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 1.5× 10/kg recipient body weight and 4.0× 10/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 $\alpha=0.10$ and $\beta=0.20$, 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-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 2.7×10/kg (range, 1.3×10 − 5.2×10) whereas the median dose of infused TNC per patient was 5.4×10/kg (range, 3.4×10 − 9.2×10). 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 mismatches24 were absent in 43% and 45% of unit *versus* recipient and unit *versus* unit combinations, respectively. The median followup of surviving patients was 25 months (range, 9–49).

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.

Table 1. Patients' characteristics.

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 2×10/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 20×10/L, 50×10/L and 100×10/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).

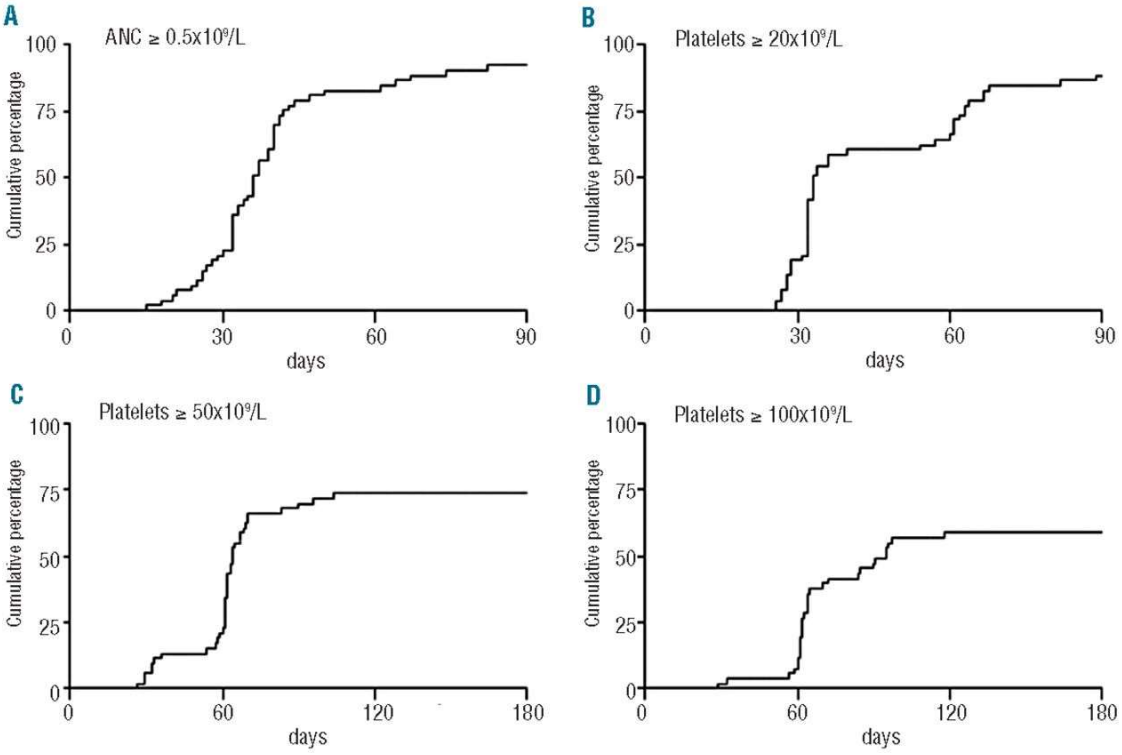

Figure 1. Recovery of peripheral blood cells. (A) Absolute neutrophil count (ANC) recovery (B–D): platelet recovery.

	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, DPB1 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 x10⁷/kg; TNC viability: 65%; CFU-GM: 0.31x10⁴/kg; viable CD34+ cells: 0.35 x10⁶/kg; viable CD3+ cells 4.5x10⁶/kg; viable natural killer (NK)-cells: 9.3x10⁶/kg; viable CD19+ lymphocytes 8.7x10⁶/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.*

Table 2. Prognostic factors: results of univariate analysis.

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 0.18×10/L and 0.27×10/L, respectively (Figure 2).

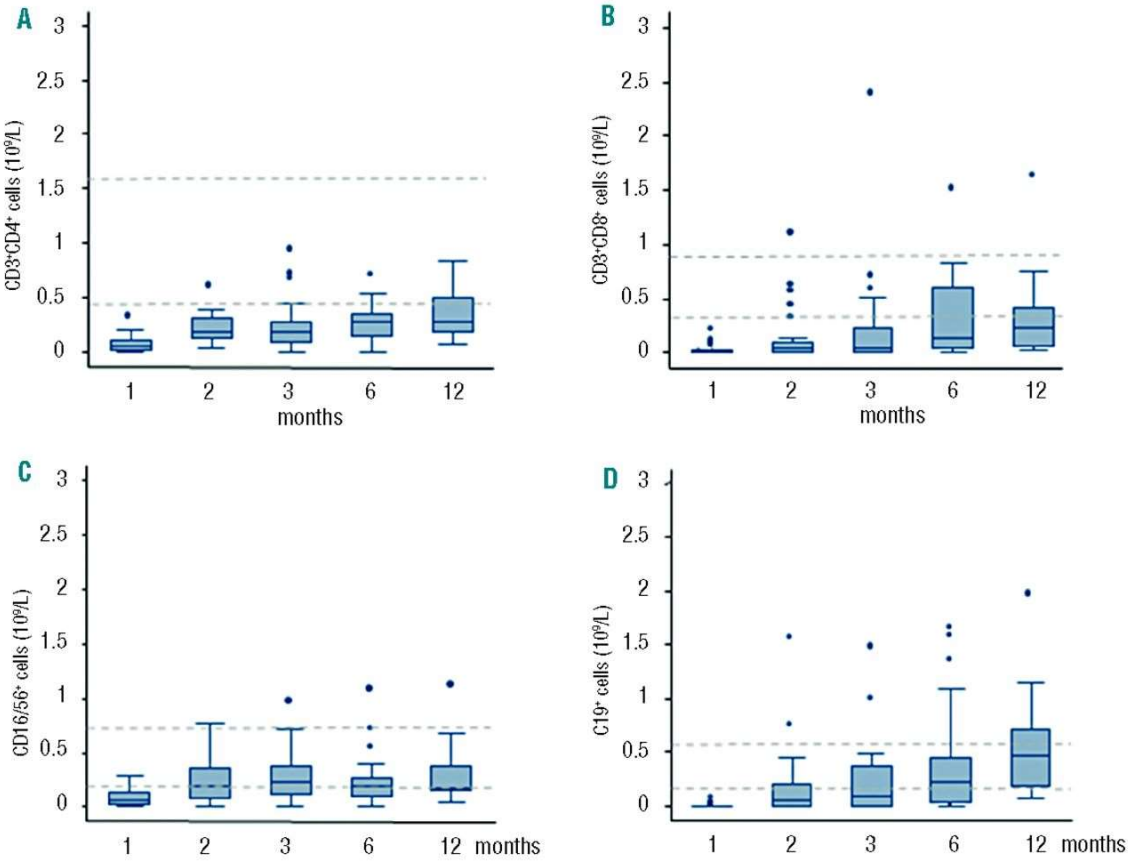

Figure 2. Immune recovery. (A) $CD4^+$ lymphocytes, (B) $CD8^+$ lymphocytes, (C) natural killer cells, (D) B-lymphocytes. Dashed lines represent the upper and lower limits of reference values.

Chimerism 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 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*).

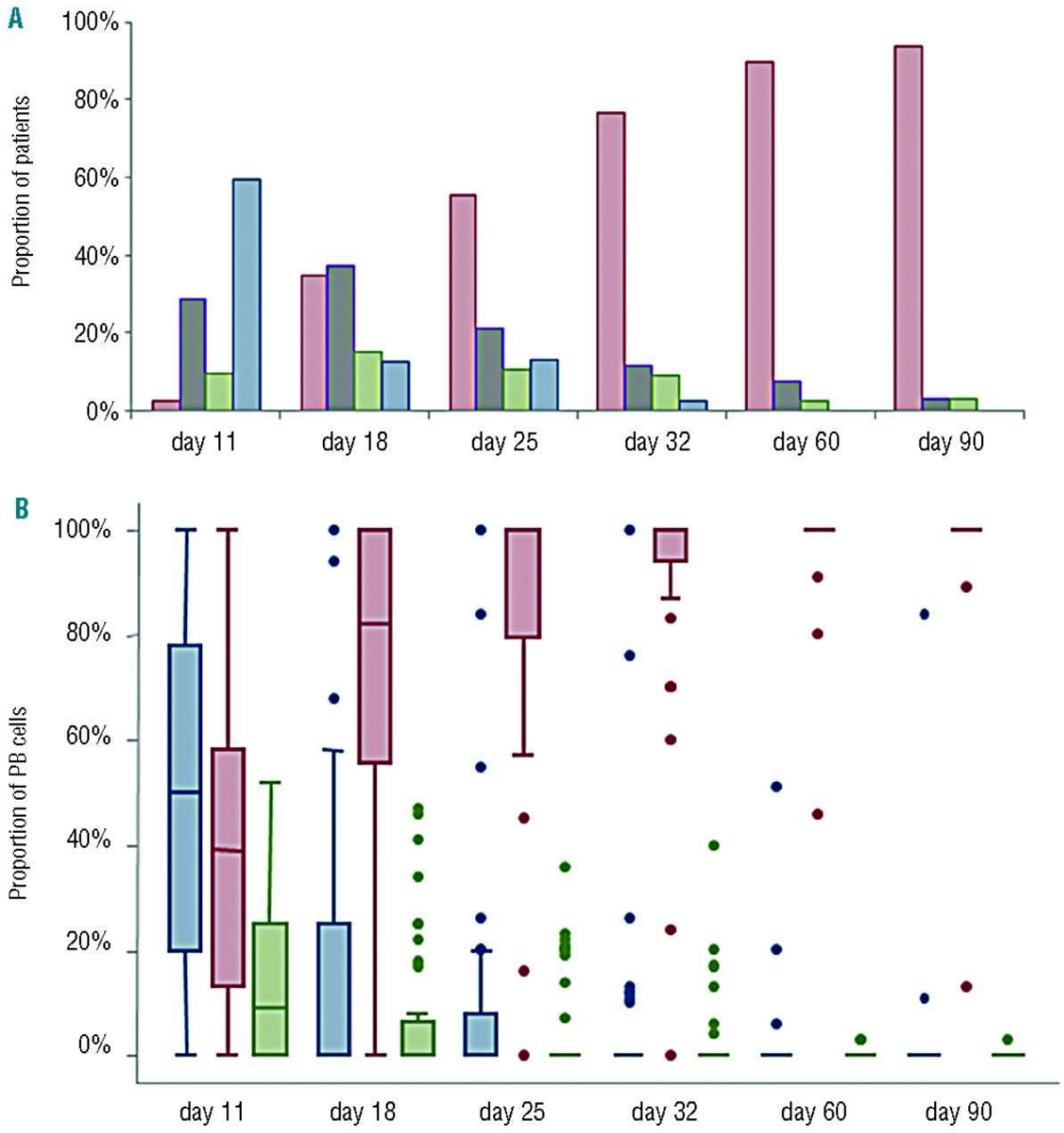

Figure 3. Chimerism. (A) Development of single donor chimerism. Complete chimerism (1 unit); mixed chimerism (recipient + 1 unit); complete chimerism (2 units); mixed chimerism (recipient + 2 units) (B) Peripheral blood chimerism in patients without graft failure. Contribution of recipient, surviving 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.

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% (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.

	Surviving unit			Non-engrafting unit		
	n.[1]	median	range	median	range	*P*
TNC, x10⁷/kg*	49	2.6	(1.3-5.2)	2.8	(1.5-5.1)	0.84
TNC after thawing, x10⁷/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, x10⁴/kg	30	0.30	(0-2.8)	0.31	(0-1.5)	0.46
Viable CD34⁺ cells, x10⁵/kg	49	0.35	(0-1.7)	0.35	(0-1.7)	0.47
Viable CD3⁺ cells, x10⁶/kg	47	4.5	(1.0-74)	6.5	(0.3-43)	0.85
Viable CD19⁺ cells, x10⁵/kg	45	8.7	(0.4-80)	9.9	(0.4-51)	0.94
Viable NK cells, x10⁶/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. [1]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.*

Table 3. Graft characteristics.

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).

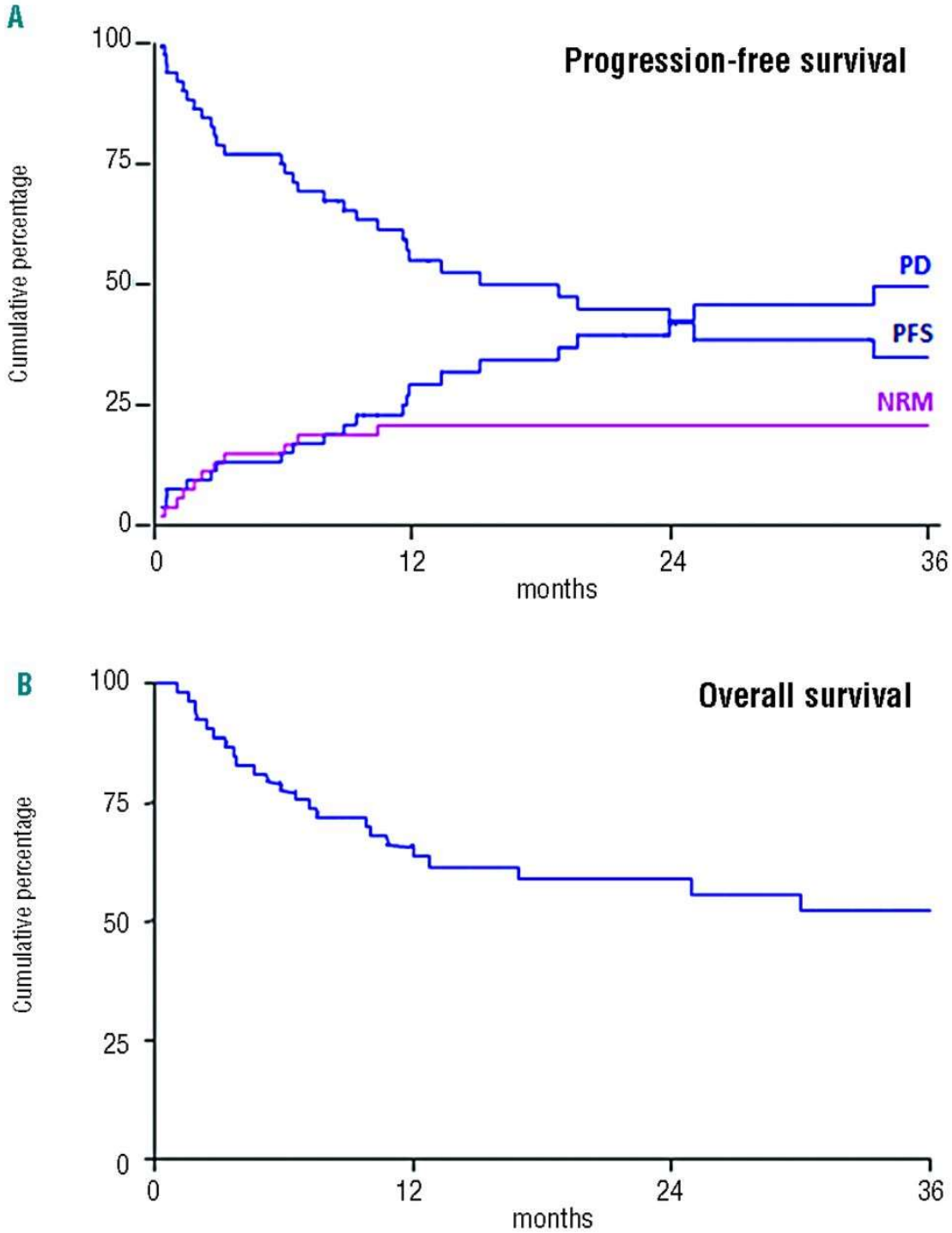

Figure 4. Survival. (A) Progression-free survival (PFS), non-relapse mortality (NRM) and progressive disease (PD). (B) Overall survival.

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).