

## Development of a New Third-Party Unit for Adult Stem Cell Transplantation using Clinical-Grade Rejected Cord Blood Units

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### Abstract

Cord blood is a valuable alternative source of Hematopoietic Stem Cells (HSC) for the transplantation of patients without a related stem cell donor. Even though the number of banked units has increased in recent years, units containing sufficient stem cells for transplantation into larger adult recipients are still very limited. Different approaches have been used to increase the number of infused HSC. However, most approaches have the disadvantage of increasing the cost of graft procurement or rely on a related haplo-identical donor who may not be available. We developed an alternative approach by creating a third-party unit of enriched CD34<sup>+</sup> cells from a pool of multiple, human leukocyte antigen (HLA)-blind, cryopreserved CBUs. These pooled units were rejected by the public cord blood bank due to insufficient volumes and/or low nucleated cell counts. Seven recipients with hematological cancers received myeloablative conditioning followed by co-infusion of a  $\geq 4/6$  HLA-compatible CBU and a third-party source of CBUs consisting of CD34<sup>+</sup> cells selected from a pool of cryopreserved CBUs. Six patients were engrafted with a median neutrophil engraftment time of 19.5 (15-29) days. All engrafted patients had 100% HLA-matched CBU chimerism on day +14. All recipients had grades I-III acute graft-versus-host-disease (GVHD) that responded promptly to treatment, and no patients developed chronic GVHD. Two patients died, one on day +28 due to disease relapse, and one on day +360 due to multisystem organ failure. This new method not only supports the goal of increasing the number of HSCs to enable rapid engraftment, but it also demonstrates that clinical-grade rejected CBUs can be used to create a third-party, HLA-blind source of enriched CD34<sup>+</sup> cells to support a small,  $\geq 4/6$  HLA-compatible CBU.

**Keywords:** Stem cells; Cord blood; Bone marrow; Transplantation

### Introduction

Since the first HSC transplantation in 1988, Cord Blood Unit (CBU) have been primarily used to treat patients suffering from hematological and oncologic disorders. This stem cell source not only has the advantage of being rapidly available compared with Bone Marrow (BM) and Peripheral Blood Stem Cells (PBSCs), but it also has a greater proliferative capacity, significantly reducing the number of Nucleated Cells (NCs) required for transplantation. When transplanting a single unit, a count of  $2.5 \times 10^7$  NCs/kg of recipient weight is recommended for successful engraftment. More recently, researchers have recommended increasing the number of NCs to  $5 \times 10^7$  NCs/kg in the presence of two Human Leukocyte Antigen (HLA) incompatibilities, to accelerate engraftment, especially for patients with malignancies [1,2].

Based on the minimum number of NCs/kg of the recipient weight, the probability of finding a CBU with a 6/6 and 4/6 HLA compatibility for a patient weighing less than 50 kg is 23% and 100%, respectively. However, the probability of finding a CBU with a 6/6 and 4/6 HLA compatibility for a recipient weighing more than 70 kg is only 2% and 76%, respectively [3]. Additionally, donor availability varies according to ethnicity. Half of all Caucasians will find a donor, while only 35% of Hispanics and 20% of African-Americans will find suitable donors [4,5]. To overcome this limitation, especially in adult recipients, some centers choose to perform Double Cord Blood Units Transplantation (dCBUs). Unfortunately, this method requires two CBUs with at least a 4/6 HLA compatibility with the recipient, where each selected CBU must contain a minimum of  $1.5 \times 10^7$  NCs/kg for a total of  $\geq 3.5 \times 10^7$  NCs/kg [6]. This increase in nucleated cells improved donor neutrophil engraftment with a median of 32 days (18-53) and platelet recovery by day 91 (56-381), which compares favorably with the results of single CBU transplantation in lower-weight patients [6-9]. Post-

transplantation outcomes also showed that dCBUs resulted in a lower rate of leukemia relapse, as well as a similar incidence of acute Graft-Versus-Host-Disease (GVHD) and chronic GVHD, when compared to single CBU transplants [10].

Another method that is used to increase the number of nucleated cells without increasing the number of T and B cells is the infusion of CD34<sup>+</sup> selected haplo-related PBSCs as a third-party source with  $\geq 4/6$  HLA-mismatched CBUs containing at least  $1.5 \times 10^7$  NCs/kg [11]. The average time required to achieve neutrophil counts of  $\geq 0.5 \times 10^9$ /L with this method is 14 days (9-36), which is followed by CBU engraftment on day +21 (12-57 days) with sustained platelet counts of  $\geq 20 \times 10^9$ /L after a median time of 32 days [12-14]. Even when mixed chimerism was observed during the first 97 days (11-186), most of the recipients exhibited full CBU chimerism by day +100, with a low incidence of GVHD [15]. However, this method requires the availability of a haplo-related donor and has a higher cost related to PBSC collection and CD34<sup>+</sup> selection, compared to single CBU transplantation.

Studies of both methods showed that the infusion of more than

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one unit with a similar or lower HLA compatibility does not increase GVHD. In addition, there is a bias for the long-term engraftment of the first infused CBU. The need to increase cell counts was not considered in discussions regarding the appropriate number of CD34<sup>+</sup> cells/kg. However, these methods clearly support the consensus that infusing  $\geq 2 \times 10^5$  CD34<sup>+</sup> cells/kg to best enable the engraftment of a single CBU. The availability of units containing a greater number of nucleated cells will require that the public cord blood banks increase the cut-off value of the collected units, which will increase the number of discarded units to more than the current amount of 70%. The present pilot study was performed to demonstrate that clinical-grade rejected CBUs can be pooled to create an HLA-blind third-party unit consisting of purified CD34<sup>+</sup> cells that can support a single  $\geq 4/6$  HLA-matched CBU that alone is too small for transplantation.

## Methods

### Matched cord blood unit

The selection of the “matched” CBU required  $\geq 4/6$  HLA compatibility with the recipient and NCs criteria. The unit was HLA-typed, at the antigen level, for HLA-A and HLA-B and, at the allele level, for HLA-DR $\beta$ 1 by the donor cord blood bank. Preference was given to full (6/6) compatibility over one or two mismatches; in the presence of a mismatch, HLA-A and HLA-B mismatches were preferred over HLA-DR $\beta$ 1 mismatch. The CBU had to contain a minimum of  $1.5 \times 10^7$  NCs per kg of the recipient’s body weight without exceeding  $3 \times 10^7$  NCs/kg in the presence of complete HLA compatibility,  $4 \times 10^7$  NCs/kg in the presence of one HLA mismatch, or  $5 \times 10^7$  NCs/kg in the presence of two HLA mismatches.

### Adult recipients

Adult recipients aged from 18 and 60 years old, with high risk of relapse were eligible for the study. All participants had a performance status of Eastern Cooperative Oncology Group (ECOG)  $<3$  or Karnofsky  $\geq 60\%$  and hematological malignancies that would normally be considered for myeloablative allogeneic transplantation at the McGill University Health Centre (MUHC). No adult Matched Unrelated Donors (MUDs) were available or potentially available 3 to 6 months after the initiation of the search for donors. The myeloablative conditioning regimen consisted of low-dose i.v. fludarabine ( $20 \text{ mg/m}^2/\text{day}$ ) for three consecutive days immediately prior to the administration of the myeloablative regimens of i.v. busulfan  $0.8 \text{ mg/kg}$  every 6 hours on days -7 to -4 and i.v. cyclophosphamide  $60 \text{ mg/kg}$  per day on day -3 and -2. All recipients received GVHD prophylaxis consisting of combined therapy with Mycophenolate Mofetil (MMF) 1 gram twice per day on days -3 to +30 and Tacrolimus (FK506) on days -3 to +180 to maintain a serum concentration in the range of 10-15 ng/mL.

## Laboratory Assays

### Pooled cord blood units

All CBUs were collected *in utero* from mothers previously consented by an obstetrician at the MUHC Birthing Centre. CBUs were processed and cryopreserved within 48 hours of collection in compliance with Health Canada’s Safety of Human Cells, Tissues and Organ for Transplantation Regulations [16].

### Analysis

A 1 mL sample of the cord blood was obtained before and after volume reduction using an Opti System as per manufacturer requirement (Fenwal, IL, USA). Both samples were assessed for TNC

counts using an electric Beckman Coulter® LH 750 Hematology Analyzer. The sample obtained after volume reduction was diluted 1:1 with a Coulter rinsing solution (BD, cat: 8547167). The TNC was calculated by multiplying the White Blood Cell (WBC) number ( $10^9/\text{L}$ ) by the volume (mL) of the CBU pre- and post-processing. The percentage of nucleated red blood cells (nRBCs) was determined by a manual differential count of Wright-Giemsa-stained blood (Beckman Coulter cat: 754181). CD34, CD45, and 7-Amino-Actinomycin D (7AAD) viability were assessed in both samples using flow cytometry. In two tubes, 20  $\mu\text{L}$  of CD45 (FITC), 20  $\mu\text{L}$  of phycoerythrin (PE) for CD34, 20  $\mu\text{L}$  of 7-AAD, and 100  $\mu\text{L}$  of Stem-Count fluorospheres were added. In each tube, 100  $\mu\text{L}$  of blood was added. Samples were then incubated at room temperature and protected from ambient light for 20 minutes, followed by lysis of RBCs for 10 minutes of fresh CB using 2 mL of 1x ammonium chloride solution (Beckman Coulter Stem-kit). Flow cytometry studies on thawed samples were done in a similar manner as for fresh CB samples, with the exception that cell lysis was done using only 2 mL of Hank’s Balanced Salt Solution (HBSS; Bio Whittaker, Lonza cat: 10-547F) to ensure cell viability and accuracy of the cell count.

### Short Tandem Repeats (STRs)

The identity of each cell source (HLA  $\geq 4/6$  matched CBU, all pooled CBUs and transplant recipients) were molecularly determined by Short Tandem Repeat (STR) pattern analysis using PCR with fluorescent primers and capillary electrophoresis. Each individual cell source was tested at 9 different STR loci (D1S1660, D3S2387, D5S1456, D5S818, D7S820, D8S1179, D13S317, D16S539, and D20S481) using a semi-quantitative (PCR) assay. This method was used to obtain a unique molecular “fingerprint” for each cell source. The STR analysis was performed on DNA extracted from the cord blood leuko-platelet layer cells and on the recipient leucocytes collected from a peripheral blood sample. Chimerism analysis was performed at days +14, +21, +28, +56 and +100 post-transplant.

### DNA Extraction for STR determination

A 200-400  $\mu\text{L}$  sample of buffy coat was thawed and transferred to a 15 mL conical tube with 3 mL of RBC Lysis Solution (5 Prime GmbH Hamburg, Germany). The sample was kept 20 minutes at room temperature to allow lysis of the RBCs, and then centrifuged at 720xg for 2 minutes to pellet the nucleated cells. The supernatant, consisting of lysed RBCs, was discarded; the white pellet containing the nucleated cells was gently disrupted by slow vortex mixing; 3 mL of cell lysis solution was added. All cells were lysed, and the DNA content was released into the suspension. The tube was placed on ice for 2 minutes, after which 1 mL of Protein Precipitation Solution was added, and mixed by vortexing at a high speed for 20-30 seconds; the tube was then left on ice for 5 minutes, precipitating the protein while leaving the DNA in suspension. The tube was then centrifuged at 1440xg at 4°C for 15 minutes, and the supernatant was transferred into a clean 15 mL conical tube. DNA was precipitated by adding 3ml of isopropanol to the pellet using a sterile transfer pipette; the precipitated DNA was transferred into a clean 15 mL conical tube. The DNA was washed with 3ml of 70% ethanol. The tube was centrifuged at 1440xg and 4°C for 3 minutes, the supernatant was gently decanted, and the tube was inverted for 5 minutes to drain all excess liquid. The DNA pellet was resuspended in DNA Hydration Solution (10 mM Tris, 1 mM EDTA buffer, pH 7.6). The DNA concentration and purity were assessed by measuring the UV absorption of the sample at 260 and 280 nm using 2  $\mu\text{L}$  of the DNA solution on a Nano Drop ND-1000 (Nano Drop,

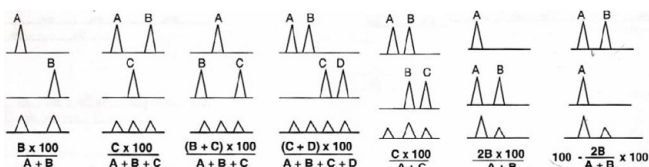
Wilmington, DE, USA) spectrophotometer. DNA was then diluted to a final concentration of 0.1 µg/µL using DNA Hydration Solution.

### PCR Amplification of STR loci

Nine STR loci were amplified under identical PCR conditions. The loci used were D1S1660, D3S2387, D5S1456, D5S818, D7S820, D8S1179, D13S317, D16S539, and D20S481. PCR was performed in a 200 µL PCR tube using the manufacturer's recommended conditions, 4 nM fluorescent primer pair 0.1 µg of DNA, 0.1 U Taq DNA polymerase, 1.5 mM MgCl<sub>2</sub>, 4 nMdNTPs, and PCR buffer (Perkin-Elmer 10X buffer). The PCR conditions were as follows: 94°C for 5 minutes followed by 35 cycles at 95°C for 30 seconds, 57°C for 30 seconds, and 72°C for 45 seconds. The PCR reaction was analyzed on an ABI PRISM 3100 Genetic Analyzer (automated capillary electrophoresis sequencer) according to the manufacturer's recommendations. The fragment lengths were determined by the number of STRs present at each locus for each individual. In a mixture of two or more samples (chimerism) in a post-transplantation event, each CBU contributes a PCR fragment with a unique length.

### DNA chimerism interpretation

Calculation of the percentage of DNA chimerism was done using the area under the curve of the STR amplification peaks of the CBU. These peaks were preferably chosen to be 4-8 or up to 12 base pairs apart to facilitate analysis. Different equations were used, depending on peak patterns, which may vary between homozygous and/or heterozygous states. The following formulas were used:



(Fernandez-Avilés et al, *Leukemia* (2003) 17, 613-620)

### Y Chromosome assay

The Quantifiler Y Human Male DNA Quantification Kit (Applied Biosystems, Inc., cat. no. 4343906) was used to identify the presence of any Y chromosomes in the peripheral blood sample of the recipient at days +14 and +21 post-transplantation. Varying DNA concentrations (0.1, 0.01, 0.001, and 0.0001 µg/µL) were used to verify the sensitivity of the analysis. In a 25µL capillary PCR tube, 2 µL of each DNA sample, at different concentrations; sterile water as a negative control; and

female DNA as a negative DNA control were mixed with 10.5 µL of the Quantifiler Y Human Male Primer Mix, which contained target-specific primers and a FAM dye-labeled probe, and with 12.5 µL of the Quantifiler PCR Reaction Mix, containing dNTPs, buffer, and Ampli Taq Gold DNA polymerase (Applied Biosystems) according to the manufacturer's instructions. Samples were amplified and analyzed on the Roche Light Cycler<sup>®</sup> 2.0 Instrument. After an initial incubation at 95°C for 10 minutes to denature the genomic DNA, 40 cycles were performed using the following profile: denaturation at 95°C for 15 seconds and amplification at 60°C for 1 minute. After completion, an absolute quantification analysis, using a standard curve, was performed using the Roche Light Cycler<sup>®</sup> Software 4.05 (Roche).

### CB pooling

The number of pooled CBUs was based on the sums of the pre-cryopreservation CD34<sup>+</sup> counts, ensuring a minimum of 1 x 10<sup>6</sup> CD34<sup>+</sup> cells/kg of recipient weight. For example, a recipient weighing 80 kg would require a total of ≥ 80 x 10<sup>6</sup> CD34<sup>+</sup> cells before thawing. The pooling method consisted of a semi-closed system using a 600 mL transfer bag (MacoPharma cat: VSE4110XA), one transfer bag containing an equal mixture of Gentran 40 and 5% human albumin, and large tubing (Figure 1). The first cryopreserved CBU was inserted into a sterile plastic bag and thawed in a 37°C water bath for 2 minutes. The unit was pierced, and an equal volume of a dilution solution was slowly infused into the thawed CBU and kept at 4 ± 2°C for a period of at least 5 minutes. The content was then transferred to a 600 mL pooling bag (VES4110XA, Maco Pharma; 4C5628, Fenwal). The unit was rinsed up to three times with the mixed solution of 5% human albumin/Dextran 40 and transferred into the same pooling bag. The second and third units were thawed and diluted using the same method. The 600 mL transfer bag with the pooled CBUs was centrifuged at 600xg at 4°C for 9 minutes. A 600 mL bag was then pierced and joined with the pooling bag in order to expel the supernatant using a Fenwal Optipress blood component extractor up to a final volume of 20 and 30 mL. Each pooled bag of three CBUs was then transferred into another 600-mL bag using 10-in-1 tubing (Maco Pharma, EPE 10177). Forty (40) to 60 mL of phosphate-buffered saline (PBS)/EDTA (Miltenyi Biotech, cat: 700-25), with 0.5% human albumin was used to rinse all pooled bags. A final centrifugation at 600xg at 4°C for 9 minutes was performed, followed by expulsion of the supernatant in the Optipress to reduce the final pooled CBU bag to a volume of 95 ± 5 mL.

### CD34 Selection

The final pooled CBU bag was injected with 100 mL of air, followed

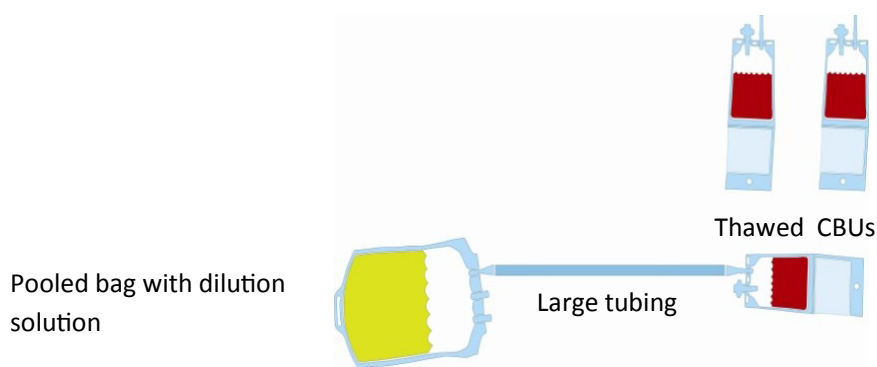


Figure 1: Pooling Method.



by the slow addition of the 7.5 mL of Clini MACS CD34 Reagent (Miltenyi Biotech cat: 171-01), drawn from the vials using a 10-mL syringe with a 16 G needle. The bag was placed on a rocking platform for 45 minutes at room temperature, and turned twice to enhance binding of the magnetic antibodies to CD34. A final wash and centrifugation at 600xg at 4°C for 9 minutes was performed after adding 400 mL of PBS/EDTA with 0.5% human albumin. The supernatant was expelled on the Optipress to obtain a final volume of  $\leq$  300 mL. A Cell Separation Column, placed in the Clini MACS Magnetic Separation Unit, was used according to the manufacturer's instructions to isolate the magnetic antibody-bound CD34-expressing cells contained in the 300 mL bag. The positive volume fraction of 40 mL was transferred by the Clini MACS into a 150 mL transfer bag. At the end of the selection, the 150 mL transfer bag containing the CD34<sup>+</sup> cells was taken from the equipment and transferred to the biological safety cabinet, where a 0.5 mL sample was taken for CFU assay, CBC, CD34, CD45, and CD3 count analysis, and 7AAD viability assessment. The negative fraction was verified for its content of CD34<sup>+</sup> cells by the retrieval of a 2 mL sample after the insertion, of a sampling site coupler. The negative fraction bag was then centrifuged at 600xg at 4°C for 9 minutes, which was followed by the expulsion of the supernatant using the Optipress to concentrate the leucocytes to a volume varying between 30 and 40 mL. Samples taken for analysis included 10 mL for bacterial and fungal cultures, 5 mL for DNA extraction (for STR by PCR), and the remainder was preserved as two 10-13 mL samples in 15 mL polystyrene tubes at -80°C.

## Statistical Analysis

Descriptive statistics were used to compare the TNC and CD34<sup>+</sup> cell counts, viability, and cell count recovery. All statistical analyses were conducted using software from the Statistical Package for the Social Sciences (SPSS, Chicago) and with Microsoft Excel version 2010. Probability (p) values of less than 0.05 were considered to be statistically significant. Descriptive statistics were used to compare the TNC and CD34<sup>+</sup> cell counts, viability, and Colony-Forming Units (CFUs) pre- and post-processing. Results are presented as the mean and Standard Deviation (SD). Analyses of the variables following the normal *t*-test distribution were performed with a one-way analysis of variance (ANOVA). In the presence of an asymmetric distribution, non-parametric analyses and correlations were performed. Two independent-sample *t*-tests ensured the comparison of two groups based on one variable with the Mann-Whitney U test. If ANOVA was not applicable, an independent-samples *t*-test was used for categorical variables and the Mann-Whitney U-test was used for continuous variables. The power and analysis of the sample size were performed using the software Power and Precision 4.1 (2011).

## Ethics

The research protocol was approved by the Research Ethics Board of the McGill University Health Center (GEN#06-018). Informed consent for the donation of CBUs was obtained from mothers before the onset of active labor by obstetrical nurses with the understanding that units would not be reserved for family use and would be de-identified once selected for use. General transplant and study-specific consent was obtained from eligible transplant candidates by a transplant physician.

## Results

### Transplant recipients

Seven patients received 4/6 or 5/6 HLA-matched CBUs. HLA Class I, A, and B loci were analyzed using a low-resolution molecular typing

method, and Class II HLA loci were analyzed using high-resolution molecular typing for DR $\beta$ 1. The HLA typing was performed by an external laboratory. All patients had high-risk disease with no related or unrelated HLA-compatible adult donors available. The CBUs that were available were too small for transplantation as a single CBU.

Patient #1 was a 41-year-old, CMV-positive male with blood type A weighing 81 kg. He was diagnosed in 2007 with an unclassifiable CD5-negative B-cell lymphoproliferative disorder that was initially morphologically similar to CLL. His disease course included successive Partial Remissions (PRs) of short duration after multiple treatment regimens, including rituximab with cyclophosphamide, vincristine and prednisolone (R-CVP), fludarabine, clophosphamide and rituximab (FCR), and high-dose etoposide/cyclophosphamide (VP16/CTX), with late transformation (Richter's Syndrome). The patient had progressive stage IV disease at the time of allogeneic stem cell transplantation.

Patient #2 was a 32-year-old, CMV-positive male with blood type B who weighed 56 kg. He was diagnosed in 2009 with Philadelphia chromosome-positive chronic myeloid leukemia (CML) in myeloid blast crisis, presenting with extramedullary chloromas. He was initially treated with imatinib mesylate and later switched to nilotinib due to intolerance. He was treated with the "7+3" regimen combined with nilotinib. Six months later he required salvage treatment with FLAG-Ida for disease progression without evidence of an Abl kinase domain mutation while on nilotinib. Each course of chemotherapy was followed by more than 2 months of pancytopenia, which was complicated by aspergillus infection. At the time of transplantation, the patient was incomplete cytogenetic remission with no evidence of extramedullary disease or active aspergillus infection after treatment, but he had rising BCR/ABL expression measured by RQ-PCR.

Patient #3 was a 30-year-old, CMV-positive female with blood type O who weighed 88 kg. She was initially diagnosed with Acute Promyelocytic Leukemia (APL) in 2007, and was treated with combination chemotherapy and all-trans-retinoic acid. Two years after achieving a CR she developed a therapy-related AML with a non t(15;17) karyotype and without retinoic acid receptor alpha locus rearrangement. At the time of transplantation, she had a near aplastic bone marrow with no evidence of leukemia, following three treatments with high-dose cytarabine (HiDAC) and etoposide (VP16)/arsenic trioxide.

Patient #4 was a 57-year-old, CMV-positive female with blood type O who weighed 72 kg. This patient was diagnosed with high-risk Philadelphia-chromosome negative (Ph-negative) B-acute lymphoblastic leukemia (ALL) in 2010 (high WBC count, high-risk cytogenetics). The patient was transplanted in first complete remission (CR1) after treatment with the Dana Farber Cancer Institute (DFCI) ALL 4 regimen.

Patient #5 was a 46-year-old, CMV-positive male with blood type O who weighed 76 kg. The patient was diagnosed with B-cell Small Cell Lymphoma (SLL) in 2007 and achieved a CR1 after treatment with FCR. He relapsed with bulky multi-site extramedullary disease in 2010. The patient was transplanted in second complete remission (CR2) following treatment with Rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP).

Patient #6 was a 37-year-old, CMV-positive female with blood type B who weighed 48 kg. The patient was diagnosed with T-cell acute lymphoblastic leukemia in 2011. Twenty-eight days post-induction, she had persistent lymphoblasts (8%). She achieved a CR1 after treatment with 6-MP and methotrexate. The patient went on protocol after failure

to find an unrelated donor after 6 months.

Patient #7 was a 53-year-old, CMV-positive male with blood type B who weighed 143 kg. He was diagnosed in 2011 with Ph-positive CML. He was initially treated with imatinibmesylate and was then switched to dasatinib after developing resistance with cytogenetic relapse and transformation to CML myeloid blast crisis. He later developed resistance to dasatinib with presence of a T315I Abelson kinase domain mutation. The recipient also suffered from much comorbidity including ischemic heart disease, respiratory syncytial virus, and morbid obesity.

### Clinical grafts

The selection of CBUs for pooling was restricted to those that were ABO blood group compatible with the matched CBU. Additionally, maternal-fetal ABO compatibility was maintained between the pooled CBUs to minimize potential interference by passively transferred maternal anti-A and anti-B IgG in the pool. The clinical-grade CBUs rejected by the public bank contained between  $0.20 \times 10^6$  and  $28.4 \times 10^6$  CD34<sup>+</sup> cells. The weight of the recipients varied between 48 and 143 kg, requiring a pool of up to 45 cryopreserved CBUs to arrive at a pre-thaw cell dose of  $1 \times 10^6$  CD34<sup>+</sup> cells/kg recipient weights per protocol. The pooled cords contributed between 25 and 54% of the total CD34<sup>+</sup> cells infused into the recipients. The percentage of each CBU within the pool ranged from 0.01% to a maximum of 8% of all CD34<sup>+</sup> cells administered to the recipient.

### Day of transplantation

The seven recipients of this pilot study were 30-57 years of age with a median weight of 76 (48-143) kg. Four recipients were ABO blood type-incompatible with their transplanted  $\geq 4/6$  HLA-matched CBU. Two patients had a single HLA class I A locus mismatch, four had both an HLA class I A and B locus mismatch, and only one patient had an HLA class II DR mismatch. On the day of transplantation the matched CBUs were infused first, followed by the pooled CBUs within an average of 66.6 minutes. Up to 45 CBUs of different volumes and different TNC and CD34<sup>+</sup> counts were pooled to create the “third party unit”, containing  $\geq 1 \times 10^6$  CD34<sup>+</sup> cells/kg of recipient weight before thawing. As shown in Table 1, the average number of CD34<sup>+</sup> cells contained in the matched CBU was  $1.2 \times 10^5$ /kg, and the “third party unit” derived from the pooled CBUs contained a mean of  $2.5 \times 10^5$ /kg for a total of  $3.7 \times 10^5$  CD34<sup>+</sup> cells/kg.

### Engraftment and chimerism

The median time to neutrophil recovery (ANC  $\geq 0.5 \times 10^9$ /L) was 19.5 (15-29) days post-infusion for all recipients except one, who had primary graft failure (Figure 2). The median time to platelet recovery ( $\geq 20 \times 10^9$ /L) was 53 (29-175) days. One patient had platelet engraftment delayed until day +175, which was probably secondary to a concurrent CMV infection and treatment with gancyclovir. The median time to platelet recovery of the other four recipients was 48 days. On day +14, DNA chimerism analysis revealed that 100% of the CD3<sup>+</sup> and CD33<sup>+</sup> cells derived from the matched CBU donor chimerism, including the cells in the patient who had primary graft

Pt #	Diagnosis	Age (Sex)	Weight (kg)	CMV status		Patient ABO/Match CBU ABO	Ethnicity	HLA Locus Mismatch	TNC 7 (x10 / kg) Matched CBU	CD34 (x105 /kg) Matched + 3rd Party	Total CD34 cells infused (x105/kg)	3 rd Party CD3 (x103/kg)	# HLA MM	# Pooled CBUs	Pooled CBUs Ethnicity	Infusion Delay between matched and pooled CBUs	Time To ANC $\geq 0.5 \times 10^9$ /L (days)	Time to Platelet $> 20,000 \times 10^9$ /L (days)
1	B-LPD	41 (M)	81	pos	pos	A/O	Arabic	A and B	2.9	1.1 + 3.0	4.1	8.6	2	45	33 Cauc; 2 Arabic; 10 other	66 min	15	42
2	CML-BC	32 (M)	56	pos	pos	B/A	Asian	A	2.7	0.7 + 2.0	2.67	1.4	1	30	26 Cauc; 2 Asian; 2 other	96 min	26	175
3	AML	30 (F)	88	pos	pos	O/O	Cauc	A and B	2.6	1.6 + 2.5	4.13	0.4	2	42	23 Cauc; 9 other	60 min	18	ND
4	B-ALL	57 (F)	72	pos	pos	O/O	Cauc	A	2.5	1.3 + 1.9	3.19	3.6	1	39	27 Cauc; 12 other	72 min	21	53
5	B-SLL	45 (M)	76	pos	pos	O/O	African descent	A and B	3	2.1 + 1.7	3.8	2.4	2	42	25 Cauc; 1 African descent; 16 other	74 min	n/a	ND
6	ALL	37 (F)	48	pos	pos	B/A	Pakistani	A and B	4	1.1 + 3.8	4.9	0.03	2	30	18 Cauc; 6 Asian; 4 Arabic; 2 other	18 min	18	29
7	CML-BC	53 (M)	143	pos	pos	B/A	Polish	DrB1	1.7	0.3 + 2.7	3.02	0.27	1	30	22 Cauc; 4 Arabic; 3 Asian; 1 African Descent	80 min	D+29	53
	Mean	42	81	-	-	-	-	-	2.8	1.2 + 2.5	3.69	2.39	-	37	-	66.6 min	21.2	70
	Median	41	76	-	-	-	-	-	2.7	1.1 + 2.5	3.8	1.4	-	39	-	72	19.5	53

ND: not done; MM: mismatch; Cauc: Caucasian; B-LPD = B-lymphoproliferative disorder, CML-BC = chronic myelogenous leukemia in blast crisis, AML = acute myelogenous leukemia, ALL = acute lymphoblastic leukemia, SLL = small cell lymphocytic lymphoma.

Table 1: Summary of Patients, Graft Characteristics, and Engraftment.

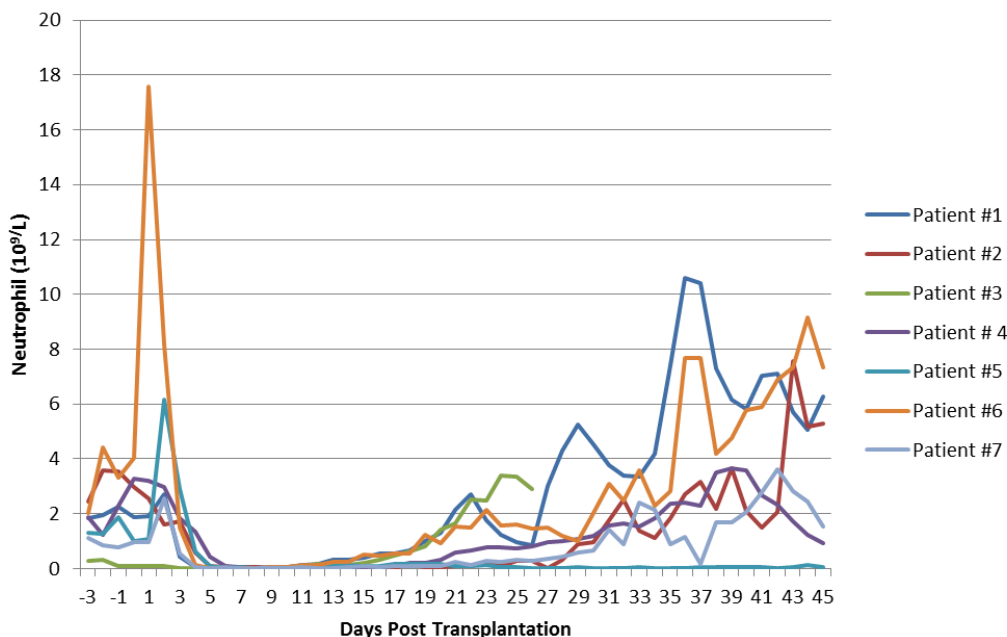


Figure 2: Recipients' Neutrophil Recovery Post-Transplantation.

Pt #	Diagnostic	Age (Sex)	Weight (kg)	DNA Chimerism (CD3 and CD33 subpopulations) % of Matched CBU						GVHD	Length of Hospital Stay	Overall Survival Days post-transplant			
				14	21	28	56	100	At death			100	6/19/2014		
1	B-LPD	41 (M)	81	CD3	100%	100%	100%	100%	90%	100%	Grade III (S/GI/K)	94	Alive	Died +360	N/A
				CD33	100%	100%	100%	100%	96%	100%					
2	CML-BC	32 (M)	56	CD3	100%	100%	100%	100%	100%		Grade II (S/GI)	99	Alive	N/A	Alive +1521
				CD33	100%	100%	100%	100%	100%						
3	AML	30 (F)	88	CD3	100%	100%	100%	N/A	N/A	100%	Grade I (S)	27	Died +28	Died +28	N/A
				CD33	100%	100%	100%	N/A	N/A	100%					
4	B-ALL	57 (F)	72	CD3	100%	100%	100%	100%	100%		Grade II (S/GI)	57	Alive	N/A	Alive +1081
				CD33	100%	100%	100%	100%	100%						
5	B-SLL	45 (M)	76	CD3	94%	35.50%	0%	N/A	N/A		N/A	58	Alive	N/A	Alive +975
				CD33	100%	75%	0%	N/A	N/A						
6	ALL	37 (F)	48	CD3	100%	100%	100%	100%	100%		Grade I (S)	47	Alive	N/A	Alive +597
				CD33	100%	100%	100%	100%	100%						
7	CML-BC	53 (M)	143	CD3	100%	100%	100%	100%	N/A		Grade II (S)	64	Alive	N/A	Alive +507
				CD33	100%	100%	100%	100%	N/A						
	Mean	42	81	-	-	-	-	-	-	-	-	64	-	-	-
	Median	41	76	-	-	-	-	-	-	-	-	58	-	-	-

S = Skin; GI = gastrointestinal; L = Liver; K = Kidney; N/A = Not applicable

B-LPD = B-lymphoproliferative disorder, CML-BC = chronic myelogenous leukemia in blast crisis, AML = acute myelogenous leukemia, ALL = acute lymphoblastic leukemia, SLL = small cell lymphocytic lymphoma.

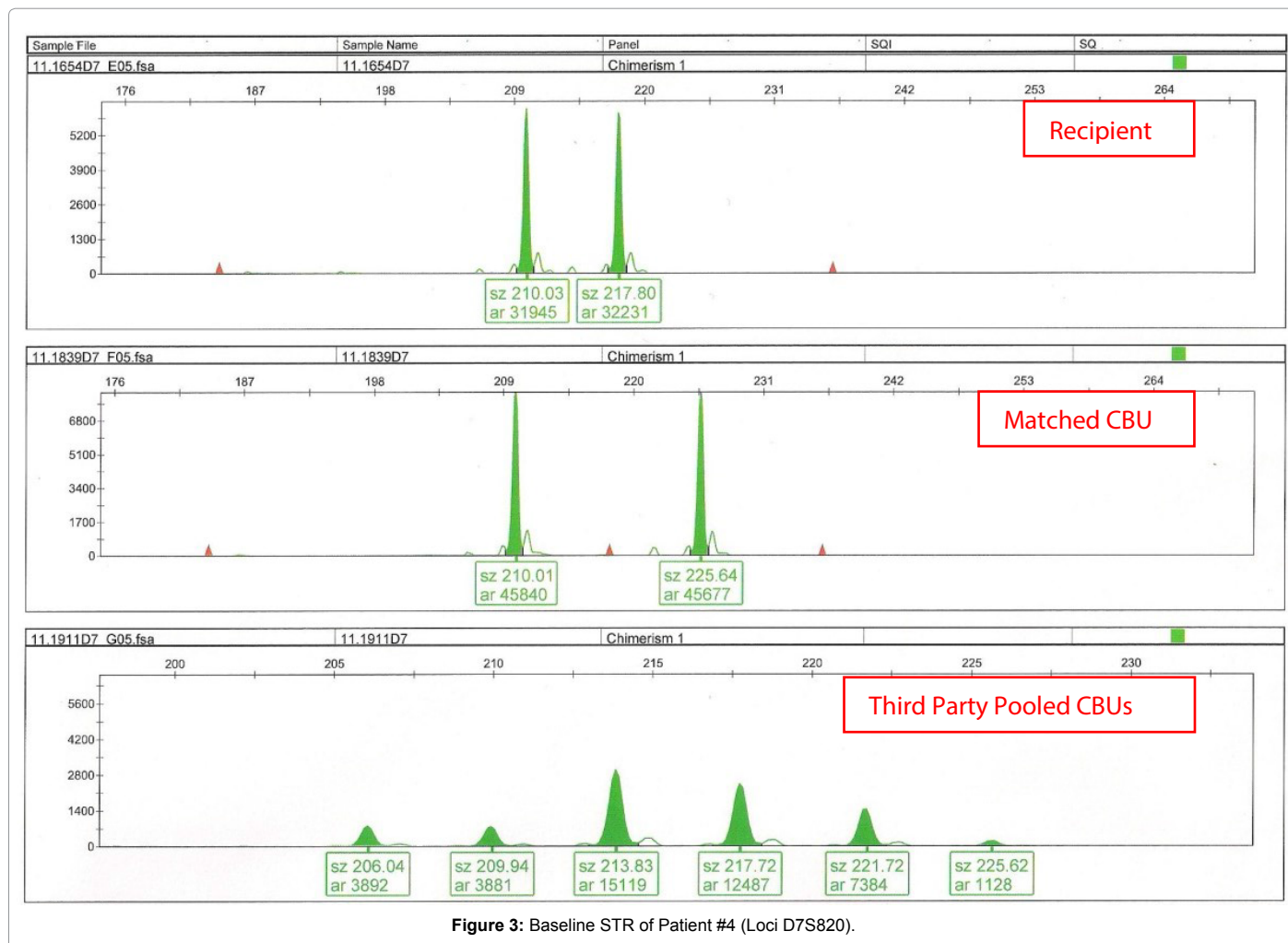
Table 2: Summary of Transplant Outcomes.

failure (Table 2). Only two recipients had a reduced matched CBU donor chimerism in the first 100 days post-transplantation. The first transplanted recipient had a matched CBU/recipient chimerism at day +100 of 90%, which returned to 100% in the subsequent weeks. In contrast, patient #5 never achieved a 100% matched CBU chimerism. The CD3 and CD33 chimerism of the matched CBU were both found at a level of only 35.5% on day +21, suggesting primary graft failure, which was later confirmed on day +40. Given the apparent absence of bridging engraftment by 3<sup>rd</sup> party CBUs by STR analysis (Figure 3), a more sensitive method for detecting the engraftment of these units was performed. One patient (recipient #4) was a female who had received

a female matched CBU with pooled CBUs composed of 21 male and 18 female donors. Real-time PCR of Y chromosome sequences (Table 3) from day +14 and day +21 peripheral blood samples failed to detect pooled CBUs indicating that these either failed to engraft or were present at levels below the sensitivity of Y-chromosome RT-PCR.

### Patient outcomes

Five of the seven transplanted patients are still alive at the time of writing. Four are off immunosuppression, and only one patient has relapsed post-transplantation. Patient #1 died on day +360 from multi-organ failure following months of antiviral therapy for



reactivation of resistant CMV. He was clinically in complete remission at the time of death. Patient #3, who had refractory therapy-related AML, had an acute relapse after day +14 and died on day +28 from disease progression. Patient #5 who had primary graft failure, was later salvaged by double-matched cord blood transplantation and remains in complete remission. Five patients developed transient cutaneous grade I/II acute graft-versus host disease (aGVHD), while one patient (patient #1) developed multi-organ grade III aGVHD (skin, kidney and gut) that responded promptly to steroid therapy. To date, none of the patients have developed chronic GVHD. There were no transplant-related complications or negative patient outcomes that were felt to be related to the third-party cells infused into this group of very high-risk patients.

## Discussion

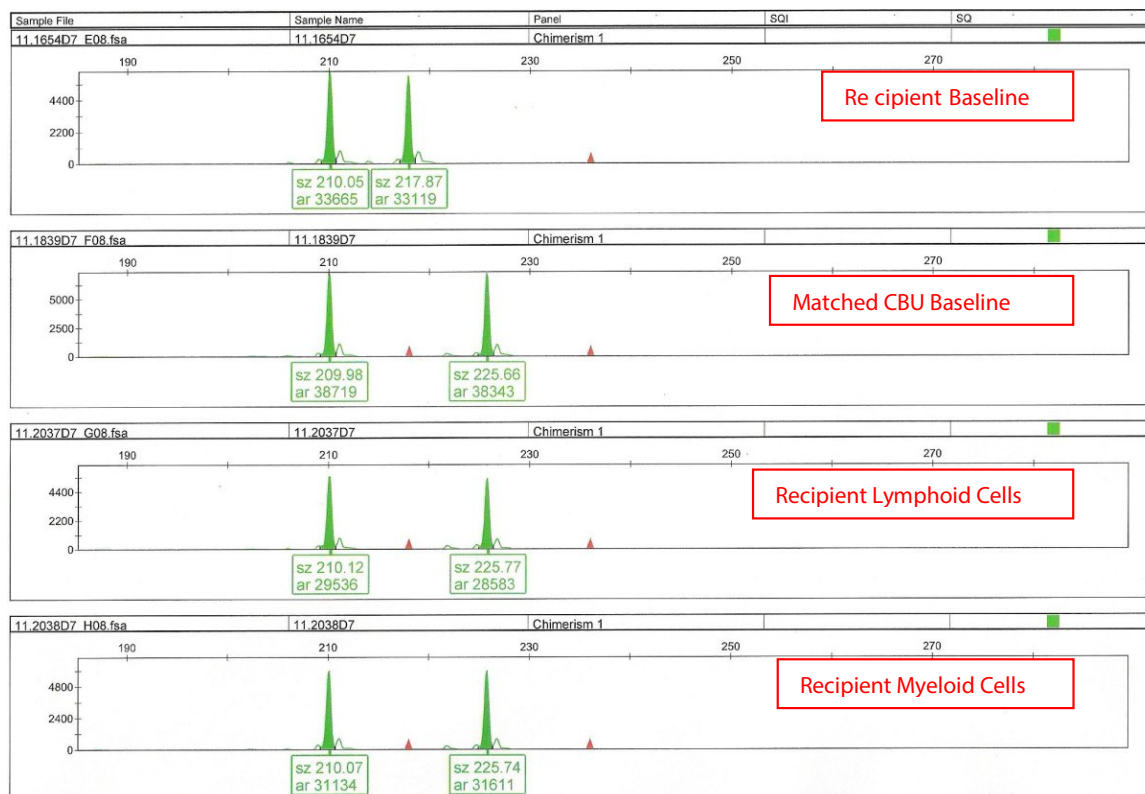
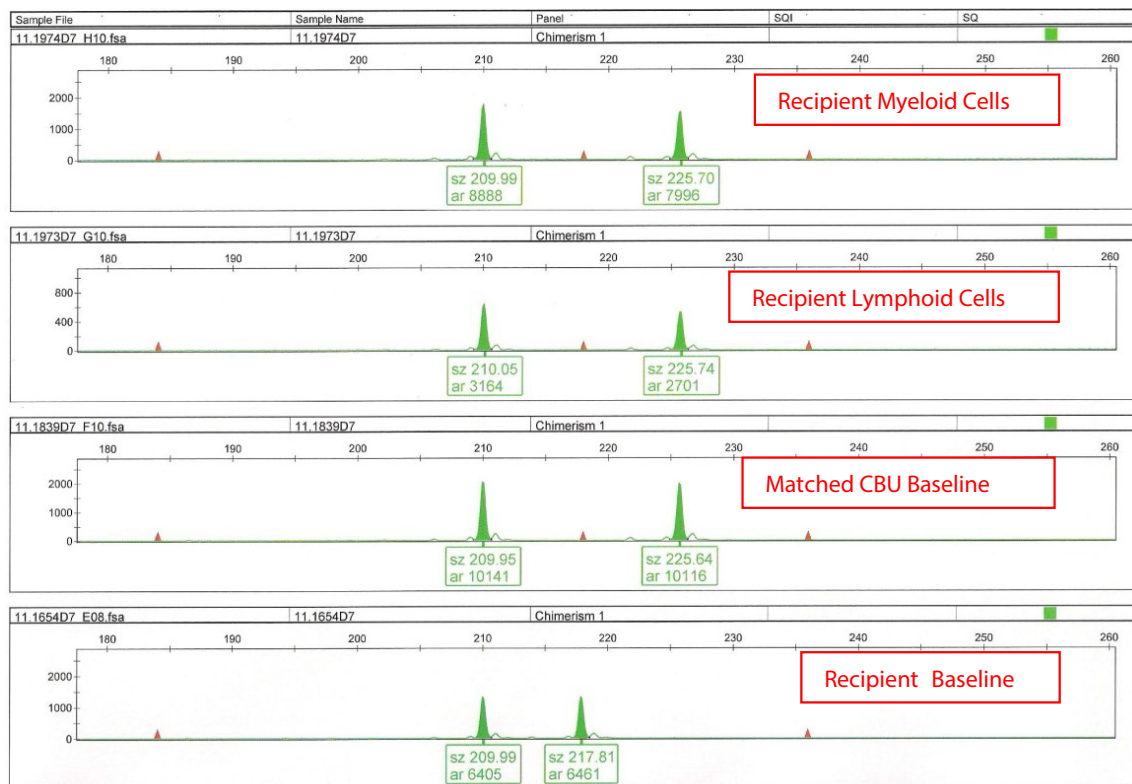
Hematopoietic Stem Cell (HSC) transplantation is an accepted treatment for life-threatening hematological diseases. The principal HSC sources for the adult population are bone marrow and peripheral blood. However, only 25 to 30% of recipients have a compatible related donor, and approximately 65% will find an unrelated adult donor through the international registry [17,18]. In the absence of a suitably matched adult donor, alternative graft sources are cord blood stem cells or a related haplo-identical donor. Cord blood is rapidly accessible and may allow for reduced HLA compatibility. However, one constraining

factor persists, namely, the limited number of nucleated cells present in a single CBU, which is necessary for ensuring engraftment, especially for larger recipients.

The most frequently used method to overcome this limitation is the infusion of two CBUs each containing a minimum of  $1.5 \times 10^7$  NCs/kg of recipient weight (i.e. double cord transplant or dCBUs) [5,10]. Using this method, a mean neutrophil recovery at 21 days for 93% of the recipients was obtained by the University of Minnesota with a relapse rate lower than that associated with single CBU transplantation [19]. The selection of a smaller CBU increases the probability of finding a second  $\geq 4/6$  HLA-matched CBU in 75% of the searches. Despite this increase, 25% of the launched searches fail to find donors due to the difficulty of finding two CBUs with the minimum TNC required that are HLA 4-6/6-compatible with the recipient [2]. The cost of such a method can also be considered a limitation in many countries, raising it from \$35,000 (USD) for a single CBU to \$70,000 for dCBUs.

As an alternative to using a second CBU to increase the number of CD34<sup>+</sup> cells, co-transplantation of a  $\geq 4/6$  HLA-matched CBU with a third-party unit created by CD34<sup>+</sup> selection from a HLA haplo-identical apheresis-related donor has been shown to enhance engraftment [20]. Similar to dCBUs, the matched CBU must contain a minimum TNC count of  $1.5 \times 10^7$ /kg of the recipient weight. Fernandez, the first to use this method, selected matched CBUs with a mean TNC of  $2.39 \times$







Position	Name	CP*	Position	Name	CP*
1	Neg Control Water	26.91	15	Day +21	0.01
2	Neg Control Water	26.89	16	Day +21	0.01
3	Neg Control Female	27.05	17	Pos Control Male 0.001	29.28
4	Neg Control Female	27.22	18	Pos Control Male 0.001	29.37
5	Pos Control Male 0.1	24.18	19	Day +14	0.001
6	Pos Control Male 0.1	24.53	20	Day +14	0.001
7	Day +14	0.1	21	Day +21	0.001
8	Day +14	0.1	22	Day +21	0.001
9	Day +21	0.1	23	Pos Control Male 0.0001	> 35
10	Day +21	0.1	24	Pos Control Male 0.0001	> 35
11	Pos Control Male 0.01	27.41	25	Day +14	0.0001
12	Pos Control Male 0.01	27.21	26	Day +14	0.0001
13	Day +14	0.01	27	Day +21	0.0001
14	Day +14	0.01	28	Day +21	0.0001

\*CP: crossing point

**Table 3:** Absolute Quantification of the Y Chromosome on Days +14 and +21 with Regard to the Chimerism of Recipient #4.

$10^7$ /kg (range 1.14-4.3) and CD34 of  $1.1 \times 10^5$ /kg (range 0.35-3.7) of recipient weight [20]. The infused third-party unit contained a mean number of  $24 \times 10^5$ /kg (10.5-33.4) CD34<sup>+</sup> cells and a reduced number of CD3<sup>+</sup> cells of  $2.4 \times 10^3$ /kg (0.6-15.6) [14]. The neutrophil engraftment time and Overall Survival (OS) were similar to those seen with dCBUs transplantation. Although this method is promising, the availability of a related haplotype donor remains an issue. According to the 2011 Canadian Census, the number of siblings per family was only 1.5 [21]. In addition, the increasingly older age of transplant recipients and an aging populace reduces the probability of finding a healthy parental donor younger than 60 years of age.

The common factor in all of these methods is the supplementation of a single  $\geq 4/6$  HLA-compatible CBU with an additional unit with equal or lower HLA-compatibility, containing some nucleated and/or CD34<sup>+</sup> cells, to enhance neutrophil engraftment. We investigated another method of achieving this by using clinical-grade rejected CBUs of unknown HLA type. This pilot study demonstrates the feasibility and safety of performing CD34<sup>+</sup> selection from multiple, clinical-grade rejected, HLA-blind CBUs that were pooled post-thawing to create a supportive third-party unit.

Seven patients, aged 32 to 57 years old (mean of 42 years), with different hematological malignancies were transplanted in this pilot

study between 2009 and 2013 at the McGill University Health Centre. All recipients received an initial  $\geq 4/6$  HLA-matched CBU containing a mean of  $2.8 \times 10^7$  ( $1.7 - 4 \times 10^7$ ) TNC/kg of the recipient, followed by infusion of a newly developed third-party unit containing a mean of  $2.5 \times 10^5$  ( $1.7-3.0 \times 10^5$ ) CD34<sup>+</sup>/kg. The creation of this third-party unit required thawing, pooling, and CD34<sup>+</sup> selection of a median of 39 (30-45) small clinical-grade rejected CBUs. Median times to neutrophil and platelet engraftment were 19.5 days (15-29) and 53 days (29-175) respectively, similar to those reported with dCBUs and third-party haplo-related transplantation. One patient had a longer platelet engraftment time (day +175), which was attributed to early reactivation of CMV and treatment with ganciclovir. One patient, who relapsed and died on day +28, had 100% matched donor chimerism on days +14 and +21 (patient #3). A second patient failed to engraft (patient #5). The neutrophil count of this recipient transiently increased to  $0.38 \times 10^9$ /L on day +20 before declining. He was later successfully salvaged on day +71 with two  $\geq 4/6$  HLA-matched CBUs and had neutrophil engraftment on day +35 with a single unit.

Recently, Rocha (2010) demonstrated that a high CD34<sup>+</sup> cell count post-thaw can improve engraftment depending on the transplant indication [22]. A CD34<sup>+</sup> cell count of  $\geq 1.7 \times 10^5$ /kg was recommended post-thaw when recipients had a malignancy, and a count of  $\geq 2-2.5 \times 10^5$ /kg was

recommended for non-malignant disorders. In our study, all recipients received an average of  $2.5 \times 10^5$  CD34<sup>+</sup> cells/kg from the third-party unit, to which was added a mean of  $1.1 \times 10^5$  CD34<sup>+</sup> cells/kg pre-thaw from the matched CBU. This infusion of a large number of CD34<sup>+</sup> cells (mean  $3.6 \times 10^5$ /kg) may be the most important determinant of the early neutrophil engraftment that we obtained, one week earlier than typically reported after single CBU transplantation [19]. Only one patient did not show evidence of myeloid or lymphoid engraftment by day +40 and salvaged 71 days after the first transplantation using dCBUs and engrafted.

In our study, the matched CBU was infused before the pooled third-party unit. Using this infusion sequence we achieved full matched CBU chimerism on day +14 in all engrafted recipients, which was possibly due to the sequence of cell infusions. According to Haspel, the first unit infused in dCBUs transplants was favored to achieve long-term engraftment in 76% of cases [23]. This bias in favor of the first infused unit appears to occur when infusion of the second unit is sufficiently delayed to enable engraftment of the first. Quiescent stem cells in G<sub>0</sub>/G<sub>1</sub> preferentially adhere to the bone marrow vascular endothelium within 5 minutes post-infusion and undergo transendothelial migration beginning about 20 to 40 minutes after the initiation of stem cell rolling, continuing for up to 3 hours [24,25]. In our cohort, the third-party unit was infused after 66.6 minutes on average (range 18-96 minutes). A greater CD3<sup>+</sup> cell dose and CD34<sup>+</sup> cell viability are also determinants of the winning unit when transplanting dCBUs [6,26]. The pooled CD34<sup>+</sup>-selected third-party unit in our study contained a total of only  $2.39 \times 10^3$  CD3<sup>+</sup> cells/kg, and the number of CD3<sup>+</sup> cells originating from individual units in the pool was even lower by an order of magnitude. The significantly delayed infusion of the third party units and their low CD3<sup>+</sup> content compared to the matched CBU may have contributed to the 100% day +14 matched CBU chimerism that we observed [6,27]. The extent of this bias in favor of the matched unit was greater than reported for dCBUs and third-party haplo-donor transplants where transient mixed graft chimerism is generally observed in the early stages of engraftment. The sensitivity of STR analysis to detect third-party cells in our study may have been reduced and below the limit of detection given that each unit of the pool represented a relatively small fraction of the total cells infused. We were able to show however that in the one female patient who received a female matched CBU, no third party cells could be detected on day +14 and +21 by Y-chromosome PCR, suggesting that preferential engraftment of the matched CBU may be particularly rapid in our protocol.

An important feature of this study was the utilization of pooled HLA-blinded CBUs to support the HLA  $\geq$  4/6 matched CBU. The possibility of finding one or several units within the pooled CBUs that have a  $\geq$  4/6 HLA compatibility with the recipient is extremely unlikely, considering the number of alleles in HLA classes I A, B, and C, which varies from 1788 to 2934 per class, class II DR $\beta$ 1 (1418 alleles), and DQ (50-323 alleles) (HLA Nomenclature website, 2013). A study performed in 1899 unrelated American-European individuals showed that the DRB1-DQA1-DQB1 haplotypes were shared in less than 1% of these individuals [28]. This low probability of HLA compatibility is further decreased by the presence of non-Caucasian units in the pooled CBUs. The low probability of compatibility and the very low cell dose of each pooled cell unit should translate into a very low chance of third-party cell engraftment. Rare exceptions could occur, as reported by Lister (2007) [29], who described long-term engraftment of a cord unit with a single compatible HLA locus (HLA B) in a study of non-CD34<sup>+</sup>-selected pooled cord unit transplantation.

Acute GVHD is less likely to occur and less severe after the transplantation of mismatched cord blood than after the transplantation of mismatched bone marrow [30,31]. However, the rate of acute GVHD was higher among recipients receiving dCBUs and haplo-related third-party grafts compared with recipients receiving a single unit or the co-transplantation of CBUs [2,20]. All engrafted recipients in our study developed acute GVHD (2 grade I, 3 grade II, 1 grade III) that responded promptly to corticosteroid therapy and no patients developed chronic GVHD. The T cells contained in the  $\geq$  4/6 HLA-matched CBUs may have eliminated the residual host T cells, and the depletion of CD3<sup>+</sup> cells in the pooled unit may have contributed to limiting acute GVHD [32-35]. It is thought that the Graft Versus Leukemia (GVL) effect may be enhanced in dual cord transplants with both units potentially contributing to the overall effect. Even though all 7 of our patients had advanced disease, a high risk of relapse, and low expected survival, only one patient with refractory AML relapsed quickly after engraftment. This may indicate that the third-party units contribute to the GVL effect despite CD3<sup>+</sup> cell depletion.

Allogeneic transplantation is a costly procedure with the source of donor cells independently contributing to differences in cost-effectiveness. The cost of cord blood transplantation is generally greater than that of bone marrow or peripheral blood stem cell transplantation [36,37]. We are not aware of any study that examines the cost-effectiveness of different types of cord blood transplants though such a consideration could affect the decision to perform dCBUs transplantation, CD34<sup>+</sup>-selected third-party haplo-related transplantation, or the novel third-party pooled cord method outlined in this pilot study. Although our series is small, the engraftment time, low incidence of acute GVHD, and the median length of hospital stay observed in this pilot study are comparable to those reported for dCBUs. The cost of a 40 unit pooled third-party graft was approximately \$24,000, including \$350/unit for processing, freezing, serological/mycological/microbiological testing, and \$10,000 for pooling and CD34<sup>+</sup>-selection. This compares favorably with the purchase of two CBUs at a cost of \$35,000 each for a total of \$70,000 (USD). Public cord blood banking is an inefficient and expensive endeavor in part due to the cost associated with the collection of units, the majority of which are discarded owing to insufficient cell content, the need to maintain large inventories, and the low annual turn-over of stored units. Using our approach, some of these costs could be recovered and result in significant savings.

The preliminary results of this pilot study suggest that the use of HLA-blind, clinical-grade rejected pooled cord blood CD34<sup>+</sup>-selected third party cells for allogeneic cord blood stem cell transplantation is feasible, safe and may result in time to neutrophil engraftment comparable to dCBUs transplants. These results need to be confirmed in a larger series of patients and the preparation of pooled CBUs further simplified. Our approach could allow some patients to undergo a transplant who would otherwise not be eligible due to lack of a suitable graft.

## Funding

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## References

1. Barker JN, Byam C, Scaradavou A (2011) How I treat: the selection and acquisition of unrelated cord blood grafts. *below Blood* 117: 2332-2339.
2. Stanevsky A, Shimoni A, Yerushalmi R, Nagler A (2010) Double umbilical cord blood transplant: more than a cell dose? *below Leuk Lymphoma* 51: 975-982.

3. Querol S, Mufti GJ, Marsh SG, Pagliuca A, Little AM, et al. (2009) Cord blood stem cells for hematopoietic stem cell transplantation in the UK: how big should the bank be? *belowHaematologica* 94: 536-541.
4. Barker JN, Byam CE, Kernan NA, Lee SS, Hawke RM, et al. (2010) Availability of cord blood extends allogeneic hematopoietic stem cell transplant access to racial and ethnic minorities. *belowBiol Blood Marrow Transplant* 16: 1541-1548.
5. Wagner JE (2009) Should double cord blood transplants be the preferred choice when a sibling donor is unavailable? *below Best Pract Res ClinHaematol* 22: 551-555.
6. Avery S, Shi W, Lubin M, Gonzales AM, Heller G, et al. (2011) Influence of infused cell dose and HLA match on engraftment after double-unit cord blood allografts. *below Blood* 117: 3277-3285.
7. Bradstock K, Hertzberg M, Kerridge I, Svennilson J, George B, et al. (2009) Single versus double unrelated umbilical cord blood units for allogeneic transplantation in adults with advanced haematological malignancies: a retrospective comparison of outcomes. *below Intern Med J* 39: 744-751.
8. Ballen KK, Spitzer TR, Yeap BY, McAfee S, Dey BR, et al. (2007) Double unrelated reduced-intensity umbilical cord blood transplantation in adults. *belowBiol Blood Marrow Transplant* 13: 82-89.
9. Brunstein CG (2011) Umbilical cord blood transplantation for the treatment of hematologic malignancies. *below Cancer Control* 18: 222-236.
10. Brunstein CG, Gutman JA, Weisdorf DJ, Woolfrey AE, Defor TE, et al. (2010) Allogeneic hematopoietic cell transplantation for hematologic malignancy: relative risks and benefits of double umbilical cord blood. *below Blood* 116: 4693-4699.
11. Fernández MN, Regidor C, Cabrera R, García-Marco J, Briz M, et al. (2001) Cord blood transplants: early recovery of neutrophils from co-transplanted sibling haploidentical progenitor cells and lack of engraftment of cultured cord blood cells, as ascertained by analysis of DNA polymorphisms. *below Bone Marrow Transplant* 28: 355-363.
12. Fernández MN, Regidor C, Cabrera R, García-Marco JA, Forés R, et al. (2003) Unrelated umbilical cord blood transplants in adults: Early recovery of neutrophils by supportive co-transplantation of a low number of highly purified peripheral blood CD34+ cells from an HLA-haploidentical donor. *belowExpHematol* 31: 535-544.
13. Andersson BS, Gajewski J, Donato M, Giralt S, Gian V, et al. (2000) Allogeneic stem cell transplantation (BMT) for AML and MDS following i.v.busulfan and cyclophosphamide (i.v.BuCy). *below Bone Marrow Transplant* 25 Suppl 2: S35-38.
14. Fernández MN (2009) Improving the outcome of cord blood transplantation: use of mobilized HSC and other cells from third party donors. *below Br J Haematol* 147: 161-176.
15. Magro E, Regidor C, Cabrera R, Sanjuán I, Forés R, et al. (2006) Early hematopoietic recovery after single unit unrelated cord blood transplantation in adults supported by co-infusion of mobilized stem cells from a third party donor. *belowHaematologica* 91: 640-648.
16. Guidance Document for Cell, Tissue and Organ Establishment - Safety of Human Cells, Tissues and Organs for Transplantation. Minister of Health
17. Zlatev A, Mihaylova A, Baltadjieva D, Ivanova M, Naumova E (2008) Cord blood stem cell transplantation. Why it is necessary to establish a Bulgarian cord blood bank? *below Cell Tissue Bank* 9: 343-346.
18. Cutler C, Ballen KK (2012) Improving outcomes in umbilical cord blood transplantation: state of the art. *below Blood Rev* 26: 241-246.
19. Avery S, Voss MH, Gonzales AM, Lubin M, Castro-Malaspina H, et al. (2012) Importance of day 21 BM chimerism in sustained neutrophil engraftment following double-unit cord blood transplantation. *below Bone Marrow Transplant* 47: 1056-1060.
20. Bautista G, Cabrera JR, Regidor C, Fores R, Garcia-Marco JA, et al. (2009) Cord blood transplants supported by co-infusion of mobilized hematopoietic stem cells from a third-party donor. *Bone Marrow Transplant* 43:365-373.
21. Canadian Census. 2011 Census & National Household Survey. Statistics Canada. 2011. Canadian Government.
22. Rocha V, Broxmeyer HE (2010) New approaches for improving engraftment after cord blood transplantation. *belowBiol Blood Marrow Transplant* 16: S126-132.
23. Haspel RL, Ballen KK (2006) Double cord blood transplants: filling a niche? *below Stem Cell Rev* 2: 81-86.
24. Yaniv I, Stein J, Farkas DL, Askenasy N (2006) The tale of early hematopoietic cell seeding in the bone marrow niche. *below Stem Cells Dev* 15: 4-16.
25. Frimberger AE, Sterling AI, Quesenberry PJ (2001) An in vitro model of hematopoietic stem cell homing demonstrates rapid homing and maintenance of engraftable stem cells. *below Blood* 98: 1012-1018.
26. Newell LF, Milano F, Nicoud IB, Pereira S, Gooley TA, et al. (2012) Early CD3 peripheral blood chimerism predicts the long-term engrafting unit following myeloablative double-cord blood transplantation. *belowBiol Blood Marrow Transplant* 18: 1243-1249.
27. Ciurea SO, Mulanovich V, Jiang Y, Bassett R, Rondon G, et al. (2011) Lymphocyte recovery predicts outcomes in cord blood and T cell-depleted haploidentical stem cell transplantation. *belowBiol Blood Marrow Transplant* 17: 1169-1175.
28. Klitz W, Maiers M, Spellman S, Baxter-Lowe LA, Schmeckpeper B, et al. (2003) New HLA haplotype frequency reference standards: high-resolution and large sample typing of HLA DR-DQ haplotypes in a sample of European Americans. *Tissue Antigens* 62:296-307
29. Lister J, Gryn JF, McQueen KL, Harris DT, Rossetti JM, et al. (2007) Multiple unit HLA-unmatched sex-mismatched umbilical cord blood transplantation for advanced hematological malignancy. *below Stem Cells Dev* 16: 177-186.
30. Hwang WY, Samuel M, Tan D, Koh LP, Lim W, et al. (2007) A meta-analysis of unrelated donor umbilical cord blood transplantation versus unrelated donor bone marrow transplantation in adult and pediatric patients. *belowBiol Blood Marrow Transplant* 13: 444-453.
31. Eapen M, Klein JP, Sanz GF, Spellman S, Ruggeri A (2011) Effect of donor-recipient HLA matching at HLA A, B, C, and DRB1 on outcomes after umbilical-cord blood transplantation for leukaemia and myelodysplastic syndrome: a retrospective analysis. *Lancet Oncol* 2045 :70260-70261
32. Szabolcs P, Park KD, Marti L, Deoliveria D, Lee YA, et al. (2004) Superior depletion of alloreactive T cells from peripheral blood stem cell and umbilical cord blood grafts by the combined use of trimetrexate and interleukin-2 immunotoxin. *belowBiol Blood Marrow Transplant* 10: 772-783.
33. Fernández-Avilés F, Urbano-Ispizua A, Aymerich M, Colomer D, Rovira M, et al. (2003) Serial quantification of lymphoid and myeloid mixed chimerism using multiplex PCR amplification of short tandem repeat-markers predicts graft rejection and relapse, respectively, after allogeneic transplantation of CD34+ selected cells from peripheral blood. *below Leukemia* 17: 613-620.
34. Bordignon C, Keever CA, Small TN, Flomenberg N, Dupont B, et al. (1989) Graft failure after T-cell-depleted human leukocyte antigen identical marrow transplants for leukemia: II. In vitro analyses of host effector mechanisms. *below Blood* 74: 2237-2243.
35. Brown JA, Boussiotis VA (2008) Umbilical cord blood transplantation: basic biology and clinical challenges to immune reconstitution. *belowClinImmunol* 127: 286-297.
36. Costa V, McGregor M, Laneville P, Brophy JM (2007) The cost-effectiveness of stem cell transplantations from unrelated donors in adult patients with acute leukemia. *below Value Health* 10: 247-255.
37. Majhail NS, Mothukuri JM, Brunstein CG, Weisdorf DJ (2009) Costs of hematopoietic cell transplantation: comparison of umbilical cord blood and matched related donor transplantation and the impact of posttransplant complications. *belowBiol Blood Marrow Transplant* 15: 564-573.