

Long-Term Donor Chimerism Monitoring for Relapse Risk Assessment After Pediatric Allo-HSCT

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Objective: Allo-HSCT is a well-established treatment for several hematological malignancies. Relapse after allogeneic hematopoietic stem cell transplantation (allo-HSCT) remains a significant problem and is associated with a poor prognosis and low overall survival. Chimerism analysis is one of the tools applied in post-transplant monitoring, as an increasing fraction of recipient cells after HSCT has been linked to a higher risk of relapse.

Study Design: In this retrospective, single-centre study we have analysed the data of patients treated with allo-HSCT for a range of hematological malignancies in the Department of Paediatric Haematology, Oncology and Transplantology, Medical University of Lublin, Poland, between years 2002–2018.

Results: For all 103 patients 3-years OS was 72.6 (95% CI: 64.3–82.0) and 3-years EFS was 72.0 (95% CI: 63.5–81.6). There were no differences in 3-years OS and EFS in group of patients who achieved FDC <14 and >14 days: 67.9 (95% CI: 57.4–80.3) vs 81.9 (95% CI: 69.7–96.2), $p = 0.220$ and 66.0 (95% CI: 55.3–78.7) vs 84.1 (95% CI: 72.3–97.9), $p = 0.073$, respectively. Early FDC achievement was not significantly associated with risk of relapse, $p = 0.181$. Based on multivariate Cox regression analysis AML/MDS increased risk of relapse 3x compared to ALL, HR = 2.72, CI95 [1.24–5.98], $p = 0.013$; PB/CB increased the risk nearly 3x compared to cells from BM, HR = 2.52, CI95 [1.12–5.65], $p = 0.025$.

Conclusion: In presented study, achieving early FDC was not associated with lower risk of relapse and had no impact on overall and event-free survival. However, the study presents a unique data of very early chimerism in a large cohort of paediatric patients with hematological malignancies treated within a single unit. Possible extensions to this study, to include analysing more data from a larger patient cohort, may allow us to determine the exact prognostic value of very early chimerism analysis to establish relevant cutoff values and risk thresholds for intervention.

Keywords: hematopoietic chimerism, allo-HSCT, children, relapse

Introduction

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a well-established treatment for several haematological malignancies. However, HSCT may be associated with many complications. These include toxicities related to conditioning treatment, infections, in it severe fungal infections, and a range of endothelial origin complications (HSCT-related thrombotic microangiopathy – TMA, veno-occlusive disease/sinusoidal obstructive syndrome – VOD/SOS, capillary leak syndrome – CLS), graft versus host disease (GvHD) and relapse of the underlying disease.^{1–8} Relapse after allo-HSCT remains a significant problem and is associated with a poor prognosis and low overall survival. Preventing the risk of relapse involves monitoring the disease. This is important because treatment can sometimes be given at a subclinical stage of the underlying disease, which results in fewer complications and increases the chance of

complete remission. It is of particular importance to monitor the presence of recipient-derived cells after allo-HSCT, as an increase in this cell fraction is associated with a higher risk of disease relapse.^{9–11}

Posttransplant haematopoietic chimerism refers to the presence of two genetically distinct cell populations within an individual's bone marrow cells after allo-HSCT. Different degrees of chimerism can be distinguished, including full donor chimerism (FDC), in which recipient cells are completely replaced by donor cells; mixed chimerism (MC), where the population of cells consists of a mix of donor and recipient cells; and full recipient chimerism, which is an undesired state where the donor cells do not engraft successfully, and the recipient bone marrow cells continue to dominate. This finding implies that an increasing level of recipient chimerism may be suggestive of relapse. The state of hematopoietic chimerism may be dynamic. Patients with an FDC may develop an MC at a later time point or vice versa. This is particularly important for patients treated with allo-HSCT for hematologic malignancies, where persisting or reappearing recipient cells might be survival malignant cells.^{9,10,12}

Chimerism status monitoring can be performed after allo-HSCT as a screening method for relapse and graft-versus-host disease (GvHD) in various haematopoietic malignancies. Bader et al. study confirmed the relationship between increased recipient chimerism and the risk of relapse in patients with acute lymphoblastic leukaemia (ALL) and acute myeloblastic leukaemia (AML).^{11,12}

However, the chimerism test alone will not answer the question of whether the surviving population of recipient cells is malignant, it may be a clue to the need for MRD testing as soon as possible, especially since time between the onset of MC and relapse is often short.

But in patients with diseases lacking a disease-specific marker like MDS or AML, chimerism analysis is of particular importance.^{9,13} In long-term follow-up, any change from FDC to increasing MC should be an indication for intervention, eg to check MRD as early as possible or even to try preventive immunotherapy.^{14–16} Kinsella et al study showed that in patients with myeloid diseases MC at day +50 posttransplant was associated with a higher relapse rate compared to FDC.¹⁷

Chimerism is routinely assessed after the transplant engraftment (+28/30 days post HSCT). In presented study, we have added early results from chimerism analyses, because its predictive value is not as strongly proven.¹⁸

Short tandem repeat polymerase chain reaction (STR-PCR) assessing the proportion of recipient/donor DNA present in peripheral blood is considered the gold standard for chimerism monitoring, with a sensitivity between 1–5%.¹⁷ The lowest detection limit reported in the literature for short tandem repeat (STR)-based chimerism monitoring is 1%.^{10,12,19} Higher precision within a specific concentration range is demonstrated by the NGS-based chimerism monitoring assay. With an analytical limit of detection of 0.3%, the assay allows for the identification of extremely low levels of recipient DNA, which is crucial for the early detection of relapse or donor failure.²⁰ Kakodkar et al successfully validated and implemented a next-generation sequencing (NGS)-based assay for monitoring cell subset chimerism in patients undergoing allo-HSCT. The robustness of this NGS-based mixed chimerism (MC) assay was demonstrated through a series of proficiency tests, where none of the samples required re-evaluation due to low DNA yields or inadequate read counts that would impede interpretation.²⁰

HLA locus mismatches between donor and recipient may affect transplant outcomes in patients receiving mismatched-donor (MMD) or unrelated-donor (UD) HSCT.^{21–23} Genomic loss of the mismatched human leukocyte antigen (HLA) is a described mechanism of leukemia immune escape and relapse after allo-HSCT.²⁴ It was initially identified in post-haploidentical HSCT but also can occur after 8/8 MUD transplants.²² According to Tie et al meta-analysis of HLA locus mismatching in unrelated donor hematopoietic cell transplantation, HLA-DPB1 locus mismatches in unrelated donor HSCT recipients significantly protected against leukemia relapse.²³ However, in our study, the analysis of individual HLA locus mismatches at the antigen level was not performed in the absence of sufficient data.

Leukemic relapse remains a significant cause of treatment failure and is associated with high mortality rate. As novel targeted therapies enter treatment protocols, it is particularly important to understand the reliability of chimerism assessment and design an appropriate testing schedule to obtain the most reliable predictive value in terms of prognosis and relapse risk. This study is an extension of a previous study that evaluated the impact of very early chimerism status on the risk of relapse in paediatric ALL. A wide range of malignancies, including acute myeloblastic leukaemia (AML),

myelodysplastic syndrome (MDS), non-Hodgkin lymphoma (NHL), and chronic myeloid leukaemia (CML), have been reported.

Very early chimerism (<30 days) monitoring is not routinely performed. Chen et al in their study on ALL patients suggest that the achievement of early FDC (30 days after allo-HSCT) can be used as a significant predictor of relapse free survival.¹⁸

In presented study, chimerism analysis has been assessed over the long term, including the early period and extending observations up to 60 months post-transplant.

The aim of this study was to analyse whether achieving very early (<14 days) full donor chimerism (FDC) is associated with lower risk of relapse after allo-HSCT in children transplanted for haematological malignancies. The second objective was to investigate the impact of achieving early FDC on survival.

Patients and Methods

Patients Characteristics

The total group consisted of 103 patients who underwent HSCT at the Department of Pediatric Hematology, Oncology, and Transplantology, Medical University, Lublin, between 2002 and 2018, during treatment for different hematological malignancies. Of these patients, 59,2% were male and 40,8% female. The median age at transplant was 9.47 years and ranged, 0.61 to 18.61 years. The most common diagnosis was ALL (53.3%), followed by AML (25.7%), and MDS (11.4%). Patients diagnosed with NHL, or CML constituted less than 10% of the analysed group. Most patients were transplanted from matched unrelated donors (MUD) (61.2%), one in three patients (33.9%) received transplant from matched sibling donor (MSD) and 5 patients (4.9%) from mis-matched family donor (MMFD). In analysed group of patients, 62 (60.2%) were transplanted in 1 complete remission (1CR); 30 patients (29.1%) in 2CR; 4 patients (3.9%) in 3CR, and 7 patients (6.8%) in partial remission (PR). In our analyses, 2CR and 3CR were considered as >1CR. In majority of patients, (72pts., 69.9%) conditioning was myeloablative (MAC) and standard regimens were based on fractionated total body irradiation (FTBI) in 46 patients (44.7%) or busulfan in 26 patients (25.2%). In reduced toxicity conditioning (RTC) applied in 31 patients (30.1%), treosulfan was used instead of busulfan.

The majority of donors were male (56.3%). Anti-Thymocyte Globulin (ATG) was administered to 64 of patients (62.1%). Bone marrow (BM) was the dominant cell source (75 pts.; 72.8%), the second most common source was peripheral blood (PB) (27 pts.; 26.2%), cord blood (CB) was used in one patient (1%). Characteristics of patients are presented in Table 1.

Table 1 Characteristics of Study Group (n = 103)

Variable	Total Group	Time to Chimerism ≥ 95%		MD (95% CI)	p
		≤14 days (n = 69)	>14 days (n = 34)		
Age at transplant, years, median (IQR)	9.47 (4.49;14.11)	8.53 (4.38;14.01)	9.57 (4.60;15.38)	-1.03 (-3.44;1.08)	0.331 ²
Patient gender, n (%)					
Female	42 (40.8)	32 (46.4)	10 (29.4)	-	0.151
Male	61 (59.2)	37 (53.6)	24 (70.6)		
Diagnosis					
ALL	56 (54.4)	35 (50.7)	21 (61.8)	-	0.108
AML/MDS	39 (37.9)	26 (37.6)	13 (38.2)		
Other*	8 (7.8)	8 (11.6)	0 (0.0)		

(Continued)

Table 1 (Continued).

Variable	Total Group	Time to Chimerism $\geq 95\%$		MD (95% CI)	p
		≤ 14 days (n = 69)	>14 days (n = 34)		
Remission type, n (%)					
I CR	62 (60.2)	38 (55.1)	24 (70.6)	–	0.194
> ICR	41 (39.8)	31 (44.9)	10 (29.4)		
Type of transplant, n (%)					
MUD	63 (61.2)	47 (68.1)	16 (47.1)	–	0.0103
MSD	35 (34.0)	17 (24.6)	18 (52.9)		
MMFD	5 (4.9)	5 (7.2)	0 (0.0)		
Donor gender, n (%)					
Female	45 (43.7)	27 (39.1)	18 (52.9)	–	0.264
Male	58 (56.3)	42 (60.9)	16 (47.1)		
Conditioning, n (%)					
TBI/MAC	72 (69.9)	47 (68.1)	25 (73.5)	–	0.738
RTC	31 (30.1)	22 (31.9)	9 (26.5)		
ATG, yes, n (%)	64 (62.1)	47 (68.1)	17 (50.0)	–	0.117
Cell source, n (%)					
BM	75 (72.8)	45 (65.2)	30 (88.2)	–	0.026
PB/CB**	28 (27.2)	24 (34.8)	4 (11.8)		
Relapse/progression, n (%)	20 (19.4)	14 (20.3)	6 (17.6)	–	0.957
Patient status, n (%)					
Alive	72 (69.9)	46 (66.7)	26 (76.5)	–	0.429
Dead	31 (30.1)	23 (33.3)	8 (23.5)		
Cause of death, n (%)***					
Relapse/progression	18 (58.1)	13 (56.5)	5 (62.5)	–	$>0.999^3$
Other	13 (41.9)	10 (43.5)	3 (37.5)		
aGvHD, n (%)****	42 (41.2)	30 (44.1)	12 (35.3)	–	0.522
cGvHD, n (%)	10 (9.7)	7 (10.1)	3 (8.8)	–	$>0.999^3$

Notes: ¹Groups compared with t-Student test, ²Mann–Whitney U-test, ³Pearson's chi-square test or Fisher exact test, as appropriate. *NHL/CMR. **Includes n = 27 (PB) and n = 1 (CB). ***Proportions calculated to all patients with status dead. ****Proportions in group ≤ 14 days calculated to n = 68 due to missing information on aGvHD for one patient.

Abbreviations: MD, mean/median difference; CI, confidence interval; IQR, interquartile range; SD, standard deviation.

Transplant Procedures

Conditioning regimens were based on the current European Bone Marrow Transplantation (EBMT) Guidelines and the respective treatment protocols for each case.²⁵ Myeloablative conditioning (MAC) regimens were based on fractionated total body irradiation (fTBI) or busulfan. In reduced toxicity conditioning (RTC), treosulfan was used instead of busulfan.

Graft-versus-host disease (GVHD) prophylaxis regimens in MSD recipients consisted of Cyclosporine and in MUD transplant recipients also included posttransplant methotrexate (day +1; +3; +6) and antithymocyte globulin – ATG (Thymoglobulin®; Genzyme) according to EBMT and institutional guidelines. Mismatched related transplant recipients received ex-vivo T-cell depleted grafts. Engraftment was diagnosed when an absolute neutrophil count (ANC) of 500 or more was present for 2 days.

Chimerism Analysis

Peripheral blood samples were collected into EDTA anticoagulant tubes, and chimerism was analysed on days +14, +21, +28, +60, +90, +120, +150, +180, +270, +365, +455, +545, +730 after HSCT. Depending on clinical indications, chimerism was monitored irrespective of the scheduled time points.

A standardised STR-PCR method was used based on the Eurochimerism recommendations.²⁶ Mononuclear cells were isolated from blood using Ficoll-Paque PLUS aqueous solution at a density of 1.077 ± 0.001 g/mL density (Amersham Biosciences, Inc., Piscataway, NJ, USA). DNA was isolated using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). The details of the analysis have been previously described.²⁷

The study was approved by the Ethics Review Committee of Medical University of Lublin (KE-0254/70/2010).

Statistical Analysis

Nominal variables were presented with number of observations and % frequency. Numeric variables were presented with mean and standard deviation or median and interquartile range, depending on normality of distribution. Distribution normality was verified with Shapiro–Wilk test and further with skewness and kurtosis. Two groups were formed and compared: patients with early chimerism (chimerism $\geq 95\%$ by or at 14 days after transplant) and other patients. Groups were compared with t-Student test, Mann–Whitney *U*-test, Pearson's chi-square test or Fisher exact test, as appropriate. Survival analysis was conducted with Kaplan–Meier method. Survival curves were compared with log rank test. Cox proportional hazard 2-step regression was performed to verify the impact of early chimerism on relapse. The covariates for the multivariate analysis were selected based on clinical rationale, results of comparisons between patients with early chimerism and others, as well as the univariate analysis. The final multivariate model includes 5 independent variables, fulfilling the rule of at least 20 events per variable. Significance level was assumed at $\alpha = 0.05$. Statistical R software in version 4.1.2 was used to perform the analyses.

Results

Characteristic and Comparison of Patients with Early Chimerism and Other Patients

The study group comprised 103 patients. Median observation time was 38.50 months (IQR: 12.81;60.95). The median age of patients with early FDC was 8.53 years, whereas that of patients who did not show FDC on day 14 it was 9.57 years. The proportion of females was 46.4% and 29.4%, respectively. The groups did not differ significantly in terms of age or sex ($p = 0.331$ and $p = 0.151$, respectively). The characteristics are presented in Table 1.

The groups differed significantly according to the transplant type ($p = 0.010$). Among the patients with early FDC, 68.1% received MUD transplants, whereas for the group that achieved FDC later (>14 days), it was 47.1%. Patients with early FDC received MSD transplants in 24.6% of cases, compared to 52.9% of cases among the patients who achieved chimerism later. Early FDC was observed in all 5 patients transplanted from MMFD (7.2%).

The source of cells was significantly different between the groups ($p = 0.026$). Bone marrow was the most common graft source. Most of PBSC recipients (24/27 pts.; 88.9%) achieved rapid FDC compared to 45/75 (60%) BM recipients. In a patient who received CB ($n = 1$; 2.9%), time to achieve FDC was above 14 days.

In the whole group, 20 patients (19.4%) had disease relapse; no statistical difference was observed between the groups of patients with early FDC and patients who achieved FDC > 14 days (14 and 6 patients, respectively; n.s).

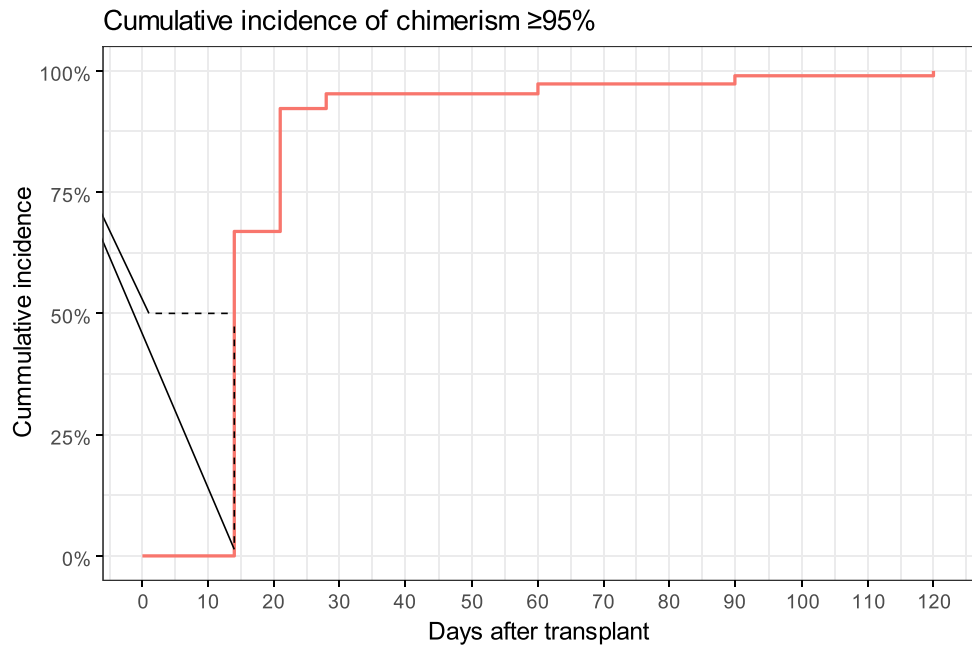


Figure 1 Cumulative incidence of chimerism $\geq 95\%$ median time to chimerism $\geq 95\%$: 14 days; all patients attained chimerism $\geq 95\%$ by day 120.

Chimerism $\geq 95\%$ in Study Group

Cumulative Incidence of Chimerism

The median time to achieve full donor chimerism $\geq 95\%$ (FDC) was 14 days. Of all patients 69 achieved chimerism $>95\%$ 14 days after transplantation (67%). Most patients By day +90 all patients achieved FDC at any time point. The cumulative incidence of chimerism $\geq 95\%$ is shown in [Figure 1](#). The dynamics of patients' chimerism during observation time is presented in [Table 2](#).

Cumulative Incidence of Relapse

The cumulative incidence of relapse was 17% and is shown on [Figure 2](#).

Table 2 Dynamics of Patients' Chimerism (n = 103)

Chimerism Information, n (%)	Day After Transplant												
	14	21	28	60	90	120	150	180	270	365	455	545	730
Chimerism $\geq 95\%$ not achieved by this day	32 (31.1)	11 (10.7)	7 (6.8)	2 (1.9)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Achieved chimerism $\geq 95\%$ by this day without decrease in chimerism	69 (67.0)	92 (89.3)	96 (93.2)	92 (89.3)	91 (88.3)	91 (88.3)	88 (85.4)	86 (83.5)	77 (74.8)	69 (67)	67 (65)	63 (61.2)	58 (56.3)
Death/relapse (chimerism $<95\%$)	0 (0.0)	0 (0.0)	0 (0.0)	9 (8.7)	11 (10.7)	10 (9.7)	12 (11.7)	13 (12.6)	18 (17.5)	22 (21.4)	23 (22.3)	24 (23.3)	26 (25.2)
No data for reason other than death or relapse (shorter observation time)	2 (1.9)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.0)	2 (1.9)	3 (2.9)	4 (3.9)	8 (7.8)	12 (11.7)	13 (12.6)	16 (15.5)	19 (18.4)

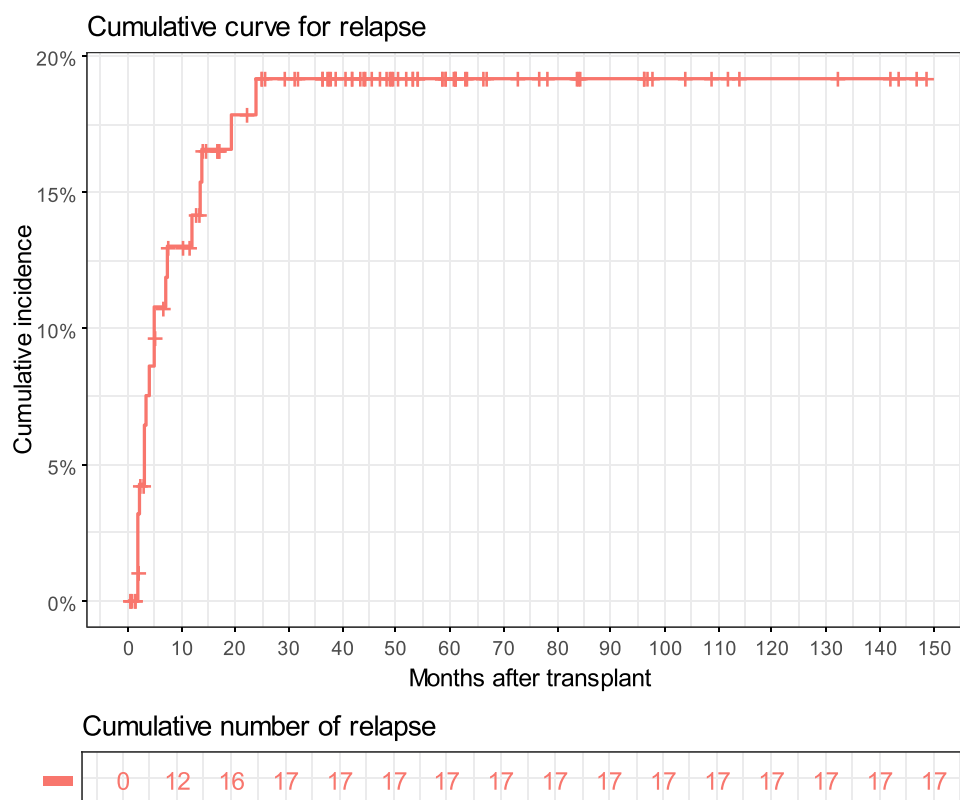


Figure 2 Cumulative incidence of relapse.

Overall Survival and Event-Free Survival Analysis

Overall Survival Analysis for All Patients

For all analysed patients 1 year OS was OS = 80.2 (95% CI: 72.8–88.4); 2 year OS was OS = 73.8 (95% CI: 65.6–83.0) and 3 years OS was 72.6 (95% CI: 64.3–82.0). The proportion of patients who survived for a given number of months is shown in [Figure 3A](#).

Overall Survival Analysis – Patients with Early Chimerism vs Other Patients

Three years OS in group of patients with early chimerism (≤ 14 days) was 67.9 (95% CI: 57.4–80.3) and in patients with FDC (> 14 days) was 81.9 (95% CI: 69.7–96.2). Statistical difference in survival curves was not confirmed, $p = 0.220$. Proportion of patients who survived after given number of months is displayed in [Figure 3B](#).

Event-Free Survival Analysis for All Patients

For all analysed patients 1 year EFS was 77.6 (95% CI: 69.7–86.3); 2 years EFS was 72.0 (95% CI: 63.5–81.6) and 3-years EFS was 72.0 (95% CI: 63.5–81.6). The proportion of event-free patients after a given number of months is shown in [Figure 3C](#).

Event-Free Survival Analysis – Patients with Early Chimerism vs Other Patients

Three years EFS in group of patients with early chimerism (≤ 14 days) was 66.0 (95% CI: 55.3–78.7) and in patients with FDC (> 14 days) was 84.1 (95% CI: 72.3–97.9). Statistical difference in survival curves was not confirmed, $p = 0.073$, [Figure 3D](#).

Due to p value suggesting that a trend might exist towards lower EFS among patients with early chimerism, an additional view in subgroups of patients with ALL and with diagnosis other than ALL was performed. In the subgroup of patients with ALL, median 3-year EFS in group with early chimerism (≤ 14 days) was 75.2 (95% CI: 61.6–91.8) and in patients with FDC > 14 days was 100.0, (log rank: $p = 0.063$), [Figure 4A](#)). Statistical difference in survival curves was

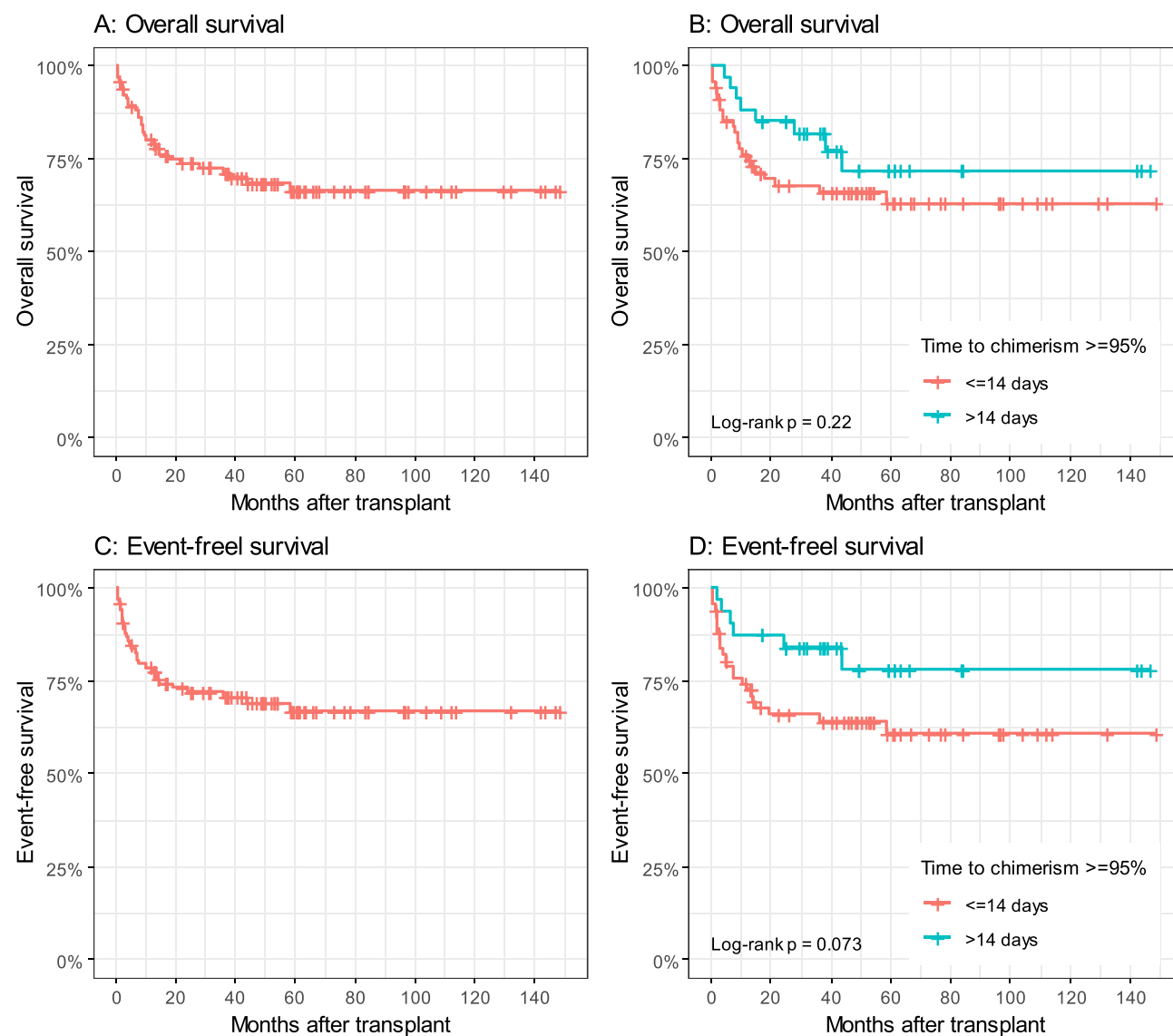


Figure 3 Kaplan–Meier survival curves, (A) overall survival for all patients, (B) overall survival for patients with early chimerism and other patients, (C) event-free survival for all patients, (D) event-free survival for patients with early chimerism and other patients.

not confirmed, yet the p value suggested that the trend might exist. In subgroup of patients with diagnosis other than ALL, median 3-year EFS in group with early chimerism (≤ 14 days) was 56.4 (95% CI: 41.5–76.8) and in patients with FDC > 14 days was 60.6 (95% CI: 38.7–94.7). Statistical difference in survival curves was not confirmed, $p = 0.590$, Figure 4B.

Multivariate Cox regression model for EFS was performed incorporating key clinical confounders: diagnosis, cell source, type of transplant, and conditioning regimen, while aGVHD and cGVHD were excluded due to non-significant associations in univariate analyses ($p = 0.427$ and $p = 0.943$, respectively). Early chimerism remained non-significant in both univariate ($p = 0.081$) and multivariate analysis ($p = 0.181$), consistent with Kaplan–Meier estimates ($p = 0.073$).

Cox Proportional Hazard Regression Analysis for Death (Corresponding to OS)

Based on univariate Cox regression analysis, following predictors impacted risk of death in statistically significant way: diagnosis, status before transplant, donor gender and cell source. AML/MDS increased the risk 2x compared to ALL, HR = 2.29, CI₉₅ [1.08–4.85], $p = 0.030$. PR status increased the risk 3x compared to CR, HR = 3.32, CI₉₅ [1.36–8.14], $p = 0.009$. Male donor was associated with 56% lowered risk compared to female donor, HR = 0.44, CI₉₅ [0.21–0.90], $p =$

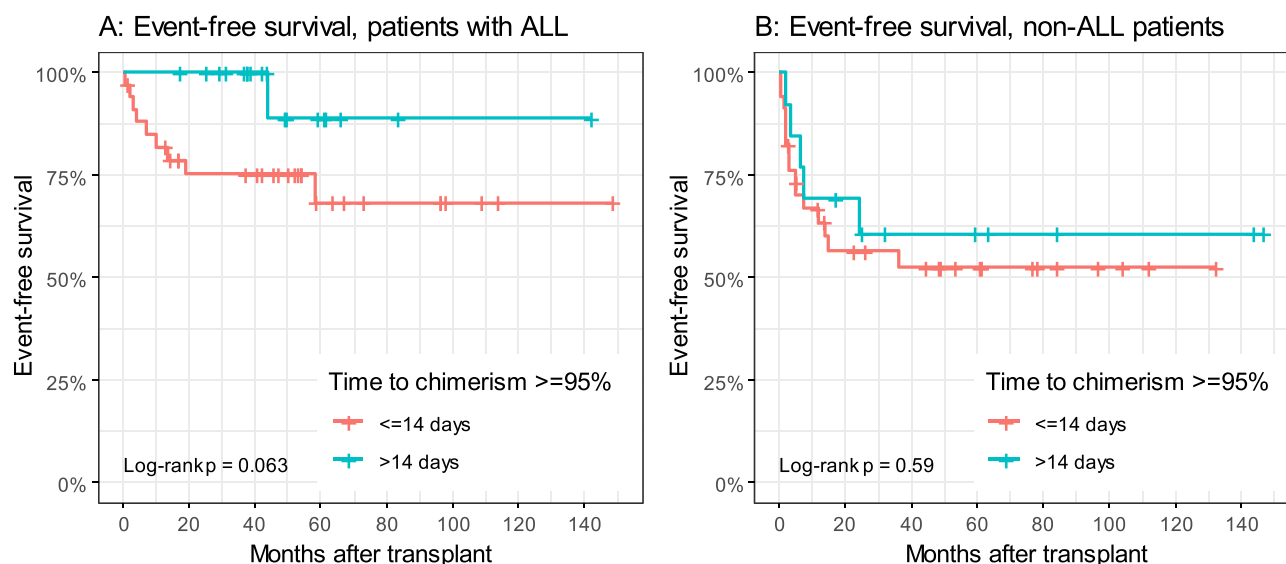


Figure 4 Kaplan–Meier event-free survival curves, **(A)** for patients with ALL, **(B)** for patients with diagnosis other than ALL.

0.026. Cells from PB/CB increased the risk 2x compared to cells from BM, HR = 2.36, CI₉₅ [1.15–4.82], $p = 0.019$. Obtaining chimerism $\geq 95\%$ before or at 14 days was not significantly associated with risk of death, $p = 0.220$, Table 3.

Adjustment for confounding factors confirmed that obtaining chimerism $\geq 95\%$ before or at 14 days was not significantly associated with risk of death, $p = 0.358$.

Based on multivariate model, AML/MDS increased the risk of relapse 2x compared to ALL, HR = 2.25, CI₉₅ [1.05–4.80], $p = 0.036$. Cells from PB/CB increased the risk nearly 2x compared to cells from BM, HR = 2.49, CI₉₅ [1.14–5.45], $p = 0.022$, Table 4.

Cox Proportional Hazard Regression Analysis for Relapse

Based on univariate Cox regression analysis, following predictors impacted risk of relapse in statistically significant way: diagnosis, disease status before transplant and cell source. AML/MDS increased the risk 3x compared to ALL, HR = 2.80, CI₉₅ [1.28–6.13], $p = 0.010$. PR status increased the risk 3x compared to CR, HR = 3.36, CI₉₅ [1.36–8.29], $p =$

Table 3 Outcomes of Univariate Cox Proportional Hazard Regression for Death and for Relapse

Variable	Cox Univariate Regression for Death, n = 103			Cox Univariate Regression for Relapse, n = 100		
	HR	95% CI for HR	p	HR	95% CI for HR	p
Age at transplant, years	1.03	0.96–1.10	0.459	1.01	0.95–1.08	0.670
Patient gender						
Female	Reference	–	–	Reference	–	–
Male	0.64	0.31–1.29	0.208	0.62	0.30–1.27	0.193
Diagnosis						
ALL	Reference	–	–	Reference	–	–
AML/MDS	2.29	1.08–4.85	0.030	2.80	1.28–6.13	0.010
Other*	2.53	0.71–9.00	0.152	2.69	0.74–9.83	0.133

(Continued)

Table 3 (Continued).

Variable	Cox Univariate Regression for Death, n = 103			Cox Univariate Regression for Relapse, n = 100		
	HR	95% CI for HR	p	HR	95% CI for HR	p
Status before transplant						
CR	Reference	–	–	Reference	–	–
PR	3.32	1.36–8.14	0.009	3.36	1.36–8.29	0.008
Remission type						
1 CR	Reference	–	–	Reference	–	–
2 CR	1.24	0.56–2.72	0.600	1.19	0.53–2.69	0.676
3 CR	1.92	0.44–8.36	0.387	2.79	0.64–12.20	0.174
PR	1.94	0.56–6.67	0.295	1.91	0.55–6.57	0.307
Type of transplant						
MUD	Reference	–	–	Reference	–	–
MSD	0.64	0.29–1.40	0.261	0.51	0.22–1.21	0.129
MMFD	0.49	0.07–3.69	0.492	1.07	0.25–4.56	0.929
Donor gender						
Female	Reference	–	–	Reference	–	–
Male	0.44	0.21–0.90	0.026	0.49	0.24–1.00	0.051
Conditioning						
TBI/MAC	Reference	–	–	Reference	–	–
RTC	1.34	0.64–2.80	0.434	1.38	0.66–2.91	0.394
Cell source						
BM	Reference	–	–	Reference	–	–
PB/CB**	2.36	1.15–4.82	0.019	2.88	1.40–5.91	0.004
aGVHD***	0.56	0.13–2.34	0.427	0.97	0.47–2.01	0.931
cGVHD	1.03	0.50–2.10	0.943	0.55	0.13–2.32	0.417
Time to chimerism $\geq 95\%$						
≤ 14 days	Reference	–	–	Reference	–	–
> 14 days	0.60	0.27–1.35	0.220	0.45	0.18–1.10	0.081

Notes: *NHL/CMR. **Includes n = 27 (PB) and n = 1 (CB) in models for death and n = 26 (PB) and n = 1 (CB) in models for relapse. ***Based on n = 102 (death) and n = 99 (relapse) due to missing information on aGVHD for one patient.

Abbreviations: HR, hazard ratio; CI, confidence interval.

0.008. Cells from PB/CB increased the risk 3x compared to cells from BM, HR = 2.88, CI₉₅ [1.40–5.91], p = 0.004. Obtaining chimerism $\geq 95\%$ before or at 14 days was not significantly associated with risk of relapse, p = 0.081, [Table 3](#).

Adjustment for confounding factors confirmed that obtaining chimerism $\geq 95\%$ before or at 14 days was not significantly associated with risk of relapse, p = 0.181. Based on multivariate model, AML/MDS increased risk of relapse 3x compared to ALL, HR = 2.72, CI₉₅ [1.24–5.98], p = 0.013. Cells from PB/CB increased the risk nearly 3x compared to cells from BM, HR = 2.52, CI₉₅ [1.12–5.65], p = 0.025, [Table 4](#).

Table 4 Outcomes of Multivariate Cox Proportional Hazard Regression for Death and for Relapse

Variable	Cox Multivariate Regression for Death, n = 103			Cox Multivariate Regression for Relapse, n = 100		
	HR	95% CI for HR	p	HR	95% CI for HR	p
Diagnosis						
ALL	Reference	–	–	Reference	–	–
AML/MDS	2.25	1.05–4.80	0.036	2.72	1.24–5.98	0.013
Other*	1.79	0.48–6.65	0.388	1.72	0.45–6.58	0.431
Cell source						
BM	Reference	–	–	Reference	–	–
PB/CB**	2.49	1.14–5.45	0.022	2.52	1.12–5.65	0.025
Type of transplant						
MUD	Reference			Reference		
MSD	0.85	0.36–2.00	0.711	0.69	0.27–1.76	0.441
MMFD	0.22	0.03–1.75	0.153	0.46	0.10–2.12	0.319
Conditioning						
TBI/MAC	Reference			Reference		
RTC	1.34	0.63–2.86	0.445	1.34	0.63–2.87	0.449
Time to chimerism ≥95%						
≤14 days	Reference	–	–	Reference	–	–
>14 days	0.67	0.29–1.57	0.358	0.53	0.21–1.35	0.181

Notes: *NHL/CMR. **Includes n = 27 (PB) and n = 1 (CB) in models for death and n = 26 (PB) and n = 1 (CB) in models for relapse.

Abbreviations: HR, hazard ratio; CI, confidence interval.

Statistical power for key parameters was verified in the comparative analyses between early chimerism and other parameters: diagnosis, remission type, type of transplant, ATG, and cell source. Statistical power for key parameters was verified and is as follows: 0.46 for the diagnosis; 0.33 for the remission type; 0.97 for the type of transplant; 0.69 for the graft source; 0.43 for ATG use and 0.09 for the conditioning type.

Discussion

The early diagnosis of relapse is a fundamental objective in the long-term management of patients treated for haematological malignancies. With a good overall prognosis for most common neoplasms, such as ALL, management of high-risk relapsed cases offers the most area for improvement. Early introduction of treatment upon recognising imminent relapse is fundamental for improving prognosis; hence, it is important to seek reliable prognostic parameters. Chimerism monitoring after allo-HSCT has become increasingly important in the risk assessment for relapse, with increasing post-transplant MC being a negative prognostic factor.^{10,12} A. Lassaletta et al showed in their study that pediatric patients with ALL treated with allo-HSCT, had a lower probability of relapse (20% vs 54%, $p = 0.004$) if FDC was achieved by day 30.²⁸

In this single-centre retrospective study, a diverse group of paediatric patients treated with allo-HSCT for a range of haematological malignancies was observed during a 12-year period to determine the influence of chimerism level on relapse risk, as well as overall and event-free survival.

Although below the assumed significance level, we observed a trend to lower event-free survival in patients achieving >95% donor chimerism early (<14 days) (Figure 3D). We tried to explain this finding by considering that the most

prevalent transplant source among the early chimerism group was a matched unrelated donor. MUD-HSCT generally results in more complications and poorer overall outcome. This has a particularly visible effect in our study, where we examined data from as early as 2002, when the management of complications was significantly less effective.

An additional interesting effect was observed, which may have contributed to this trend. Among the children who achieved early FDC, 15 showed a temporary decrease in chimerism levels on days 21 and 28 in subsequent tests. Of these, 5 (33%) relapsed and died. Interestingly, in three of the five patients who died, the decrease in chimerism was intermittent and not directly linked to immediate disease relapse/progression.

The apparent mechanism of MC dynamics observed in this study may be that relapse is often preceded by a series of events that shift the proportions of specific donor/recipient immune cells, which can be detected months before full BM relapse is diagnosed.²⁹ This can be explained by partial graft rejection, resulting in a loss of the graft versus leukaemia (GvL) effect and making the patient more prone to relapse.³⁰ Interestingly, previous study on AML patients has suggested that even if MC observed after the period of engraftment later evolved into full donor chimerism, the risk of relapse was still higher than that in patients who achieved FDC earlier.³¹ This makes chimerism analysis a valuable tool, as even a single observed decrease in donor chimerism percentage suggests the need to classify patients as potentially high-risk, although, based on limited sensitivity to detect minor cell population of about 1%, chimerism analysis by STR-PCR in the whole blood is not suitable to serve alone as an MRD marker. MRD monitoring in patients with leukemias is now becoming standard after HSCT in combination with chimerism studies.^{14–16} Our retrospective study with a long-term follow-up, includes patients transplanted since 2002, when MRD monitoring was not yet routinely performed in all patients after HSCT, and therefore we focused only on the analysis of the significance of chimerism testing in the diagnosis of relapse after transplantation.

One of the challenges is determining the appropriate cutoff points both in terms of chimerism level and time post-transplant, which would allow the screening of relapse with appropriate sensitivity and specificity.³² Clinically relevant cutoffs are necessary to ensure the best prognosis for imminent relapse, while avoiding unnecessary treatment. Lindahl et al suggested that chimerism data should be primarily used to point to relapse with 73–88% specificity, rather than rule it out, with a sensitivity of 63%–67%.³⁰

Although several studies have arrived at similar conclusions regarding the influence of chimerism level on relapse risk,³³ there is little evidence concerning chimerism monitoring in the context of overall and event-free survival rates.³⁴ In our analysis, OS did not differ statistically between patients who achieved early FDC < 14 days and those who achieved later (>14 days) posttransplant, in contrast, with the literature demonstrating that full donor chimerism is one of the strongest predictors of relapse-free survival after allo-HSCT.^{35,36} Koreth et al in a heterogeneous study on a diverse group of haematological malignancies found that low levels of donor chimerism +100 days posttransplant were linked to significantly impaired overall survival, additionally pointing out that later (+100 days) chimerism analyses had a higher predictive value than analysis performed at +30 days.³⁵ This may also be an explanation for the results we obtained.

In our analysis, no difference was observed in EFS between groups of patients with FDC <14 days and >14 days posttransplant, although the EFS trend is approaching significance ($p = 0.073$). We explored this further by analysing EFS separately in patients with ALL and those with other diagnoses. Among patients with ALL, the p -value was closer to significance ($p = 0.063$), suggesting a possible trend. In contrast, no such trend was observed among non-ALL patients ($p = 0.590$). We tried to connect this finding with TBI-based conditioning, which was applied to most ALL patients, assuming that it may be connected to more toxicities, but this assumption has not been confirmed by previously published studies and meta-analyses Yalcin.^{37–40} According to meta-analysis performed by Ansari et al comparing the TBI- and no-TBI-based conditioning regimens in paediatric ALL patients, there was no difference in OS in both group of patients, but the occurrence of latent toxicity was higher with TBI conditioning regimens. On the other hand, TBI-based regimens were superior to non-TBI conditioning regimens regarding disease-free survival (DFS).³⁷ Also in Khimani et al meta analysis on ALL patients showed that the TBI-based regimen did not increase the likelihood of NRM or aGvHD.³⁹ In Abdelaty et al study on patients with ALL, despite the high relapse rate in the non-TBI regimen (based on busulfan and cyclophosphamide), no statistically significant differences were observed in OS, DFS, and non-relapse mortality (NRM) comparing to TBI-based regimens.³⁸ Yalcin et al study on paediatric ALL patients confirmed that TBI in conditioning

had no impact on survival rates.⁴⁰ In order to find factors influencing EFS, it would be necessary to extend the analyses to a larger group of patients and other factors.

In our study, based on multivariate Cox regression analysis, the risk factors associated with death and relapse in statistically significant way were type of disease (AML/MDS increased risk of death 2x and risk of relapse 3x compared to ALL), and graft source (PB/CB increased the risk of death nearly 2x and the risk of relapse nearly 3x compared to BM). This is consistent with the results of other studies, where PBSCT was associated with higher non-relapse mortality.^{36,41,42}

Univariate analysis showed that disease status (1CR vs >1CR) was associated with death and relapse, but it was not confirmed in multivariate analysis, unlike other authors' findings. Zhou et al in the study on AML patients found that allo-HSCT performed in 1CR was associated with a significantly lower post-transplant 3-year cumulative incidence of relapse.⁴³ Also in Molina et al study, risk factors associated with relapse in the multivariate analysis were disease status and the presence of GVHD.⁴⁴

HLA locus mismatches between donor and recipient may also affect transplant outcomes, especially in patients receiving mismatched-donor (MMD) HSCT.^{21–23} However, in our study, the analysis of individual HLA locus mismatches at the antigen level was not performed in the absence of sufficient data.

This study had several limitations inherent to retrospective studies, such as the potential for selection bias. This was a single-centre study, which means that the results might be more appropriate for regional demographics, and drawing general conclusions might be difficult.

As retrospective, this study included patients treated since 2002, when posttransplant MRD testing was not yet routinely performed in our department. Therefore, MRD could not be analyzed as a risk factor for relapse. We currently know that lineage-specific chimerism (myeloid vs lymphoid) has greater prognostic value and our department is introducing lineage-specific chimerism monitoring in clinical routine, starting with non-malignant disease monitoring,¹⁹ but the study analysed data collected when such an analysis was not available. The analysis of the influence of HLA mismatches was not performed in the absence of sufficient data and due to small number of patients transplanted from MMD.

Another limitation is the method of chimerism analysis by short tandem repeat (STR) PCR, particularly in the context of its sensitivity constraints. Kakodkar et al²⁰ successfully validated and implemented a next-generation sequencing (NGS)-based assay for monitoring cell subset chimerism in patients undergoing allo-HSCT. The robustness of this NGS-based mixed chimerism (MC) assay was demonstrated through a series of proficiency tests, where none of the samples required re-evaluation due to low DNA yields or inadequate read counts that would impede interpretation. This robustness is reflected in a remarkable concordance rate of 99.9% when measuring mixed chimerism within a concentration range of 0.3% to 50%. With an analytical limit of detection of 0.3%, the assay allows for the identification of extremely low levels of recipient DNA, which is crucial for the early detection of relapse or donor failure. In contrast, the lowest detection limit reported in the literature for STR-based chimerism monitoring is 1%.

Another limitation of our study is the varying statistical power across compared parameters between early chimerism and chimerism achieved >14 days. While some outcomes were sufficiently powered (eg type of transplant: 0.97), others showed low power (eg remission type: 0.33), which may limit the ability to detect true differences and should be interpreted with caution.

However, this study offers important strengths, such as a unique data of very early chimerism (<14 days posttransplant) in large cohort of paediatric patients with haematological malignancies treated within a single unit, which limits the effect of some external variables regarding treatment and conditioning protocols. Possible extensions to this study, to include analysing more data from a larger patient cohort, may allow us to determine the exact prognostic value of very early chimerism analysis to establish relevant cutoff values and risk thresholds for intervention. Additionally, new methods of lineage-specific chimerism analysis can be applied in the future to better understand the chimerism dynamics and its value as predictor of relapse.

Many factors can influence the maintenance of remission after HSCT. All of them together must be considered when estimating the risk of the transplant procedure and the risk of relapse after HSCT. Based on using artificial intelligence in research, Asteris et al developed a machine learning (ML) model to predict the long-term survivorship of patients who

receive allo-HSCT based on clinical pre- and post-allo-HSCT variables, and on transplantation-related characteristics. They used the eight parameters included in the algorithm were the following: CD34+ cells infused, patients' age and gender, conditioning regimen toxicity, disease risk index (DRI), graft source, and platelet and neutrophil engraftment.⁴⁵ Adding the results from the chimerism analysis would be highly advisable to these studies in our opinion. We did not use this model of ML in our research, but it inspires us for the future.

Conclusions

In this retrospective, single-centre study on pediatric patients treated with allo-HSCT for hematological malignancies, we found that achieving early (<14 days) FDC was not associated with lower risk of relapse after allo-HSCT in children transplanted for haematological malignancies. Early achievement of FDC had no impact on overall and event-free survival in analysed group of patients. However, the study presents a unique data of very early chimerism in a large cohort of paediatric patients with haematological malignancies treated within a single unit. Possible extensions to this study, to include analysing more data from a larger patient cohort, may allow us to determine the exact prognostic value of very early chimerism analysis to establish relevant cutoff values and risk thresholds for intervention.

The study may highlight the importance of routine chimerism testing in clinical practice, fields that still need improvement, especially since in Poland posttransplant chimerism is tested in individual centers according to EBMT and institutional guidelines and there is no single protocol that would apply nationwide.

Data Sharing Statement

The medical history of the patients, including test results and information on the procedures performed, is not publicly available, but access can be requested by contacting the corresponding author of the manuscript.

Ethics Approval and Consent to Participate

The study was performed in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of the Medical University of Lublin (Komisja.bioetyczna@umlub.pl). Further information and documentation are available to the editor upon request, by contacting the corresponding author. Informed Consent for participation in the study was granted by patients' legal guardians.

Author Contributions

Agnieszka Zaucha-Prażmo and Monika Lejman contributed equally as senior and supervisor authors. All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that there were no competing interests.

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