Impact of length of cryopreservation and origin of cord blood units on hematologic recovery following cord blood transplantation

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Impact of length of cryopreservation and origin of cord blood units on hematologic recovery following cord blood transplantation.

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Running Heads

Impact of long-term cryopreservation of cord blood

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Abstract

As the history of the cord-blood banking system has lengthened, the number of cord-blood units (CBUs) cryopreserved for years has increased. The global expansion of cord-blood banking resulted in active international exchange of CBUs. To determine whether long-term cryopreservation and international shipment of CBUs affect the quality of the units and outcome after transplantation, we retrospectively analyzed the quality of 95 CBUs and the hematologic recovery of 127 patients with hematological malignancy following single-unit cord-blood transplantation. Of the 127 CBUs used to transplant, 42 units were cryopreserved for long periods (5-11.8 years), and 44 units were shipped from distant countries. We found that length of cryopreservation and origin of CBUs did not affect the ratio of viable total-nucleated cells after thawing. Also, neutrophil engraftment was not affected by long-term cryopreservation (> 5 years) or origin (from distant countries), (hazard ratio, 0.91 and 1.2; P = 0.65 and 0.41; respectively). The number of $CD34^+$ cells before freezing (> 1.4 cells/kg recipient) was the only factor which enhanced neutrophil engraftment (hazard ratio, 1.8; P < 0.01). This suggests that length of cryopreservation and origin need not be prioritized over the $CD34^+$ cell dose when selecting CBUs.

Introduction

Recent studies have shown that the number of umbilical cord-blood transplantations (CBT) has been steadily increasing, and the outcomes of CBT are getting closer to those obtained from bone marrow transplantation.[1-3] Over the past 20 years, a worldwide large-scale cord-blood banking system has enabled immediate access to cryopreserved cord-blood units (CBUs) for patients who require an alternative stem cell source for transplantation. As the history of the cord-blood banking system becomes longer, the number of cord-blood units which are cryopreserved for years has increased.[4] Global expansion of this banking has resulted in active international exchange of CBUs; > 40% of CBUs are shipped beyond country borders.[5]

Although long-term preservation of cord blood was shown to not influence hematopoietic reconstitution potential in the mouse model,[6,7] it is unclear whether preservation length has an impact on hematologic recovery following CBT in humans. The fact that not all banks have adopted international guidelines,[4] such as NetCord-FACT International Standards,[8] raises an additional issue, the potential difference among banks in quality control, which might result in impairment of reconstitution potential during a prolonged period of cryopreservation or international shipment. Moreover, incomplete

60 standardization of the processing method for cord blood[9] provokes questions about whether
61 bank-provided information such as number of $CD34^+$ cells reflects clinical outcomes after
62 CBT.

63 The aim of this study is to evaluate the effect of long-term cryopreservation of
64 CBUs and the region of the banks from which the cord blood originates on the quality of
65 CBUs and hematologic recovery after CBT. We retrospectively analyzed the quality of 95
66 units obtained from various countries, and hematological recovery in 127 CBTs. Also, we
67 investigated whether information about the pre-freezing CBUs that is issued by banks stays
68 reliable and can predict the clinical outcome regardless of the length of cryopreservation or
69 the origin of the units.

70

71 **Subjects and Methods**

72 *Cord blood units*

73 CBUs were selected to infuse the most closely matched donor unit/recipient pair:
74 minimum requirements were 4/6 considering difference for HLA-A, B, and DRB1. CBUs
75 with greater than 1.0×10^7 cells/kg recipient-body weight of total nucleated cells (TNCs)
76 were selected. Units from the following countries were used in San Martino Hospital (Genoa,

Italy): the United States (40), Italy (25), Germany (10), Australia (8), France (5), Belgium (3), Spain (2), Brazil (1), and Taiwan (1). Units of domestic origin were transplanted in University of Tsukuba Hospital (Tsukuba, Japan).

Measurement of TNCs and $CD34^+$ cells

95 CBUs used in San Martino Hospital were analyzed. Before transplant, each cord blood unit was thawed at 37°C and cells were washed according to the Rubinstein method.[10] Then, cells were resuspended in 20 mL of thawing solution (saline solution + 5% dextran + 2.5% human albumin). A sample of the final volume was used for quality controls: TNC count and $CD34^+$ cell numbers. Nucleated cells were counted using a Neubauer chamber for the WBC counting; $CD34^+$ cell numbers were evaluated by flow cytometry. Samples were stained with the following antibodies: PE-conjugated anti-CD34 and FITC-conjugated anti-CD45. Nucleic acid dye 7-aminoactinomycin D was used to distinguish dead cells. Flow cytometry was performed using a FACSCalibur instrument (Beckton Dickinson, San Jose, CA, USA), and the Cell Quest software was used for analysis. The $CD34^+$ subpopulation was identified by co-staining of CD45, according to the single platform guidelines of the International Society of Hematotherapy and Graft Engineering (ISHAGE).[11] The recovery rates of TNCs

and $CD34^+$ cells were determined as the ratios of each post-thawing cell number measured in San Martino Hospital and the pre-freezing cell number provided by each bank.

Patients and Transplant Procedures

127 consecutive CBTs performed on adult patients with hematologic malignancies from April 2007 to September 2014 were retrospectively analyzed. 83 were transplanted in San Martino Hospital and 44 were transplanted in University of Tsukuba Hospital. Patients were prepared for transplant with myeloablative conditioning for younger patients or reduced-intensity conditioning for older patients or those with comorbidities. Cord blood were transplanted into the bone marrow in 92 cases, or intravenously in 35 cases. Granulocyte colony-stimulating factor was given after transplant until neutrophil recovery. The time of neutrophil engraftment was defined as the first day of three consecutive days after transplantation when the absolute neutrophil count was maintained at 0.5×10^9 /L or higher. The time of platelet engraftment was defined as the first day of seven consecutive days when the platelet count was maintained at 20×10^9 /L or higher without transfusion support. Graft failure was defined as no sign of hematological recovery by post-transplant day 100.

Statistical analysis

Recovery rates of TNCs and $CD34^+$ cells were evaluated with the student-t for length of cryopreservation and bank of origin. Cumulative incidence of neutrophil and platelet recovery was assessed with the Gray test, with deaths from other causes as competing risk factors.[12] Multivariate analyses were performed with the Fine and Gray proportional hazards regression model. All p values were two-sided with type I error fixed at 0.05. Statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan),[13] a graphical user interface for R (R Foundation for Statistical Computing, Vienna, Austria. Version 3.0.2).

Result

The median cryopreservation period of 127 CBUs was 3.2 (range, 0.1 to 11.8) years. The median number of TNCs and $CD34^+$ cells before cryopreservation were 2.0×10^9 (range, 0.9 to 4.5) cells and 7.5×10^6 (range, 2.1 to 27), respectively.

To analyze the influence of the length of cryopreservation, we divided the evaluable 95 cord blood units into 50 “younger” units, which were cryopreserved for less than 5 years, and 45 “older” units, which were cryopreserved for more than 5 years. The TNC recovery rate

of younger and older units was 74.2 ± 18.9% and 76.1 ± 15.6%, respectively (p = 0.61, Figure 1A). The mean difference was 1.9 (95% CI, -5.5 to 9.2). Also, the $CD34^+$-cell recovery rate of older units (74.3 ± 28.1%) was not different from that of younger units (76.2 ± 34.6%) (p = 0.79, Figure 2A). The mean difference was -1.9 (95% CI, -15.9 to 12.1). Next, we analyzed the influence of region of bank on the quality of CBUs. TNC recovery rates of units from European countries, and other distant countries were 74.8 ± 17.4%, and 75.3 ± 17.5%, respectively. The mean difference was 0.5 (95% CI, -6.9 to 7.9). $CD34^+$-cell recovery was 76.7 ± 30.7%, and 73.9 ± 32.7%, respectively. The mean difference was -2.8 (95% CI, -16.7 to 11.1). Hence, distance between the transplant facility and banks did not have statistically significant impact on recovery of TNCs and $CD34^+$ cells (p = 0.89 and 0.69, respectively). The correlation coefficients between pre-freezing and post-thawing numbers of TNCs and $CD34^+$ cells were not different regardless of the length of cryopreservation and the origin (data now shown).

Overall cumulative incidence of neutrophil engraftment was 86% (95% CI, 78 to 91) and median neutrophil engraftment day was day 23 in 127 evaluable patients following CBT performed in Genoa and Tsukuba. Of the 127 CBUs used to transplant, 42 units were cryopreserved over 5 years (5 to 11.8 years), and 44 units were shipped from distant countries.

When we compared the cumulative incidence of neutrophil engraftment after CBT, length of cryopreservation did not have a significant impact (84% vs 91%; p = 0.95, Figure 2A). Moreover, neutrophil recovery after CBT with units from distant countries was not different from that with domestic and neighboring country-origin units (European-origin units used in Genoa and Japanese-origin units used in Tsukuba) (84% vs 87%; p = 0.66, Figure 2B). In our series of transplants, the pre-freezing number of TNCs per recipient body weight did not influence neutrophil recovery, namely, cumulative engraftment of patients receiving units of larger and smaller than 2.5×10^7 /kg TNCs were 86% and 85% respectively (p = 0.36; Figure 2C). On the other hand, a pre-freezing $CD34^+$ cell dose larger than 1.4×10^5 /kg significantly promoted neutrophil recovery (p = 0.002, Figure 2D), namely, cumulative incidence and median day of engraftment were 92% (95% CI, 79 to 97) and day 21 in the larger $CD34^+$ cell dose group, and 81% (95% CI, 70 to 88) and day 25 in the smaller $CD34^+$ cell dose group, respectively.

In the multivariate models, more than 1.4×10^5 /kg recipient body weight of pre-freezing $CD34^+$ cell dose was the unique variable affecting neutrophil recovery (hazard ratio 1.8; 95% CI, 1.2 to 2.8, p = 0.005, Table 1), and platelet recovery (hazard ratio, 2.0; 95% CI, 1.3 to 3.0, p = 0.002, data not shown). HLA compatibility or intensity of conditioning

regimens did not have any impact on neutrophil and platelet recovery in both univariate and multivariate analysis (data not shown).

Discussion

As the history of CB banking has become longer, the number of cord blood units stored for more than a decade has increased,[14] while whether or not there is an expiration date for cord blood units has been unclear. In addition, the system of cord blood banking have spread worldwide, and over 40% of cord blood units is currently exported to another country.[5] Not all banks are yet accredited by global standards such as NetCord-FACT International Standards,[8] and, moreover, long-distant transportation from banks could affect cord blood quality.[15] Marked differences in the $CD34^+$ cell viability of units obtained from different individual CB banks were demonstrated[16] and more than 10% of units was reported to have quality problems that might be a risk for patients undergoing CBT.[15] Thus the influence of long-term cryopreservation and bank origin need to be investigated to know whether the units remain useful for clinical use.

The number of TNCs and $CD34^+$ cells is a good indicator of cord blood quality, because these have been reported to be associated with engraftment.[17,18] However, is the

information about the pre-freezing number of cells reliable? The information provided by banks might not reflect actually infused viable cells, because various factors could influence the cell viability, such as long-term cryopreservation, impaired quality control during cryopreservation, and long-distance shipment of cord blood units. Previous studies showed that long-term cryopreservation did not compromise the number of hematopoietic progenitor cells for up to 12 years,[19] nor recovery of TNCs and $CD34^+$ cells of CBUs.[20] But in those studies conditions of cryopreservation were homogeneous, which may not mimic the actual banking system in which preservation conditions may differ from bank to bank. We measured post-thawing TNC and $CD34^+$-cell doses in each unit by a standardized method. Deterioration of viability and dispersion of the bank-provided cell dose can result in alteration in the recovery rate of TNCs and $CD34^+$ cells, which is the ratio between the pre-freezing and post-thawing values. That is why we chose the recovery rate as a quality indicator of cord blood units. Consequently, recovery rates of TNCs and $CD34^+$ cells were not statistically different regardless of length of cryopreservation or distance between the transplant facility and banks

With regard to the function of long-term cryopreserved units, the hematopoietic reconstitution potential of CB cells stored for 15 years,[6] and for up to 23.5 years[7] has been proved by in vitro assay and transplantation in immunodeficient mice. It has been reported

196 that long-term cryopreservation did not influence hematological recovery after CBT using
197 units of at most 5 years old[21] and by analysis of child recipients,[22] although these data are
198 based on a limited number of cases. Our retrospective study provides confirmative
199 information that length of cryopreservation for up to 11.8 years, and bank of origin had little
200 impact on engraftment ability in adult recipients. In addition, these results implied that the
201 quality control of banks is working well.

202 Since cord blood was transplanted directly into the bone marrow in majority of our
203 cases,[23] the homing capacity of hematopoietic stem cells could not fully be evaluated by this
204 study. Although equivalence of TNC recovery rate could be shown, our sample size might not
205 be large enough to strictly prove equivalence in $CD34^+$ cell recovery in Figure 1, judged from
206 the relative wideness of 95% CIs of the mean differences. Moreover, we could not exclude
207 potential selection bias because our study was a retrospective analysis. Further large-scale
208 prospective multicenter analysis is needed.

209 In conclusion, the factor that had impact on hematological recovery after CBT in
210 adults was neither length of cryopreservation nor bank of origin, but instead the number of
211 pre-freezing $CD34^+$ cells provided by the cord blood bank. Given that the recovery rate of
212 TNCs and $CD34^+$ cells after thawing, and engraftment serve as indicators of cord blood

quality, quality control is working well regardless of length of cryopreservation or bank of origin. These results provide the useful information that the number of pre-freezing $CD34^+$ cells provided by banks is reliable and can serve as a basis for selection of suitable cord blood units.

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Conflict of interest

The authors report no potential conflicts of interest.

Figure Legends

Figure 1. Influence of length of cryopreservation and banks of origin on recovery rate of TNCs and $CD34^+$ cells

Recovery rates of total nucleated cells and $CD34^+$ cells were calculated as the post-thawing cell number divided by the pre-freezing counterpart provided by the cord blood bank. Influence of the length of cryopreservation (more or less than 5.0 years, A and B) and influence of bank of origin (European countries or other distant countries, C and D) are shown. TNCs, total nucleated cells.

Figure 2. Univariate analysis of effect of cord blood units-characteristics on neutrophil engraftment

Influence of the length of cryopreservation (A), the bank of origin (B), the number of pre-freezing total nucleated cells per recipient's body weight (C), and number of pre-freezing $CD34^+$ cells per recipient's body weight (D) on neutrophil engraftment after cord blood transplant. The "domestic" group includes neighboring country-origin units in B. TNCs, total nucleated cells.

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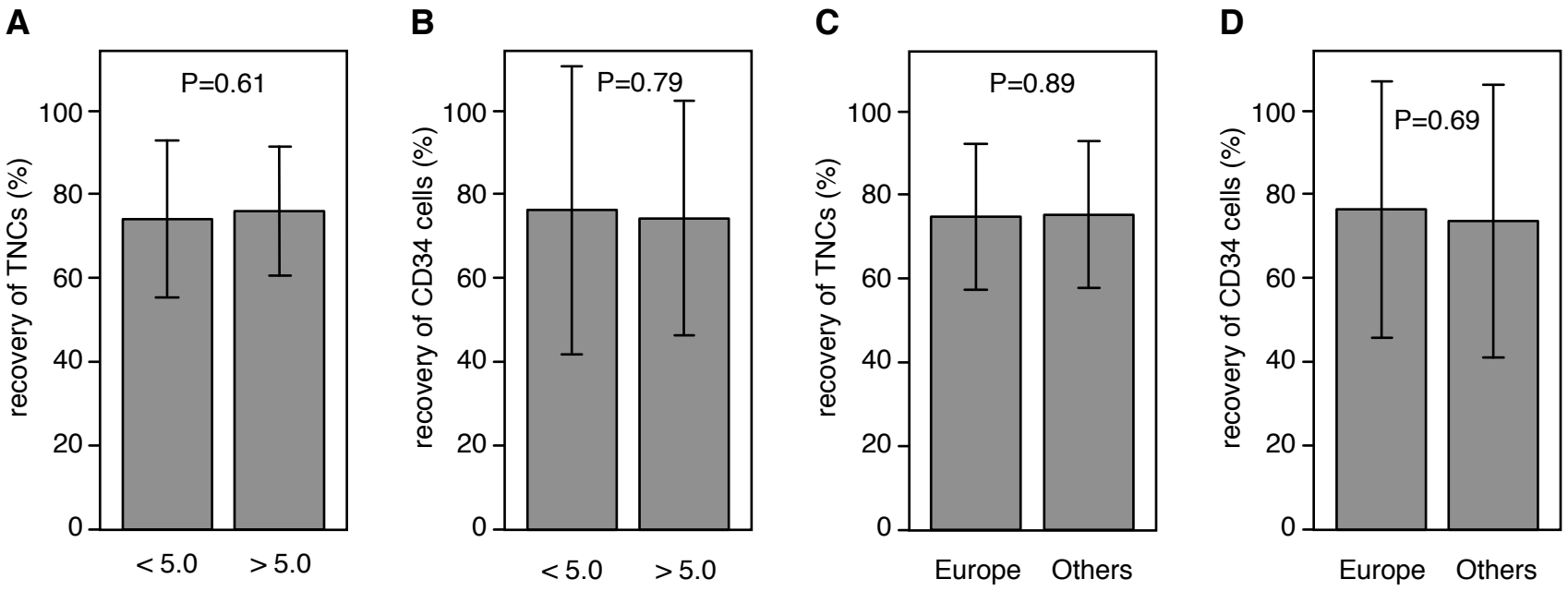
A
P=0.61
recovery of TNCs (%)
100
80
60
40
20
0
< 5.0
> 5.0
B
P=0.79
recovery of CD34 cells (%)
100
80
60
40
20
0
< 5.0
> 5.0
C
P=0.89
recovery of TNCs (%)
100
80
60
40
20
0
Europe
Others
D
P=0.69
recovery of CD34 cells (%)
100
80
60
40
20
0
Europe
Others

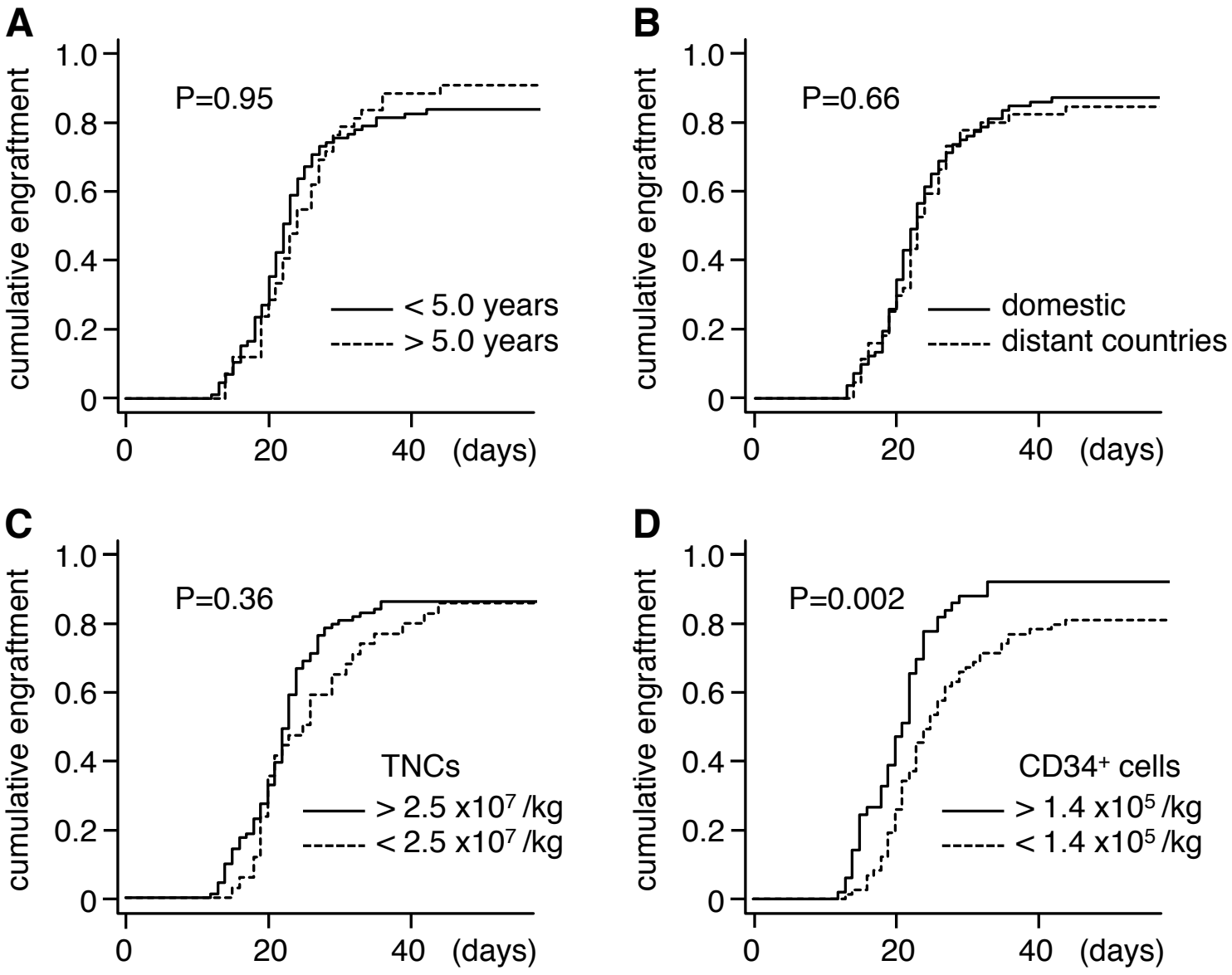
A
cumulative engraftment
1.0
0.8
0.6
0.4
0.2
0
P=0.95
< 5.0 years
> 5.0 years
0
20
40
(days)
B
cumulative engraftment
1.0
0.8
0.6
0.4
0.2
0
P=0.66
domestic
distant countries
0
20
40
(days)
C
cumulative engraftment
1.0
0.8
0.6
0.4
0.2
0
P=0.36
TNCs
> 2.5 x10^7 /kg
< 2.5 x10^7 /kg
0
20
40
(days)
D
cumulative engraftment
1.0
0.8
0.6
0.4
0.2
0
P=0.002
CD34+ cells
> 1.4 x10^5 /kg
< 1.4 x10^5 /kg
0
20
40
(days)

Table 1. Predictors impacting neutrophils engraftment in multivariate analysis

		Hazard ratio (95% CI)	P-value
Length of cryopreservation (years)	< 5.0	1	
	> 5.0	0.91 (0.62-1.4)	0.65
Banks of origin	domestic or neibouring countries	1	
	distant countries	1.2 (0.79-1.8)	0.41
Pre-freezing TNCs (x 10^7 /kg)	< 2.5	1	
	> 2.5	1.1 (0.73-1.6)	0.70
Pre-freezing $CD34^+$ cells (x 10^5 /kg)	< 1.4	1	
	> 1.4	1.8 (1.2-2.8)	0.005