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

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10 **Running Heads**

11 Impact of long-term cryopreservation of cord blood

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26 **Abstract**

27 As the history of the cord-blood banking system has lengthened, the number of
28 cord-blood units (CBUs) cryopreserved for years has increased. The global expansion of
29 cord-blood banking resulted in active international exchange of CBUs. To determine whether
30 long-term cryopreservation and international shipment of CBUs affect the quality of the units
31 and outcome after transplantation, we retrospectively analyzed the quality of 95 CBUs and the
32 hematologic recovery of 127 patients with hematological malignancy following single-unit
33 cord-blood transplantation. Of the 127 CBUs used to transplant, 42 units were cryopreserved
34 for long periods (5-11.8 years), and 44 units were shipped from distant countries. We found
35 that length of cryopreservation and origin of CBUs did not affect the ratio of viable total-
36 nucleated cells after thawing. Also, neutrophil engraftment was not affected by long-term
37 cryopreservation (> 5 years) or origin (from distant countries), (hazard ratio, 0.91 and 1.2; P =
38 0.65 and 0.41; respectively). The number of CD34⁺ cells before freezing (> 1.4 cells/kg
39 recipient) was the only factor which enhanced neutrophil engraftment (hazard ratio, 1.8; P <
40 0.01). This suggests that length of cryopreservation and origin need not be prioritized over the
41 CD34⁺ cell dose when selecting CBUs.

42

43 **Introduction**

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

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

60 standardization of the processing method for cord blood⁹ 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,

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

80

81 *Measurement of TNCs and CD34⁺ cells*

82 95 CBUs used in San Martino Hospital were analyzed. Before transplant, each cord
83 blood unit was thawed at 37°C and cells were washed according to the Rubinstein method.¹⁰
84 Then, cells were resuspended in 20 mL of thawing solution (saline solution + 5% dextran +
85 2.5% human albumin). A sample of the final volume was used for quality controls: TNC count
86 and CD34⁺ cell numbers. Nucleated cells were counted using a Neubauer chamber for the
87 WBC counting; CD34⁺ cell numbers were evaluated by flow cytometry. Samples were stained
88 with the following antibodies: PE-conjugated anti-CD34 and FITC-conjugated anti-CD45.
89 Nucleic acid dye 7-aminoactinomycin D was used to distinguish dead cells. Flow cytometry
90 was performed using a FACSCalibur instrument (Beckton Dickinson, San Jose, CA, USA),
91 and the Cell Quest software was used for analysis. The CD34⁺ subpopulation was identified
92 by co-staining of CD45, according to the single platform guidelines of the International
93 Society of Hematotherapy and Graft Engineering (ISHAGE).¹¹ The recovery rates of TNCs

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

96

97 *Patients and Transplant Procedures*

98 127 consecutive CBTs performed on adult patients with hematologic malignancies
99 from April 2007 to September 2014 were retrospectively analyzed. 83 were transplanted in
100 San Martino Hospital and 44 were transplanted in University of Tsukuba Hospital. Patients
101 were prepared for transplant with myeloablative conditioning for younger patients or reduced-
102 intensity conditioning for older patients or those with comorbidities. Cord blood were
103 transplanted into the bone marrow in 92 cases, or intravenously in 35 cases. Granulocyte
104 colony-stimulating factor was given after transplant until neutrophil recovery. The time of
105 neutrophil engraftment was defined as the first day of three consecutive days after
106 transplantation when the absolute neutrophil count was maintained at 0.5×10^9 /L or higher.
107 The time of platelet engraftment was defined as the first day of seven consecutive days when
108 the platelet count was maintained at 20×10^9 /L or higher without transfusion support. Graft
109 failure was defined as no sign of hematological recovery by post-transplant day 100.

110

111 *Statistical analysis*

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

120

121 **Result**

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

125 To analyze the influence of the length of cryopreservation, we divided the evaluable
126 95 cord blood units into 50 “younger” units, which were cryopreserved for less than 5 years,
127 and 45 “older” units, which were cryopreserved for more than 5 years. The TNC recovery rate

128 of younger and older units was $74.2 \pm 18.9\%$ and $76.1 \pm 15.6\%$, respectively ($p = 0.61$, Figure
129 1A). The mean difference was 1.9 (95% CI, -5.5 to 9.2). Also, the CD34⁺-cell recovery rate of
130 older units ($74.3 \pm 28.1\%$) was not different from that of younger units ($76.2 \pm 34.6\%$) ($p =$
131 0.79 , Figure 2A). The mean difference was -1.9 (95% CI, -15.9 to 12.1). Next, we analyzed
132 the influence of region of bank on the quality of CBUs. TNC recovery rates of units from
133 European countries, and other distant countries were $74.8 \pm 17.4\%$, and $75.3 \pm 17.5\%$,
134 respectively. The mean difference was 0.5 (95% CI, -6.9 to 7.9). CD34⁺-cell recovery was
135 $76.7 \pm 30.7\%$, and $73.9 \pm 32.7\%$, respectively. The mean difference was -2.8 (95% CI, -16.7
136 to 11.1). Hence, distance between the transplant facility and banks did not have statistically
137 significant impact on recovery of TNCs and CD34⁺ cells ($p = 0.89$ and 0.69 , respectively).
138 The correlation coefficients between pre-freezing and post-thawing numbers of TNCs and
139 CD34⁺ cells were not different regardless of the length of cryopreservation and the origin
140 (data now shown).

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

145 When we compared the cumulative incidence of neutrophil engraftment after CBT, length of
146 cryopreservation did not have a significant impact (84% vs 91%; $p = 0.95$, Figure 2A).
147 Moreover, neutrophil recovery after CBT with units from distant countries was not different
148 from that with domestic and neighboring country-origin units (European-origin units used in
149 Genoa and Japanese-origin units used in Tsukuba) (84% vs 87%; $p = 0.66$, Figure 2B). In our
150 series of transplants, the pre-freezing number of TNCs per recipient body weight did not
151 influence neutrophil recovery, namely, cumulative engraftment of patients receiving units of
152 larger and smaller than 2.5×10^7 /kg TNCs were 86% and 85% respectively ($p = 0.36$; Figure
153 2C). On the other hand, a pre-freezing CD34⁺ cell dose larger than 1.4×10^5 /kg significantly
154 promoted neutrophil recovery ($p = 0.002$, Figure 2D), namely, cumulative incidence and
155 median day of engraftment were 92% (95% CI, 79 to 97) and day 21 in the larger CD34⁺ cell
156 dose group, and 81% (95% CI, 70 to 88) and day 25 in the smaller CD34⁺ cell dose group,
157 respectively.

158 In the multivariate models, more than 1.4×10^5 /kg recipient body weight of pre-
159 freezing CD34⁺ cell dose was the unique variable affecting neutrophil recovery (hazard ratio
160 1.8; 95% CI, 1.2 to 2.8, $p = 0.005$, Table 1), and platelet recovery (hazard ratio, 2.0; 95% CI,
161 1.3 to 3.0, $p = 0.002$, data not shown). HLA compatibility or intensity of conditioning

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

164

165 **Discussion**

166 As the history of CB banking has become longer, the number of cord blood units
167 stored for more than a decade has increased,¹⁴ while whether or not there is an expiration date
168 for cord blood units has been unclear. In addition, the system of cord blood banking have
169 spread worldwide, and over 40% of cord blood units is currently exported to another country.⁵
170 Not all banks are yet accredited by global standards such as NetCord-FACT International
171 Standards,⁸ and, moreover, long-distant transportation from banks could affect cord blood
172 quality.¹⁵ Marked differences in the CD34⁺ cell viability of units obtained from different
173 individual CB banks were demonstrated¹⁶ and more than 10% of units was reported to have
174 quality problems that might be a risk for patients undergoing CBT.¹⁵ Thus the influence of
175 long-term cryopreservation and bank origin need to be investigated to know whether the units
176 remain useful for clinical use.

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

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

193 With regard to the function of long-term cryopreserved units, the hematopoietic
194 reconstitution potential of CB cells stored for 15 years,⁶ and for up to 23.5 years⁷ has been
195 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 old²¹ 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

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

217

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224

225 **Conflict of interest**

226 The authors report no potential conflicts of interest.

227

228 **Figure Legends**

229

230 **Figure 1. Influence of length of cryopreservation and banks of origin on recovery rate of**

231 **TNCs and CD34⁺ cells**

232 Recovery rates of total nucleated cells and CD34⁺ cells were calculated as the post-thawing

233 cell number divided by the pre-freezing counterpart provided by the cord blood bank.

234 Influence of the length of cryopreservation (more or less than 5.0 years, A and B) and

235 influence of bank of origin (European countries or other distant countries, C and D) are

236 shown. TNCs, total nucleated cells.

237

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

239 **engraftment**

240 Influence of the length of cryopreservation (A), the bank of origin (B), the number of pre-

241 freezing total nucleated cells per recipient's body weight (C), and number of pre-freezing

242 CD34⁺ cells per recipient's body weight (D) on neutrophil engraftment after cord blood

243 transplant. The "domestic" group includes neighboring country-origin units in B. TNCs, total

244 nucleated cells.

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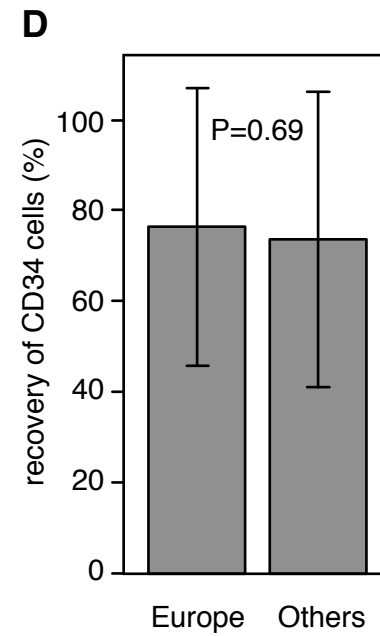
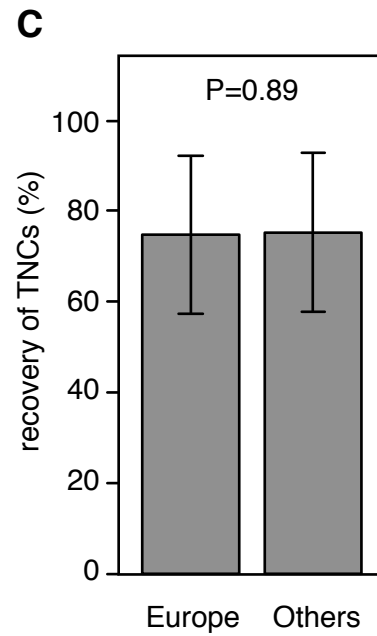
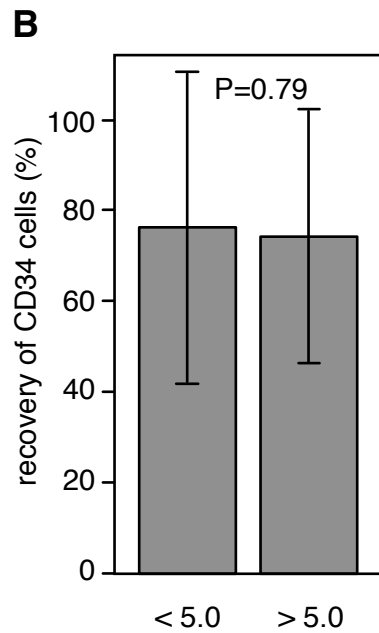
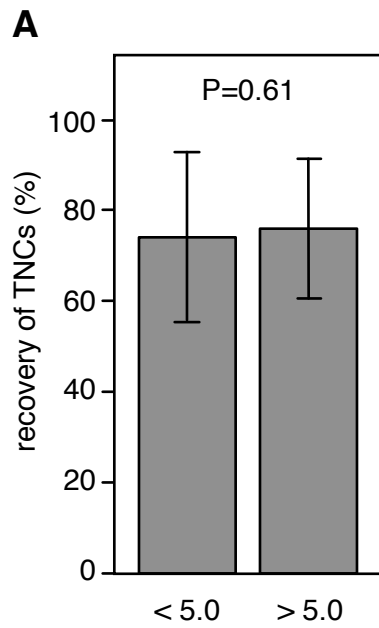
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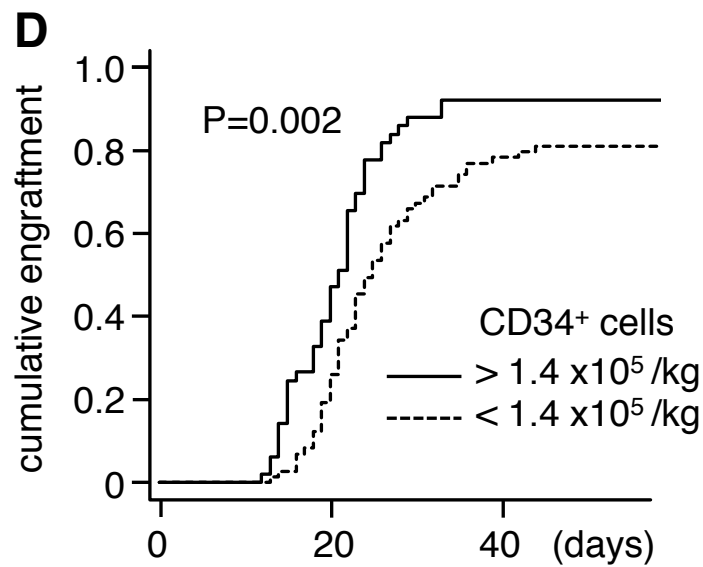
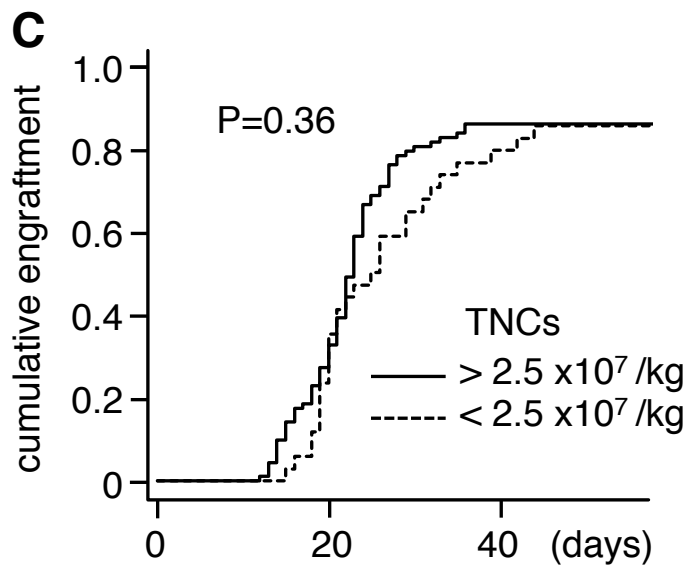
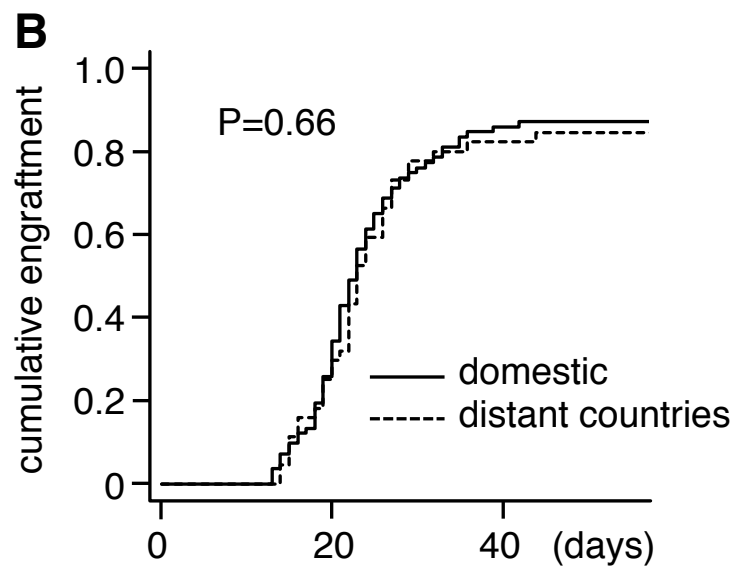
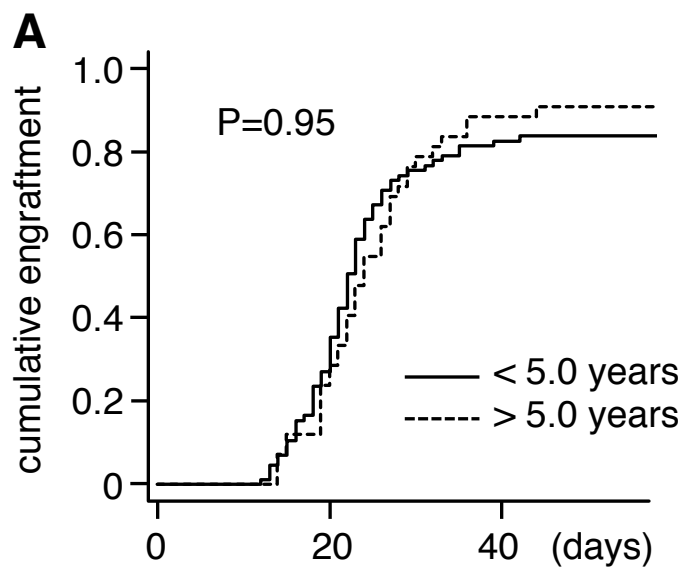


Table 1. Predictors impacting neutrophils engraftment in multivariate analysis

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