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Total Colony-Forming Units Are a Strong, Independent Predictor of Neutrophil and Platelet Engraftment after Unrelated Umbilical Cord Blood Transplantation: A Single-Center Analysis of 435 Cord Blood Transplants

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Graft failure occurs in approximately 20% of patients after unrelated umbilical cord blood transplantation (UCBT). This could be because of inadequate potency of the cord blood unit (CBU). To this end, we investigated the impact of graft characteristics on engraftment and survival of 435 primarily pediatric (median age: 5.3 years) patients receiving a single-unit unrelated UCBT after myeloablative conditioning from 2000 to 2008. Pre-cryopreservation (pre-cryo) graft characteristics were provided by the banks. Post-thaw parameters were measured on dextran/albumin-washed grafts. Post-thaw recovery of the colony-forming unit (CFU), a biological assay reflecting functional viability of the cord blood cells was the lowest percent age (median 21.2%, mean 36.5%) of the pre-cryo value, regardless of the bank of origin. The cumulative incidences of neutrophil and platelet engraftment were 76.9% (95%, confidence interval [CI], 71.3%-82.5%) and 55% (95% CI, 49.3%-60.7%), respectively. Univariate and separate multivariate models using pre-cryo and post-thaw datasets including clinical parameters identified predictors of engraftment and survival. In multivariate modeling, higher CFU dosing was the only pre-cryo graft characteristic predictive of neutrophil ($P = .0024$) and platelet engraftment ($P = .0063$). In the post-thaw model, CFU dose best predicted neutrophil and platelet engraftment (both $P < .0001$). Comparatively, $CD34^+$ and total nucleated cell (TNC) were only weakly predictive in post-thaw neutrophil and platelet engraftment models, respectively. In conclusion, CFU dose is a strong independent predictor of engraftment after unrelated UCBT and should be used to assess potency when selecting CBUs for transplantation.

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INTRODUCTION

Using current graft selection criteria, primary graft failure and engraftment delays are major obstacles to the overall success of unrelated donor umbilical cord blood transplantation (UCBT). Up to 20% of patients receiving a UCBT experience primary graft failure [1-4], which results in part from inadequate potency of their donor cord blood unit (CBU). Current practice in most transplant centers is to base selection of a donor CBU on total nucleated cell (TNC) dose and human leukocyte antigen (HLA) matching. This strategy is based on multiple reports demonstrating that TNC dose is a critical determinant of engraftment and survival after unrelated UCBT [1,5-9]. $CD34^+$ cell dose has also been shown, in a smaller number of studies, to predict engraftment and survival [10-13], but the use of $CD34^+$ as a selection parameter is limited by lack of pre-cryopreservation (pre-cryo) $CD34^+$ enumeration on a significant proportion of CBUs in the public banking inventories and lack of standardization and reproducibility of $CD34^+$ enumeration methodology among banks. In addition, measuring TNC and $CD34^+$ cell dose on the CBU before cryopreservation may not necessarily reflect the overall quality or potency of the CBU after cryopreservation and storage. Identification of additional criteria to assess CBU potency should allow for optimization of selection of CBUs likely to engraft, improving overall outcomes of unrelated UCBT.

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Since performing the first unrelated UCBT in 1993, our center has been integrally involved in the evolution of the field, transplanting more than 900 patients with umbilical cord blood (UCB) to date. The establishment of the Carolinas Cord Blood Bank (CCBB) at Duke, a member of the National Cord Blood Inventory (NCBI), through the Cord Blood Transplantation (COBLT) program in 1997 has allowed us to develop expertise in the collection, processing, testing, and banking of UCB for unrelated donor transplantation. Thus, with dual expertise, we are well suited to evaluate the role of UCB graft parameters on engraftment after UCBT as they relate to characteristics of the graft at collection, after processing and before cryopreservation, and after thawing for infusion into the patient. In this report, we present a retrospective analysis of unrelated donor UCBT performed at our center that identifies important graft parameters influencing engraftment. We show that the dose of colony-forming units (CFU) is the most important CBU parameter influencing engraftment, and that the number of CFUs in a unit is changed significantly by cryopreservation and thawing. These results have important implications for understanding the utility of inventory in public cord blood (CB) banks. We have also used these results to construct a metric of CBU potency useful in optimization of UCB graft selection for transplantation [14].

MATERIALS AND METHODS

Study Design and Eligibility

We performed a retrospective analysis of 435 consecutive unrelated UCBTs using a single, nonmanipulated CBU after full myeloablative conditioning as a first transplant between 1/1/00 and 12/31/08. Study subjects were selected from a pool of consecutive patients meeting these criteria transplanted at Duke University Medical Center for a malignancy, bone marrow failure syndrome, hemoglobinopathy, immunodeficiency, or metabolic disorders. Transplants were eligible for this analysis if identified technical graft characteristics, including TNC, mononuclear cell count (MNC), $CD34^+$, and total CFU content, were available for the CBU before cryopreservation and after thawing. All patients were enrolled in a Duke University Medical Center institutional review board–approved protocol or treatment plan. Written informed consent was obtained from all patients or legal guardians. Of the patients included in this study, 132 patients were previously reported as part of the COBLT study [1,2,15], and 10 patients are enrolled on the current Blood and Marrow Transplant Clinical Trials Network single versus double unrelated donor UCBT trial (BMT CTN 0501). A subset of the study population has been previously reported by Prasad et al. [16] (n = 159) and in other reports from our center [17-21].

Selection of Donors

Intermediate-resolution typing for HLA class I (A and B) and high resolution for HLA-DRB1 was used for initial donor screening. High-resolution HLA typing was also obtained on the patient and donor for confirmatory typing and used for final unit selection. The CBU with the highest number of nucleated cells/recipient body weight and closest HLA match (minimum 3/6 HLA loci match) was selected. ABO typing and information about donor gender, race, and ethnicity were provided for all units pre-cryopreservation. A cell dose of $>3 \times 10^7$ TNC/kg and a minimum of a 4/6 HLA match were prioritized for final donor selection whenever possible. Matching for race/ethnicity and ABO types were also prioritized when feasible.

CBU Collection, Processing, Thawing, and Infusion

CBUs were collected and processed according to local bank procedures. Upon selection for transplantation, they were shipped to the Duke Stem Cell Laboratory (SCL) in a dry shipper that maintained temperatures below −150°C. Upon receipt at the SCL, units were removed from the dry shipper and quickly transferred to a liquid nitrogen freezer where they were stored under liquid nitrogen until the day of transplant. One to 2 hours before transplant, all CBUs were thawed using a modified version of the dextran/albumin wash per Rubinstein et al. [22]. The washed cells were resuspended in dextran 40/5% albumin in a final volume of the adjusted not to exceed 5 mL/kg of the patient's body weight. Thirty minutes prior to CBU infusion, patients were premedicated with acetaminophen, diphenhydramine, and methylprednisolone. The CBU was infused through the patient's central venous line over 15 minutes. Aliquots (0.5 mL) of the product were obtained after washing (post-thaw) and analyzed for sterility, TNC, MNC, viability, CFU, $CD3^+$, and $CD34^+$ content.

CBU Characteristics

Data were provided by the CB bank supplying the unit for transplantation as part of routine reporting procedures. The available data included pre-cryo TNC, MNC, $CD34^+$ cell dose, CFU content, and cell viability. Post-thaw testing was performed at Duke SCL using samples obtained after thawing and washing with dextran/albumin, and included TNC, MNC, $CD34^+$, CFU (total), $CD3^+$, and cell viability. CFU subsets were also enumerated, but not included in this analysis. ABO typing and microbial cultures (bacterial and fungal) were provided for all CBUs pre-cryo and post-thaw.

Graft Characterization

TNC and MNC

For CBUs collected and processed by the CCBB, and all thawed units, TNC counts were obtained on an automated cell counter (Sysmex K-1000 from 1/00-4/08, Sysmex XE5000 for pre-cryo analysis from 4/08 to present; Sysmex XS-1000i for post-thaw analysis from 4/08 to present, Sysmex America, Inc. Mundelein, IL). Manual differentials were performed by personnel of the CCBB at Duke and Duke SCL for calculation of the total MNC count, which was represented by (%lymphocytes + %monocytes + %blasts) × TNC.

$CD34^+$

Enumeration of $CD34^+$ cells was determined by flow cytometry (ProCOUNT, BD Biosciences, San Jose, CA). ProCOUNT reagent (20 μL) was added to 12 × 75 mm test tubes followed by 50 μL of CB sample. Samples were diluted with phosphate-buffered saline/bovine serum albumin (PBS/BSA) wash (Gibco, Invitrogen, Carlsbad, CA) as needed for a concentration $<4 \times 10^7$ cells/mL. Tubes were mixed gently and incubated in the dark at room temperature for 15 minutes. Red blood cells (RBCs) were further lysed using 450 μL of a 1:10 dilution of FACS Lysing Solution (BD Biosciences, San Jose, CA) for samples and incubated in the dark at room temperature for 15 minutes. A 2-laser, 4-color flow cytometer was used to analyze samples. Results were analyzed by CellQuest Pro (BD Biosciences) using ProCOUNT gating strategies.

Precryo CFU progenitor cell assay

CFU progenitor cells, including granulocyte macrophage (CFU-GM), granulocyte, erythrocyte, macrophage, and megakaryocyte (CFU-GEMM), and burst-forming unit erythroid (BFU-E), were enumerated on precryo samples of CB. Initial samples of 2.5×10^5 CB cells were removed for testing and diluted in 0.5-mL Iscove's modified Dulbecco's Medium (IMDM) plus 2% fetal bovine serum (FBS) (StemCell Technologies, Vancouver, BC, Canada). Cells were further diluted by adding 5×10^4 cells in a total of 0.5 mL IMDM plus 2% FBS to achieve a final cell count of 2.5×10^4/mL when mixed with 1.5-mL of MethoCult medium 4434 (StemCell Technologies). Cells were plated in triplicate (0.5 mL/well) at 1.25×10^4 cells/well in 24-well tissue culture plates and incubated in a humidified, 37°C, 5% CO_2 incubator for 11 to 14 days. Colony growth was scored by trained personnel using an inverted, phase-contrast microscope and reported as the mean colony count $\times 10^5$ nucleated cells. These numbers were then used to calculate the number of progenitors in the entire graft by using the pre-cryo TNC.

Postthaw CFU progenitor cell assay

CFU progenitors were enumerated on post-thaw samples of CB obtained at time of unit thaw and a total of all CFU progenitors were calculated for each graft infused. From the thawed graft, CB samples containing 4×10^5 cells were diluted in IMDM plus 2% FBS. A total of 4×10^4 cells in 0.1-mL IMDM plus 2% FBS were mixed with 0.5 mL of MethoCult medium (4434, StemCell Technologies). Cells were plated in duplicate (0.3 mL/well) at 2×10^4 cells/well in 24-well tissue culture plates and incubated in a humidified, 37°C, 5% CO_2 incubator for 11 to 14 days. Colony growth was reported as the mean colony count $\times 10^5$ nucleated cells. Total CFU progenitors were calculated by summing the mean CFU-GM, CFU-GEMM, and BFU-E totals for each CB sample and then calculating the total number of CFU progenitors in the infused graft based on the TNC in the infused graft.

Conditioning, Graft-versus-Host Disease Prophylaxis and Supportive Care

Conditioning regimens were based on patient age, diagnosis, and disease state. Total-body irradiation (TBI) was used in conditioning for 190 patients (43.6%). Graft-versus-host disease (GVHD) prophylaxis was cyclosporine + methylprednisolone (n = 330, 75.8%) or cyclosporine + mycophenolate mofetil (n = 90, 20.6%) or tacrolimus ± mycophenolate mofetil (n = 15, 3.6%). All patients were nursed in reverse isolation rooms under positive pressure and HEPA filtration. Standard prophylaxis was used against pneumocystis carinii, acyclovir for viral, and voriconazole for fungal infections. Empiric antibiotic treatment was started with the first febrile episode and continued through engraftment. Intravenous immune globulin (500 mg/kg/dose) was administered weekly through day 100 then monthly through 1 year, then weaned as tolerated. As prophylaxis against veno-occlusive disease, a continuous infusion of low-dose heparin was used through day 28. Patients received transfusions of leukocyte-depleted, irradiated-packed RBCs and platelets. Granulocyte colony-stimulating factor (G-CSF) was administered from day 0 until hematopoietic recovery and then was weaned.

Engraftment and GVHD

Neutrophil engraftment was defined as the first day of 3 consecutive days of an absolute neutrophil count of ≥500 donor cells/mm^3. Platelet engraftment was defined as the first day of achieving an untransfused count of ≥50,000 platelets/mm^3 for 7 days. Confirmation of donor cell chimerism was performed using fluorescence in situ hybridization (FISH) or RFLP. Acute GVHD and chronic GVHD (aGVHD, cGVHD) were scored as the maximum grade in all patients at the highest level per consensus criteria [23].

Statistical Analysis

Pearson's correlation coefficient was used for correlation analyses between pre-cryo and post-thaw graft characteristics. Survival probabilities at 180 days and 1 year were estimated using Kaplan-Meier method [24]. Graft and patient characteristics were examined to determine factors predictive of survival using Log rank test in the univariate analysis, and Cox proportional hazards model for multivariate analysis. Time to neutrophil and platelet engraftment were estimated using the cumulative-incidence-function method, from patients surviving past day 14 and treating death, relapse, and graft failure as competing risks [25]. Relapse was considered as a competing risk for engraftment to account for those patients who relapsed prior to engraftment. Associations between graft/patient characteristics and kinetics of neutrophil or platelet engraftment were evaluated univariately by Gray's test [26], and multivariate analysis was performed using Fine and Gray model [27] with backward selection. A P value of $\leq .05$ was considered significant.

Graft and patient characteristics examined in both univariate and multivariate analysis for engraftment and survival included pre-cryo and post-thaw graft parameters (TNC, MNC, $CD34^+$, and CFU cell doses, volume collected, bank, etc.), patient characteristics (age, gender, diagnosis, degree of HLA match, recipient and unit ethnicity, recipient weight, use of TBI, GVHD prophylaxis, and cytomegalovirus [CMV] status), and matching status of recipient to unit in terms of gender, ABO, and ethnicity. Separate multivariate analysis was performed for pre-cryo and post-thaw graft parameters whereas clinical characteristics were evaluated together with graft parameters. Continuous parameters were categorized by corresponding medians or quartiles. All analyses were completed using the SAS system, version 9.2, and R, version 2.7.

RESULTS

Patient and Treatment Characteristics

From 2000 to 2008, 435 consecutive patients referred to our center were treated with unrelated donor UCBT using a single, nonmanipulated CBU after a myeloablative preparative regimen. The median age of the group was 5.3 years (range: 3 weeks to 55.8 years) including 43 adults (>18 years of age; 9.8%). The median patient weight was 19.8 kg (range: 2.7-118.4 kg) at time of transplant. Of the 435 patients, 58.2% had malignancies, 61.6% were males, 70.3% were Caucasian, and 37.7% were CMV seropositive pretransplant. Patients and donors were matched for ABO, gender, and race/ethnicity in 53.1%, 50.3%, and 70.1% of cases, respectively. Patients and CBUs were matched at 6/6 (7.6%), 5/6 (33.6%), 4/6 (55.9%), or ≤3/6 (2.9%) HLA loci using antigen-level typing at HLA- Class I and allelic typing at HLA-Class II, DRB1. According to practices at our transplant center, which transplants a large number of small children with inborn errors of metabolism, approximately half of the patients received non–TBI-containing preparative regimens, and the majority received cyclosporine-based GVHD prophylaxis (Table 1).

Graft Characteristics

Donor CBUs were obtained from 16 public banks in the United States. Almost half (46%, n = 199) were obtained from the CCBB at Duke. The other CBUs were obtained from the National Cord Blood Program (22%, n = 97) or 14 other U.S. CB banks who distribute CBUs through the National Marrow Donor

Table 1. Patient and Treatment Characteristics of the Overall Study Population, Cord Blood Units, and Transplants (N = 435)

Patient Characteristics	Median (Range)
Age, years	5.3 (3 weeks-55.8)
Weight, kg	19.8 (2.7-118.4)
	N (%)
Age, >18 years old	43 (9.8)
Gender	
Male	268 (61.6)
Female	167 (38.4)
Race/ethnicity	
Caucasian	306 (70.3)
Non-Caucasian	129 (29.7)
Diagnosis	
Malignant	253 (58.2)
Metabolic	132 (30.3)
Other	50 (11.5)
CMV serostatus	
Positive	164 (37.7)
Negative	269 (61.8)
Indeterminate	2 (0.4)
Patient—CBU match	
HLA match	
6/6	33 (7.6)
5/6	146 (33.6)
≤4/6	255 (58.8)
ABO match	
Matched	231 (53.1)
Mismatched	204 (46.9)
Gender match	
Matched	219 (50.3)
Mismatched	216 (49.7)
Race/ethnicity match	
Matched	305 (70.1)
Mismatched	130 (29.9)
Treatment characteristics	
Preparative regimen	
TBI containing	190 (43.6)
Non-TBI containing	245 (56.4)
GVHD prophylaxis	
Cyclosporine/methylprednisolone	330 (75.9)
Cyclosporine/mycophenolate mofetil	90 (20.7)
Tacrolimus-based regimens	15 (3.4)

CMV indicates cytomegalovirus; CBU, cord blood unit; HLA, human leukocyte antigen; GVHD, graft-versus-host disease; TBI, total-body irradiation.

Table 2. Characteristics of Transplanted Cord Blood Units

Graft Characteristic	Precryo		Post-thaw		Median Recovery Post-thaw		Pearson Correlation Coefficient (r)
	n	Median (Range)	n	Median (Range)	n*	%	
TNC, $\times 10^7$/kg	434	6.7 (1.4-67.3)	435	5.7 (0.9-50.3)	434	81.1	0.96
MNC, $\times 10^7$/kg	216	2.8 (0.21-30.3)	383	2.1 (0.2-15.1)	197	82.8	0.83
$CD34^+$, $\times 10^5$/kg	381	2.1 (0.1-29.3)	433	1.7 (0.1-18.3)	379	84.5	0.87
CFU, $\times 10^4$/kg	279	19.1 (0-294.0)	435	3.3 (0-105.3)	279	21.2	0.40

TNC indicates total nucleated cell; MNC, mononuclear cell count; CFU, colony-forming unit.
*Refers to n available for paired analysis.

Program (NMDP) (32%, n = 139). Eighty-three (19%) units were banked through the COBLT study funded by the National Heart, Lung, and Blood Institute. The majority of CBUs were volume reduced and RBC depleted before cryopreservation; 10 (2%) contained RBCs as they were processed via plasma depletion alone. All CBUs were washed with dextran/albumin before administration to the patient.

Grafts were characterized pre-cryo and post-thaw (after washing) to enumerate TNC, MNC, $CD34^+$, and CFU (Table 2). There was good correlation ($r > .9$) between TNC and MNC in both pre-cryo and post-thaw datasets. Correlations between CFU and $CD34^+$ ($r = .71$) and CFU and MNC ($r = .76$) were moderate. Comparison of the pre-cryo and post-thaw values for all CBUs from all banks showed excellent correlations for TNC ($r = .96$, $P < .0001$) and good correlations for MNC ($r = .83$, $P < .0001$) and $CD34^+$ ($r = .87$, $P < .0001$). Of note, much weaker correlations were noted for pre-cryo and post-thaw CFU ($r = .40$, $P < .0001$). The median post-thaw recoveries of TNC, MNC, $CD34^+$, and CFU were 81.1%, 82.8%, 84.5%, and 21.2%, respectively. Similar correlations (TNC r = .95, MNC r = .83, $CD34^+$ r = .91, and CFU r= .37) were observed when evaluating CBUs both collected and transplanted at Duke.

Impact of Graft and Clinical Characteristics on Engraftment

Neutrophil engraftment

In the overall study group, neutrophil engraftment occurred in a median of 26 days (range: 7-104 days) with an overall probability of neutrophil engraftment of 76.9% at 42 days (95% confidence interval [95% CI], 71.3%-82.5%) posttransplant, which is consistent with previous reports of outcomes of UCBT [1,2,7,28]. We first examined in univariate analysis the influence of graft and clinical characteristics on the time to neutrophil engraftment. In univariate analysis of the pre-cryo dataset, CFU and $CD34^+$ cell doses were most predictive of engraftment, followed by TNC and MNC (Table 3 and Figure 1). We also examined the influence of post-thaw graft characteristics on neutrophil engraftment. In univariate analysis of post-thaw graft characteristics, CFU, followed by MNC, $CD34^+$, and TNC doses were favorably associated with neutrophil engraftment; post-thaw CFU dose had the strongest hazard risk (Table 3 and Figure 2). Multiple clinical variables, most notably recipient pretransplant CMV serostatus, metabolic disease, and younger age, were predictive in univariate analysis of neutrophil engraftment (Supplemental Table S1).

Analysis of neutrophil engraftment in multivariate analysis

Separate multivariate analyses were performed using a pre-cryo and post-thaw datasets for neutrophil engraftment. Clinical characteristics were considered in all multivariate analyses. In the pre-cryo neutrophil engraftment model, negative recipient CMV was the strongest predictor of engraftment followed closely by higher precryo CFU dose, male donor, and metabolic diagnosis, a group of smaller and younger patients who generally received higher cell dosed grafts (Table 4). Post-thaw CFU dose was the most highly correlated characteristic in the post-thaw multivariate model followed by metabolic diagnosis and male donor. Recipient CMV, post-thaw MNC, and $CD34^+$ doses were predictive of engraftment in the post-thaw model, but to a much lesser degree (Table 4).

Platelet engraftment

Platelet engraftment, defined as ≥50,000 platelets/mm^3 untransfused, occurred at a median 134 days (range: 22-471 days) posttransplant with an overall probability of 55% at 180 days (95% CI, 49.3%-60.7%). In univariate analysis of pre-cryo parameters, patients receiving higher doses (above the median) of CFU, $CD34^+$, and TNC were more likely to engraft platelets. Higher doses were also associated with more rapid platelet engraftment (Table 3 and Figure 3). Pre-cryo MNC dose was also predictive of overall platelet engraftment, but to a lesser degree than the other graft variables. In the post-thaw dataset, all of the graft characteristics (TNC, MNC, $CD34^+$, and CFU doses) were predictive of platelet engraftment in univariate analysis (Table 3 and Figure 4). Multiple clinical variables were predictive in univariate analysis of platelet engraftment including recipient CMV status, diagnosis, race/ethnicity, age, and HLA matching (Supplemental Table S1).

Table 3. Graft Characteristics Affecting Neutrophil and Platelet Engraftment (≥50,000 Platelets/mm^3) in Univariate Analysis*

	Hazard Ratios (95% CI)	
	Neutrophil Engraftment	Platelet Engraftment (≥50,000 Platelets/mm^3)
Pre-cryopreservation graft characteristics		
TNC, $\times 10^7$/kg		
4.0 or less	1.00	1.00
>4.0-6.7	1.19 (0.91-1.57)	1.26 (0.90-1.78)
>6.7-10.6	1.70 (1.27-2.27), $P = .0004$	1.76 (1.25-2.49), $P = .0010$
>10.6	2.07 (1.53-2.78), $P < .0001$	2.09 (1.48-2.96), $P < .0001$
MNC, $\times 10^7$/kg		
1.7 or less	1.00	1.00
>1.7-2.8	1.08 (0.76-1.55)	0.97 (0.62-1.51)
>2.8-4.7	1.06 (0.73-1.53)	1.15 (0.74-1.79)
>4.7	2.10 (1.43-3.08), $P = .0001$	1.75 (1.12-2.72), $P = .0130$
CD34$^+$, $\times 10^5$/kg		
1.0 or less	1.00	1.00
>1.0-2.1	1.12 (0.84-1.50)	1.50 (1.05-2.14), $P = .0250$
>2.1-3.6	1.56 (1.14-2.14), $P = .0050$	1.64 (1.12-2.40), $P = .0110$
>3.6	2.49 (1.81-3.44), $P < .0001$	2.39 (1.63-3.50), $P < .0001$
CFU, $\times 10^4$/kg		
10.5 or less	1.00	1.00
>10.5-19.1	1.40 (0.99-1.97)	1.42 (0.92-2.18)
>19.1-33.4	1.54 (1.10-2.15), $P = .0110$	1.94 (1.29-2.91), $P = .0013$
>33.4	2.47 (1.71-3.58), $P < .0001$	2.37 (1.54-3.63), $P < .0001$
Post-thaw graft characteristics		
TNC, $\times 10^7$/kg		
3.4 or less	1.00	1.00
>3.4-5.7	1.29 (0.99-1.68)	1.67 (1.18-2.36), $P = .0039$
>5.7-8.7	1.61 (1.20-2.17), $P = .0017$	2.16 (1.50-3.10), $P < .0001$
>8.7	2.10 (1.56-2.83), $P < .0001$	2.43 (1.68-3.51), $P < .0001$
MNC, $\times 10^7$/kg		
1.3 or less	1.00	1.00
>1.3-2.1	1.43 (1.06-1.93), $P = .0180$	1.30 (0.91-1.87)
>2.1-3.5	1.56 (1.17-2.08), $P = .0023$	1.52 (1.08-2.16), $P = .0180$
>3.5	2.36 (1.72-3.24), $P < .0001$	1.84 (1.27-2.67), $P = .0013$
CD34$^+$, $\times 10^5$/kg		
0.9 or less	1.00	1.00
>0.9-1.7	1.35 (1.04-1.78), $P = .0260$	2.02 (1.42-2.88), $P = .0001$
>1.7-3.0	1.69 (1.27-2.25), $P = .0004$	2.21 (1.54-3.17), $P < .0001$
>3.0	2.32 (1.71-3.14), $P < .0001$	2.55 (1.76-3.71), $P < .0001$
CFU, $\times 10^4$/kg		
1.3 or less	1.00	1.00
>1.3-3.3	1.51 (1.16-1.95), $P = .0020$	1.57 (1.11-2.20), $P = .0100$
>3.3-6.8	1.90 (1.42-2.53), $P < .0001$	1.56 (1.09-2.22), $P = .0150$
>6.8	3.64 (2.61-5.07), $P < .0001$	3.23 (2.24-4.66), $P < .0001$

TNC indicates total nucleated cell; MNC, mononuclear cell count; CFU, colony-forming unit; CI, confidence interval.
*Significant P values are indicated (as defined by a value of ≤.05).

Analysis of platelet engraftment in multivariate analysis

Multivariate analyses including clinical characteristics were performed using a pre-cryo and post-thaw datasets for platelet engraftment. In the pre-cryo multivariate model of platelet engraftment, higher CFU dosing, the bank that supplied the CBU, and negative recipient CMV status were the only significant predictors of engraftment (Table 4). Post-thaw CFU dose had the strongest correlation followed by metabolic patient diagnosis in the post-thaw multivariate platelet engraftment analysis. Receiving a graft containing a TNC of $>5.7 \times 10^7$/kg, negative recipient CMV serostatus, and closer HLA matching were also predictive of platelet engraftment in the post-thaw multivariate model with similar hazard ratios (Table 4).

Impact of Clinical and Graft Characteristics on Overall Survival

The probability of overall survival (OS) in the study group was 58.9% (95% CI, 54.2%-63.6%) at 1 year. There were 221 patient deaths with primary causes because of progressive disease (28.5%), graft failure (5.9%), GVHD (9.1%), infection (31.8 %), organ failure (18.1 %), or other causes, including secondary malignancy and hemorrhage (6.6%). We examined factors predictive of OS in univariate and multivariate analyses at 180 days posttransplant and 1 year (Tables 5 and 6). In the univariate analysis, TNC, CD34$^+$, and CFU were associated with OS in both the pre-cryo and post-thaw settings (Table 5). Multiple clinical characteristics were predictive in univariate analysis of overall survival at both time points, most notably age, diagnosis, and recipient CMV serostatus.

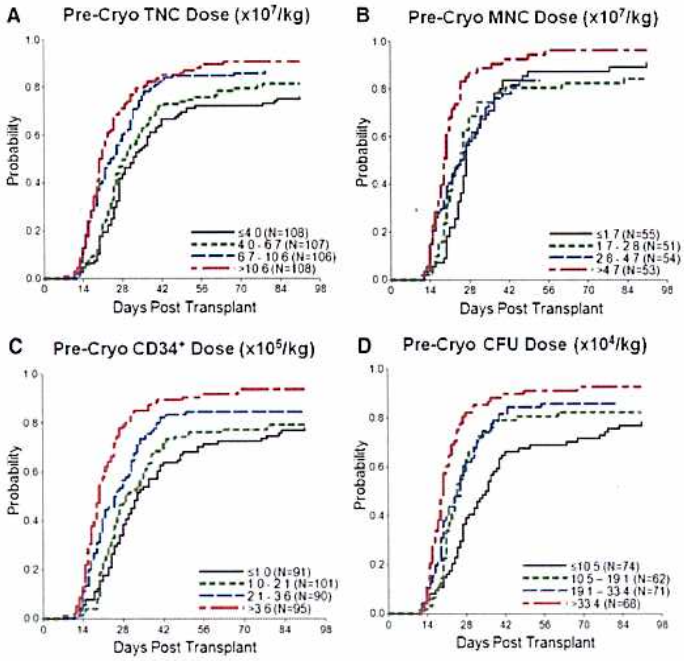

Figure 1. Impact of precryo graft characteristics on the probability of neutrophil engraftment. Probability plots are shown for each of the 4 quartiles. Panels A-D depict the impact of precryo TNC ($\times 10^7$/kg recipient weight), MNC ($\times 10^7$/kg recipient weight), $CD34^+$ ($\times 10^5$/kg recipient weight), and CFU ($\times 10^4$/kg recipient weight) doses, respectively, on neutrophil engraftment.

In multivariate models of OS that included both clinical and graft characteristics, negative recipient CMV status, metabolic diagnosis and, to a lesser degree, male gender were predictive of OS at 180 days posttransplant. At 1 year, OS in both the pre-cryo and post-thaw models was strongly impacted by recipient CMV status, followed by gender and HLA match (5/6 or 6/6). A metabolic diagnosis was also predictive in the pre-cryo model (data not shown), but it was not predictive when considering post-thaw graft characteristics. This is likely because of the small size of these patients who typically received higher cell dosed grafts. Although no graft characteristic strongly predicted OS in multivariate analysis, $CD34^+$ (both precryo and post-thaw) and CFU (pre-cryo) had weak predictive value at 180 days and 1 year posttransplant, respectively (Table 6).

How Do These Observations Impact Graft Selection from a Public CB Bank?

Given the observation that both pre-cryo and post-thaw CFU doses were the graft parameters most closely correlated with engraftment, we asked what proportions of the inventory (n = 22,559) of a large public CB bank (the CCBB at Duke) could deliver acceptable doses (at or above the median of 19.1×10^4 CFU/kg calculated on the pre-cryo CBU content) to theoretical patients of various weights. As shown in Figure 5, approximately 49% of the inventory would deliver an optimal CFU dose for a small patient (20 kg), but only 2.8% would deliver an adequate dose for a larger patient (>50 kg). We observed similar findings when we examined the CCBB inventory more recently banked (ie, since 2005 and since 2007). This highlights the need for continued efforts to increase the inventory of publically banked CBUs to provide CBU grafts likely to engraft to patients undergoing UCBT.

DISCUSSION

Primary graft failure and engraftment delays continue to be significant barriers to the overall success of unrelated donor UCBT. Both clinical and graft-related factors may contribute to graft failure. We are interested in developing methods for measuring graft potency that will minimize the chance of that a patient will not engraft because of the graft he or she receives. Patients that fail to engraft often will engraft when re-transplanted with a different CBU. This highlights that conventional methods of graft selection, using TNC and HLA match, do not discriminate CBUs that will be unlikely to engraft with sufficient sensitivity. In this large, single-center retrospective study, we present evidence that the dose of pre-cryo and post-thaw CFUs best predicts CBU engraftment after transplantation compared to other measures routinely obtained during the processing, cryopreservation, and thawing of CBUs. Because of this, we propose that any measure of CBU potency should give heavy weighting to the number of CFUs present in and delivered to the patient by a selected CBU.

Most transplant centers use TNC/kg dosing for CBU selection, based on multiple single-center and registry series demonstrating strong correlation with cell dosing and engraftment [4,6,7,9]. With this approach,

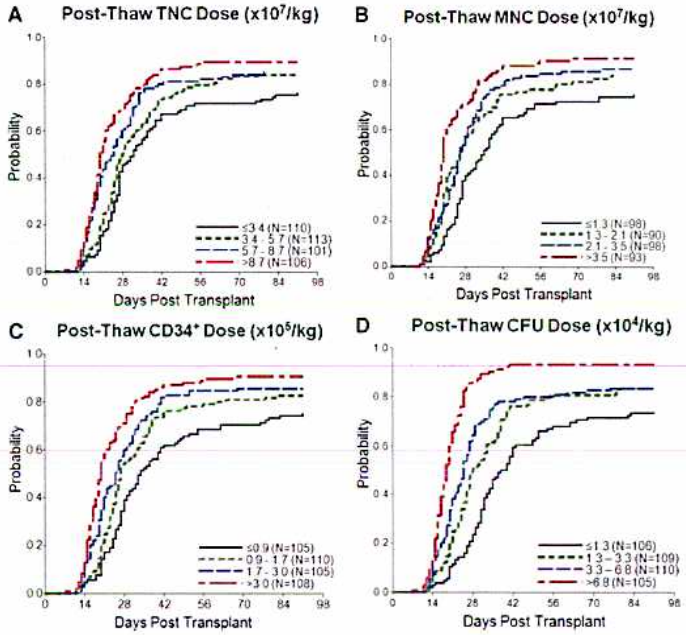

Figure 2. Impact of postthaw graft characteristics on the probability of neutrophil engraftment. Probability plots are shown for each of the 4 quartiles. Panels A-D depict the impact of post-thaw TNC ($\times 10^7$/kg recipient weight), MNC ($\times 10^7$/kg recipient weight), $CD34^+$ ($\times 10^5$/kg recipient weight), and CFU ($\times 10^4$/kg recipient weight) doses, respectively, on neutrophil engraftment.

however, approximately 20% of patients still fail to engraft. In this report, which evaluated an expanded set of graft characteristics in a patient group receiving CBUs delivering higher cell doses, we found that neither pre-cryo nor post-thaw TNC dose correlated with neutrophil engraftment in the multivariate analysis, although post-thaw TNC was predictive in the platelet engraftment model. This also held true when conventional TNC quartiles were considered (Supplemental Table S2). Post-thaw MNC dose was predictive in multivariate analysis of neutrophil engraftment, but was not predictive of platelet engraftment. Ideally, a measure of CBU potency should predict both neutrophil and platelet engraftment.

Table 4. Positive Predictors in Multivariate Analyses of Neutrophil and Platelet Engraftment

Characteristic	Favorable Characteristic	HR (95% CI)	*P* Value*
Neutrophil engraftment precryo model†			
Recipient CMV	Negative	1.65 (1.23-2.20)	.0008
CFU	>19.1 × 10^4/kg	1.49 (1.15-1.93)	.0024
Unit gender	Male	1.49 (1.16-1.93)	.0020
Diagnosis‡	Metabolic	1.47 (1.11-1.94)	.0064
Neutrophil engraftment post-thaw model§			
CFU	>3.3 × 10^4/kg	1.70 (1.34-2.14)	<.0001
Diagnosis	Metabolic	1.55 (1.21-1.97)	.0004
Unit gender	Male	1.45 (1.15-1.83)	.0016
MNC	>2.1 × 10^7/kg	1.43 (1.11-1.85)	.0060
Recipient CMV	Negative	1.34 (1.05-1.71)	.0190
$CD34^+$	>1.7 × 10^5/kg	1.30 (1.01-1.67)	.0400
Platelet engraftment precryo model			
Recipient CMV	Negative	1.96 (1.38-2.70)	.0001
CFU	>19.1 × 10^4/kg	1.56 (1.13-2.15)	.0063
Bank	CCBB	1.54 (1.06-2.23)	.0240
Platelet engraftment post-thaw model			
CFU	>3.3 × 10^4/kg	1.66 (1.29-2.13)	.0007
Diagnosis	Metabolic	1.58 (1.20-2.07)	.0010
Recipient CMV	Negative	1.44 (1.08-1.91)	.0120
TNC	>5.7 × 10^7/kg	1.43 (1.11-1.84)	.0062
HLA match	5/6 or 6/6	1.39 (1.07-1.79)	.0130

HR indicates hazard ratio; CI, confidence interval; CMV, cytomegalovirus; CFU, colony-forming unit; MNC, mononuclear cell count; TNC, total nucleated cell; HLA, human leukocyte antigen.

*Significant *P* values were defined as a *P* value of ≤ .05.

†Precryo model included clinical characteristics and precryo graft characteristics.

‡Diagnosis refers to malignancy versus metabolic versus other.

§Post-thaw models considered clinical characteristics and post-thaw graft characteristics.

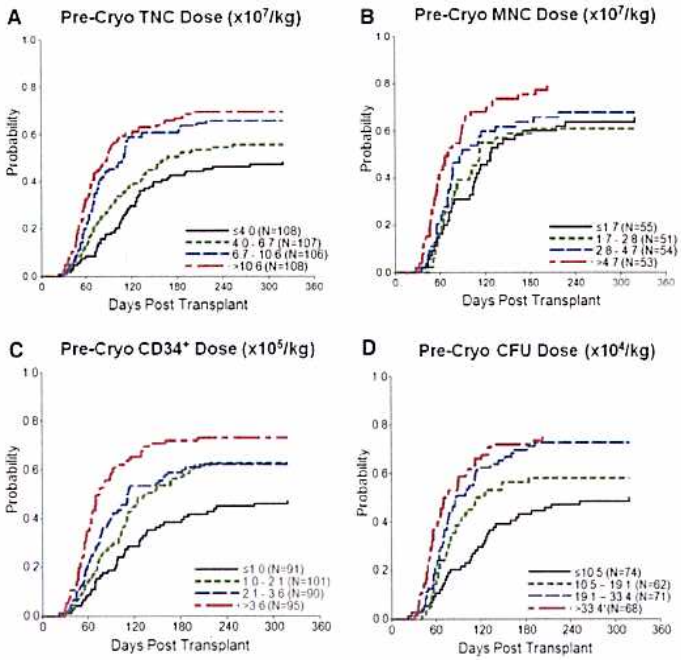

Figure 3. Impact of precryo graft characteristics on the probability of platelet engraftment. Probability plots are shown for each of the 4 quartiles. Panels A-D depict the impact of precryo TNC ($\times 10^7$/kg recipient weight), MNC ($\times 10^7$/kg recipient weight), $CD34^+$ ($\times 10^5$/kg recipient weight), and CFU ($\times 10^4$/kg recipient weight) doses, respectively, on platelet engraftment.

In addition to TNC, $CD34^+$ dose has been reported in several studies as being an important predictor of engraftment [10-13]. Some transplant centers utilize the $CD34^+$ cell dose in CBU selection recognizing that significant interlaboratory variability exists [28,29]. In our series, post-thaw $CD34^+$ dose was a significant predictor of neutrophil engraftment in multivariate analysis ($P = .04$), but to a lesser degree than post-thaw CFU ($P < .0001$). $CD34^+$ dose did not correlate with platelet engraftment, but did correlate weakly with OS.

In this large, single-center series, CFU dose was most closely associated with both neutrophil and platelet engraftment among the graft parameters considered in a multivariate model. The importance of CFU dosing was previously recognized in a report from the New York Blood Center in 2001 showing that pre-cryo CFU dose was more closely correlated with engraftment of both neutrophils and platelets than TNC [30]. In a smaller series of 42 patients transplanted from 1995 to 2001, CFU-GM was the only graft characteristic impacting OS ($P = .003$) and event-free survival (EFS) ($P = .02$) [31]. Another study more recently showed that post-thaw CFU-GM along with TNC and $CD34^+$ cell dose correlated with the kinetics of neutrophil engraftment, but only CFU-GM dose correlated with platelet engraftment [32]. This report also confirms our earlier observations reported by Prasad et al. [16] demonstrating that post-thaw CFU dose had the highest correlation with survival and engraftment after UCBT in a series of 159 young pediatric patients with inherited metabolic disease. In this series, a post-thaw CFU dose above 5.7×10^4/kg was associated with the most favorable outcomes. Our findings in this larger series of predominately pediatric patients (<10% were older than 18 years old), many with inherited metabolic diseases, confirm that CFU dose is a strong predictor of engraftment. However, the pre-cryo CFU dose was only weakly predictive of OS, which we believe is because of insufficient power to detect differences in OS. Further analysis of an adult dataset should be performed to confirm these findings and define dosing threshold for an older population.

HLA has been well described previously as an important predictor of engraftment and OS. The current standard of CBU selection matches at the antigen level for HLA-A and -B and at the allelic level for HLA-DRB1. In addition to functioning as an independent variable, there is some evidence that there is mutual interaction between HLA and TNC. Gluckman et al. [9] suggested that HLA mismatching can be overcome by higher TNC. Eapen et al. [33] also analyzed the interaction between HLA matching and cell dose. Although no differences were observed for fully matched or 2-antigen mismatch UCBT, higher cell dose defined as $>3.0 \times 10^7$/kg in 1-antigen mismatched UCBT showed improved neutrophil engraftment compared to lower cell dose with the same mismatch. In a recent large series from Barker et al. [4], patients receiving a 6/6 matched CBU regardless of TNC had lower transplant-related mortality, followed by patients receiving a 1-mismatched unit with a TNC of $\geq 2.5 \times 10^7$/kg or a 2-mismatched unit with a TNC of $\geq 5.0 \times$

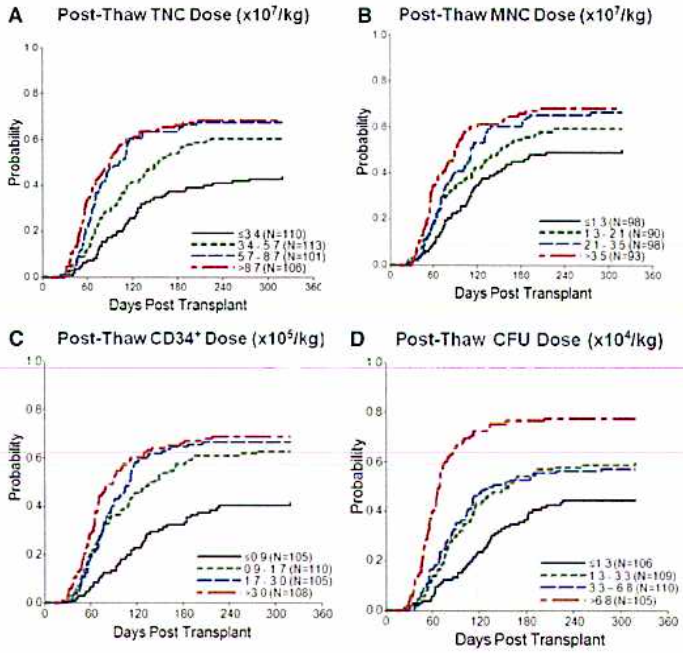

Figure 4. Impact of post-thaw graft characteristics on the probability of platelet engraftment. Probability plots are shown for each of the 4 quartiles. Panels A-D depict the impact of post-thaw TNC ($\times 10^7$/kg recipient weight), MNC ($\times 10^7$/kg recipient weight), $CD34^+$ ($\times 10^5$/kg recipient weight), and CFU ($\times 10^4$/kg recipient weight) doses, respectively, on platelet engraftment.

10^7/kg. There was no difference in survival outcomes in patients receiving a 1-mismatched unit with a TNC of 2.5-4.9 $\times$ 10^7/kg compared to patients receiving a 2-mismatched CBU that delivered a TNC of $\geq 5.0 \times 10^7$/kg. We found HLA matching was predictive of platelet but not neutrophil engraftment in multivariate analysis. HLA matching was also predictive of OS at 1 year, but not of earlier survival (day 180). Thus, although our findings support the role of HLA in outcomes after UCBT, the effects were less strong than those detected in other studies and less strong than graft-related parameters in our study.

Given the strong correlation of CFU dose with engraftment, we asked whether the current inventory in a large public CB bank would provide adequate dosing of CFUs for various patient weights. Although there appears to be a sufficient number of potential units available for smaller patients, there were very few units providing more than 19.1 $\times$ 10^4/kg CFUs among larger patients. To overcome this issue, additional emphasis may need to be placed on assessing CFU growth on the postprocessed CBU to expand the inventory to include more potent units. Consideration of the CFU growth in unit selection is appropriate.

Neither the existing Health Resources and Services Administration (HRSA) standards for banking CBU nor the final U.S. Food and Drug Administration (FDA) guidance setting out standards for units intended for allogeneic UCBT specify that quantitative CFU assays be performed to characterize CBU dosing. Our results demonstrate that measurements of pre-cryo and post-thaw CFU doses are likely to contribute strongly to an improved assay for the potency of a CBU. However, this assay is difficult to standardize, and interlaboratory variability is an issue [34-36]. In this regard, our study, which presents the largest examination of CFUs to date, benefitted from having a large, single-center dataset. Additionally, at our center, consistent personnel have performed almost half of the pre-cryo CFUs and all of the post-thaw CFUs during the duration of the study, which, perhaps, allowed for these findings to emerge. Post-thaw CFU measurements reflect potential damage to the CBU during cryopreservation, shipping to a transplant center, and thawing. Indeed, our data show that only about 20% of the CFU in a CBU typically survive banking, shipping, thawing, and washing using standard clinical techniques. Because we noticed this phenomenon also in units that were not shipped (ie, collected, processed, banked by the CCBB and thawed at Duke SCL), the contribution of shipping to cell loss does not appear to be extensive. Although reports of CFU recoveries are limited, our observation is similar to Wagner et al. [37], who reported 89% loss compared to pre-cryo for 20 thawed units transplanted at their center. The low CFU recovery in this study is contradictory to the excellent post-thaw clonogenic potential observed by Broxmeyer et al. [38], who has been following the stability of progenitor cell growth from a single cord over more than a decade, but this cord was processed, cryopreserved, and thawed under different conditions than those used for standard CB banking. Alonso et al. [39] noted a median of 76% CFU recovery in

Table 5. Positive Graft Characteristics Predictive of Overall Survival (OS) at 180 Days and 1 Year Posttransplant in Univariate Analysis

	OS at Day 180	OS at 1 Year
	Hazard Risk (95% CI), P*	
Pre-cryopreservation graft characteristics		
TNC, $\times 10^7$/kg		
4.0 or less	1.00	1.00
>4.0-6.7	0.79 (0.52-1.21)	0.84 (0.58-1.23)
>6.7-10.6	0.53 (0.33-0.85), P = .0081	0.57 (0.37-0.87), P = .0089
>10.6	0.47 (0.29-0.76), P = .0024	0.54 (0.35-0.82), P = .0039
MNC, $\times 10^7$/kg		
1.7 or less	1.00	1.00
>1.7-2.8	1.21 (0.60-2.42)	1.45 (0.79-2.68)
>2.8-4.7	0.87 (0.42-1.84)	1.07 (0.56-2.04)
>4.7	0.72 (0.33-1.56)	0.71 (0.35-1.44)
$CD34^+$, $\times 10^5$/kg		
1.0 or less	1.00	1.00
>1.0-2.1	0.63 (0.39-1.01)	0.65 (0.43-1.01)
>2.1-3.6	0.55 (0.33-0.92), P = .0218	0.62 (0.40-0.98), P = .0399
>3.6	0.41 (0.24-0.71), P = .0013	0.54 (0.34-0.86), P = .0088
CFU, $\times 10^4$/kg		
10.5 or less	1.00	1.00
>10.5-19.1	0.83 (0.47-1.47)	0.82 (0.49-1.38)
>19.1-33.4	0.41 (0.21-0.80), P = .0094	0.55 (0.32-0.95), P = .0317
>33.4	0.63 (0.35-1.14)	0.64 (0.38-1.09)
Post-thaw graft characteristics		
TNC, $\times 10^7$/kg		
3.4 or less	1.00	1.00
>3.4-5.7	0.52 (0.34-0.81), P = .0034	0.61 (0.42-0.89), P = .0109
>5.7-8.7	0.52 (0.33-0.81), P = .0042	0.51 (0.34-0.77), P = .0013
>8.7	0.39 (0.24-0.62), P = .0001	0.45 (0.29-0.68), P = .0002
MNC, $\times 10^7$/kg		
1.3 or less	1.00	1.00
>1.3-2.1	0.84 (0.52-1.36)	0.99 (0.64-1.54)
>2.1-3.5	0.71 (0.44-1.16)	0.82 (0.53-1.28)
>3.5	0.62 (0.37-1.04)	0.73 (0.46-1.17)
$CD34^+$, $\times 10^5$/kg		
0.9 or less	1.00	1.00
>0.9-1.7	0.61 (0.39-0.93), P = .0223	0.61 (0.41-0.90), P = .0126
>1.7-3.0	0.44 (0.28-0.71), P = .0008	0.45 (0.29-0.69), P = .0003
>3.0	0.44 (0.27-0.70), P = .0006	0.54 (0.36-0.81), P = .0030
CFU, $\times 10^4$/kg		
1.3 or less	1.00	1.00
>1.3-3.3	0.83 (0.53-1.29)	0.89 (0.60-1.32)
>3.3-6.8	0.84 (0.54-1.31)	0.84 (0.57-1.25)
>6.8	0.46 (0.28-0.771), P = .0033	0.52 (0.33-0.81), P = .0042

TNC indicates total nucleated cell; MNC, mononuclear cell count; CFU, colony-forming unit; CI, confidence interval; OS, overall survival.
*Significant P values are indicated (as defined by a value of ≤.05).

11 CBUs thawed for transplantation (n = 11), although details of the thawing and post-thaw CFU methods were not described. We believe that the data from our larger dataset showing low CFU recovery are accurate and that it is the best measure of CBU potency. The loss of CFU post-thaw is likely explained by damage incurred by the CBU during cryopreservation, long-term storage, and thawing. Further consideration of this finding is warranted.

The post-thaw CFU is an important reflection of the overall potency health of the CBU transplanted into patients. At our institution, we use the following strategy for patients undergoing UCBT. At time of initial unit selection, an additional backup unit is identified, typed, and reserved for the patient. For all transplants, CFUs are performed on a sample of the thawed transplant product on day 0 with results available on day 14-16 posttransplant. If CFUs do not grow but the patient is engrafting, no action is taken. If CFUs do not grow and the patient is aplastic, the availability of a second unit is confirmed, and the patient is monitored more closely. If count recovery has not occurred by day 42 posttransplant, we perform a bone marrow biopsy and aspirate to determine cellularity and to document donor chimerism. In the cases where graft failure has been documented, we proceed to a second transplant using a reduced-intensity conditioning regimen.

Post-thaw CFU dose was a very strong predictor of engraftment, suggesting that measurement of post-thaw CFU dose, or a more reliable surrogate, should be an important part of graft selection. However, the CFU assay is limited by a long readout time and issues with standardization between centers. Development of shorter CFU assays and automatic methods for

Table 6. Predictors Impacting Overall Survival (OS) at 180 Days and 1 Year Posttransplant in Multivariate Analysis

Characteristic	Risk Factor*	HR (95% CI)	*P* Value†
Precryo model of OS at 180 days			
Recipient CMV	Positive	2.11 (1.45-3.05)	<.0001
$CD34^+$, $\times 10^5$/kg	$\leq 2.1 \times 10^5$/kg	1.55 (1.06-2.26)	.0244
Post-thaw model of OS at 180 days			
Recipient gender	Female	1.40 (1.00-1.97)	.0490
Diagnosis‡	Malignant	1.74 (1.11-2.74)	.0168
Recipient CMV	Positive	1.72 (1.22-2.43)	.0021
$CD34^+$, $\times 10^5$/kg	$\leq 1.7 \times 10^5$/kg	1.47 (1.03-2.10)	.0340
Precryo model of OS at 1 year			
Recipient gender	Female	1.76 (1.19-2.60)	.0050
HLA match§	≤4/6	1.92 (1.23-2.94)	.0033
Recipient CMV	Positive	2.04 (1.37-3.03)	.0005
CFU, $\times 10^4$/kg	$\leq 19.1 \times 10^4$/kg	1.49 (1.01-2.22)	.0471
Post-thaw model of OS at 1 year			
Diagnosis	Malignant	1.73 (1.18-2.55)	.0054
HLA match§	≤4/6	1.44 (1.04-2.00)	.0282
Recipient CMV	Positive	1.94 (1.43-2.64)	<.0001
Recipient gender	Female	1.39 (1.03-1.87)	.0329

CMV indicates cytomegalovirus; CFU, colony-forming unit; HR, hazard ratio; CI, confidence interval.
*Risk refers to increased risk of death.
†Significant *P* values are indicated (as defined by a value of < or =.05).
‡Diagnosis refers to malignant versus metabolic versus other.
§HLA (human leukocyte antigen) match ≤4/6 versus 5/6 or 6/6.

enumeration are being investigated to address these limitations. Additionally, CFUs could be assayed on an attached segment prior to release from the CB-bank. Previous reports have correlated CFU content from an attached sample with the overall thawed graft [40,41]. At our institution, we have developed a potency assay, including CFU, measured on the attached segment. We are currently studying whether potency measured on an attached segment correlates with engraftment and recoveries of TNC, $CD34^+$, and CFU from the actual transplant bag. We hypothesize the assay will be predictive of outcomes after unrelated donor UCBT and would be used to evaluate potency prior to final unit selection. Such innovations, coupled with development of metrics for potency based on the most important graft parameters influencing engraftment that we have identified, should help reduce CBU-related graft failure.

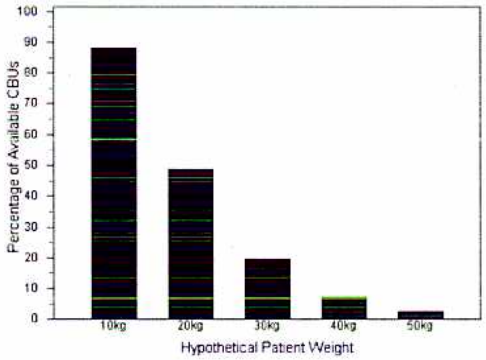

Figure 5. Availability of CBUs in a large public bank (n = 22,559), which contain high CFU cell doses for various patient weights. For various theoretical patient weights, we have presented the percentage of CBUs available in a large, public cord blood bank (Carolinas Cord Blood Bank at Duke). CBUs all met criteria for banking as specified by the National Cord Blood Inventory.

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SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.bbmt.2011.01.011

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