). Comparisons between different OS and EFS probabilities were performed using the Log-Rank test, (Klein *et al*, 2001a), while Gray's test was used to assess, in univariable analyses, differences between cumulative incidences (Gray, 1988). Multivariable analysis was performed using the Cox proportional hazard regression model (Klein *et al*, 2001b). All results were expressed as 10-year probabilities or 10-year cumulative incidences (%) and 95% confidence interval (95% CI). $P < 0{\cdot}05$ were considered to be statistically significant. Statistical analysis was performed using NCSS [NCSS 10 Statistical Software (2015). NCSS, LLC. Kaysville, Utah, ncss.com/software/ncss.] and Stata MP/14 (StataCorp LP, College Station, TX, USA, www.stata.com).

Results

Overall outcome

The median observation time for surviving patients was 7·8 years (range, 1·2–13·4 years). All patients engrafted. Grade II–IV acute GVHD developed in 57 out of the 119 patients, with a cumulative incidence of 50% (95% CI, 40–58). Grade III–IV acute GVHD was observed in 13 patients (CI 11%; range 7–18). Chronic GVHD developed in 17 of the 111 patients surviving in remission for at least 100 days (CI 15%; 95% CI, 10–24), with 12 of them experiencing the extensive form of the disease (CI 11%; 95% CI, 6–18).

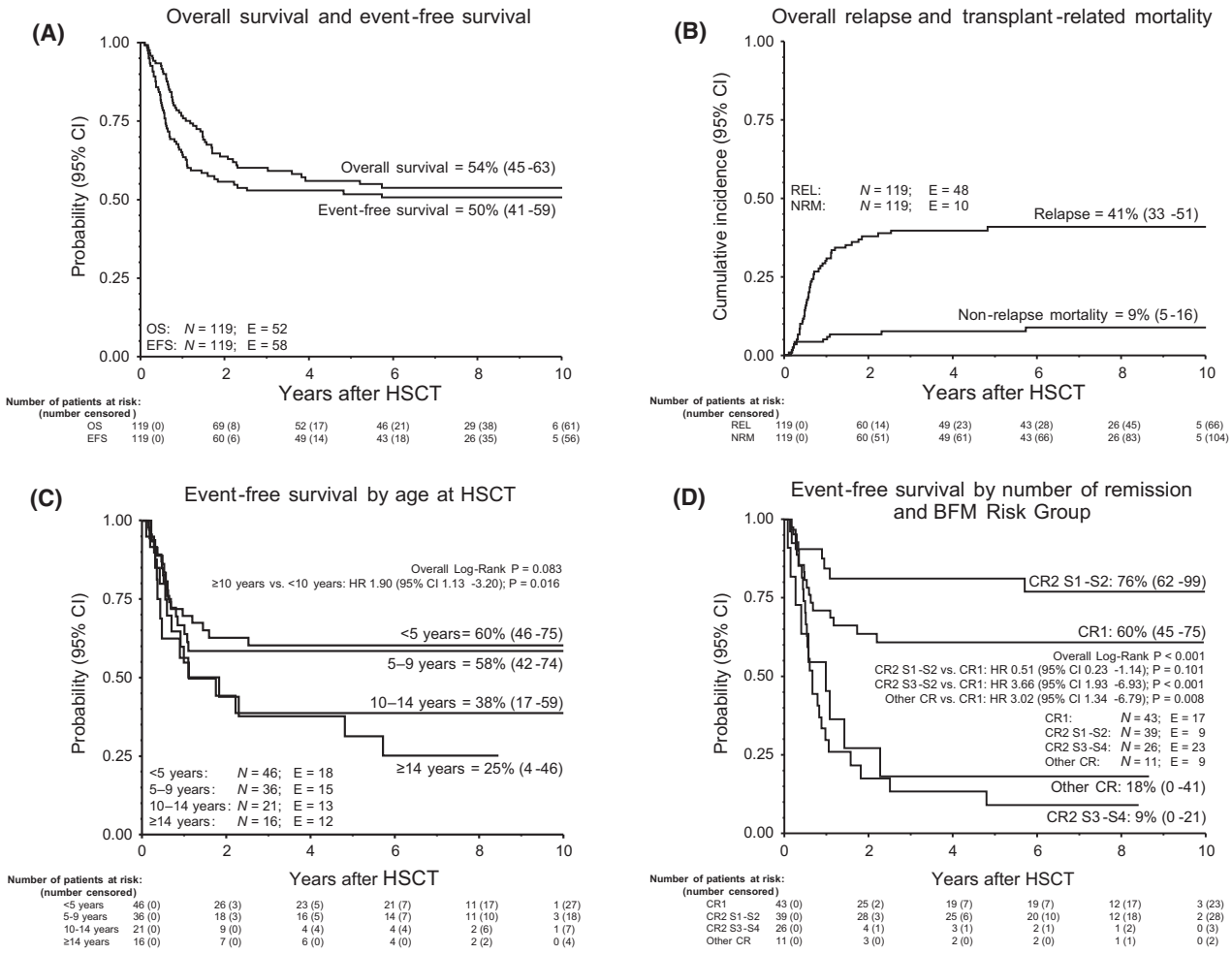

Fig 1. Probability curves of the study population by time from transplantation. (A) Overall probability of survival (OS) and event-free survival (EFS). (B) Overall cumulative incidence of relapse and non-relapse mortality. (C) Overall probability of EFS according to age at transplantation. (D) Overall probability of EFS according to the disease phase and BFM risk group. 95% CI, 95% confidence interval; CR, complete remission; CR1, first complete remission; CR2, second complete remission; EFS, event-free survival; HR, hazard ratio; HSCT, haematopoietic stem cell transplantation; NRM, non-relapse mortality; OS, overall survival; REL, relapse; S1–S4, Berlin-Frankfürt-Münster (BFM) standard risk groups.

Overall, 67 of the 119 patients (56%) are alive, 61 of whom are disease-free after transplantation, resulting in an estimated 10-year OS and EFS probability of 54% (95% CI, 45–63) and 50% (95% CI, 41–59), respectively (Fig 1A). Forty-eight patients relapsed (REL 41%; 95% CI, 33–51) at a median of 7 months after HSCT (range, 1·8–58 months). Ten patients died in remission from transplantation-related causes, at a median of 7 months after transplantation (range, 1–68 months), resulting in a NRM of 9% (95% CI, 5–16) (Fig 1B).

Table II summarizes the results of the univariate analysis for EFS. Only 3 variables were found to be associated with a statistically different EFS: age <10 years at HSCT [EFS = 59% (95% CI, 49–70%) vs. 30% (95% CI, 15–46) age >10 years at HSCT; $P = 0{\cdot}01$] (Fig 1C); disease phase at HSCT [EFS = 60% (95% CI, 45–75) for patients transplanted in CR1, 76% (95% CI, 62–90) for patients transplanted in CR2 and belonging to the S1–S2 BFM risk groups, 9% (95% CI, 0–21) for those transplanted in 2nd CR and belonging to the S3–S4 risk groups and 18% (95% CI, 0–41) for children transplanted in subsequent CR; $P < 0{\cdot}0001$] (Fig 1D); the use of TBI during the conditioning regimen [EFS = 52% (95% CI, 43–61) vs. no TBI 17% (95% CI, 0–46); $P = 0{\cdot}04$].

Results of MRD analysis

A total of 172 RQ-PCR targets were used for MRD assessment. Most of them were *IGH* rearrangements (60%), followed by *TRD* and *TRG* (16% and 10%, respectively). MRD could be evaluated by 2 markers in 53/119 patients, and in 30/53 cases the PCR results were concordant. In cases with discordant results, the highest MRD value was considered for patient categorization into the appropriate MRD group.

Table II. Univariate analysis of event-free survival (EFS) according to patient and transplant characteristics.

Variable	Patients (*n*)	Events (*n*)	EFS (%)	EFS (95% CI)	Log-rank *P*	Hazard ratio*	(95% CI)	*P*
Overall EFS	119	58	50%	(41–59)				
Gender:								
Male	74	39	46%	(35–58)	0·179			
Female	45	19	57%	(42–72)		0·69	(0·40–1·19)	0·182
Age at HSCT:†								
<5 years	46	18	60%	(46–75)	0·083†			
5–9 years	36	15	58%	(42–74)		1·10	(0·56–2·19)	0·781
10–14 years	21	13	38%	(17–59)		1·78	(0·87–3·64)	0·114
≥14 years	16	12	25%	(4–46)		2·27	(1·09–4·71)	0·028
Phenotype:								
B cell precursor ALL	105	49	52%	(43–62)	0·413			
T cell precursor ALL	14	9	34%	(9–60)		1·34	(0·66–2·74)	0·416
t(9;22):								
No	102	50	50%	(40–60)	0·878			
Yes	17	8	53%	(29–77)		1·06	(0·50–2·24)	0·878
Disease phase at HSCT:								
CR1	43	17	60%	(45–75)	<0·001			
CR2 BFM S1–S2	39	9	76%	(62–90)		0·51	(0·23–1·14)	0·101
CR2 BFM S3–S4	26	23	9%	(0–21)		3·66	(1·93–6·93)	<0·001
Other CR	11	9	18%	(0–41)		3·92	(1·34–6·79)	0·008
Donor:								
MFD	45	21	53%	(38–67)	0·971			
UD	59	30	48%	(35–61)		1·07	(0·61–1·87)	0·809
PMFD	15	7	53%	(27–78)		1·04	(0·44–2·46)	0·921
Stem cell source:								
Bone marrow	77	36	53%	(42–64)	0·611			
Peripheral blood	34	19	42%	(25–59)		1·17	(0·67–2·05)	0·571
Cord blood	8	3	63%	(29–96)		0·66	(0·20–2·13)	0·482
Conditioning regimen:								
TBI	113	53	52%	(43–61)	0·036			
Chemotherapy	6	5	17%	(0–46)		2·59	(1·03–6·53)	0·043
GVHD prophylaxis								
Cs-A	28	14	50%	(31–68)	0·803			
Cs-A + MTX	18	7	60%	(37–83)		0·63	(0·25–1·56)	0·318
Cs-A + MTX + ATLG	49	26	46%	(31–60)		1·00	(0·52–1·92)	0·994
Cs-A + Steroids + ATLG	9	4	56%	(23–88)		0·69	(0·23–2·11)	0·520
Ex vivo T-cell depletion	15	7	53%	(27–28)		0·90	(0·36–2·23)	0·821
Acute GVHD								
Grade 0–I	62	32	48%	(35–60)	0·534			
Grade II–IV	57	26	53%	(40–67)		0·85	(0·51–1·42)	0·535
Chronic GVHD‡								
Absent	94	43	53%	(43–64)	0·642			
Present	17	7	51%	(22–80)		0·83	(0·37–1·84)	0·642

95% CI, 95% confidence interval; ALL, acute lymphoblastic leukaemia; BFM S1–S4, Berlin-Frankfürt-Münster standard risk groups; CR, complete remission; CR1, first complete remission; CR2, second complete remission; CsA, ciclosporin A; EFS, event-free survival; GVHD, graft-versus-host disease; HSCT, haematopoietic stem cell transplantation; MFD, matched family donor; MTX, short-term methotrexate; APMFD, partially matched family donor; TBI, total body irradiation; TLG, anti-T lymphocyte globulin; UD, unrelated donor.

*The first value of each variable was considered as reference value to estimate the hazard ratio.

†Age at HSCT <10 years vs. ≥10 years: Log-rank $P = 0{\cdot}014$.

‡For chronic GVHD analysis, only the 111 patients surviving in remission at least 100 days post-transplantation were considered.

Pre-HSCT MRD was negative (MRD-neg) in 51/119 patients (43%), positive $<1 \times 10^{-3}$ (MRD-low) in 46 (31%), and positive $\geq 1 \times 10^{-3}$ (MRD-high) in 22 (18%). As shown in Table III, we observed a strong correlation between disease phase and pre-transplant MRD level. Negative MRD was observed more frequently in patients transplanted in CR1 or

Table III. Association between disease phase at HSCT and pre-HSCT MRD level.

	Pre-HSCT MRD level			
Disease phase at HSCT	Negative	Positive $<1 \times 10^{-3}$	Positive $\geq 1 \times 10^{-3}$	Total
CR1	20 (47%)	19 (44%)	4 (9%)	43 (100%)
CR2 BFM S1–S2	24 (61%)	10 (26%)	5 (13%)	39 (100%)
CR2 BFM S3–S4	5 (19%)	10 (39%)	11 (42%)	26 (100%)
≥CR3	2 (18%)	7 (64%)	2 (18%)	11 (100%)
Total	51 (43%)	46 (39%)	22 (18%)	119 (100%)

Chi-square $P = 0{\cdot}0009$. BFM S1–S4, Berlin-Frankfürt-Münster standard risk groups; CR1, first complete remission; CR2, second complete remission; CR3, third complete remission; HSCT, haematopoietic stem cell transplantation; MRD, minimal residual disease.

in those transplanted in CR2 and belonging to the S1–S2 risk groups, while MRD $\geq 1 \times 10^{-3}$ was more frequent in patients transplanted in CR2 and belonging to the S3–S4 risk groups ($P = 0{\cdot}0009$).

MRD was also assessed after HSCT in 109/119 patients (92%) either during the first trimester (post-HSCT1) or the third trimester (post-HSCT3). MRD at post-HSCT1 was analysed in 98 patients: 71 were negative, 23 were MRD-low (22/23 with not-quantifiable MRD levels) and 4 were MRD-high. BM aspirates at post-HSCT3 were available for 59 patients (32 patients relapsed or died in remission before post-HSCT3, while the BM aspirate was not performed or not available in 28 cases). MRD was negative in 38 patients (64%), MRD-low in 16 (27%; not quantifiable levels in 12/16) and MRD-high in 5 (9%).

BM aspirate was consecutively analysed at the first 2 time points (before HSCT and at post-HSCT1) in 71 of the 119 patients, and at all the 3 time points in 48. Details on the evolution of MRD in these 71 patients are presented in Fig 2.

Twenty-six of these patients were MRD-neg before HSCT, 20 of whom (77%) remained negative both at post-HSCT1 and post-HSCT3. Two (2%) patients were MRD-neg at post-HSCT1, but one became MRD-low at post-HSCT3 and subsequently relapsed, and one had an overt relapse between post-HSCT1 and post-HSCT3. Four additional patients (4%) became MRD-low already at post-HSCT1: 2 relapsed shortly after, while the other 2 remained MRD-low at post-HSCT3 and are alive in complete remission at the time of last follow-up (9 and 11 years after HSCT, respectively).

Thirty-one patients were MRD-low before HSCT; 19 of them (61%) became MRD-neg, 11 (36%) remained MRD-low and 1 (3%) presented a very early marrow relapse at post-HSCT1. Nine of the 19 patients who were MRD-neg at post-HSCT1 remained MRD-neg at post-HSCT3; 7 remained in remission at last follow-up. The MRD level of the other 10 patients increased at post-HSCT3 and only one of them is still in remission. Of the 11 patients who remained stable MRD-low at post-HSCT1, only 4 remained MRD-low or became negative at post-HSCT3 (1 subsequently relapsed) while 7 ultimately relapsed.

Fourteen of these 71 children were MRD-high at the pre-transplant evaluation. Seven (50%) became negative at post-HSCT1, but only 1 remained negative at post-HSCT3 and is currently alive and in remission, while the MRD level of other 6 patients increased at post-HSCT3 and they ultimately relapsed. Of the other 7 children who were pre-transplant MRD-high, 5 improved to MRD-low and 2 remained MRD-high. Only one patient was still MRD-low at post-HSCT3

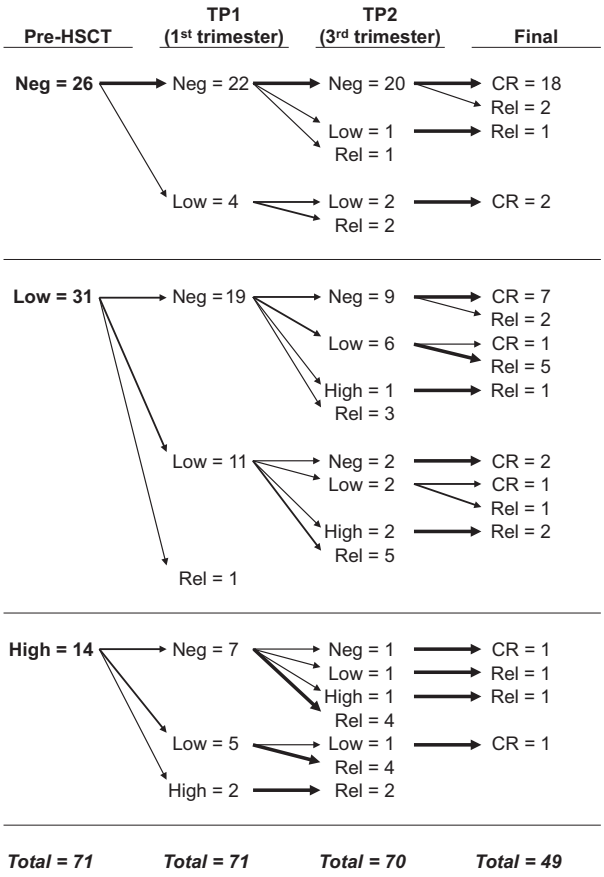

Fig 2. Prospective evolution of minimal residual disease (MRD) before HSCT and at post-HSCT1 and post-HSCT3 time points. Only patients with MRD evaluated at least 2 time points (pre-HSCT and post-HSCT1) are included. The width of the arrows is proportional to the percentage of patients. CR, complete remission; HSCT, haematopoietic stem cell transplantation; Neg, negative; Rel, relapse; TP1, 1st trimester (first 3 months post-HSCT);TP2, 3rd trimester (month 7–9 post-HSCT).

and is currently alive and in remission, while the remaining 6 patients ultimately relapsed.

Prognostic significance of pre-transplant MRD

Considering the whole study population, a negative MRD evaluation before transplantation was associated with better outcome: 38 out of 51 patients (75%) with negative MRD at time of HSCT are still alive in complete remission. Persistence of any MRD level at pre-HSCT was associated with a lower probability to be alive and in remission: 19/46 patients (41%) with MRD-low are alive and disease free, while only 4/22 patients (18%) with MRD-high values are alive in complete remission. The 10-year EFS probability was 73% (95% CI, 61–86) for MRD-neg patients, 39% (95% CI, 25–54) for MRD-low patients and 18% (95% CI, 2–34) for MRD-high patients, $P < 0{\cdot}001$ (Fig 3A). The difference in EFS was entirely due to a different relapse risk, the cumulative incidence of relapse being 20% (95% CI, 11–35) for MRD-neg patients, 50% (95% CI, 37–67) for MRD-low patients and 73% (95% CI, 56–94) for MRD-high patients, $P < 0{\cdot}001$ (Fig 3B). No difference in NRM was observed among the 3 MRD groups (Table IV).

As shown in Table IV and Fig 3C, D, the predictive value of pre-HSCT MRD level was confirmed also when patients were analysed according to disease phase at HSCT (first, second or subsequent CR). However, the impact of pre-HSCT MRD level was different in patients transplanted in CR1 or CR2. In detail, considering patients transplanted in CR1, the 10-year EFS probability was similar for MRD-neg and MRD-low patients [74% (95% CI, 55–94) vs. 63% (95% CI, 41–85), respectively] while it was 0% for MRD-high patients ($P < 0{\cdot}0001$). Conversely, for patients transplanted in CR2, EFS probability was significantly better for MRD-neg patients [78% (95% CI, 62–94), $P = 0{\cdot}001$], while it was almost identical for MRD-low and MRD-high patients [24% (95% CI, 3–45) vs. 25% (95% CI, 4–46), respectively].

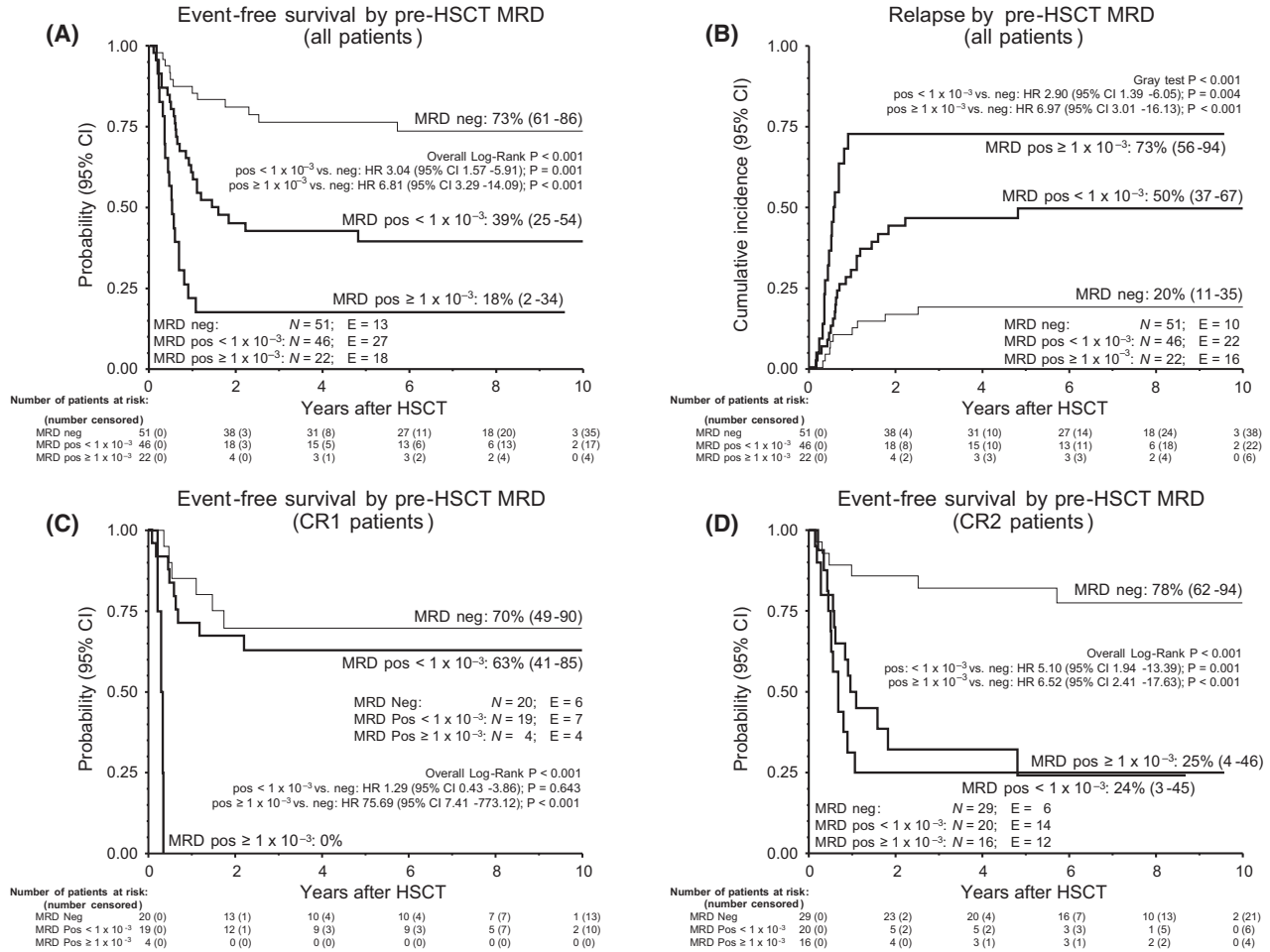

Fig 3. Prognostic significance of MRD levels before HSCT. Event-free survival (EFS) (A) and cumulative incidence of relapse (B) according to pre-HSCT MRD in the whole study population and EFS for patients transplanted in CR1 (C) or in CR2 (D). 95% CI, 95% confidence interval; CR1, first complete remission; CR2, second complete remission; HR, hazard ratio; HSCT, haematopoietic stem cell transplantation; MRD, minimal residual disease.

Grade II-IV acute GVHD demonstrated a protective effect against relapse, especially in patients with pre-transplant low-level MRD positivity, where the effect was statistically significant [(relapse incidence = 67% (95% CI, 50–88) vs. 27% (95% CI, 14–54) in grade 0–I and grade II–IV patients respectively, $P = 0{\cdot}018$] (Table V). Also, chronic GVHD seems to have a protective impact against relapse in pre-HSCT low-level MRD patients, even though the advantage associated with chronic GVHD occurrence was not statistically significant (Table V).

Prognostic significance of post-transplant MRD

The probability of EFS was evaluated according to MRD level at post-HSCT1 and post-HSCT3. Considering the 98 patients evaluated at post-HSCT1, the 71 who had a negative MRD had a 10-year EFS of 63% (95% CI, 50–74), while EFS was 30% (95% CI, 12–49) and 25% (95% CI, 0–67) for the 23 and 4 patients with low positive MRD and high positive MRD, respectively ($P < 0{\cdot}001$) (Fig 4A). Likewise, considering the 59 children evaluated at post-HSCT3, the 38 patients with a negative MRD had an EFS probability of 84% (95% CI, 72–97), while EFS was 44% (95% CI, 19–69) for the 16 patients with MRD-low and 0% (95% CI, 0–67) for the 5 children with MRD-high ($P < 0{\cdot}001$) (Fig 4B).

The impact of MRD change from pre-HSCT to post-HSCT is shown in Fig 4C. Patients with unchanged negative MRD (i.e., MRD-neg both before HSCT and at post-HSCT1 time point) had the best EFS probability, 80% (95% CI, 67–93). Children whose MRD decreased from pre-HSCT to post-HSCT1 had an EFS probability of 37% (95% CI, 21–52) if they reached MRD-neg and of 14% (95% CI, 0–40) if they

Table IV. Impact of pre-transplant MRD on patient outcome.

			EFS					
Variable	Patients (*n*)	Events (*n*)	(%)	(95% CI)	Log-rank *P*	Hazard ratio*	(95% CI)	*P*
EFS, all patients:								
MRD-negative	51	13	73%	(61–86)	<0·0001			
MRD-low	46	27	39%	(25–54)		3·04	(1·57–5·91)	0·001
MRD-high	22	18	18%	(2–34)		6·81	(3·29–14·09)	<0·001
Relapse, all patients:								
MRD-negative	51	10	20%	(11–35)	<0·0001			
MRD-low	46	22	50%	(37–67)		2·90	(1·39–6·05)	0·004
MRD-high	22	16	73%	(56–94)		6·97	(3·01–16·13)	<0·001
NRM, all patients:								
MRD-negative	51	3	7%	(2–21)	0·648			
MRD-low	46	5	11%	(5–25)		1·95	(0·47–8·03)	0·356
MRD-high	22	2	9%	(2–34)		1·58	(0·27–9·26)	0·614
EFS, CR1 patients								
MRD-negative	20	5	74%	(55–94)	<0·0001			
MRD-low	19	7	63%	(41–85)		1·29	(0·43–3·86)	0·643
MRD-high	4	4	0%	–		75·69	(7·41–773·12)	<0·001
EFS, CR2 patients								
MRD-negative	29	6	78%	(62–94)	0·0001			
MRD-low	20	14	24%	(3–45)		5·10	(1·94–13·39)	0·001
MRD-high	16	12	25%	(4–46)		6·52	(2·41–17·63)	<0·001
EFS, CR2 S1–S2 patients								
MRD-negative	24	3	86%	(72–100)	0·115			
MRD-low	10	4	60%	(30–90)		3·95	(0·88–17·85)	0·074
MRD-high	5	2	60%	(17–100)		3·94	(0·66–23·71)	0·134
EFS, CR2 S3–S4 patients								
MRD-negative	5	3	30%	(0–77)	0·380			
MRD-low	10	10	0%			2·26	(0·62–8·32)	0·219
MRD-high	11	10	9%	(0–26)		2·40	(0·65–8·93)	0·191
EFS, other CR patients								
MRD-negative	2	1	50%	(0–100)	0·131			
MRD-low	7	6	14%	(0–40)		4·21	(0·48–37·18)	0·196
MRD-high	2	2	0%			12·65	(0·85–187·18)	0·065

95% CI, 95% confidence interval; CR1, first complete remission; CR2, second complete remission; CR3, third complete remission; EFS, event-free survival; HSCT, haematopoietic stem cell transplantation; MRD, minimal residual disease; MRD-high, MRD positive $\geq 1 \times 10^{-3}$;MRD-low, MRD positive $<1 \times 10^{-3}$; MRD-negative, MRD negative; NRM, non-relapse mortality; S1–S4, Berlin-Frankfürt-Münster standard risk groups.
*The first value of each variable was considered as reference value to estimate the hazard ratio.

only achieved a low level of positivity. Patients with unchanged positive MRD (i.e., a positive MRD before HSCT that remained at the same level also at post-HSCT1) had an EFS of only 23% (95 CI, 0–46).

The effect of MRD variation from post-HSCT1 to post-HSCT3 is shown in Fig 4D. EFS was 88% (95% CI, 75–100) for patients with an unchanged negative MRD, 80% (95% CI, 45–100) for those with an unchanged low-positivity MRD and 100% for the 2 children whose MRD decreased from positive to negative (P = N.S.). In contrast, EFS was only 8% (95% CI, 0–24) for those whose MRD increased between post-HSCT1 and post-HSCT3 ($P < 0{\cdot}001$).

Multivariable analysis

Table VI presents the results of multivariable analysis of EFS. As expected, disease status at HSCT had a significant association with EFS probability. The risk ratio of treatment failure was 2·59 (95% CI, 1·25–5·36; $P = 0{\cdot}011$) for CR2 patients belonging to the S3–S4 groups vs. CR1 patients, and 2·44 (95% CI, 1·00–5·91; $P = 0{\cdot}049$) for other CR patients vs. CR1 patients. Pre-HSCT MRD confirmed its strong predictive value also in multivariable analysis. The risk ratio of treatment failure was 2·18 (95% CI, 1·10–4·31; $P = 0{\cdot}025$) for MRD-low vs. MRD-neg patients and 4·14 (95% CI, 1·84–9·32; $P = 0{\cdot}001$) for MRD-high vs. MRD-neg patients.

Discussion

The probability of cure for children affected by ALL exceeds 80% with current front-line chemotherapy (Schrappe *et al*, 2011; Pui *et al*, 2012). For this reason, the indication for allogeneic HSCT in CR1 has been progressively restricted and, nowadays, only patients with very high risk genetic features or those with suboptimal response to initial treatment are offered transplantation in CR1. Likewise, considering patients who experience leukaemia relapse, allogeneic HSCT is reserved for those with high-risk characteristics, namely those with BM relapse of B-cell precursor (BCP) ALL occurring within 6 months from treatment discontinuation or with T-cell ALL, or to children with standard risk disease, but with persistently positive MRD at the end of induction therapy. Unfortunately, despite the use of a fully myeloablative conditioning regimen, often including TBI, 20–40% of children given an allogeneic HSCT ultimately relapse (Paganin *et al*, 2008; Dini *et al*, 2011; Fagioli *et al*, 2012, 2013;

Table V. Effect of acute and chronic GVHD on the cumulative incidence of relapse, stratified by pre-transplant MRD level.

Variable	Patients (*n*)	Events (*n*)	Cumulative incidence (%)	(95% CI)	Log-rank *P*	Hazard ratio*	(95% CI)	*P*
Acute GVHD								
All patients								
Grade 0–I	62	30	49%	(38–64)	0·054			
Grade II–IV	57	18	32%	(22–47)		0·57	(0·32–1·01)	0·053
MRD-negative								
Grade 0–I	26	5	20%	(9–43)	0·990			
Grade II–IV	25	5	20%	(9–44)		0·99	(0·29–3·38)	0·990
MRD-low								
Grade 0–I	24	16	67%	(50–88)	0·018			
Grade II–IV	22	6	27%	(14–54)		0·33	(0·13–0·85)	0·021
MRD-high								
Grade 0–I	12	9	75%	(54–100)	0·354			
Grade II–IV	10	7	70%	(47–100)		0·62	(0·24–1·61)	0·327
Chronic GVHD								
All patients								
Grade 0–I	94	38	41%	(32–53)	0·646			
Grade II–IV	17	6	36%	(19–69)		0·82	(0·36–1·89)	0·640
MRD-negative								
Grade 0–I	44	8	19%	(10–35)	0·472			
Grade II–IV	6	2	33%	(11–100)		1·87	(0·43–8·20)	0·404
MRD-low								
Grade 0–I	34	18	55%	(40–75)	0·171			
Grade II–IV	8	2	27%	(8–89)		0·39	(0·09–1·58)	0·186
MRD-high								
Grade 0–I	16	12	75%	(57–100)	0·628			
Grade II–IV	13	2	67%	(30–100)		0·69	(0·18–2·58)	0·580

95% CI, 95% confidence interval; GVHD, graft-versus-host disease; MRD, minimal residual disease; MRD-high, MRD positive $\geq 1 \times 10^{-3}$; MRD-low, MRD positive $<1 \times 10^{-3}$; MRD-negative, MRD negative.

*The first value of each variable was considered as reference value to estimate the hazard ratio.

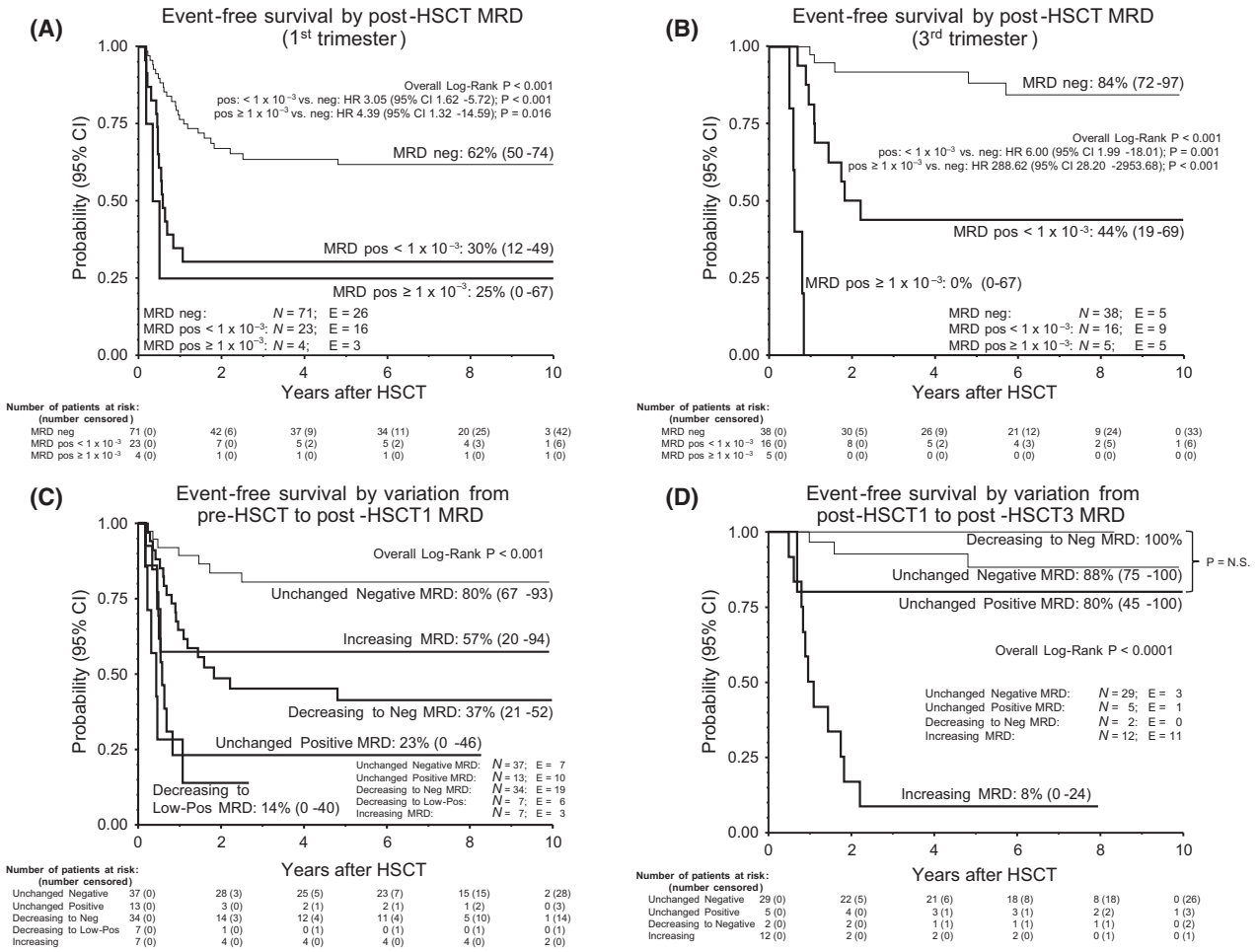

Fig 4. Event free survival (EFS) according to MRD post-transplantation. EFS according to post-transplant MRD level at post-HSCT1 (A) and post-HSCT3 (B) time points and according to the variation from pre-HSCT to post-HSCT1 time points (C) and from post-HSCT1 to post-HSCT3 (D). MRD variation is classified as unchanged negative (an already negative MRD that remains negative), unchanged positive (a positive MRD that remain positive at the same level), decreasing to negative (a positive MRD that becomes negative), decreasing to low-positive (a high-positivity MRD that becomes low-positive) and increasing (from negative to positive or from low-positive to high-positive). 95% CI, 95% confidence interval; HR, hazard ratio; HSCT, haematopoietic stem cell transplantation; HSCT1, 1st trimester (first 3 months post-HSCT); HSCT3, 3rd trimester (month 7–9 post-HSCT); MRD, minimal residual disease; N.S., not significant.

Conter *et al*, 2014; Locatelli *et al*, 2017), with disease recurrence remaining the most frequent cause of treatment failure (Balduzzi *et al*, 2005). Previous reports have shown that pre-transplant MRD level can predict the risk of post-transplant relapse of patients with ALL (Knechtli *et al*, 1998a,b; Bader *et al*, 2009; Leung *et al*, 2012; Ruggeri *et al*, 2012; Balduzzi *et al*, 2014; Umeda *et al*, 2016). Pre-transplant intensification chemotherapy aimed at achieving MRD negativity or significant reduction has been suggested as a potential strategy in order to prevent leukaemia relapse after HSCT (Balduzzi *et al*, 2014). Furthermore, extensive clinical and experimental data support the concept of an immune-mediated graft-versus-leukaemia (GVL) effect after allogeneic HSCT (Kolb, 2008), suggesting that immunological interventions, such as less intensive GVHD prophylaxis, early discontinuation of immunosuppression or administration of DLI, could have an effect in preventing relapse and improving transplant outcome (Locatelli *et al*, 2000; Pulsipher *et al*, 2009; Balduzzi, 2017).

In this study, we retrospectively evaluated the outcome of a large cohort of children and adolescents with ALL given allogenic HSCT in first, second or subsequent CR and correlated the outcome with pre- and post-transplant MRD. Overall, we observed an EFS probability of 50%, a value comparable to that of previous reports (Dini *et al*, 2011; Fagioli *et al*, 2012; Bader *et al*, 2015), with a low NRM of 9%. The cumulative incidence of relapse exceeded 40%, and disease recurrence was confirmed to be the most important cause of treatment failure. This high relapse rate was mainly due to the very poor outcome of high-risk patients, namely those transplanted in CR2 and belonging to the S3–S4 BFM risk group (EFS = 9%) or those transplanted in more advanced disease (EFS = 18%). On the contrary, children in S1 and S2 risk groups who were

Table VI. Results of multivariable analysis of pre-transplant patient characteristics and pre-transplant MRD on event-free survival (EFS).

Independent variable	Hazard ratio	(95% CI)	*P*
Age at HSCT:			
≥10 years vs. <10 years	1·62	(0·94–2·78)	0·080
Disease status at HSCT:			
CR2 S1–S2 vs. CR1	0·57	(0·24–1·33)	0·195
CR2 S3–S4 vs. CR1	2·59	(1·25–5·36)	0·011
Other CR vs. CR1	2·44	(1·00–5·91)	0·049
TBI			
No vs. Yes:	1·29	(0·47–3·57)	0·618
Pre-HSCT MRD			
Pos $<1 \times 10^{-3}$ vs. Neg	2·18	(1·10–4·31)	0·025
Pos $\geq 1 \times 10^{-3}$ vs. Neg	4·14	(1·84–9·32)	0·001

95% CI, 95% confidence interval; CR, complete remission; CR1, first complete remission; CR2, second complete remission; HSCT, haematopoietic stem cell transplantation; MRD, minimal residual disease; S1–S4, Berlin-Frankfürt-Münster standard risk groups; TBI, total body irradiation.

transplanted in CR1 or in CR2 had an EFS probability of 60% and 76%, respectively.

We found a strong association between pre-transplant MRD and disease phase at transplantation, with the highest pre-HSCT MRD being observed in children transplanted in CR2 and belonging to the S3–S4 BFM risk group. Indeed, 42% of these patients had a MRD level $\geq \times 10^{-3}$ at time of HSCT, as compared to less than 20% observed in the other subgroups. Our data confirm that the S3–S4 BFM relapse risk group has a poorer molecular response to conventional chemotherapy and, to optimize the efficacy of transplantation as final consolidation treatment, patients in this risk group are candidates for new therapeutic approaches, including experimental immunotherapies based on the use of bispecific T-cell engager (BiTE) antibodies targeting the CD19 antigen ubiquitously present on Bcp-ALL (von Stackelberg *et al*, 2016).

As expected, pre-transplant MRD was a strong predictor of outcome, thus confirming previously reported studies on the value of pre-transplant MRD in children affected by ALL (Knechtli *et al*, 1998a,b; Bader *et al*, 2009; Leung *et al*, 2012; Balduzzi *et al*, 2014; Umeda *et al*, 2016). We observed that the prognostic significance of pre-transplant MRD was consistent in all disease phases at HSCT. Nevertheless, a new and, in our opinion, important finding was that the level of MRD positivity had a different impact on EFS according to disease phase at HSCT. In patients transplanted in CR1, only high MRD ($\geq 1 \times 10^{-3}$) was associated with an increased the risk of relapse. On the contrary, considering patients transplanted in CR2, a low-level MRD positivity ($<1 \times 10^{-3}$) was also associated with a high relapse rate and poor outcome (Fig 3C). Our finding differs from the observation of Eckert *et al* (2015), of a negative impact only of an MRD $\geq 1 \times 10^{-3}$ and supports the concept that, in contrast to CR1 patients, for those who relapse, low level MRD positivity also suggests an intrinsic resistance of the leukaemic cells to chemo- and radiotherapy.

MRD was also evaluated during the first and third trimester after transplantation. Patients with a negative MRD early post-transplant had a good EFS probability, which was even better for those who were still negative at the third trimester assessment, although one relapse was observed in this subgroup at more than 4 years after transplantation. However, as previously suggested (Balduzzi *et al*, 2014; Rettinger *et al*, 2017), low level MRD positivity after transplantation was not invariably associated with relapse. Indeed, children with MRD $\leq 1 \times 10^{-3}$ at the first and third trimester post-transplant had an EFS of 30% and 44%, respectively. Conversely, only one out of the 4 patients with high MRD positivity at post-HSCT1 and none of the 5 with high MRD positivity at post-HSCT3 is surviving in remission.

Our data show that patients with pre-transplant low-level positive MRD and grade II-IV acute GVHD or chronic GVHD have a lower risk of relapse as compared to those without GVHD. For this reason, considering that this analysis was retrospective and that no clinical investigator received information regarding MRD results before transplantation or during the post-transplant follow-up, we believe that a low-level MRD positivity can be controlled by the GVL effect of the transplant, while the finding of a high-level MRD warrants a prompt and more aggressive intervention, such as the immediate discontinuation of all immunosuppressive therapy or the use of DLI. High-risk patients with early low-level MRD who are transplanted in CR2 may also benefit greatly from such prompt interventions of immune modulation, also considering that DLI did not seem to be associated with an increased rate of acute GVHD in a paediatric cohort treated with pre-emptive DLI for positive MRD after HSCT (Rettinger *et al*, 2017).

Furthermore, adoptive cell therapy with chimeric antigen receptor (CAR) T cells (Grupp *et al*, 2013; Maude *et al*, 2014) might be even more effective and with less severe side effects if used in patients with only MRD positivity post-HSCT, before progression to an overt haematological relapse.

Our data also provide support to the results published by Bader *et al* (2015), showing that MRD after HSCT is a dynamic process and that variations of MRD over time are important. In our experience, the change between pre-HSCT and post-HSCT1 enabled the identification of 3 categories of patients: those with good prognosis (unchanged negative MRD), those with poor prognosis (unchanged positive MRD or decreasing but still positive MRD) and those with an intermediate prognosis (MRD decreasing to negative or increasing from negative to low positive). The variation between post-HSCT1 and post-HSCT3 was even more important, identifying 2 subgroups with a dramatically different outcome: a first group of patients with very good prognosis (those with MRD remaining negative or decreasing from positive to negative and those with an unchanged low-level positivity, with a EFS probability

≥80%), and a group of patients with severe prognosis (those whose MRD increases between post-HSCT1 and post-HSCT3, who had an EFS probability of only 8%) (see Fig 4C, D).

In our study, the median time from transplant to morphological leukaemia relapse was 7 months, with a range between 1 and 68 months. Only 4 (8%) out of the 48 relapses were observed within the first 3 months, while 26 (54%) occurred between the months 3 and 9, and 18 (38%) after the third trimester. For this reason, the prospective evaluation of MRD after HSCT could identify, in advance, patients with the highest risk of relapse and with a strong indication for prompt immunological intervention, such as rapid tapering or discontinuation of the immunosuppressive treatment, infusion of DLI or other form of immune-therapy (Locatelli *et al*, 2000; Pulsipher *et al*, 2009; Maude *et al*, 2015; Balduzzi, 2017; Comoli *et al*, 2017). MRD must be cleared before the graft becomes tolerant toward the recipient (Bader *et al*, 2015). Thus, it is reasonable to hypothesize that these interventions could be more effective if performed early after HSCT and, if possible, with the lowest MRD level.

In conclusion, we confirm that pre-transplant MRD allows early identification of patients at higher risk of relapse after allogeneic HSCT. The impact of pre-transplant MRD positivity is different in patients transplanted in first, second or subsequent CR. A prospective, longitudinal evaluation of post-HSCT MRD could provide accurate information to predict impending relapse, and thus, represent a tool for implementing strategies of pre-emptive immunological intervention aimed at avoiding progression to frank relapse.

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Authorship

Contribution: F.Lov., M.Z., and G.B. conceived and designed the study, analysed results, and wrote the manuscript; B.R., M.C., E.M., executed experiments; E.G. managed biological samples processing and storage; G.C. and S.S. collected/provided molecular data; T.M., G.A., B.B., F.F., P.Q. and F.Loc. provided clinical care, collected patient data, and commented on the manuscript.

Disclosures

The authors declare no competing financial interests.

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