Rapid induction of single donor chimerism after double umbilical cord blood transplantation preceded by reduced intensity conditioning: results of the HOVON 106 phase II study

Judith A.E. Somers,[1,2] Eric Braakman,[1] Bronno van der Holt,[3] Eefke J. Petersen,[4] Erik W.A. Marijt,[5] Cynthia Huisman,[6] Kees Sintnicolaas,[2] Machteld Oudshoorn,[7,8] Marlies E. Groenendijk-Sijnke,[3] Anneke Brand,[2,7,8] and Jan J. Cornelissen[1]

[1]Erasmus MC-Daniel Den Hoed Cancer Center, Dept. of Hematology, Rotterdam; [2]Sanquin Blood Supply, Dept. of Transfusion Medicine, Rotterdam/Leiden; [3]Erasmus MC-Daniel Den Hoed Cancer Center, Clinical Trial Center, HOVON Data Center, Rotterdam; [4]University Medical Center Utrecht, Dept. of Hematology; [5]Leiden University Medical Center, Dept. of Hematology; [6]Academic Medical Center, Dept. of Hematology, Amsterdam; [7]Europdonor Foundation, Leiden; and [8]Leiden University Medical Center, Dept. of Immunohematology and Blood Transfusion, the Netherlands.

ABSTRACT

Double umbilical cord blood transplantation is increasingly applied in the treatment of adult patients with high-risk hematological malignancies and has been associated with improved engraftment as compared to that provided by single unit cord blood transplantation. The mechanism of improved engraftment is, however, still incompletely understood as only one unit survives. In this multicenter phase II study we evaluated engraftment, early chimerism, recovery of different cell lineages and transplant outcome in 53 patients who underwent double cord blood transplantation preceded by a reduced intensity conditioning regimen. Primary graft failure occurred in one patient. Engraftment was observed in 92% of patients with a median time to neutrophil recovery of 36 days (range, 15-102). Ultimate single donor chimerism was established in 94% of patients. Unit predominance occurred by day 11 after transplantation and early CD4$^+$ T-cell chimerism predicted for unit survival. Total nucleated cell viability was also associated with unit survival. With a median follow up of 35 months (range, 10-51), the cumulative incidence of relapse and non-relapse mortality rate at 2 years were 39% and 19%, respectively. Progression-free survival and overall survival rates at 2 years were 42% (95% confidence interval, 28-56) and 57% (95% confidence interval, 43-70), respectively. Double umbilical cord blood transplantation preceded by a reduced intensity conditioning regimen using cyclophosphamide/fludarabine/4 Gy total body irradiation results in a high engraftment rate with low non-relapse mortality. Moreover, prediction of unit survival by early CD4$^+$ lymphocyte chimerism might suggest a role for CD4$^+$ lymphocyte mediated unit-*versus*-unit alloreactivity. *www.trialregister.nl NTR1573.*

Introduction

Umbilical cord blood is a valuable alternative stem cell source for patients in need of an allogeneic stem cell transplant but lacking a matched unrelated donor. However, cell dose is the major limitation in single umbilical cord blood transplantation (UCBT) for adult patients, as the small number of hematopoietic progenitor cells in a single umbilical cord blood unit is associated with protracted or insufficient neutrophil recovery.[1-5]

The lack of cord blood units containing a sufficient cell dose has been overcome by the development of the double UCBT concept.[6] However, the higher cell dose of two units is not a sufficient explanation of the better engraftment, because only one cord blood unit ultimately survives. Recently it has been shown that the outcome of single UCBT using units with adequate cell doses is comparable to that of double UCBT.[7] Moreover, it has been suggested that double UCBT results in less relapse as compared to single UCBT[8] but a recent report did not confirm that conclusion.[7] These observations suggest that the non-engrafting unit plays a facilitating role in transplant outcome. However, neither the mechanisms of unit predominance and survival nor the presence and significance of unit-*versus*-unit interactions have been consistently demonstrated yet. Consequently, optimal selection criteria for a double unit combination have not been clearly defined.

Various reduced intensity conditioning (RIC) regimens have been developed to enable the use of UCBT to be extended to adult patients.[9-16] To date, the regimen proposed by the Minneapolis group yields superior results regarding neutrophil recovery and non-relapse-related mortality (NRM). Indeed, it was reported that UCBT preceded by that non-myeloablative regimen resulted in a similar survival as compared to RIC peripheral blood stem cell transplantation using an 8/8 or 7/8 HLA-matched donor.[17]

The benefit of antithymocyte globulin (ATG) in RIC regimens preceding UCBT is still unclear.[18] ATG might prevent rejection, especially in patients who do not receive chemotherapy within 3 months prior to transplantation.[9,18] On the other hand, ATG induces *in vivo* T-cell depletion of the graft. T-cell depletion of cord blood units might interfere with engraftment potential, thereby resulting in mixed chimerism.

 doi:10.3324/haematol.2014.106690
The online version of this article has a Supplementary Appendix.
Manuscript received on March 3, 2014. Manuscript accepted on August 4, 2014.
Correspondence: j.cornelissen@erasmusmc.nl

Persistent dual donor chimerism after double UCBT has been attributed to the use of ATG as well[19,20] (*own unpublished data*). Furthermore, delayed immune reconstitution may potentially lead to more infections and higher NRM. A high incidence of Epstein-Barr virus (EBV)-related complications was reported after ATG-containing non-myeloablative conditioning regimens.[15,21,22]

We hypothesized that a 4 Gy total body irradiation (TBI)-based conditioning regimen without the use of ATG would result in rapid and complete donor chimerism with early T-cell recovery by inducing sufficient recipient immune suppression to prevent rejection without a negative effect on cord blood engraftment potential. In this prospective study we, therefore, evaluated the clinical outcome of double UCBT preceded by a RIC regimen consisting of cyclophosphamide, fludarabine and 4 Gy TBI. In addition, we analyzed the pattern of early chimerism and the influence of graft characteristics on engraftment kinetics and unit survival.

Methods

Patients and cord blood units

Patients were eligible for inclusion if they met the following criteria: (i) a diagnosis of a high-risk hematologic malignancy (for definitions: see *Online Supplementary File*) or severe or very severe aplastic anemia relapsing after or failing to respond to immunosuppressive therapy in need of an allogeneic stem cell transplant but lacking a matched unrelated donor; (ii) age 18-65 years inclusive; (iii) absence of severe organ dysfunction; (iv) absence of active infections; and (v) World Health Organization performance status 0-2. All patients gave written informed consent to enrollment in the study. Six Dutch transplant centers participated in this study. The trial protocol was approved by the Dutch Central Committee on Research Involving Human Subjects (CCMO) and was conducted according to the principles of the Declaration of Helsinki.

The selection of cord blood units was based on total nucleated cell dose (TNC) and HLA match. The required minimum TNC dose for each individual unit was $1.5x10^7$/kg recipient body weight and $4.0x10^7$/kg for both units together. HLA matching was performed at split antigen level for HLA-A and -B and at high resolution level for HLA-DRB1. The minimal match grade required was 4/6 between individual units and recipient as well as between both units. The presence of unit-directed anti-HLA-A, -B or -DRB1 antibodies was excluded in all patients. Red blood cell- and plasma-reduced units were selected preferably. Transplant procedures, methods of detecting early chimerism, definitions and additional analyses of cord blood units are described in the *Online Supplementary File*.

Study endpoints

The primary endpoint of the study was the proportion of patients with primary graft failure, defined as persistent cytopenia and bone marrow hypoplasia with <10% donor hematopoiesis at day +60. Secondary endpoints included the time to peripheral blood cell recovery, cumulative incidence of acute and chronic graft-*versus*-host disease (GVHD), NRM, progression-free survival and overall survival.

Statistical analysis

This study was designed as a non-randomized, prospective, multicenter phase II trial, following an optimal Simon two-stage design.[23] The primary endpoint was the proportion of patients with primary graft failure. A true percentage of 25% patients with a primary graft failure at day 60 after transplantation would be considered too high, while 10% or less would be desirable. With α=0.10 and β=0.20, a sample size of 34 patients would be required. However, in order to allow for dropouts, it was planned to include 40 patients. Adverse events and infections were scored according to the National Cancer Institute's Common Terminology Criteria for Adverse Events (CTCAE), version 3.0. Progression-free and overall survival rates were estimated by the Kaplan-Meier method, and 95% confidence intervals (95% CI) were constructed. Kaplan-Meier survival curves were generated to illustrate progression-free survival and overall survival. The cumulative incidences of progressive disease and NRM were calculated using competing risk analyses.

Further exploratory analyses were also performed. The Wilcoxon matched-pairs signed rank test was used to evaluate variables associated with unit dominance. Univariate Cox regression analysis was performed to study the association of unit characteristics of the engrafting unit with outcome. All reported *P* values are two-sided, and a significance level α=0.05 was used, without correction for multiple testing.

Results

Characteristics of the patients and cord blood units

Sixty patients were registered for the study between October 2008 and January 2012. Four patients were not eligible because of availability of a matched unrelated donor (n=1), liver dysfunction (n=1) or refractory disease (n=2), and another three patients did not proceed to transplantation because of early relapse (n=2) or death (n=1). These seven patients were excluded from the analysis. The patients' characteristics are shown in Table 1. Fifty-

Table 1. Patients' characteristics.

Number of patients	53	
Diagnosis, n. (%)		
Acute myeloid leukemia CR1/CR2	30	(57)
Acute lymphoblastic leukemia CR1/CR2	10	(19)
Chronic myeloid leukemia, 2nd chronic phase	2	(4)
Severe or very severe aplastic anemia	4	(8)
Non-Hodgkin lymphoma	4	(8)
Chronic lymphocytic leukemia	3	(6)
Age, years, median (range)	51	(20-65)
Weight, kg, median (range)	73	(49-119)
Sex		
Male	28	(53)
Female	25	(47)
Cytomegalovirus serostatus		
Negative, n. (%)	17	(33)
Positive, n. (%)	34	(63)
Unknown, n. (%)	2	(4%)
EBV serostatus		
Negative, n. (%)	4	(8)
Positive, n. (%)	49	(92)
HCT-CI score, median (range)	1	(0-6)
Conditioning regimen		
Cy/Flu/TBI 2x2 Gy	51	(96)
Cy/Flu/TBI 2 Gy	2	(4)

CR1: first complete remission; CR2: second complete remission; HCT-CI: Hematopoietic Cell Transplant Comorbidity Index; Cy: cyclophosphamide; Flu: fludarabine; TBI: total body irradiation.

three patients underwent double UCBT. Acute leukemia was the most common diagnosis. The patients' median age was 51 years (range, 20-65) and their median weight was 73 kg (range, 49-119). The median TNC of individual units was $2.7x10^7$/kg (range, $1.3x10^7$ - $5.2x10^7$) whereas the median dose of infused TNC per patient was $5.4x10^7$/kg (range, $3.4x10^7$ - $9.2x10^7$). Most patients received a 4/6 + 4/6 (n=19, 36%) or 5/6 + 4/6 (n=24, 45%) matched unit combination whereas 13%, 4% and 2% of patients received a 5/6 + 5/6 (n=7), 5/6 + 6/6 (n=2) and 6/6 + 6/6 (n=1) matched combination, respectively. Additional high-resolution HLA typing revealed the presence of multiple allele mismatches (*Online Supplementary Table S1*). The median numbers of unit-recipient HLA class I (A, B, C) and class II (DRB1, DQB1, DPB1) allele mismatches were two (range, 0-6) and three (range, 0-5), respectively. Killer-cell immunoglobulin-like receptor ligand mismatches[24] were absent in 43% and 45% of unit *versus* recipient and unit *versus* unit combinations, respectively. The median follow-up of surviving patients was 25 months (range, 9-49).

Hematologic recovery

Primary graft failure occurred in one patient (2%). The cumulative incidence of neutrophil recovery at days +60 and +90 was 83% and 92%, respectively (Figure 1A). The cumulative incidence of engraftment was 92% with a median time to neutrophil recovery of 36 days (range, 15-102). Three patients did not meet the criteria for engraftment at the time of death due to persistent pancytopenia (n=1) or because the neutrophil count had still not been measured despite a leukocyte count above $2x10^9$/L (n=2). However, single donor chimerism had been established in all three patients. Secondary graft failure occurred in one patient at 3 months after transplantation. The cumulative incidence of platelet recovery to $20x10^9$/L, $50x10^9$/L and $100x10^9$/L at day +60 was 66%, 23% and 11%, respectively (Figure 1B-1D). Neutrophil and platelet recovery were not associated with allele level or class II unit-unit HLA-match (Table 2).

A higher TNC content of the surviving unit was associated with a faster neutrophil recovery (32 *versus* 39 days; *P*=0.04) (Table 2). A higher granulocyte-macrophage colony-forming unit (CFU-GM) dose of the surviving unit resulted in neutrophil recovery in 32 days as compared to 39 days with a CFU-GM below the median; this difference was not, however, statistically significant. TNC dose infused was not associated with neutrophil recovery (*data not shown*).

Natural killer cells recovered to normal values within 2 months. B cells recovered to normal values after 6 months. T-cell recovery was slow, with absolute cell counts reaching the lower limits of normal by 12 months. Strikingly, $CD4^+$ T-cell recovery was somewhat faster than $CD8^+$ T-cell recovery during the first months after transplantation, with median cell counts at 3 and 6 months of $0.18x10^9$/L and $0.27x10^9$/L, respectively (Figure 2).

Chimerism assessment

Complete single donor chimerism (as determined by short tandem repeat polymerase chain reaction analysis) was present in 77% of patients at day +32 (Figure 3A), increasing to 94% at 3 months. Complete donor

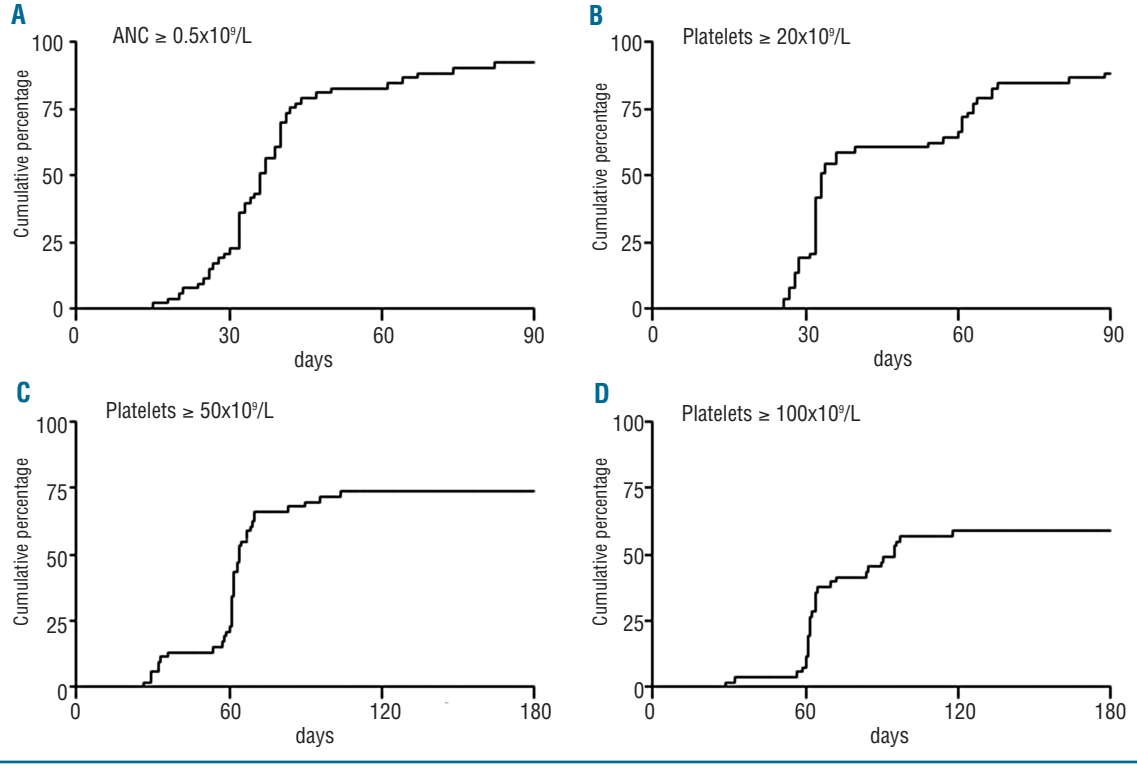

Figure 1. Recovery of peripheral blood cells. (A) Absolute neutrophil count (ANC) recovery (B-D): platelet recovery.

Table 2. Prognostic factors: results of univariate analysis.

	Neutrophil recovery			PFS			Relapse			NRM			OS		
	HR	(95% CI)	P	HR	(95% CI)	P	HR	(95% CI)	P	HR	(95% CI)	P	HR	(95% CI)	P
TNC infused*	2.19	(1.15-4.16)	0.016	1.25	(0.61-2.58)	0.54	0.88	(0.35-2.26)	0.80	2.29	(0.66-7.94)	0.18	1.03	(0.44-2.38)	0.95
TNC viability*	1.41	(0.66-3.02)	0.37	1.26	(0.53-2.99)	0.60	3.64	(0.98-13.58)	0.037	0.30	(0.06-1.48)	0.11	0.85	(0.33-2.21)	0.74
CFU-GM infused*	1.91	(0.89-4.13)	0.10	1.69	(0.71-4.05)	0.23	1.34	(0.43-4.20)	0.61	2.36	(0.58-9.55)	0.21	2.08	(0.77-5.64)	0.14
Viable CD34$^+$ cells*	1.27	(0.70-2.28)	0.43	0.90	(0.44-1.85)	0.77	0.77	(0.31-1.93)	0.58	1.16	(0.35-3.81)	0.80	1.20	(0.52-2.78)	0.67
Viable CD3$^+$ lymphocytes*	1.49	(0.81-2.72)	0.20	0.84	(0.41-1.75)	0.65	0.85	(0.34-2.15)	0.73	0.83	(0.25-2.74)	0.76	0.79	(0.34-1.85)	0.59
Viable NK-cells*	1.22	(0.66-2.25)	0.52	1.66	(0.79-3.52)	0.18	2.54	(0.96-6.72)	0.056	0.86	(0.26-2.83)	0.80	1.12	(0.48-2.60)	0.80
Viable CD19$^+$ lymphocytes*	1.46	(0.79-2.69)	0.23	1.09	(0.52-2.25)	0.83	1.01	(0.40-2.54)	0.99	1.23	(0.37-4.07)	0.73	0.94	(0.41-2.18)	0.89
HLA match grade surviving unit-recipient															
A, B, DRB1 selection criteria[1]	0.81	(0.44-1.51)	0.51	1.26	(0.61-2.59)	0.53	2.01	(0.80-5.02)	0.13	0.53	(0.14-1.99)	0.32	0.77	(0.32-1.84)	0.55
A, B, C, DRB1 allele level[2]	1.52	(0.81-2.87)	0.20	1.67	(0.80-3.51)	0.18	3.14	(1.24-7.95)	0.015	0.44	(0.09-2.08)	0.26	1.32	(0.56-3.12)	0.53
A, B, C, DRB1, DQB1, DPB[1] allele level[3]	0.73	(0.36-1.48)	0.39	1.13	(0.51-2.49)	0.76	2.17	(0.78-6.05)	0.13	0.32	(0.07-1.55)	0.12	0.86	(0.34-2.17)	0.75
DRB1, DQB1, DPB1 allele level[4]	0.97	(0.40-2.34)	0.94	1.71	(0.64-4.57)	0.31	1.73	(0.49-6.09)	0.42	1.67	(0.34-8.12)	0.54	1.42	(0.47-4.27)	0.54
HLA match grade unit-unit															
A, B, DRB1 selection criteria[1]	1.05	(0.56-1.99)	0.88	0.58	(0.25-1.35)	0.18	0.84	(0.33-2.16)	0.71	0.18	(0.02-1.51)	0.053	0.43	(0.15-1.26)	0.09
A, B, C, DRB1 allele level[2]	0.66	(0.35-1.22)	0.17	0.60	(0.27-1.35)	0.20	0.50	(0.18-1.37)	0.16	0.88	(0.23-3.43)	0.86	0.72	(0.30-1.74)	0.46
A, B, C, DRB1, DQB1, DPB1 allele level[3]	0.92	(0.48-1.77)	0.80	0.76	(0.36-1.63)	0.48	0.83	(0.33-2.08)	0.70	0.62	(0.16-2.50)	0.50	1.00	(0.43-2.31)	1.00
DRB1, DQB1, DPB1 allele level[4]	0.91	(0.43-1.92)	0.79	0.88	(0.35-2.21)	0.79	0.73	(0.21-2.50)	0.60	1.17	(0.28-4.83)	0.83	1.41	(0.55-3.63)	0.53

** Surviving unit; values above the median were compared to values below the median. Median values: TNC infused: 2.6 x10^7/kg; TNC viability: 65%; CFU-GM: 0.31x10^4/kg; viable CD34$^+$ cells: 0.35 x10^5/kg; viable CD3$^+$ cells 4.5x10^5/kg; viable natural killer (NK)-cells: 9.3x10^5/kg; viable CD19$^+$ lymphocytes 8.7x10^5/kg. [1] ≥5/6 vs. 4/6; [2] ≥6/8 vs. ≤5/8; [3] ≥8/12 vs. ≤7/12; [4] ≥5/6 vs. ≤4/6. PFS: progression free survival; OS: overall survival; HR: hazard ratio.*

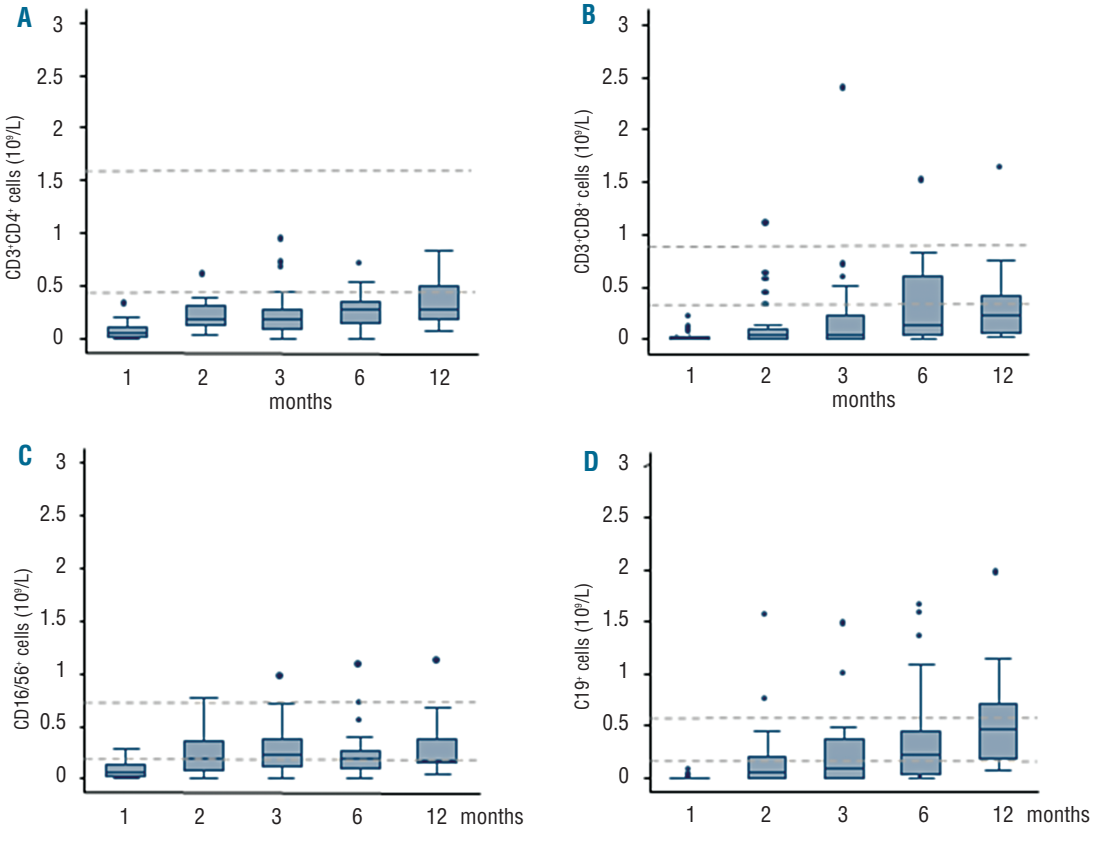

Figure 2. Immune recovery. (A) CD4$^+$ lymphocytes, (B) CD8$^+$ lymphocytes, (C) natural killer cells, (D) B-lymphocytes. Dashed lines represent the upper and lower limits of reference values.

chimerism with presence of both units was observed in four (9%) patients at day +32. However, one unit was predominant at that time point and single donor chimerism was established at day +60 in three of these patients. They had received units with an 8/12 (n=1; 4 mismatches at HLA class II), 11/12 (n=2; mismatch at HLA-DPB1) or 12/12 (n=1) unit-unit match. Double donor chimerism without a clear unit predominance persisted until disease relapse beyond day +180 in the patient who had received a 12/12 matched unit combination. Mixed chimerism was present in five patients (11%) at day +32 and persisted in one patient beyond day +90. The results of T-cell chimerism were concordant with results of unseparated peripheral blood in the majority of patients at all time points (*data not shown*).

Early chimerism was analyzed by short tandem repeat polymerase chain reaction in 44 patients (Figure 3B). A median of 40% (range, 0-100) peripheral blood cells originated from the ultimately surviving unit at day +11, with this value increasing to 82% (range, 0-100) at day +18. Predominance of the ultimately surviving unit over the non-engrafting unit was present in 78% and 89% of patients at days +11 and +18, respectively. The non-engrafting unit had completely disappeared beyond day +18 in most patients. Recipient hematopoiesis did not contribute substantially to peripheral blood cell recovery as recipient peripheral blood cells had disappeared before neutrophil recovery.

Simultaneous three-donor-origin detection of leukocyte subpopulations based on HLA mismatches using HLA monoclonal antibodies was possible in ten patients. Results were reported earlier as a part of a larger group of patients.[25] In brief, at day +11, the median percentages of cells derived from the surviving unit within $CD4^+$ T cells, $CD8^+$ T cells, natural killer cells, monocytes and granulocytes in peripheral blood were 89% (range, 46-100), 98%

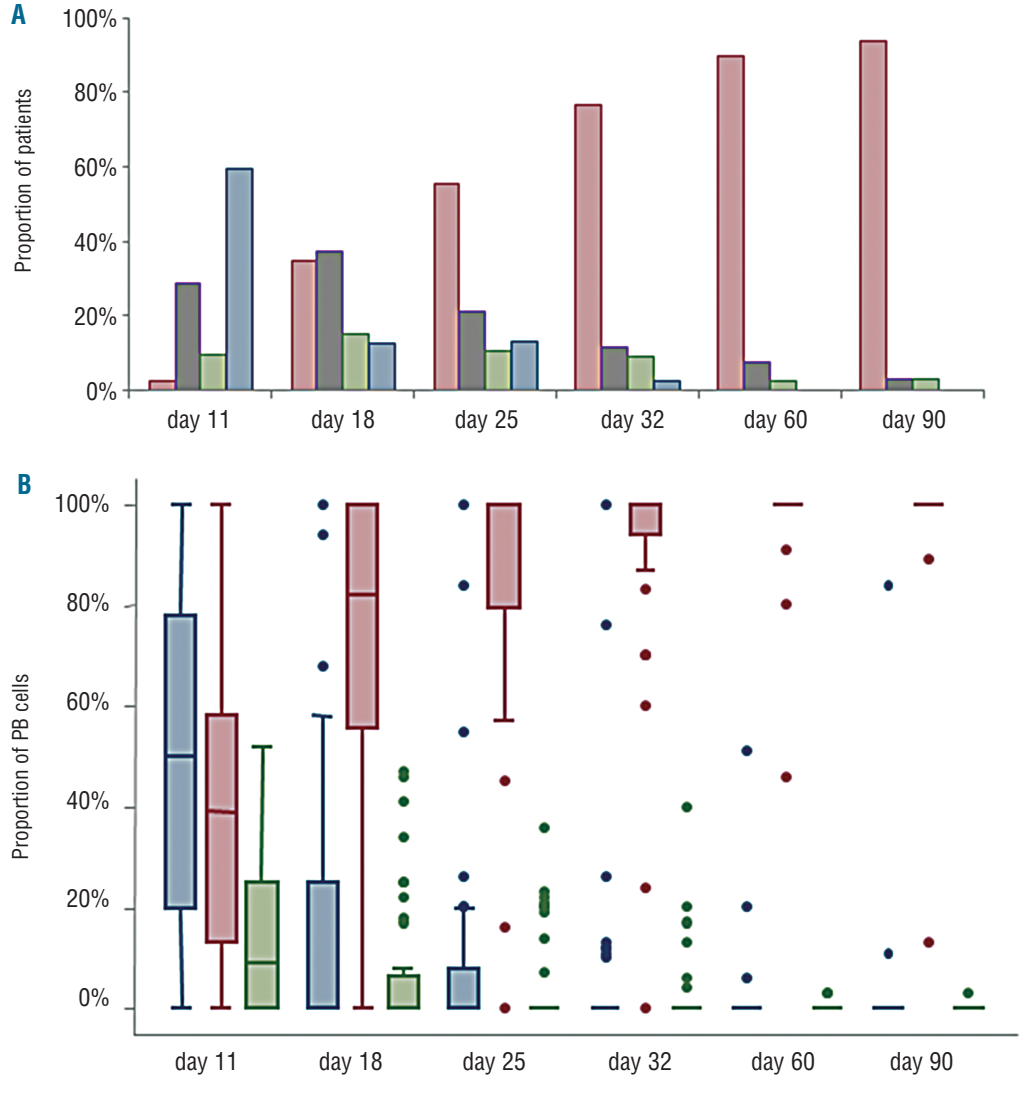

Figure 3. Chimerism. (A) Development of single donor chimerism. Complete chimerism (1 unit); mixed chimerism (recipient + 1 unit); complete chimerism (2 units); mixed chimerism (recipient + 2 units) (B) Peripheral blood chimerism in patients without graft failure. Contribution of recipient, surviving unit and non-engrafting unit to peripheral blood counts at different time points. N=44 at days 11, 18 and 25. Recipient; dominant unit; non-engrafting unit.

(range, 66-100), 61% (range, 36-93), 42% (range, 9-84) and 19% (range, 0-98), respectively (*results not shown*). Ultimate unit survival was predicted by chimerism within $CD4^+$ and natural killer cell subsets in 89% (8/9 evaluable patients) and 89% (8/9 evaluable patients) of patients, respectively, at this time point. $CD8^+$ T-cell numbers were below the detection limit in 7/10 patients, precluding the prediction of donor chimerism, but chimerism in the three remaining patients was concordant with ultimate graft predominance.

Absolute cell counts, as measured by the different participating transplant centers, showed a high inter-laboratory variation. The analyses regarding absolute cell counts were, therefore, performed separately on a subgroup of 33 patients from a single transplant center as well. Analyses of TNC viability and CFU-GM were only performed within that subgroup. TNC viability was associated with unit survival (median 64.5% in surviving units *versus* 56.5% in non-engrafting units, P=0.003) (Table 3). Neither absolute counts of TNC, $CD34^+$ cells, T cells, B cells and natural killer cells nor degree of unit-recipient allele level HLA matching were associated with unit survival.

Patients' outcome

EBV reactivation requiring therapy and EBV post-transplant lymphoproliferative disease (PTLD) developed in 10% and 4% of EBV-seropositive patients, respectively. Cytomegalovirus reactivation requiring therapy occurred in 40% of cytomegalovirus-seropositive patients while cytomegalovirus disease was not observed. Viral upper respiratory infections were reported in 26% of patients whereas BK virus reactivation and infections with norovirus or adenovirus were reported in 19%, 6% and 6% of patients, respectively.

The cumulative incidence of NRM at day +90 was 9% [standard error (s.e.) 4%]; the 2-year NRM was 19% (s.e. 5%) (Figure 4A). Causes of death were infections (viral and fungal infection, n=1; bacterial sepsis, n=1), infections in a GVHD setting (viral and/or fungal infection, n=3), multi-organ failure (n=2), cardiac complications (n=2), secondary graft failure (n=1) and leukoencephalopathy (n=1).

The cumulative incidences of acute GVHD grades II-IV and grades III-IV at day +90 were 53% and 11%, respectively. The cumulative incidence of chronic GVHD at 1 year was 38%, whereas this was 19% for extensive chronic GVHD. The cumulative incidence of relapse or progression of disease was 23% (s.e. 6%) at 1 year and 39% (s.e. 7%) at 2 years (Figure 4A). The probabilities of progression-free survival and overall survival at 2 years were 42% (95% CI, 28-58%) and 57% (CI, 43-70%), respectively (Figures 4A and 4B). A Hematopoietic Cell Transplantation Comorbidity Index score of >2 was associated with overall survival (hazard ratio 3.14, 95% CI 1.42-6.93, P=0.006).

Absolute cell counts, TNC viability and CFU-GM of the surviving unit were not associated with NRM, relapse, progression-free survival or overall survival nor was the degree of six loci (A, B, C, DRB1, DQB1, DPB1) or HLA class II (DRB1, DQB1, DPB1) allele level matching (Table 2). However, a higher unit-recipient HLA-A,-B,-C,-DRB1 allele level match (≥6/8) was associated with higher relapse, but not with overall survival.

A better (≥5/6) HLA-A, -B, -DRB1 unit-unit match (selection criteria) was associated with lower NRM but not with time to neutrophil recovery, relapse or progression-free survival (Table 2). The lower NRM translated into a trend for better overall survival (92% *versus* 57% at 1 year and 75% *versus* 51% at 2 years). Apart from HLA-matching, mismatching for natural killer cell inhibitory receptors (KIR) was evaluated for possible association with relapse. However, KIR mismatching for surviving units with the recipient (in the graft-*versus*-leukemia direction) did not appear to be significantly associated with relapse (hazard ratio=1.78; 95% CI 0.52-6.04; P=0.36).

Discussion

We studied the outcome and early chimerism of double UCBT preceded by a RIC regimen using cyclophosphamide, fludarabine and 4 Gy TBI. This regimen resulted in a high engraftment rate, a low NRM and an approximately 20% incidence of chronic extensive GVHD.

Complete single donor chimerism was observed within 1 month in bone marrow as well as in unseparated peripheral blood and in peripheral blood T cells in most patients,

Table 3. Graft characteristics.

		Surviving unit		Non-engrafting unit		
	n.†	median	range	median	range	*P*
TNC, x10^7/kg*	49	2.6	(1.3-5.2)	2.8	(1.5-5.1)	0.84
TNC after thawing, x10^7/kg	47	2.3	(1.4-5.5)	2.2	(1.4-5.3)	0.72
TNC viability, %	29	64.5	(36.5-79.5)	56.5	(35-82)	0.003
CFU-GM, x10^4/kg	30	0.30	(0-2.8)	0.31	(0-1.5)	0.46
Viable $CD34^+$ cells, x10^5/kg	49	0.35	(0-1.7)	0.35	(0-1.7)	0.47
Viable $CD3^+$ cells, x10^6/kg	47	4.5	(1.0-74)	6.5	(0.3-43)	0.85
Viable $CD19^+$ cells, x10^6/kg	45	8.7	(0.4-80)	9.9	(0.4-51)	0.94
Viable NK cells, x10^6/kg	45	9.3	(0.2-38)	8.5	(0.3-45)	0.08
HLA match *vs.* recipient A B C DRB1, allele level	50	5/8	(3/8-7/8)	5/8	(2/8-7/8)	0.10
HLA match *vs.* recipient A B C DRB1 DQB1 DPB1, allele level	40	7/12	(4/12-10/12)	7/12	(3/12-9/12)	0.27
HLA match *vs.* recipient DRB1 DQB1 DPB1, allele level	40	3/6	(1/6-6/6)	3/6	(1/6-6/6)	0.40

Graft characteristics of dominant vs. *non-engrafting units.* †*In four patients a predominant unit could not be assigned because of lack of follow up due to relapsed disease (n=2), primary graft failure (n=1) or persistent dual chimerism (n=1). TNC: total nucleated cell count; CFU-GM: granulocyte-macrophage colony-forming unit.* **as reported by cord blood bank.*

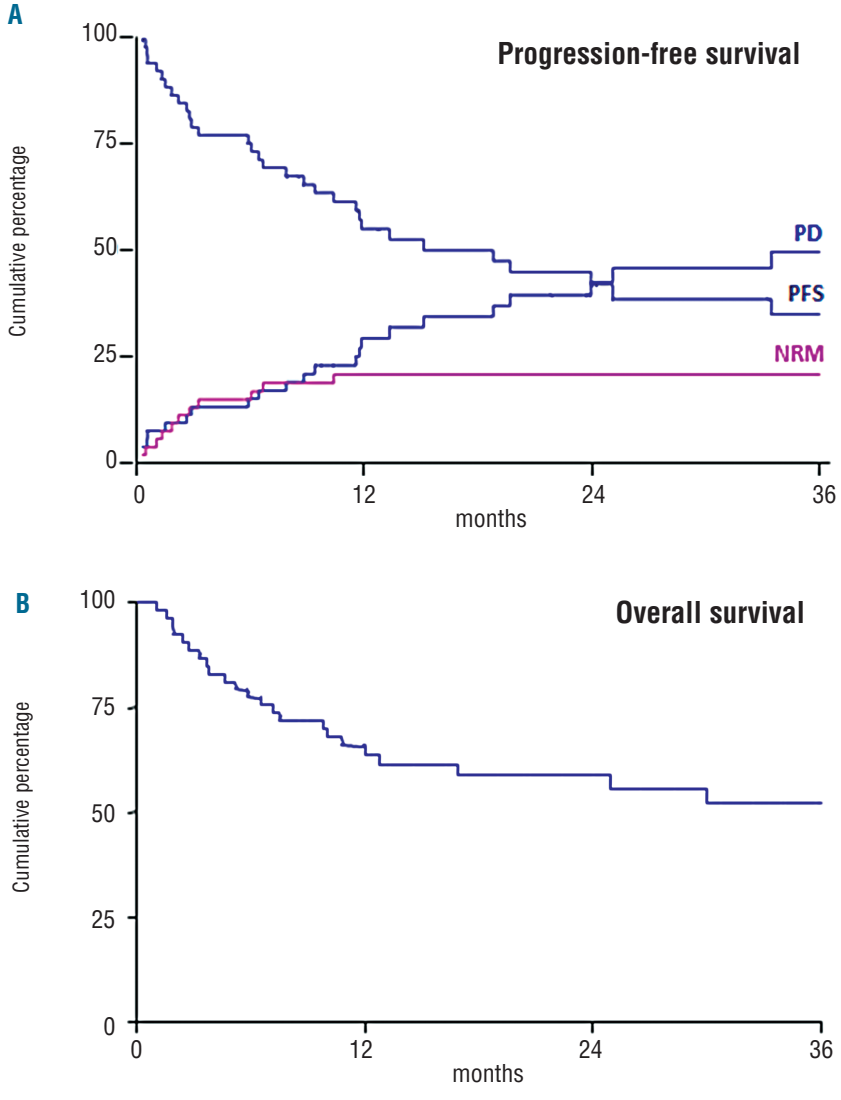

Figure 4. Survival. (A) Progression-free survival (PFS), non-relapse mortality (NRM) and progressive disease (PD). (B) Overall survival.

which is faster than that reported after an ATG-containing RIC regimen.[12,15,22,26] Early establishment of complete donor chimerism might be favorable as persistent mixed chimerism after RIC stem cell transplantation is associated with relapse and graft rejection.[27] Predominance of one grafted unit over another was established within 11 days in most patients. In a previous study in a subcohort of these patients using flow cytometry with HLA-monoclonal antibodies[25] we observed that early CD4$^+$ T-lymphocyte chimerism was predictive of ultimate graft predominance, which may suggest a role for CD4$^+$ lymphocytes in a graft-*versus*-graft reaction. This is in line with several reports suggesting the presence of a T-cell-mediated unit-*versus*-unit allo-immune reaction.[28,29] Moreover, a graft-facilitating role for the non-engrafting unit has been suggested from findings in experimental models in mice and dogs.[30,31] In addition to this, it was shown that alloreactive CD4$^+$ T cells directed against mismatched HLA class II molecules were capable of directly eliminating leukemic cells in adult patients.[32] As many unit-recipient HLA class II mismatches were present, we hypothesize that HLA class II-specific CD4$^+$ T cells of the ultimately surviving unit elicit an immediate and targeted alloreactive immune response towards mismatched HLA class II molecules of the non-engrafting unit in the absence of ATG. This hypothesis may be supported by the observation of a relatively rapid CD4$^+$ T-cell recovery, by the abundance of class II mismatches, and also by the protracted presence of the non-engrafting unit in patients who had received a unit combination with a high inter-unit level of HLA matching. The last observation might point to the absence of a unit-*versus*-unit allo-immune reaction between highly matched units. The delay in CD4$^+$ T-cell recovery and the concomitant protracted time to develop single chimerism as observed after an ATG-based RIC regimen implies that ATG might reduce that allo-immune reaction. Furthermore, specificity of surviving cord blood unit-derived CD4$^+$ T cells towards mismatched HLA-class II alleles of the non-engrafting unit was demonstrated in two patients (*results not shown*) and this observation provided the basis for a prospective study of T-cell specificity in double UCBT (HOVON 115, *www.trialregister.nl NTR 3535*).

Only one case of primary graft failure was observed, but the time to neutrophil recovery was prolonged (36 days) compared to that reported by others for RIC[12-16,33,34] or myeloablative regimens.[6,8,33,35] Our chimerism results revealed that recipient hematopoiesis disappeared early after trans-

plantation and did not, therefore, contribute to neutrophil recovery, which differs from less myelotoxic conditioning regimens. In contrast to most other studies, granulocyte colony-stimulating factor (G-CSF) was not applied in this study. The duration of neutropenia may largely be attributed to the lack of G-CSF, as Ponce *et al.* reported a median time to neutrophil recovery of 26 days using a comparable 4 Gy-based RIC regimen supported with G-CSF[34] while use of the less myelotoxic cyclophosphamide/fludarabine/TBI 2 Gy regimen with G-CSF resulted in a median time to neutrophil recovery of 9-12 days.[13,17] In our study, the prolonged neutropenia resulted in a protracted stay in hospital but did not result in early NRM.

Despite the lack of ATG as well as the expected higher toxicity of a 4 Gy TBI dose, rates of NRM and GVHD were similar to those reported by the Minneapolis group, using a lower TBI dose with or without ATG.[13,17] In addition, the incidence of EBV-PTLD was low, in contrast to that reported after ATG-containing RIC regimens.[12,15,21,22,26] These findings suggest that ATG can be safely omitted using our 4 Gy-based conditioning regimen. An ATG-free conditioning regimen might permit the development of unit-*versus*-unit allo-immune reactions thereby leading to a rapid establishment of single donor chimerism and disease control. Moreover, suppression of transient autologous recovery by 4 Gy TBI might lead to a stronger antileukemic effect as well.

We did not find an association between graft characteristics and unit survival except post-thawing TNC viability. To date, TNC, $CD34^+$ cell content and degree of HLA matching have not been found to predict unit survival, but $CD3^+$ cell dose[36,37] and $CD34^+$ cell viability[36,38] may be associated with unit predominance. Our observation that post-thawing TNC viability was associated with unit survival supports the hypothesis that, apart from immunological unit-*versus*-unit reactions, unit quality is important in unit survival.

We observed a higher relapse rate if the surviving unit had a ≥6/8 unit-recipient match compared to a ≤5/8 HLA match. Recently, an association was found between degree of allele level HLA matching and risk of NRM, but not of relapse, after single unit myeloablative UCBT.[39] Moreover, it was suggested that allele level HLA matching of the surviving unit was associated with GVHD severity in double UCBT preceded by an ATG-free conditioning regimen.[40] Malignant cells express class II HLA antigens and are, therefore, susceptible targets for $CD4^+$ T cells. The lower relapse rate in our ≤5/8 matched transplants may thus be explained by a higher frequency of allo-immune $CD4^+$ T cells in the engrafting unit resulting in a graft-*versus*-disease reaction. In double UCBT, the transient presence of the non-engrafting unit might fuel that allo-immune reaction of higher mismatched units leading to better disease control at the cost of more severe GVHD.

In conclusion, double UCBT after RIC using cyclophosphamide, fludarabine and 4 Gy TBI was associated with a high engraftment rate, but slow recovery. However, the protracted neutropenic phase was not associated with NRM. Despite a relatively slow recovery, rapid establishment of unit predominance leading to single donor chimerism was observed in 94% of patients. These results suggest that G-CSF is not necessary for engraftment and establishment of chimerism. Furthermore, early $CD4^+$ T-lymphocyte chimerism was predictive for ultimate graft predominance, suggesting a role for HLA class II in unit-*versus*-unit reactions, leading to single donor chimerism, which has provided a basis for an ongoing, prospective study.

Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

References

1. Laughlin MJ, Barker J, Bambach B, Koc ON, Rizzieri DA, Wagner JE, et al. Hematopoietic engraftment and survival in adult recipients of umbilical-cord blood from unrelated donors. N Engl J Med. 2001;344(24):1815-22.
2. Rocha V, Labopin M, Sanz G, Arcese W, Schwerdtfeger R, Bosi A, et al. Transplants of umbilical-cord blood or bone marrow from unrelated donors in adults with acute leukemia. N Engl J Med. 2004;351(22):2276-85.
3. Laughlin MJ, Eapen M, Rubinstein P, Wagner JE, Zhang MJ, Champlin RE, et al. Outcomes after transplantation of cord blood or bone marrow from unrelated donors in adults with leukemia. N Engl J Med. 2004;351(22):2265-75.
4. Rocha V, Mohty M, Gluckman E, Rio B, Eurocord; Reduced-Intensity Conditioning Subcommittee of the Acute Leukaemia Working Party; French Society of Bone Marrow Transplantation and Cellular Therapy. Reduced-intensity conditioning regimens before unrelated cord blood transplantation in adults with acute leukaemia and other haematological malignancies. Curr Op Oncology. 2009;21 (Suppl 1):S31-4.
5. Barker JN, Scaradavou A, Stevens CE. Combined effect of total nucleated cell dose and HLA match on transplantation outcome in 1061 cord blood recipients with hematologic malignancies. Blood. 2010;115(9):1843-9.
6. Barker JN, Weisdorf DJ, DeFor TE, Blazar BR, McGlave PB, Miller JS, et al. Transplantation of 2 partially HLA-matched umbilical cord blood units to enhance engraftment in adults with hematologic malignancy. Blood. 2005;105(3):1343-7.
7. Scaradavou A, Brunstein CG, Eapen M, Le-Rademacher J, Barker JN, Chao N, et al. Double unit grafts successfully extend the application of umbilical cord blood transplantation in adults with acute leukemia. Blood. 2013;121(5):752-8.
8. Verneris MR, Brunstein CG, Barker JN, MacMillan ML, DeFor T, McKenna DH et al. Relapse risk after umbilical cord blood transplantation: enhanced graft-versus-leukemia effect in recipients of 2 units. Blood. 2009;114:4293-9.
9. Barker JN, Weisdorf DJ, DeFor TE, Blazar BR, Miller JS, Wagner JE. Rapid and complete donor chimerism in adult recipients of unrelated donor umbilical cord blood transplantation after reduced-intensity conditioning. Blood. 2003;102(5):1915-9.
10. Miyakoshi S, Yuji K, Kami M, Kusumi E, Kishi Y, Kobayashi K, et al. Successful engraftment after reduced-intensity umbilical cord blood transplantation for adult patients with advanced hematological diseases. Clin Cancer Res. 2004;10(11):3586-92.
11. Misawa M, Kai S, Okada M, Nakajima T, Nomura K, Wakae T, et al. Reduced-intensity conditioning followed by unrelated umbilical cord blood transplantation for advanced hematologic malignancies: rapid engraftment in bone marrow. Int J Hematol. 2006;83(1):74-9.
12. Ballen KK, Spitzer TR, Yeap BY, McAfee S, Dey BR, Attar E, et al. Double unrelated reduced-intensity umbilical cord blood transplantation in adults. Biol Blood Marrow Transplant. 2007;13(1):82-9.
13. Brunstein CG, Barker JN, Weisdorf DJ, DeFor TE, Miller JS, Blazar BR, et al. Umbilical cord blood transplantation after nonmyeloablative conditioning: impact on transplantation outcomes in 110 adults with hematologic disease. Blood. 2007;110(8):3064-70.
14. Uchida N, Wake A, Takagi S, Yamamoto H,

Kato D, Matsuhashi Y, et al. Umbilical cord blood transplantation after reduced-intensity conditioning for elderly patients with hematologic diseases. Biol Blood Marrow Transplant. 2008;14(5):583-90.
15. Cutler C, Stevenson K, Kim HT, Brown J, McDonough S, Herrera M, et al. Double umbilical cord blood transplantation with reduced intensity conditioning and sirolimus-based GVHD prophylaxis. Bone Marrow Transplant. 2011;46(5):659-67.
16. Ostronoff F, Milano F, Gooley T, Gutman JA, McSweeney P, Petersen FB, et al. Double umbilical cord blood transplantation in patients with hematologic malignancies using a reduced-intensity preparative regimen without antithymocyte globulin. Bone Marrow Transplant. 2013;48(6):782-6.
17. Brunstein CG, Eapen M, Ahn KW, Appelbaum FR, Ballen KK, Champlin RE, et al. Reduced-intensity conditioning transplantation in acute leukemia: the effect of source of unrelated donor stem cells on outcomes. Blood. 2012;119(23):5591-8.
18. Mohty M, Gaugler B. Advances in umbilical cord transplantation: the role of thymoglobulin/ATG in cord blood transplantation. Best Pract Res Clin Hematol. 2010:23(2):275-82.
19. Berglund S, Okas M, Gertow J, Uhlin M, Mattsson J. Stable mixed donor-donor chimerism after double cord blood transplantation. Int J Haematol. 2009;90(4):526-31.
20. Gertow J, Berglund S, Okas M, Uzunel M, Berg L, Karre K, et al. Characterization of long-term mixed donor-donor chimerism after double cord blood transplantation. Clin Exp Immun. 2010;162(1):146-55.
21. Brunstein CG, Weisdorf DJ, DeFor T, Barker JN, Tolar J, van Burik JA, et al. Marked increased risk of Epstein-Barr virus-related complications with the addition of antithymocyte globulin to a nonmyeloablative conditioning prior to unrelated umbilical cord blood transplantation. Blood. 2006;108(8):2874-80.
22. Chen Y-B, Aldridge J, Kim HT, Ballen KK, Cutler C, Kao G, et al. Reduced-intensity conditioning stem cell transplantation: comparison of double umbilical cord blood and unrelated donor grafts. Biol Blood Marrow Transplant. 2012;18(5):805-12.
23. Simon R. Optimal two-stage designs for phase II clinical trials. Control Clin Trials. 1989;10(1):1-10.
24. Robinson J, Mistry K, McWilliam H, Lopez R, Marsh SG. IPD--the Immuno Polymorphism Database. Nucleic Acids Res. 2010;38:D863-9.
25. Somers JA, Brand A, van Hensbergen Y, Mulder A, Oudshoorn M, Sintnicolaas K, et al. Double umbilical cord blood transplantation: a study of early engraftment kinetics in leukocyte subsets using HLA-specific monoclonal antibodies. Biol Blood Marrow Transplant. 2013;19(2):266-73.
26. Jacobson CA, Turki AT, McDonough SM, Stevenson KE, Kim HT, Kao G, et al. Immune reconstitution after double umbilical cord blood stem cell transplantation: comparison with unrelated peripheral blood stem cell transplantation. Biol Blood Marrow Transplant. 2012;18(4):565-74.
27. Baron F, Sandmaier BM. Chimerism and outcomes after allogeneic hematopoietic cell transplantation following nonmyeloablative conditioning. Leukemia. 2006;20(10):1690-700.
28. Gutman JA, Turtle CJ, Manley TJ, Heimfeld S, Bernstein ID, Riddell SR, et al. Single-unit dominance after double-unit umbilical cord blood transplantation coincides with a specific CD8+ T-cell response against the nonengrafted unit. Blood. 2010;115(4):757-65.
29. Newell LF, Milano F, Nicoud IB, Pereira S, Gooley TA, Heimfeld S, et al. Early CD3 peripheral blood chimerism predicts the long-term engrafting unit following myeloablative double-cord blood transplantation. Biol Blood Marrow Transplant. 2012;18(8):1243-9.
30. Eldjerou LK, Chaudhury S, Baisre-de Leon A, He M, Arcila ME, Heller G, et al. An in vivo model of double-unit cord blood transplantation that correlates with clinical engraftment. Blood. 2010;116(19):3999-4006.
31. Georges GE, Lesnikov V, Baran SW, Aragon A, Lesnikova M, Jordan R, et al. A preclinical model of double- versus single-unit unrelated cord blood transplantation. Biol Blood Marrow Transplant. 2010;16(8):1090-8.
32. Stevanovic S, Griffioen M, Nijmeijer BA, van Schie MLJ, Stumpf AN, Rutten CE, et al. Human allo-reactive CD4+ T cells as strong mediators of anti-tumor immunity in NOD/scid mice engrafted with human acute lymphoblastic leukemia. Leukemia. 2012;26:312-22.
33. Ponce DM, Zheng J, Gonzales AM, Lubin M, Heller G, Castro-Malaspina H, et al. Reduced late mortality risk contributes to similar survival after double-unit cord blood transplantation compared with related and unrelated donor hematopoietic stem cell transplantation. Biol Blood Marrow Transplant. 2011;17(9):1316-26.
34. Ponce DM, Sauter C, Devlin S, Lubin M, Gonzales AM, Kernan NA, et al. A novel reduced-intensity conditioning regimen induces a high incidence of sustained donor-derived neutrophil and platelet engraftment after double-unit cord blood transplantation. Biol Blood Marrow Transplant. 2013;19(5):799-803.
35. Brunstein CG, Gutman JA, Weisdorf DJ, Woolfrey AE, Defor TE, Gooley TA, et al. Allogeneic hematopoietic cell transplantation for hematologic malignancy: relative risks and benefits of double umbilical cord blood. Blood. 2010;116(22):4693-9.
36. Avery S, Shi W, Lubin M, Gonzales AM, Heller G, Castro-Malaspina H, et al. Influence of infused cell dose and HLA match on engraftment after double-unit cord blood allografts. Blood. 2011;117(12):3277-85.
37. Ramirez P, Wagner JE, DeFor TE, Blazar BR, Verneris MR, Miller JS, et al. Factors predicting single-unit predominance after double umbilical cord blood transplantation. Bone Marrow Transplant. 2012;47(6):799-803.
38. Scaradavou A, Smith KM, Hawke R, Schaible A, Abboud M, Kernan NA, et al. Cord blood units with low CD34+ cell viability have a low probability of engraftment after double unit transplantation. Biol Blood Marrow Transplant. 2010;16(4):500-8.
39. Eapen M, Klein JP, Ruggeri A, Spellman S, Lee SJ, Anasetti C, et al. Impact of allele-level HLA matching on outcomes after myeloablative single unit umbilical cord blood transplantation for hematologic malignancy. Blood. 2014;123(1):133-40.
40. Ponce DM, Gonzales A, Lubin M, Castro-Malaspina H, Giralt S, Goldberg JD, et al. Graft-versus-host disease after double-unit cord blood transplantation has unique features and an association with engrafting unit-to-recipient HLA match. Biol Blood Marrow Transplant. 2013;19(6):904-11.