Development of Third Party Grafts from Pooled CD34-Selected Cryopreserved Cord Blood Units for Stem Cell Transplantation

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Abstract

Umbilical cord blood (CB) is a valuable alternative source of stem cells for patients who do not have a compatible donor for allogeneic stem cell transplantation (SCT). Unfortunately, few banked cord blood units (CBUs) contain sufficient hematopoietic stem cells (HSC) to transplant adult patients. Different approaches have been used to increase the number of infused HSC including the use of two or three HLA compatible CBUs, co-transplantation with related HLA haploidentical grafts to provide "third party" support, and *ex vivo* cell expansion. These alternative approaches have the disadvantage of increasing the cost of graft procurement or relying on a related haploidentical donor that may not be available.

I have developed an alternative approach that aimed to increase the number of HSC available for transplantation by creating a third-party graft from pooled HLA-blind cryopreserved CBUs enriched for CD34-positive cells by immune-selection. By using only CBUs rejected by the public cord blood bank (based on their low nucleated cell counts, low volume or late registration), the cost of developing this mixed CBUs graft has been minimized. In my hospital, over 60% of all collected CBUs do not qualify for public banking and could potentially be salvaged to create third-party grafts for clinical use.

In order to pursue this project, it was necessary to establish a clinical grade research cord blood bank [the McGill University Health Center Clinical Research Cord Blood Bank (MUHC CRCBB)] to ensure the availability of CBUs for experimental graft

development. This project covered all aspects of cord blood banking from screening potential mothers, to CBU collection, processing, cryopreservation, and release of CBUs for transplantation. From October 2007 to December 2012, a total of 2313 CBUs have been collected. Approximately 38% of the collected CBUs qualified for the Québec public cord blood bank at Héma-Québec and 1418 CBUs were retained in the MUHC CRCBB for research. A small volume (8.6%) of CBUs did not qualified for any banking mainly due to a volume of less than 20 mL, CBUs being more than 96 hours post collection or positive bacteriology/serology testing.

Some of the key accomplishments that permitted the successful creation of mixed cord blood grafts were the development of pooling methods to minimize the toxicity of the cryopreservative dimethyl sulfoxide (DMSO) while simultaneously thawing and combining multiple CBUs. I also observed that passive transfer of anti-A and anti-B immunoglobulin-gamma (IgG) could bind ABO-incompatible fetal leukocytes and potentially interfere with cell yields and function. The quality of mixed cord blood grafts was optimized by dilution of thawed CBUs to reduce DMSO concentration from 10% to 1% and selecting CBUs that had maternal-fetal ABO compatibility. Mixed cord blood grafts using from 12 to 45 CBUs were pooled with a mean nucleated cell (NC) recovery of 87% post-thawing and 73% post-centrifugation with a yield of $CD34^+$ cells, sufficient for third party support in allogeneic cord blood transplantation.

The safety and utility of using mixed cord blood grafts for allogeneic stem cell transplantation has been demonstrated in a clinical trial developed and conducted at the

MUHC. Seven recipients with hematological cancers received, after myeloablative conditioning, a 4/6 or 5/6 HLA compatible CBU (average of 2.8 $x10^7$ NC/Kg; average of 1.2 x 10^5 $CD34^+$/Kg), followed by infusion of the third-party graft containing on average 2.5 x 10^5 $CD34^+$ cell/kg. The median neutrophil engraftment time was 19.5 (15-29) days. One patient had primary graft failure (latter rescued with a double CBUs transplant). All engrafted patients showed a 100% HLA-matched donor chimerism at first assessment on day +14. Six patients were alive at day +100 while one patient died at day +28 of disease relapse. All engrafted recipients developed grade I-III acute graft-versus-host-disease (aGVHD) that responded promptly to treatment and no patients developed chronic GVHD.

These results demonstrate that increasing the number of HSCs by using third-party cells from pooled HLA-blind CBUs to support a ≥ 4/6 HLA compatible CBU is safe, feasible, and results in rapid engraftment. Future studies are warranted to better understand the role of pooled third party units and factors involved in the homing of stem cells to their natural niche to facilitate the selection of units for engraftment.

Abrégé

Le sang du cordon ombilical (SC) est une précieuse source de cellules souches pour les patients n'ayant pas de donneur allogénique pour une transplantation de cellules souches (TCS). Malheureusement, le nombre d'unités de sang de cordon (USC) contenant suffisamment de cellules souches hématopoïétiques (CSH) pour transplanter des patients de taille adulte est limité. Différentes approches ont été utilisées pour augmenter le nombre de cellules souches transplantées, y compris l'utilisation de deux ou trois USCs HLA-compatible, la co-transplantation d'une USC avec une unité nommée « tierce partie » composée de cellules souches provenant d'un donneur apparenté HLA haplo-compatible et l'expansion *in vitro* d'USC. Ces approches comportent par contre l'inconvénient d'augmenter le coût d'acquisition du greffon ou de nécessiter la disponibilité d'un donneur apparenté haplo-compatible limitant ainsi leur application.

J'ai développé une approche alternative de transplantation qui vise à augmenter le nombre de cellules souches hématopoïétiques par la création d'une unité nommée « tierce partie » composée de cellules $CD34^+$ sélectionnées provenant du regroupement d'USCs cryopréservées dont la compatibilité HLA est inconnue. En utilisant uniquement les USCs rejetées par la banque publique de sang de cordon (en fonction de leur faible taux de cellules nucléées, d'un volume insuffisant ou d'une inscription tardive), le coût de développement de ce type de greffon est minimal tout en réduisant le gaspillage des dons de sang de cordon. La banque publique de sang de cordon a rejeté plus de 60%

des USCs prélevées dans notre établissement, malgré que ces dernières puissent potentiellement être utilisées pour créer des unités « tierce partie » à usage clinique.

Le développement d'un tel projet a exigé l'établissement d'une banque de sang de cordon [Centre universitaire de santé McGill – Banque clinico-recherche de sang de cordon (BCRSC–CUSM)] afin d'assurer la disponibilité d'USCs pour des greffes expérimentales. Tous les aspects de la banque de sang de cordon ont été mis en place incluant l'approche des futures mères, la collecte des USCs, la manipulation, la congélation, et la libération d'USCs pour la transplantation. Entre octobre 2007 et décembre 2012, plus de 2313 USCs ont été prélevées. Environ 38% de ces unités se sont qualifiées pour la banque publique québécoise de sang de cordon d'Héma-Québec et 1418 USCs sont demeurées à la BCRSC du CUSM pour la recherche. Une infime quantité d'USCs (8.6%) n'ont pu se qualifier pour aucune des deux banques, principalement dû aux raisons suivantes : un volume de moins de 20 mL, des USCs collectées de plus de 96 heures ou le résultat positif de bactériologie/sérologie.

Parmi les principales réalisations qui ont permis la création d'un mécanisme mixte de greffes de sang de cordon, j'ai développé une méthode regroupant de multiples USCs tout en minimisant la toxicité du cryoprotecteur diméthyle-sulfoxyde (DMSO) après la décongélation. Le transfert passif d'immunoglobuline-gamma (IgG) anti-A et anti-B pouvant se fixer aux leucocytes fœtaux ABO-incompatibles a également été observé, ce qui peut potentiellement interférer avec la récupération des cellules regroupées ainsi qu'avec leurs fonctions. La qualité des greffons d'USCs regroupées a été optimisée par

la dilution des USCs décongelées réduisant la concentration du DMSO de 10% à 1% et par la sélection d'USCs ayant une compatibilité ABO materno-fœtale. Les greffons ont requis le regroupement de 12 à 45 USCs, dont la récupération moyenne de cellules nucléées (CN) a été de 87% après la décongélation et de 73% après la centrifugation. Cette méthode a permis d'obtenir un nombre suffisant de cellules $CD34^+$ en tant que « tierce partie » pour soutenir la transplantation allogénique d'USC.

La sécurité et l'utilisation d'un regroupement d'USCs pour la transplantation allogénique de cellules souches ont été démontrées dans un essai clinique élaboré et mené au CUSM. Sept receveurs ont reçu, après conditionnement myéloablatif, une USC 4/6 ou 5/6 HLA-compatible (moyenne de 2.8 $x10^7$ CN/kg; moyenne de 1.2 x 10^5 $CD34^+$/Kg), suivie d'une infusion d'une unité « tierce partie » contenant en moyenne 2.5 x 10^5 cellules $CD34^+$/kg. Le temps moyen de prise de la greffe des neutrophiles était de 19.5 (15-29) jours. Un seul patient a rejeté le greffon (ce dernier fut sauvé par une double greffe d'USC). Tous les patients greffés ont démontré un chimérisme de 100% du donneur HLA-compatible lors de la première évaluation faite au jour +14. Six patients étaient vivants au 100^e jour, alors qu'un patient est décédé au 28^e jour, d'une rechute aigüe de sa leucémie. Tous les receveurs greffés ont développé une maladie aigüe de la greffe contre l'hôte de grade I à III (aGVHD), ayant répondu rapidement au traitement, et aucun patient n'a développé une GVHD chronique.

Ces résultats démontrent que l'augmentation du nombre de cellules souches hématopoïétiques (CSH) par l'utilisation d'une unité « tierce partie » provenant du

regroupement d'USCs HLA-inconnus pour soutenir une USC ≥ 4/6 HLA compatible est sécuritaire, faisable, et se traduit par une prise rapide de la greffe. Des études futures sont requises dans le but de mieux comprendre le rôle de l'unité « tierce partie » composée d'un regroupement d'USCs ainsi que les facteurs impliqués dans la domiciliation des cellules souches afin de faciliter la sélection des unités pour une greffe.

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Dedications

I dedicate this thesis to my sons, Mathieu and Jean-Pierre for whom I am blessed. They have always been supportive of my numerous projects but especially this long lasting one. They ran to me when I was discouraged, to offer me support, were happy for me when everything was going well and pushed me to obtain my goal. With their unique personalities, they provided me with different insight and astounding youthful wisdom.

I would also like to dedicate this thesis to the courage and the determination of transplant recipients, and to all those who take care of them.

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Abbreviations

7AAD	7-Amino-Actinomycin D
Ab	Antibody
ABO	Blood group
Ag	Antigen
AGM	Aorta-gonad-mesonephros
Ang	Angiopoietin
BFU-E	Burst-forming unit erythrocytes
BM	Bone marrow
CAM	Cell adhesion molecule
CB	Cord blood
CBB	Cord blood bank
CBC	Cell blood count
CBU	Cord blood unit
CCL	Chemotactic ligand
CCR, CXCR	Chemokine receptor
CD	Cluster differentiation
CFC	Colony forming cells
CFU	Colony forming unit
CMR	Central marrow region
CMV	Cytomegalovirus
DMSO	Dymethyl sulfoxide
DPD-A	Citrate/phosphate/dextrose-adenine
EPO	Erythropoietin
ER	Endosteal region
EVA	Ethylene Vinyl Acetate
FBM	Fetal bone marrow
FBS	Fetal bovine serum
Fc	Fragment crystallizable
FGF	Fibroblast growth factors

FITC	Fluorescein isothiocyanate
FLT-3	Fetal liver tyrosine kinase-3
G-CSF	Granulocyte colony-stimulating factor
GEMM	Granulocyte-erythrocyte-macrophage-megakaryocytes
GM	Granulocyte-macrophage
CSF	Colony-stimulating factor
GVHD	Graft-versus-host-disease
HA	Hyaluromic acid
HBSS	Hanks balanced salt solution
HES	Hetastarch
HLA	Human leukocytes antigen
HLDA	Human leukocytes differentiation antigen
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem progenitor cell
HTC	Hematocrit
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPA	Inherited paternal antigen
KIR	Killer immunoglobulin-like receptor
LTC-IC	Long-term culture-initiating cell
LIF	Leukemia inhibitory factor
MCP	Monocyte chemo-attractant protein
M-CSF	Monocytes-colony-stimulating factor
mL	milliliter
mg	milligram
MM	Mismatch
MP	Megakaryocytes progenitor
MSC	Mesenchymal stem cell
MUD	Match unrelated donor

NC	Nucleated cell
NIMA	Non-inherited maternal antigen
NK	Natural Killer
NMDP	National Marrow Donor Program
nRBC	Nucleated red blood cells
Opn	Osteopontin
PB	Peripheral blood
PCR	Polymerase chain reaction
RBC	Red blood cell
RCVRS	Red cell volume reduction system
SCF	Stem cell factor
SDF-1	Stromal-derived factor-1
STR	Short Tandem Repeat
TNC	Total nucleated cell
TNF-α	Tumor necrosis factor-alpha
TPO	Thrombopoietin
TRM	Transplant related mortality
UD	Unrelated donor
uL	microliter
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VLA	Very late antigen
WBC	White blood cell

CHAPTER 1: INTRODUCTION

Hematopoietic stem cells (HSC)

Origin of Stem Cell Development

Human life begins with the fusion of two gametes, sperm and egg, giving rise to an embryonic stem cell. At the top of cell hierarchy, these undifferentiated stem cells are capable of self-renewal and will differentiate into other cells lineages [1]. The differentiation process can be symmetric, where stem cell gives rise to two stem cells with identical properties, or asymmetric, where one daughter cells will retain its properties while the other differentiates [2].

In the early phase of fetal development, totipotent embryonic stem cells will regroup into blastocysts followed by gastrulation phase where three distinct cell lineages will be formed: ectoderm, endoderm and mesoderm. The mesoderm, situated in the middle of the three germ layers, develops into the musculo-skeletal and circulatory systems [3]. The following divisions are crucial for the development of the circulatory system and of specific cells which grow into different blood cells, a process called “fetal hematopoiesis”. For many years, it was believed that in the first trimester of pregnancy, hematopoiesis originated from the yolk sac and hematopoietic stem cell (HSC) would then migrated into the liver and the spleen while the bone marrow environment is developing [4]. A recent study, using long-term repopulation assays, demonstrated that human HSCs first emerge in the intra-aortic cell clusters from the floor of the dorsal aorta

of the aorta-gonad-mesonephros (AGM) region, followed by the yolk sac, the placenta, the liver and the bone marrow [5,6]. After birth, the bone marrow becomes the primary location where HSC will self-renew and begin differentiation into leukocytes, platelets and red blood cells. The spleen and the liver will be used as secondary locations to ensure long-term maintenance of hematopoiesis especially upon stress induce and during increase need of hematopoiesis [7-11].

Identification of Hematopoietic Stem Cells

The three methods most frequently used to qualify and quantify HSC are immuno-phenotyping, *in vitro* and *in vivo* proliferation. Immunophenotyping refers to a technique to identify cells based on their cell-surface antigen expression. Clusters of differentiation (CD) markers are monoclonal antibodies directed against antigen expression present on leukocytes membranes. The phenotypic marker most commonly used for human HSC is CD34, a glycophospho-protein. It has been found on HSC's isolated from umbilical cord blood, fetal liver, fetal bone marrow, adult bone marrow, and mobilized peripheral blood cells [12]. The segregation between a more differentiated lineage of $CD34^+$ cells and an earlier lineage is possible by the identification of other cell surface expressions such as CD38 (ADP-ribosyl cyclase 1) and CD90 (Thy-1)[12-14]. The $CD34^+CD38^+$ population represents committed progenitor cells, and those expressing $CD34^+CD38^-$ remains as multipotiential hematopoietic stem cells [15]. The presence of CD90 (Thy-1) expression is a specific marker of a subpopulation in the differentiation process from CD45RA cells expressed on naïve T cells. It was demonstrated that a $CD34^+CD38^-$ lineage can

subdivide into $CD90^{+}CD45RA^{-}$, $CD90^{-}CD45RA^{-}$ and $CD90^{-}CD45RA^{+}$, indicating that a multipotent progenitor would express $CD34^{+}CD38^{-}CD90^{-}CD45RA^{-}$ [16]. Once the subsets were identified, a search started to identify an earlier differentiation stage. It was found that a long-term negative lineage ($CD34^{+}Lin^{-}$) of pluripotent stem cells would express other markers such as HLA-DR (CD74) and c-kit (CD117), confirming the potential presence of earlier HSC [17].

The search for an earlier stem cell lineage brought some researchers to identify HSC expressing an early $ALDH^{hi}CD133^{+}Lin^{-}$ which, when transplanted were able to maintain a primitive hematopoietic phenotype ($CD34^{+}CD38^{-}$) and were able to repopulate cells for long-term [18,19]. Finally, a recent study in mice identified the phenotype of long-term HSC, and allowed the successful regeneration of all blood cell lineages after transplantation of a single HSC [20]. This single HSC immunophenotype expressed $Lin^{-}CD34^{+}CD38^{-}CD45RA^{-}Thy1^{+}Rho^{lo}CD49f^{+}$, and displayed multi-lineage chimerism 20 weeks after transplantation. These same stem cells can differentiate to a point where the CD34 expression will no longer be detectable [21].

The demonstration of CD34 functionality and renewal potential was made possible through an *in vivo* long-term lineage assay and by immunophenotyping; this is also possible using an *in vitro* assay. A colony-forming cells (CFC) assay measures the differentiation potential and viability of $CD34^{+}$ progenitor cells using a semisolid methylcellulose culture media containing specific growth factors such as erythropoietin (EPO), Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF), Granulocyte-

Colony Stimulating Factor (G-CSF), and Interleukine-3 (IL-3) (Stem Cell Technologies, Methocult® H4034). Studies compared $CD34^+$ cells from cord blood, peripheral blood, fetal bone marrow and adult bone marrow and observed that the clonogenic potential was similar between cord blood, peripheral blood and bone marrow HSC and significantly higher in fetal bone marrow [22]. The highest self-renewal potential was demonstrated by HSC originating from the AGM region, where a single cell generates at least 300 daughter HSCs [6]. An *in vitro* assay used on pre-cryopreservation and post-thaw cord blood samples was used to predict neutrophil and platelet engraftment [23]. HSC proliferation potential can be demonstrated using a long-term culture-initiating cell assay (LTC-IC). To ensure long-term $CD34^+$ cell expansion, co-culture with bone marrow mesenchymal cells (MSC), thrombopoietin (TPO), Fetal Liver Tyrosine Kinase 3 Ligand (Flt3-L), IL-6, IL-3 and stem cell factor (SCF) is required. After 4 weeks of incubation, it has been demonstrated that HSC can expand up to 184 fold ± 40 fold [24]. However, when expanded a significant decrease in the adhesion of the progenitor cells to fibronectin was observed, and this was associated with delayed engraftment in non-obese diabetic/severe immune-deficient mice [25]. Nonetheless, LTC-CFC was used as indicator of allogeneic stem cell transplantation using cord blood and demonstrated that compared to peripheral blood stem cell (PBSC), the number of LTC-CFC per mononucleated cell from cord blood was found to be 2.5 fold greater than those from PBSC [6,26]. Similar research done after cell sorting HSC expressing CD34 and/or CD38

cells from cord blood demonstrated that the cells expressing $CD34^{+}CD38^{-}$ would be ideal for large-scale expansion [27].

Hematopoietic Niche

The hematopoietic niche is a physical anchoring site for stem cells where adhesion molecules and different cells interact with other stem cells and their extracellular matrix to regulate the behaviour of stem cells [28,29]. The trabecular region of the bone is the principal region where the niche of HSCs is located. The central marrow, the periosteal and the endosteal regions, which are rich in vascular and nervous system, contain long-term self-renewing HSC, MSC, adipocytes, fibroblast, stromal cells and a large number of osteoblasts [30,31].

The endosteal region ensures a high HSC viability by offering a relatively hypoxic environment [32]. Its extracellular matrix molecules are osteopontin, hyaluronic acid, collagen type I, fibronectin and collagen type IV. It ensures the maintenance of the niche structure and the microenvironment by keeping the HSC quiescent [33]. The bone-forming osteoblasts are the major component of the HSC niche. Studies demonstrate that osteoblasts secrete osteopontin (Opn) and play a key role in retention of HSC in the endosteal region [34]. Similarly, hyaluronic acid (HA) expressed at the surface of primitive HSC is necessary to ensure their localization at the endosteal region of the niche [8]. Other factors responsible for the inhibition of HSC cell cycle progression are the interaction between Tie2, a tyrosine kinase receptor expressed on endothelial cells and its ligand angiopoietin (Ang-1)[8]. The chemokine CXCL12 (stromal cell-derived factor-1; SDF-1)

expressed by the reticular cells is known for its role in keeping the HSC quiescent in the endosteal surface. SDF-1 also acts as a signal indicating an hypoxic environment in the presence of tissue ischemia [35]. This chemokine is also found in the sinusoid endothelial cells, which suggests that the HSC niche may lie at the interface of the endosteal and perivascular cells [11,11,11,29,36]. An opposite function of osteoblasts is the production of hematopoietic growth factors that will impact the proliferation of HSC [37]. The central marrow region, also called the vascular niche, contains strong vascular and neural networks that favour cell proliferation. It is surrounded with sinusoidal vessels which provide a higher oxygen level contributing to HSC proliferation and differentiation [28].

Regulation of HSC

Together with oxygen, the develop and differentiation of HSCs require not only a specific cellular environment, such as the bone marrow niche, but also specific soluble proteins known as growth factor. Some regulators present in the bone marrow are required to ensure not only the quiescence or differentiation but also self-renewal, homing and migration of HSC [8]. Regulators include stem cell factor (SCF), fibroblast growth factors (FGF), and thrombopoietin (TPO), which ensure long term reconstitution potential and anchoring, are found in all niche regions [9,38]. Cytokines, including G-CSF, GM-CSF, macrophage (M)-CSF, IL-1, IL-6, IL-7, leukemia inhibitory factor (LIF), stromal-derived factor (SDF-1), tumor necrosis factor-α (TNF-α), and vascular endothelial growth factor (VEGF) are secreted by osteoblasts and are necessary for differentiation and migration [39,40].

The stem cell niche also includes elements of the sympathetic nervous system that stimulate osteoblast cells in the niche. Stimulation of the sympathetic nervous network with a β2adrenergic agonist in conjunction with a mobilizing agent such as G-CSF leads to enhanced stem cell mobilization. Extracellular matrix components and environmental elements such as calcium, oxygen concentration, and parathyroid hormone present in the endosteal and vascular niches are also involved in HSC regulation [8,41].

HSC Homing

HSC homing refers to the process by which HSC infused intravenously will migrate to the bone marrow and lodge within it. It relies primarily on adhesion molecule interactions between stem cells, stromal cells and extracellular matrix [42]. This process is composed of several overlapping steps [43]. HSC expresses L-selectin at a high concentration and epithelial venule cells expressing CD34 recognize each other, thus facilitating their movement [44]. It has been determined that homing, long term engraftment and proliferation of HSC is dependent on their cell cycle phase [45]. HSC cells in late S/early G2 phase will not engraft due to their differentiation following stimulation by IL-3, IL-6 and IL-11 cytokines [42]. Upon infusion in the blood stream, stem cells are also in contact with different cytokines released into the circulation following pre-transplantation conditioning. Cytokines such as CXCL12 play an important role in the homing and engraftment of HSC with the adhesion molecules E-selectin and the vascular cell adhesion molecule-1 (VCAM-1) [46]. CXCL12 is believed to be an important

chemokine because of its expression in both marrow stromal cells and its interaction with CXCR4 expressed on HSC [47]. CXCL12 also enhances the activity of adhesion receptors and particularly integrins present at the surface of HSC such as very late antigen-4 (VLA-4). Some integrins bind to counter-receptors on endothelial cells, particularly vascular cell adhesion molecule-1 (VCAM-1), producing firm adhesion [48]. Adhesion molecules also enhance cell viability when they interact with VCAM-1 [39,51,52]. Their binding initiates the transit of the stem cell through the endothelium and into the BM, where it may repopulate the niche microenvironment [10]. During pregnancy, LFA-1, VLA-4 and L-selecting expression is increased from 41% to 55%, 89% to 93% and 57% to 81% respectively [44,45,53]. Jointly with CD44 and VLA-5, they are implicated in the crawling and crossing into the bone marrow, where VLA-4 and VLA-5 ensure a close contact of HSC with stromal cells [33,54]. Other adhesion molecules are expressed by hematopoietic progenitor cells anchored to osteoblast cells including CD44 and CAM-1 [9,29,33,55].

Following the interaction of adhesion molecules with cytokines, stem cells transmigrate through the bone marrow endothelial cells to the endothelium region of the bone marrow [48]. Firm adhesion to the endothelium is initiated by binding of the chemokine CCL21 to its receptor CCR7 and subsequent activation of the integrin LFA-1, which in turn binds to ICAM-1 [47]. It is governed by other cell adhesion molecule–ligand interactions such as $CD34^{+}$/CXCR4 and VLA-4/VCAM-1 [48]. HSC must exhibit micro-spikes, and occasionally with branching and their cytokine-induced pseudopodial

membranes extensions (proteopodia) on their surface [56]. The presence of SDF-1 located in the bone marrow increases the number of pseudopodia from 1-2 to 3-7 on 21% of the cells [56].

The expression of E and P-selectins on endothelial cells bind the glycosylated ligands expressed on the HSC. It has been observed that fucosylation of cord blood HSC is reduced as compared to bone marrow. Since P and E-Selectins bind more strongly to fucose sites, then homing of $CD34^+$ cells expanded with fucose increases to 98% [57,58].

In addition to integrins and adhesion molecules, the homing of HSC relies on the up-regulation and activation of SDF-1 [48,59]. SDF-1 is a chemo-attractant expressed by both bone marrow stromal cells and endothelial cells and its expression is increased with chemotherapy or radiotherapy [48]. When bound to the endothelial wall, SDF- 1 induces an intracellular signaling cascade which activates integrin expression and ensures stem cell homing and retention [43,48]. Moreover, the binding of SDF-1 with CXCR4 increases the adhesion of early $CD34^+$ cells to VCAM 1, ICAM-1, fibronectin and fibrinogen, thus homing of HSC [43,60]. SDF-1 is also involved in the binding of CD44 expressed on $CD34^+$ cells with hyaluronic acid, also expressed on the endothelial cells of bone marrow [48]. Other factors which have been associated with increased homing and repopulation such as the stimulation of HPC with stem cell factors (SCF) and IL-6, results in the up-regulation of CXCR4 [48]. IL-6 and G-CSF are secreted by stromal cells and influence the homing and engraftment of HSC [37,39].

Various *in vitro* and *in vivo* studies explored the time necessary for HSC to interact with endothelial cells, crawl and home into the bone marrow, and all indicate that the HSC are capable of homing very quickly. The first homing step after infusion (i.e. the rolling on and crossing of the bone marrow endothelium) takes between 20 and 40 minutes [39]. This is dependent on the percentage of cells homing and engrafting following a partial analysis of factors influencing homing such as the adhesion molecule interactions between stem cells, sinusoidal endothelium, stromal cells, or bone marrow matrix [45]. Furthermore, homing is faster when HSC expressing negative lineage cells are in G0/G1 cell cycle phase [42].

Upon infusion, HSC will interact with stromal cells within the first 5 minutes and up to 3 hours. Engraftment occurs within 3-24 hours with a survival rate after one day of 97% [39,42]. If stimulated by cytokines more than 82% ± 7% of HSC bind to stromal cells within 24 hours of contact [56]. Other studies suggest that 18-20% of all intravenously transplanted stem cell subsets are engrafted in the marrow within 16–18 h following transplantation [48].

Adhesive interactions and lineage influence the localization of the homing of HSC [45]. Nilsson (2001) documented that in the first hour post infusion, 42% of the early lineage ($CD34^{-}38^{-}$) affixes in the endosteal region (ER) and 58% of $CD34^{+}38^{-}$ reside in the central marrow region (CMR) [61]. In the following 14 hours the number of cells in the ER diminishes for a final concentration of 24% CD34 lineage positive on day 15 and the number of cells in the CMR region increase to 76% [61,62]. On day +3 post-transplantation,

approximately 50% of the initially homing cells seed in the close proximity to the bone marrow cortex in contact with osteoblast lining, and others attach to the sinusoids of the endothelium of the bone marrow within the extravascular compartment [39]. These cells remain in this location for 4 to 5 days before migrating to a different site in the central marrow, where they remain for weeks to months [39].

Cord blood biology

Hematopoietic Stem Cells ($CD34^+$)

The frequency of $CD34^+$ cells varies depending on its sources. In normal conditions, bone marrow (BM) contains 1% to 3%, cord blood (CB) between 0.1% to 3% and non-mobilised peripheral blood (PB) 0.001% to 0.01% $CD34^+$ cells [22,63,64]. The number required for successful transplantation is approximately 2 to 4 x 10^6 per kilogram when the source is BM or PB. On the other hand, when transplanting CB, studies have demonstrated that less than 10 fold of $CD34^+$ cells (1 x 10^5) is required to ensure similar engraftment [6,65] showing a significant difference between CB and adult sources of HSC. When looking at the difference between CB, mobilised PB and BM $CD34^+$ cells, it was demonstrated that CB and PB $CD34^+$ cells contains ≥ 3% of immature progenitor cells expressing HLA^-DR^- and $CD38^-$, compared to BM $CD34^+$ cells [66,67]. UCB cells have longer telomere lengths and have a greater proliferative capacity compared to BM or PBSC [43]. There are fewer $CD34^+CD19$ or CD10 (early B cells) in CB and PB compared to BM (1.6% ± 0.6% for CB vs 1.7% ± 0.7% PB vs 18.7% ± 10% for BM) [22,67]. The most important difference of CB with adult BM is its reduced proportion of B cells and “naïve”

leukocytes classifying the immune systems as immature [68,69]. Cord blood $CD34^+$ cells also expressed more CXCR4 compared to PB inducing a more rapid migration than for BM or PB $CD34^+$ cells [70,71].

The volume of cord blood collected is a good indicator of the quantity of total nucleated cells (TNC) and $CD34^+$ cells it contains. Direct correlation of TNC count with $CD34^+$ cells is frequently referred to justify the disqualification of CBU in presence of low TNC count [72]. However, many studies have shown that cord blood contains a greater relative concentration of $CD34^+$ cell when associated with younger maternal age, larger birth weight, and shorter time from collection to processing [73]. The time of gestation seems to influence the levels of $CD34^+$ cells, with 0.94% ± 0.26% of all leukocytes at 22 weeks, reducing significantly to 0.37% ± 0.05% at 32 ± 5 weeks, and finally to 0.29% ± 0.03% at full term of pregnancy ($p = 0.004$) [53,74]. One very surprising factor which seems to influence the number of $CD34^+$ cells and TNC in cord blood is the blood group. CBU of blood group O contain a larger percentage of $CD34^+$ and even larger percentage of TNC. On the other hand, the percentage of CD34 and TNC of blood group A and B seems to be equivalent to each other but lower than blood group O and higher than AB group, the latter one with the lowest percentage of both cell types [75].

IgG Level

B-cells in newborns are capable of producing IgM antibody comparable to adult B cells, but their capacity to produce IgG is remarkably reduced [76]. This is compensated by the passive transfer of maternal IgG across the placenta by binding to the Fc receptor

during pregnancy [77]. This transfer begins at week 15 and increases from week 22 until the end of pregnancy. Cord blood serum has been found to contain IgG antibodies at titers comparable to those present in the sera of most adults [78]. This IgG helps protect the newborn against infection by gram-negative bacteria [78]. However, the amount of the blood group IgG anti-A and anti-B that is transferred varies according to the mother's ABO type [79]. IgG anti-A has been observed to be higher in newborns with a blood type O and B than with those with blood group A or AB [80,81].

nRBC

The number of nucleated red blood cells (nRBC) in an adult is approximately 1% of peripheral blood leukocytes [77]. This percentage may increase up to 84% in cord blood confounding the reported automated white blood cells count and its ratio with $CD34^+$ cells[82]. It was found that pregnant women with pre-eclampsia have up to 18% nRBC [83,83,84]. The type and length of the delivery do not affect the percentage of nRBC [85]. Another factor, which influences the percentage of nRBC is the method for enumeration. Manual enumeration using a microscope is considered laborious compared to the electronic counter; on the other hand automated systems are known to count higher numbers of nRBC with a larger standard deviation but are more precise when the nRBC percentage is more than 5% [85]. Flow cytometry can provide a more precise count [82]. The number of nRBC being a predictor of myeloid engraftment speed justifies the importance in its evaluation and reports [86]. The correlation between the

number of nRBCs and the number of hematopoietic progenitor cells reflects the involvement of early stem cells in erythroid responses [86].

Cytokines

Cytokines are produced by many different cells and are involved in growth, division, differentiation and activation of cells [87]. During pregnancy, the concentration of many cytokines is increased but will decrease significantly after week 37 of gestation [88]. This reduction is more rapid in the last two months of gestation. Even if the level has been lowered, it remains higher than in adult blood, especially for stem cell factor (SCF), FLT3 ligand, erythropoietin (EPO), G-CSF and IL-11 [74]. In contrast to bone marrow cells, cord blood cells will produce less IL-1, IL-2, IL-4, IL-8, IL-12, IL-15, Interferon-gamma (IFNγ), Tumor Growth Factor-beta1 (TGF-β1), GM-CSF cytokines and no IL-6 [89]. Graft-versus-host-disease (GvHD) is an important complication of unrelated stem cell transplantation; furthermore, the reduced production of some cytokines is likely to play a role in the immune-reactivity and low rate of this disease when transplanting CB [90,91]. IL-10, an anti-inflammatory cytokine that promotes humoral immunity for protection against extracellular pathogens, is also up-regulated [92]. IL-10 is involved in the down-regulation of the immune response during pregnancy and in the reduction of recipient GvHD [90,91]. Cohen et al. (1999) reported that cryopreservation of stem cell product lowers the production of IL-2 and increases the level of IL-10 post-thawing [90].

T Cells

During pregnancy the fetal immune system is incompetent and immunologically immature up to 22 weeks of gestation [93]. Up to 20 weeks of gestation CD4 T-cells represent 15-20% of lymphocytes, which reduces to 3-7% at birth [94]. Essential regulatory mechanisms controlling T cell responses are in place in early life ensuring suppression of fetal immune responses against invading maternal cells [77]. $CD25^{+}CD4$ T-lymphocytes that naturally develop in the human foetus inhibit the proliferation of CD4 and CD8 T-lymphocytes, thereby controlling autoimmune reactions as well as allogeneic and antimicrobial immune responses [95].

Immature lymphocytes phenotypically express a CD45RA (naive) isoform. In CB the majority of lymphocytes express CD45RA with a very low percentage of CD45RO (memory), consistent with the immaturity of the CB system compared to the analogous population in adult peripheral blood [96]. This immaturity is also responsible for the reduced production of some cytokines and for its failure to respond upon B-cell stimulation [97,98].

In addition to immature lymphocytes, only 50% of CB mononucleated cells express $CD3^{+}$ T-cells, compared to 70% in adult blood [76]. However, phenotypic analysis of T-cell subsets (CD4, helper T-cells, and CD8, suppressor T-cells) have revealed no significant differences between adult and cord blood [98]. The difference is predominantly attributed to the lower levels of both IL-2 receptors and HLA-DR than adult T-cells [99]. The initial immunologic activating event requires the engagement of TCR with allogeneic

human leukocyte antigen (HLA) and the trigger of the reactive cell population, which is dependent on the concentration of IL-2 and the presence of antigen presenting cells (APC) [33]. After activation by APC cells, cord blood $CD45RA^+$ cells will be down-regulated and begin expressing CD45RO, having similar proliferative capacity but reduced cytotoxic activity [95]. This modulation is shown with transplanted CBU regulatory T cells $CD4^+CD25^+$, which suppress allo-specific proliferation of $CD4^+CD25^-$ T cells in the mixed lymphocyte reaction thus preventing death from donor-induced GvHD [100].

Recently, Weinberg (2012) demonstrated by flow cytometry that fetal naïve $CD4^+$ and T_{reg} $CD4^+CD25^+$ cells are different from the adult naïve $CD4^+$ and T_{reg} $CD4^+CD25^+$ cells population. To better understand these differences, fetal and adult naïve T-cells were stimulated with allogeneic cells resulting in fetal cells producing 10 times more T_{reg} compared to adult cells. Given that fetal immune system promotes tolerance between foetus and mother [94], this increased production of Fetal T_{reg} may also contribute to the low grade GVHD when transplanting cord blood cells compared to bone marrow or mobilised peripheral blood stem cells [101].

Natural Killer (NK)

In CBUs more than 20% of lymphocytes are the primitive natural killer (NK) cells, possessing proliferative and cytotoxic capacities [90,102]. Identification of NK cells is done primarily by the detection of $CD3^-CD16^+CD56^+$ and $CD3^-CDl6^-CD56^+$ in adult blood [76]. Although they are functionally and phenotypically immature, they respond rapidly to IL-2, IL-12 and IL-27 [103]. IL-27 increases during pregnancy and has been observed to

enhance cytotoxicity of cord blood cells when combined with IL-12 [104]. However, because IL-12 and IL-15 are in low concentration in CBUs given that IL-2 is their activator, the immaturity and cytokine reduction would contribute to decrease the production of NK cells from CB [105]. Furthermore, when stimulating cord blood NK cells with IL-2, there is a decreased production of IFN-gamma (γ).

The ability of cord blood cells to respond to cytokines is equally important as its cytokine production. The expression of the IL-2 receptor is reduced in cord blood mononucleated cells compare to those of adult peripheral blood. Also, IL-4 and IFN-γ receptors have a decreased expression and are functional on cord blood cells, thus ensuring the production of some immunoglobulin such as IgE [96].

Platelets

CB platelet recovery following transplantation takes longer compared to platelet recovery after bone marrow or peripheral blood [106]. Schipper and al. (2003) expanded *in vitro* megakaryocyte progenitor (MP) to determine their differentiation potential in cord blood. He observed that 98% of CB immature MP expressing $CD34^+$, but lacking the platelet specific markers CD41 or CD61, have a higher proliferative capacity [107]. In contrast, most MP cells in peripheral blood are much more mature and express $CD34^+/CD41^+$, indicating different developmental stages compared to CB, which may be the contributing factor for slower recovery post CB transplantation [106,107].

Cord Blood Processing

Collection

The choice of cord blood processing in its first years was simple due to the limited number of available methods at that time. At first, the cord blood collection bag was a standard 500 mL adult blood collection bag containing 63 mL anticoagulant citrate/phosphate/dextrose-adenine (CPD-A), from which the anticoagulant was removed with a sterile needle and syringe [108]. At present, some changes have been made to this procedure: (1) the collection volume has been reduced to 250 mL, (2) the volume of anticoagulant (CPD-A or citrate/phosphate /dextrose (CPD)) varies from 15 mL to 35 mL, and (3) the number of needles reduced to one to puncture the umbilical cord blood vein [109].

Similarly, twenty years after the opening of the first Cord Blood Bank (CBB), only two distinct collection methods are still used: *in utero* and *ex utero*. The *in utero* method is described as a collection done on the umbilical cord vein while the placenta is still in the uterus. The *ex utero* method uses a similar method, however the placenta has been expelled from the uterus and the environment is recreated using a metal support. Both collection methods have their advantages and disadvantages. Some banks prefer the *ex utero* method because it ensures larger volume, less clotting with a seemingly reduced delay to processing [110]. Conversely, others prefers *in utero* collection, which ensures a TNC count above 9.5% per collection bag compared to the *ex utero*

collection [111]. This difference was explained by a larger presence of blood clots when the collection method is *ex utero* (31%) compare to a trace of blood clot (1%) when *in utero* collection is used [111]. Lasky et al. (2002) reported more clotting wth *in utero* versus *ex utero* collection (53% versus 40%, respectively) [110]. These large differences and percentage of blood clots are often explained by the inadequate mixing of the blood with the anticoagulant contained in the collection bag.

Very few researchers have studied the impact of having a collecting team using the *ex utero* method versus the impact of having the uterus contracting the placenta thereby increasing the flow of blood and the volume collected. Solves et al. (2003) demonstrated significant differences in the blood volume collected, with 108.82 ± 28.6 mL for the *in utero* method compared with only 98 ± 28.5 mL for the *ex utero* method [112]. Similar studies reached a different conclusion with the demonstration of no differences in the volume collected using any collection methods [113,114]. The COBLT Study observed a difference in the volume collected depending on ethnic background and delivery mode. For example, the African American donor contains much less volume compared to the non-African American, and a higher volume is obtained from cesarean section as compared to vaginal delivery [115].

The principal attribute of a CBU volume is its positive correlation with its nucleated cells (NC) and $CD34^+$ cell counts. In the first years of cord blood banking, CBUs with a volume of 40 mL units containing TNC count of ≥ 0.6×10^9 were processed and banked [113], which was later increased to a TNC count of ≥ 1.0×10^9 [116]. This increase in TNC

count as a qualification cut-off point brought the number of discard rates down from 30% to 50% of all collected units. Methods have been used to increase the volume collected to lower the number of discarded units. Rinsing the cord vein during an *in utero* collection increased the volume, the total number of $CD34^+$ and TNC cells in the collection bag, but the ratio of $CD34^+$ over TNC remained the same [117].

A significant correlation was demonstrated between the volume collected and the NC count in studies where the effect of some obstetric factors was analyzed. One factor studied was the delivery mode where an additional 3.5 x 10^9 TNC was observed between c-section and vaginal delivery with values of 17 x 10^9 and 14 x 10^9, respectively [118]. This large difference can be explained by larger new borns often requiring a c-section, thus providing a mean volume of 89.2 mL compared to 76.6 ml for vaginal deliveries [115,119]. Other factors identified are the gestational age, the sex of newborns, stress during delivery, difficult birth and the presence of meconium [72,120].

Volume Reduction

Before CB processing, a first qualification of the CBU is done by the public cord blood bank, where more than 30% of donors are rejected at registration and almost twice as many after collection, mainly due to insufficient volume or cell counts (61%) and other diverse reasons such as family history, personal medical history or bacterial contamination [115,121,122]. The main goal of CBU processing is to reduce its volume and to optimize storage space. At the same time, the depleted products can be used to perform the required quality testing without significant loss of TNC and $CD34^+$ cells. Some banks

prefer preserving as many cells as possible by refusing volume reduction to ensure a more rapid engraftment [123].

The first and most frequently used type of CBU processing is the “two times volume reduction”. Pioneering this methodology, Rubinstein et al. (1995) added hetastarch (HES, hydroxyethyl starch) solution as a density gradient, in a ratio of 1:5 volume, ensuring an increased TNC yield post processing [124-126]. After an incubation of 30-45 minutes, a first centrifugation was done to expel a concentration of red blood cells followed by a second centrifugation ensuring separation of the plasma from the top of the processing bag. The mean yields were 87% for TNC and 87-97% for $CD34^+$ cells [116,124,127].

Further studies verified the impact of performing the depletion of red blood cells only, or plasma only, to improve nucleated cell recovery partially using the Rubinstein method with or without the addition of HES. After red blood cell depletion the mean recovery was reduced to 78% and 84%, respectively, compared to plasma depletion, which was 96% and 99%, respectively [128]. The use of two consecutive red blood cell depletions instead of a single one demonstrated a more superior nucleated cell recovery with 92.9% instead of 78.4% [129]. A more recent system, using the antibody-based reagent called PrepaCyte-CB, was developed to increase cell-specific separation by removing erythrocytes without the use of chemicals or particles [130]. The methodology is very similar to the HES, however, using another product like Pentaspan [130]. It has been claimed that this method ensures recovery of 76% CD34 compared to 64% for HES. In

addition, they compared TNC and CD34 yield based on volume, demonstrating that the increased volume had a negative impact on both TNC and CD34 recovery despite their product recovery on par with the differing volume [130]. Red blood cell depletion is done mainly to reduce transfusion reactions secondary to blood group incompatibility. Studies also verified the engraftment impact and observed that the nucleated cell recovery was increased from plasma-depleted CBUs compared to red cell depleted CBUs [128].

The Top-bottom volume reduction technique using the Optipress System allows the use of a closed bag system and reduces the number of centrifugations to a single one [108,131]. This method also ensures the reduction of a larger number of units in the same period of time while maintaining a mean lymphocyte recovery of 89% and a mean $CD34^+$ recovery of 100% [108]. However a study looking at the impact of this methodology on low volume (40-80mL) versus high volume (≥ 80mL) of CBU showed reduced yields 51% compared to 70% for bigger volumes [132]. Nonetheless, the normally selected CBU for banking is of 65 mL and more. Comparison of the Top-bottom versus the Rubinstein method showed equivalence in TNC and CD34 recovery [131].

In the past ten years, many methodologies have been developed to reduce procedural steps and ensure a wider applicability. One of these is the Sepax system which is a fully automated system, with a TNC (80.3% ± 7.7%) and $CD34^+$ cell (86% ± 11.6%) recuperation comparable with the previous two methods. This method still requires the addition of HES at a final concentration of 20% and can process volumes ranging from a minimum of 20 mL of blood to a maximum of 200 mL [133-135].

In the early 21 century, a prototype filter made of superfine polyethylene terephthalate fibres to separate the cord blood component named Red Cell Volume Reduction System (RCVRS) [136] was invented in Japan. This simple and rapid volume reduction method is a fibrous polyester fibre containing hydroxyethyl methacrylate and methacrylic acid called a hematopoietic stem and progenitor cell (HSPC) Recovery Filter. Using gravity, the collected blood is filtered to keep the stem cells in the filter. A 9 mL aliquot of recovery solution is used to rinse the filter. The mean TNC and CD34 recovery is 65% and 84% respectively [137]. The nRBC banked with the unit can range from 0.4 to 44% contributing significantly to the TNC final count [116].

Despite these various processing methods, the National Marrow Donor Program (NMDP) registry for the USA public cord blood bank has two CBB banks performing plasma depletion and 18 doing red cell depletion [128].

Cryopreservation

In 1977, preservation of hematopoietic stem cells from peripheral blood was successfully achieved with a mean mononuclear cell recovery of 86% [138]. The cells were cryopreserved with a final concentration of 10% dimethyl sulfoxide (DMSO) in a plastic bag using a control-rate freezing method ensuring a regular decrease of 1°C per minute. Other cryopreserving solutions were analysed for long term storage, however the use of DMSO with Dextran increased cell viability by reducing the likelihood of water crystallisation [139,140]. It also preserve the expression of different adhesion molecules present at the surface of $CD34^+$ cells with the exception of L-selectin, which is reduced

post-thaw [62]. Still, when cultured for 6-12 hours, L-selectin expression increases and recovers up to 80% of its expression after 48 hours. Furthermore, these results are consistent even after three freeze-thaw cycles [141].

After reports of multiple adverse reactions in transplant patients, ranging from nausea to cardiovascular events following the infusion of 10% DMSO, different concentrations of DMSO were studied in order to lower the concentration while still ensuring a high cell viability [140]. The lowest concentration studied using a control-rate freezer and a dump freeze method was of 2% DMSO [117,142]. Significantly lower apoptotic and necrotic $CD34^+$ cells were observed compared to 10% concentration using the control-rate freezing method [143,144,144]. However, when a dump freeze method was used, lower than 10% DMSO concentration did not ensure adequate TNC and $CD34^+$ cell viability.

This difference in outcome may be due to the NC concentration. The recommended concentration of NC when cryopreserving peripheral blood stem cells may not exceed 2 x 10^8/mL when using a 10% DMSO concentration with the dump freeze method [145]. A study performed on CBUs demonstrated that more than twice the volume of peripheral blood NC can be frozen for the same 10% DMSO concentration [146]. Another factor influencing cells viability has been the storage temperature. It has been recommended to optimize storage conditions by using vapor nitrogen phase with a temperature of ≤ -150ºC [140].

Thawing

Rubinstein (1995) developed a thawing protocol for cryopreserved CBUs consisting of a dilution of 1:1 in a solution of equal volume of 2.5% human albumin and 5% Dextran 40 in an isotonic salt solution, followed by a centrifugation for the partial removal of DMSO before infusion with a reconstitution [124]. Similarly, Zinno (2010) showed that this method ensured a recovery of 76.8% of nucleated cells [147]. This first documented thawing method was compared in the following years with newly developed methods such as thawing with only a reconstitution consisting of adding the same volume of an equal volume of dextran 40 with 5% human albumin pre-infusion.

The thawing method needs to ensure adequate and viable nucleated and $CD34^+$ cells recovery. A simple centrifugation post-thawing decreases heavy clumping of cells caused by the destruction of granulocytes, and will require special attention [138]. First, clumping problems are often solved by re-suspension in a solution containing deoxyribonuclease (DNase) with or without fetal calf serum [138]. This technique showed a viability of 87.8% and a $CD34^+$ cell recovery of 70% [148]. Another technique using a more careful washing procedure is minimising cell loss from 30% to 10-15% [149]. It is also possible to directly infuse the cells called “thaw at bedside”. In 2004, comparison of unwashed and washed units was done [150]. It was demonstrated that washing reduces the number of TNC in a CBU by 27% but similar $CD34^+$ counts are observed before and after. Not washing a CBU not only increases neutrophil recovery but does not increase the chances of observing serious toxicity adverse events after infusion [123]. However, when transplanting

a pediatric population, the infusion rate must be much slower. It has been reported that the delay between thawing and infusion has an impact on engraftment time. Nucleated cells yield and recovery must not exceed 15 minutes in presence of a DMSO concentration of 10%. Also, a temperature post-thaw must be respected to ensure adequate cell viability. The NC recovery is significantly reduced post-thaw from 80% to 40% when it is kept at 22ºC versus 37ºC [151]. However, if the temperature is close to 0ºC the recovery is reduced to 60% in the same period of time [151].

An automated washing device was developed to ensure DMSO was removed after thawing following observation of mild to moderate adverse reactions in transplant recipients. After ensuring more than 96% retrieval of DMSO the mean recovery of viable $CD34^+$ and TNC was above 60%-80% [152], however the loss is more than the recommended 10 to 15%. Similar equipment was tested in Spain, ensuring the removal of 90% of DMSO with a loss of less than 10% $CD34^+$ cells with an adequate viability. However the loss of MSC and other cytokines has not been studied and this method has not been linked to engraftment time [153,154].

Positive CD34 selection on frozen product

To ensure that the stem cell product is depleted of T cells or B cells, an immune-magnetic cell separation system can be used. To date, few systems have been able to separate biological components using equipment approved for clinical application. Such devices have been evaluated on fresh or cryopreserved bone marrow, peripheral blood or CBUs.

The importance of such procedures is to ensure adequate viability, purity and mainly cell yield. The Miltenyi CliniMACS system was compared to the Baxter Isolex system on frozen cord blood products. Isolex system ensured $CD34^+$ purity of 51% when executing a positive selection with a median recovery of 34%. However, better recovery was obtained with the CliniMACS, with 80% $CD34^+$ cells with a similar median purity of 54% [155]. To increase the purity during positive selection, different solutions have been proposed. For instance, the addition of DNase or chymopapain increased the purity of $CD34^+$ cells to 80% and a $CD34^+$ cells yield of 76 ± 9% [159]. The substantial differences have not been explained. Different concentrations of NC were tested to verify its impact on recovery and purity using leukocytes and no correlation was observed [160].

Cord Blood Stem Cell Transplantation

Cord blood as an adequate source for stem cell transplantation

When a matched sibling is not available, cord blood is often used as a source of unrelated HSC and even as a first line of stem cell source for pediatric and adult patients mainly in the United States and in Europe. However, in Canada, transplant physicians are more reluctant to use CBU as first unrelated stem cell source. When searching for an allogeneic donor for stem cell transplantation (SCT), approximately 30% of patients will have a matched related donor. For those requiring an unrelated search for a suitable HLA match, more than 40% of the future recipients will not find a suitable donor due to the strict requirement of HLA compatibility [128]. In comparison with other sources of

allogeneic HSCT, CBUs have some unique advantages. One of the first advantages is the size of the donor pool. Even if the pool of CBUs is 10 times smaller, immaturity of the immune system improves the tolerance and increases the number of acceptable mismatches (MM) up to 2 HLA types. This reduced compatibility will not impact the incidence of engraftment failure or time to neutrophil recovery, which vary between 22 to 27 days [161]. The reduced HLA matching requirements increases the chance of finding an alternative stem cell donor source for patients of mixed ethnicity [43,162]. Another advantage is the rapid availability of the stem cell source being ready for infusion when listed in the registry compared to bone marrow donors who require the health status confirmation of the donor to ensure the possibility of donation. This rapid availability reduces the delay between donor identification and transplantation from 49 days when selecting a bone marrow versus 13.5 days when choosing a CBU [162,163]. CBU containing a naïve immune system offers a lower incidence and lower severity of GVHD [89]. However, when selecting a CBU for transplantation, recipient criteria must be taken in to consideration for which the impact on the recipient's overall survival or disease free survival may vary such as older age, advanced disease and positive CMV sero-status [164].

Cell Dose

In the first few years of CB transplantation the selection of a cord blood unit was based on a nucleated cell count of at least 1.5 x 10^7 per kilogram (kg) of recipient weight [165]. Analysis of many cord blood transplantation results demonstrated that

recipients had very poor outcomes, higher risk of graft failure, prolonged engraftment time and increasing infection risk when using single CBU. Very early, Rubinstein demonstrated that an increased number of nucleated cells enhanced neutrophil engraftment [166]. It was followed by a multicenter study, COBLT, designed to evaluate the use of allogeneic cord blood transplantation looking at the probability of finding suitable CBUs when increasing the number of NC per kg of recipient's weigh [167]. The probability of finding a unit containing ≥ 2.5 x 10^7 nucleated cells per kg for a recipient weighing 60 kg was only 6%, 4% for a patient of 70kg and 2% for those weighing 80kg [115,168]. The observation that pediatric transplant patients had a more rapid engraftment time when infusing ≥ 3.7x 10^7 nucleated cells per kg of the recipient's weight resulted in revision of standards to a minimum of 2.5 x 10^7 of TNC/kg [169]. Studies demonstrated that the most important factor influencing the rate of engraftment was the NC dose per kilogram of the recipient weight ensuring a median neutrophil engraftment time of 23 days [168-170]. A recent study revealed that a lower transplant related mortality (TRM) and lower relapse rate were observed in recipients receiving a TNC dose of ≥ 2.5 x10^7/kg in presence of one HLA mismatch (MM), or with a TNC dose of ≥ 5.0 x10^7/kg in presence of two HLA-MM. Importantly, there was no difference in survival outcomes between a CBU with a TNC dose of ≥ 2.5 x10^7/kg in presence of one HLA mismatch (MM), or with a TNC dose of ≥ 5.0 x10^7/kg in presence of two HLA-MM [171].

Another factor to consider when selecting a CBU is the number of $CD34^+$ cells per kg of the recipient's weight to ensure engraftment. It was observed that an infused CBU

containing a $CD34^+$ cell dose of ≤ 1.7 x 10^5/kg ensured neutrophil engraftment in only 72% of the recipients with a median time of 34 days [168,170]. The correlation between TNC and $CD34^+$ cell count being clearly defined, this observation confirms that an increased neutrophil count requires an increase in the number of $CD34^+$ cells to be infused in the recipient [172]. In addition, when more than 2.3 x 10^5 $CD34^+$ cells/kg were infused the percentage of overall survival (OS) doubled [169,173]. The current recommendations when choosing a CBU is to ensure a NC count of at least 2.5 x 10^7/kg or ≥ 2 x 10^5 $CD34^+$ cells/kg [162]. This recommendation is supported by observations of a rapid engraftment at day +18 with a CBU containing more than 2.5 x 10^7/kg nucleated cells, corresponding to 0.43 x 10^5 $CD34^+$/kg [174]. Another criterion to consider is the $CD34^+$ cell viability, since only one CBU out of 16 will engraft when the $CD34^+$ viability is less than 75% [175]. An additional benefit of the increased TNC and $CD34^+$ cell dose is a more rapid platelet recovery. The incidence of platelet recovery at 6 months is now at 0.65 (CI, 0.53-0.77) with a median engraftment time of 86 days [170].

The disadvantage of increasing the number of cells per kg of recipient's weight is the risk of infusing a larger amount of cord blood T cells. Even in presence of an immature immune system, it was observed that the infusion of more than 1 x 10^5 $CD34^+$/kg increases the incidence grade II-IV and III-IV GVHD [176]. On the other hand, higher nucleated cell dose resulted in higher development of grade III-IV acute GVHD but was also associated with lower TRM [170]. As previously suggested by Rubinstein *et al.*, a higher $CD34^+$ cell dose can partially overcome the negative impact of

HLA mismatch for each level of HLA disparity. It was suggested that in the presence of 2 HLA MM between recipient and a CBU the recipient should be infused with more than 1.7×10^5 $CD34^+$ cells/kg [170].

Human Leukocyte Antigen (HLA)

Also called major histocompatibility complex (MHC), the HLA family consists of more than 200 genes located on chromosome 6 regrouped into several classes. This complex system ensures the identification of non-self-pathogens such as viruses, bacteria or foreign organ or immune system. When transplanting CB stem cells, HLA Class I and Class II are verified for their compatibility between donor and recipient. Of all possible sub-classes, two groups of Class I genes are compared (HLA-A and HLA-B) and one of Class II (HLA-DRβ1). In allogeneic stem cell transplantation, the first stem cell source will be from an adult family member donor. The probability of finding a related family donor is about 30%, and considering the reduced number of siblings per family, most will require an unrelated donor transplantation [177]. An unrelated adult donor requires compatibility between the donor and the recipient of ≥ 9 out of 10 based on HLA Class I A, B and C and Class II Drβ1 and DQ alleles [178]. Despite the availability of more than 10 million adult donors worldwide, many patients still cannot find an adequate adult donor [163]. In addition, a donor's availability varies according to their ethnicity. One Caucasian out of two will find a match adult donor, while only 35% of Hispanics and 20% of African-Americans will find suitable donors [179,179,180]. Expanding the search by adding cord blood donors can change these probabilities, since HLA matching requirements are

lowered to HLA Class I A and B and Class II Drβ1. Based on the weight of the recipient, the chance of finding a CBU with a 6/6 HLA compatibility for a patient of less than 50 Kg is 23% and 100% for a 4/6 HLA compatibility. However, the chance for a recipient of more than 70 kg with a 6/6 HLA compatible CBU is only 2%, and 76% for finding a 4/6 match [181].

In the event that no single compatible CBU containing sufficient nucleated cells is available, double CBU transplantation is considered to be acceptable if no more than 1 HLA difference existed between the 2 units and the patient [182,183]. In this type of transplantation, the influence of unit-recipient or unit-unit HLA disparity has been shown to have no influence on engraftment or on the dominance of a unit [173]. When transplanting bone marrow or peripheral blood stem cells with full compatibility between donor and recipient, more than 40% of the recipients will develop systemic acute GVHD [177]. Also, each HLA mismatch will decrease disease-free survival by 10%. On the other hand, when transplanting a matched HLA or a 2 MM CBU, the incidence of acute GVHD is lower compared to bone marrow or peripheral blood stem cell sources [184,185].

Natural killer cells (NK) are known for their capacity to kill cancer cells and for their implication in innate immunity. Among the different NK-cell receptors is the polymorphic Killer-cell immunoglobulin-like receptor (KIR) that recognizes epitopes of HLA A, B and C [186]. Because HLA and KIR gene are located on different chromosomes, the chance of finding compatibility between a related donor and recipient is only 25%, and this chance is much reduced between an unrelated donor and recipient [177]. The importance of such

compatibility has been associated with a significant reduction of relapses in AML recipients with a B/B KIR genotype and increased disease free survival, with no impact of any genotype in ALL recipients [177,186]. When a HLA class I KIR is present in the donor but absent in the recipient, NK cells would allo-reactivate. In presence of NK allo-reactive donor a better event-free survival and 67% less relapse are observed in recipients compared to non-NK allo-reactive donor [187]. A mismatch of the HLA-C locus had no significant impact on engraftment. A significant impact was observed on TRM in the presence of one DRβ1 in mismatch unrelated transplantation [188,189].

The HLA-match level was associated with the incidence of GVHD. In the absence of HLA mismatched CBUs, an improved neutrophil engraftment was observed and a presence of an increased number of HLA MM resulted in an increased incidence of aGVHD grade III-IV [171]. This was sustained when match-related CBU was transplanted with a lower incidence of acute GVHD and chronic GVHD compared to recipients transplanted with single MM CBU followed by MM double CBUs (dCBUs) [185,190,191].

The possibility of finding a haplo-identical related donor is increased when considering the parents. However, in an adult population, it is more likely that the genetically haplo-identical donor would be a sibling, thus reducing this availability to 50%. Transplanting stem cells from adult donors with 3 MM increases significantly the rate of acute GVHD grade II-IV (6%), grade III-IV (34%) and TRM, with the exception of infusion of T-cell depleted product [192,193]. On the other hand, when T-cells are depleted in order to prevent GVHD from the haplo-identical donor, a slow immune recovery is

observed [193]. Opposite results are observed when the haplo CD34 selected cells are used as a third party, which does not increase aGVHD and cGVHD [194,195].

During fetal development, the fetus is exposed to the maternal HLA antigens and to the microchimeric cells expressing inherited paternal HLA antigens (IPA) present in the mother [196]. The traffic between mother and fetus will give rise to microchimeric cells of non-inherited maternal HLA antigen (NIMA) and inherited paternal HLA haplotype [197]. The concentration of IPA, in the mother, increases with the number of pregnancies, and microchimeric cells also transferred through breast-feeding, therefore the exposition may increase in the newborn [198]. These NIMA and IPA seems to contribute to a better stem cell transplant recipient outcome and lower risk of severe aGVHD when the donor cells are T cell-replete and if they contain NIMA or IPA corresponding to recipient HLA [94,196,199]. The presence of NIMA and IPA was measured at different donor's age. Their presence of NIMA was detected in 67% of newborns and decreases over the years with a persistence of 6% after 59 years of age [200]. Recipient exposed to IPA had no impact on morbidity and mortality of graft-versus-host disease, until recently where reduction of relapse has been observed in recipients who shared one or more HLA-A, -B or DRB1 antigens with their CB donor's IPAs [201-203].

Other transplantation method

Double CBU Transplantation

Researchers from the University of Minnesota studied the eligibility of adult recipients for CB transplantation [168]. Due to the small volume and the limited number of

TNC and $CD34^+$ per CBU, only 30% of their patients were eligible. Of those 30%, only 72% engrafted at day +34 due to the low number of NC infused per kilogram of the recipient's weight [168]. Double cord blood units (dCBUs) transplantation was a strategy used to increase the nucleated cell dose infused to a recipient; however the disadvantage was the identification of two CBUs with the same HLA MM [173]. The total number of NC must be ≥ 3.5×10^7 NC/kg and each CBU must have a minimum of 1.5×10^7 NC/kg. It was demonstrated that this method resulted in improved sustained donor neutrophil engraftment which varied between a median of 20 and 32 days, platelet recovery by day 65-91, comparable to transplantation with a single CBU [173,204-206]. Post-transplantation outcomes showed that dCBUs resulted in a lower rate of leukemia relapse, shorter neutrophil and platelet recovery time, and similar aGVHD and cGVHD, compared to single CBU transplants [185].

Interestingly, after more than 10 years of practicing such a method one cannot predict which unit will engraft based on nucleated cell dose, HLA compatibility or incompatibility, $CD34^+$ cell dose, or blood group [168,207]. However, it was demonstrated that 76% of the time, the first infused CBU was the one that engrafted [168]. An immune interaction after the infusion of dCBUs has also been observed which can originate from a T-cell mediated graft-versus-graft reaction [208]. It was observed that a higher $CD3^+$ cell dose, the percentage of $CD34^+$ cell viability and a higher CD3 count at day 7 post transplantation were associated with the engrafting unit [173]. One disadvantage of double

CBUs transplantation is persistence of both CBUs, observed in 3 to 7% of recipients [173,209].

CD34 selection on PBSC as Third party combined with a CBU

Similar to the previous method, the goal of this particular method is to increase the number of nucleated cells without increasing the number of T and B cells. A third party unit composed of peripheral stem cells from a haplo-related donor depleted of all allo-reactive cells and retaining uniquely the $CD34^+$ cells has been used. It was co-infused with ≥ 4/6 HLA mismatch CBUs containing at least 1.5×10^7 NC/kg [210]. A high engraftment rate of the CBU was obtained with full CBU chimerism within 100 days [211-213]. However, the initial predominant transient engraftment came from the third party unit ensuring a very rapid neutrophil median recovery time of 10 days followed by the CBU recovery median time of 22 days [214].

In the first trials, 11 patients with high risk leukemia were transplanted, four of which received $CD34^+$ cells from their mother's mobilised peripheral stem cells. Only two patients did not engraft, whose $CD34^+$ cells originated from their mothers, and none of them were carriers of HLA NIMA [211]. The incidence of serious acute and chronic GVHD was very low, as was the incidence of relapse suggesting an important graft versus tumour effect [215]. Their overall survival and disease free survival rates were 56% and 47%, respectively [213,216].

Multi-CBU Transplantation

To date, there are two different definitions of multiple CBU transplantation. Most frequently, double CB transplantation is considered 'multiple' with a wide range of neutrophil engraftment times varying from 12 to 26 days but with a low graft failure (0-39%) [217,218]. However, Lister et al. (2007) pushed the meaning of 'multiple' a step further by infusing one CBU per 10 kg of the recipient's weight. Ten recipients received an average of 6 HLA-blinded CBUs infused one after the other post-thaw. Three patients died before day +28, only two engrafted and one survived more than 76 days. This unique recipient had one HLA-B locus compatible with the engrafted CBU, only grade II GVHD of the skin, no evidence of cGVHD and was still alive three years post transplantation.

Cord Blood Expansion

Different cord blood components have been expanded in the past decade. The principal indication for CB expansion is to restore hematopoiesis after stem cell transplantation or to partially enhances hematopoietic recovery when transplanted [219]. However, Xu and Reems obtained significantly lower engrafted $CD34^+$ cells from cultured $CD34^+$ cells from CBU following an assay done on NOD/SCID mice [220]. Shortly after researchers have shown the opposite, when a different clinical application was done using two CBUs, where one CBU was expanded and the other CBU expanded only a fractioned and infused the remaining unit [43,210,221]. The median neutrophil engraftment varied between 10 to 28 days with an OS of 35% at two years. The cell culture medium

used for expansion contained SCF, FLT3, TPO, IL-3 and IL-6 for a period of 14-16 days. One of the studies reported aGVHD up to 66.7% and cGVHD up to 74%. All recipients infused with a higher concentration of $CD34^+$ per kilogram (5×10^4/kg) had a more rapid time to neutrophil recovery [43].

Cord Blood (CB) T cells were also successfully expanded using medium containing IL-2 for 14 days [222,223]. The goal was to verify the possibility of transplanting CB T-cells in addition to the primary graft to improve immune reconstitution and reduce infectious complications. Other CBU expansion assays demonstrated the large potential of this stem cell source and its capacity to differentiate into many hematopoietic lineages [224]. Recently, NK cells of cryopreserved cord blood units were expanded for future use to enhance its tumor cytotoxicity effect [225]. A previous study examined the contribution of the different cord blood $CD34^+$ cell populations after thrombopoietin (TPO) expansion [226]. SDF-1/CXCL12 was found to enhance expansion of CB HSC induced by the combination of SCF, Flt3-L, and TPO [161]. Finally, a recent study combined cytokines and endothelial cells contained in the bone marrow niche, ensuring a larger expansion compared to cytokine media alone [219]. This not only confirms the possible expansion of CB for future transplantation but also the important role of endothelial cells in the engraftment process.

Enhancement of Stem Cell Homing

The goal of the different CB transplantation methods developed over the years was to shorten the delay of neutrophils and platelet engraftment time. Increasing the

number of TNC and CD34 per kilogram of recipient weight has been intensely studied. Other methods attempted to increase hematopoietic stem cell homing by using diprotin A or Val-Pyr, a CD26 inhibitor, that cleaves dipeptides from the N-terminal of proline or an alanine [161,227]. Sitagliptin has been studied for its inhibition of DPP-4, normally used for the treatment of Type II Diabetes, which also increased stem cell homing. Prostaglandin-E2 is a lipid mediator which demonstrated a regulatory role in hematopoiesis. It inhibits the growth of colony-forming unit- granulocyte/macrophages (CFU-GM) and stimulates erythroid and some progenitor cells. When in contact with HSC at 37ºC, it increases CXCR4 expression with SDF-1, thus increasing homing [228,229].

Intra-bone Transplantation

Instead of increasing the homing by inhibition of cell signaling or phosphorylation, infusion of CB $CD34^+$ cells directly into the bone was suggested, based on the rationale that only a fraction of cells infused migrate to the bone marrow niche [43,230]. The engraftment seems to be 15 times better compared to intravenous infusion [161]. Using this method, the median transplanted cell dose was 2.6 x 10^7/kg. However the median neutrophil recovery time was between 15 to 23 days and no engraftment was observed [161,231,232]. Intra-bone transplantation was also done when using reduced intensity conditioning regimen for recipients and similar results were obtained with a neutrophil recovery of 17 days; platelet recovery however was obtained in 8 out of 10 recipients. The OS at 1 year was 46.7% [233].

Nonetheless, the remaining limiting factor when choosing a single CBU for adult transplantation is the limited number of nucleated cells contained in that unit. Subsequently different methods were developed to increase the number of cells in adult transplantation. Several double CBUs transplantations demonstrated success, however the drawback was the identification of two CBUs containing the same HLA mismatches added to the cost, which would double bringing it to $70 000 US ($35 000 US/CBU) per transplantation[161,168,214,235]. Nevertheless, increasing the number of stem cells by infusing two CBUs provided new possibilities, such as infusion of multiple CBUs, however this method significantly increased the cost of transplantation [236,237]. Several studies were successful in conducting *in vitro* expansion of hematopoietic stem cells (HSC) in serum-free media. However, in these studies HSC preferably differentiated into pluripotent stem cells, while reducing proliferation of HSC with the observation of an increase in stem cell apoptosis. Novel expansion methods show promise for the future but it remains a time-consuming and costly solution [238-242]. Another frequently used method is the enhancement of the engraftment of a unique compatible CBU in adults using $CD34^+$ selected nucleated cells from a haplo-identical family member as a third party unit [115,210]. However, given the low number of siblings per family (1.5 siblings), many recipients still lack donors. An additional concern is the presence in the recipient' bone marrow of both stem cell sources, the two CBUS or the CBU and the third party family donor, in the first months post-transplantation. This persistence of mixed

chimerism has been demonstrated to increases time to myeloid engraftment and lower platelet engraftment[243].

Hypothesis

That grafts from pooled HLA-blind CD34-selected cord blood stem cells, salvaged from small normally discarded cord blood units, can be used as third party support for the engraftment of matched cord blood units that are otherwise deficient or suboptimal in their content of CD34-positive stem cells.

Rationale

Insufficient $CD34^+$ stem cell is one of the main barriers to the greater utilization of cord blood for transplantation in adults. Only about 4% of cord blood units contain sufficient stem cells to safely transplant an adult of average size and up to 70% of collected units are rejected by public banks due to insufficient cell content. Third party support using related haploidentical CD34-selected peripheral blood stem cell grafts have been used successfully to enhance the engraftment of co-transplanted HLA-matched cord blood units. It is thus feasible to consider that third party support from pools of HLA-blind and CD34-selected cord blood units could similarly supply additional $CD34^+$ cells to support the engraftment of a matched (≥ 4/6 HLA compatible) that are deficient in stem cell content.

Primary Objective

To develop method to pool multiple thawed cord blood units followed by $CD34^+$-selection to produce a third party graft with adequate cell recovery and viability suitable for human trials.

Secondary Objective

To assess the feasibility and safety of this new third party graft in co-transplantation with a single ≥ 4/6 HLA-compatible CBU in adults, using time to neutrophil engraftment and 100 days post transplantation as primary end points.

Originality

This approach of using HLA-blind pooled $CD34^+$-selected cord blood units to create third party support for allogeneic cord blood transplantation is entirely unique and novel.

Contribution

The creation and management of the MUHC Research Cord Blood Bank, all methods leading to the successful creation of pooled third-party grafts from UCB units, the investigation of the potential effects of maternal-fetal ABO incompatibility on graft quality, and molecular chimerism analysis, have been done by me. The pilot allogeneic stem cell transplant study using grafts that I prepared was carried out by the MUHC Stem Cell Transplant Unit with Dr. Pierre Laneuville as Principal Investigator.

CHAPTER 2: POPULATION AND METHODOLOGY

Study Population

Adult Donors

The development of cord blood pooling technique required the build-up of a cord blood bank (CBB). The pooling technique was done using whole blood collected from donors 18 years of age and older, who required a previously scheduled phlebotomy until sufficient CBUs were banked. The unit nurse was responsible for obtaining a clear and full consent from the donor and ensuring that the donation was anonymous. The units were transported to the laboratory at room temperature to be processed the same day. The volume of each whole blood unit varied between 200 and 300 mL and was divided into smaller units of approximately 60 to 100 mL. The processing of each small unit was performed according to the cord blood volume reduction procedure described below.

Matched Cord Blood Unit

The selection of the “Matched” CBU was based on a required ≥ 4/6 HLA compatibility with the recipient. The unit was HLA-typed at the antigen level for HLA-A and HLA-B and at the allele-level for HLA-DRβ1 by the 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 were preferred and a HLA-DRβ1 mismatch was selected as a last resort.

The CBU must contains a minimum of 1.5 x 10^7 NC per Kg of the recipient’s body weight but not exceeding 3 x 10^7 NC/kg in the presence of complete HLA compatibility or 4 x 10^7

NC/kg in the presence of one HLA mismatch or 5 x 10^7 NC/kg in the presence of two HLA mismatches.

Adult Recipients

To be eligible, adult patients had to be between 18 and 60 years of age and at a high risk of relapse, with a performance status of ECOG < 3 or Karnofsky ≥ 60%, with hematological malignancies which would normally be considered for myeloablative allogeneic transplantation at my institution. A confirmation that no adult matched unrelated donors (MUD) were available or potentially available 3 to 6 months after the launch of a search was also required. The stem cell transplant physicians of my institution were responsible for the identification and consent of adult recipients. The myeloablative conditioning regimen consisted of fludarabine, cyclophosphamide, busulphan.

Material

Participants

Future mothers with a single pregnancy aged 18 and older were given information related to this research project after showing interest for the cord blood public bank of Québec. Free and clear consent for collection and processing of the cord blood unit was obtained before labor by obstetricians or by nurses. Mothers were asked to complete a medical questionnaire in compliance with Health Canada Cells, Tissue, and Organ regulations. A unique number was attributed to each potential cord blood donation.

Cord blood collection

After the cord was cut, the cord blood was collected while the placenta was still in the uterus (*in utero* method). The umbilical cord was disinfected with iodine by the physician. The cord blood vein was punctured using a 14-gauge needle attached to a collection bag (Fenwal, Inc., CGR81D2S, Lake Zurick, IL, USA). The blood contained in the umbilical cord and in the placenta was collected by gravity in the Fenwal 250 mL cord blood collection bag containing 35 mL of CPD (Citrate-Phosphate-Dextrose). A milking of the umbilical cord was performed to ensure complete drawing of all the blood contained in the umbilical cord. The tubing of the collection bag was clamped after ensuring that all the blood it contained was transferred to the bag. This was followed by gentle mixing by inverting the bag 4 to 5 times. The collection bag was labeled using a previously assigned unique number, and packaged in a biohazard plastic bag for transportation at room temperature to the Cord Blood Laboratory of the McGill University Health Centre.

CBU processing and cryopreservation

Upon reception, CBUs were weighed and their volume estimated assuming 1.053g/mL of cord blood. All units collected for the public bank contain ≥ 100 mL with ≥ 1.1 x 10^9 nucleated cells for all donors, with the exception of Caucasian which had to contain ≥ 1.3 x 10^9 nucleated cells, were sent to this bank. Unqualified CBUs and all CBUs collected for only clinical research were transferred into a triple processing bag (MacoPharma, cat: VRT0000XU) by inserting the tubing-spike into the collected CBU bag. Using a 3 mL syringe (Becton Dickinson and Company, cat #309585), a 2 mL CB

sample was taken and aliquoted as follow: 0.5 mL on blotting paper (FTA Minicard, VWR cat: 14222-804) dried for a minimum of 4 hours in the biological safety cabinet and long term preserved in a multi-barrier pouch labeled with the unit unique number (Whatman FTA™ cat: WB120355) for future HLA testing; 10 µL used for a blood smear; 0.5 mL sample for a complete blood count (CBC) and the calculation of the TNC content of the bag. All CBUs were volume reduced with the exception of units containing less than 0.3×10^9 nucleated cells, these being qualified as clinically inadequate. The CBUs that were cryopreserved within 48 hours of the collection were classified for clinical use or as clinically inadequate and used only for validation.

All units were volume reduced using the “Top-bottom Technique”. The triple MacoPharma cord bag was folded and inserted into a centrifuge bucket tightly using adapter to maintain a straight position of the unit during the centrifugation. Following the centrifugation at 2650xg for 13 minutes at room temperature, the unit was slowly retrieved from the bucket making sure that the buffy coat layer was not disturbed and was hooked on the semi-automatic Optipress (Optisystem® system, Fenwal). The program selection of the Optipress was based on the volume of the CBUs pre-processing (Table 1) to ensure a minimal loss of nucleated cells with most of the plasma and red cells expelled. After processing, the Optipress tube-seals upper and lower tubings which are snapped to separate the red cells from the plasma and buffy coat bags. The volume of each bag was measured and documented.

Table 1: Optipress Programmed Protocols

Programmed Protocol	1-1 (A)	1-2 (B)	1-3 (C)	1-4 (D)	1-5 (E)	1-6 (F)
Buffy coat volume (mL)	21	23	36	36	36	38
Buffy coat level[1]	2.5	3.0	3.3	4.0	3.9	4.2
Force[2]	20	20	18	19	18	20

1. Buffy coat level is measured by red dotted line on the pressing plate.
2. The force is controlled by different sensors which limit the flow rate of the expelling components.

A sampling site coupler (Fenwal Inc cat: 4C2405) was inserted into the red cells and plasma fraction bags and different samples were collected using a 10 mL syringe (Becton Dickinson cat: 309604) and 18G needle (Becton Dickinson cat: 305196). A first sample from the red cells bag was used to test for the presence of bacterial contamination using a minimum of 5 mL. A second sample of 4 mL was used for blood group typing and for hemoglobin chromatography. Some red blood cell bags were preserved at 4ºC for future use. A 10 mL plasma sample was used to screen for transmittable infectious diseases as required by Health Canada. Four 1-1.5 mL plasma samples were cryopreserved in 2 mL cryovials in the same condition and at the same time as the leuko-platelet layer. The buffy coat bag rested for a period of 30 minutes, followed by the use of 4 x 4 gauze to gently rub the surface of the bag and dislodge the nucleated cells adhered to the inner surface of the bag. Another 1.5 mL sample of buffy coat was collected using a 3 mL syringe with a 16G needle (Becton Dickinson cat: 305197) for the calculation of total nucleated cell (TNC), the hematopoietic stem cell

(CD34) counts and for CD34 viability. A 0.5 mL was used for colony forming cell (CFC) assay described later.

The volume of the buffy coat bag was re-verified followed by tube-welding with a 50 mL EVA cryocyte bag (MacoPharma, cat: GSR1001AU). The bags were placed at 4°C for at least 5 minutes. A solution of 50% dimethyl sulfoxide (DMSO; Alveda Pharma cat:1296CC01) and Dextran 10% LMD 40 (Hospira Cat 0741950) was prepared by mixing equal parts of DMSO and Dextran in a 5 mL syringe (BD cat: 309603) with an 18G needle and also placed at approximately 4°C for at least 5 minutes. Calculation of the DMSO volume required to ensure a final concentration of 10% was done based on four volumes of the leuko-platelet layer for one volume of 50% DMSO for cryopreservation. The DMSO/Dextran solution was slowly added through the infusion port of the buffy coat bag over a period of 10 minutes while gently rotating the bag to ensure adequate mixing with the buffy coat. A 1.5 mL sample of buffy coat mixed with the final concentration of DMSO 10% was taken and sub-aliquoted in 4 pre-identified cryotubes. The tube-weld-seal was opened to allow the transfer of the buffy coat into the EVA cryocyte bag. Using a 20 mL syringe (BD, cat: 309661) screwed onto one of the cryocyte bag tubing, all air was taken out of the cryobag before tube sealing the tubing. Two additional segments attached to the bag were sealed. The final volume was measured and labelling was done before placing the cryobag into a pre-identified aluminum cartridge. The cartridge was placed in a -80°C freezer between two Styrofoam plates for 1 ½ - 3 hours, before transfer into a vapor phase cryofreezer (-186°C ± 10°C)

for long term storage. One of the buffy coat cryotubes was used for DNA Short Tandem Repeat (STR) polymerase chain reaction (PCR) for each unit to document the DNA chimerism baseline pre-transplantation (assay describe below).

Laboratory assays

Complete blood count and nucleated red blood cell count

TNC count was done on a 0.3 mL sample taken before and after volume reduction using a method based on electric Beckman Coulter ® LH 750 Hematology Analyzer. The sample taken after volume reduction was diluted 1:1 with Coulter rinsing solution (BD, cat: 8547167). The TNC was calculated by multiplying the WBC (10^9/L) by the volume (mL) of the CBU pre-processing or post-volume reduction. The percentage of nucleated red blood cell (nRBC) was obtained by manual differential of a Wright-Giemsa stained blood (Beckman Coulter cat: 754181).

Flow cytometry

A 300 µL pre-processing sample was taken from whole cord blood and post-processing sample from the buffy coat. Three 5 mL polystyrene tubes (Becton Dickinson Falcon cat: 352008) were identified as one control and two as positive staining. In the control tube 20 µL Fluorescein isothiocyanate (FITC) for CD45 was added, with 20 µL 7-Amino-Actinomycin D (7-AAD) as a viability dye and 100 µL of Stem-Count Fluorospheres for absolute cells count (Beckman Coulter Stem-Kit). In the other two tubes 20 µL of CD45 (FITC) was added, 20 µL of phycoerythrin (PE) for CD34, with 20 µL of 7-AAD and 100 uL of Stem-Count Fluorospheres. In each tube 100 µL of blood

was added and samples incubated at room temperature and protected from ambient light for 20 minutes followed by the lysing of RBC for 10 minutes for fresh CB using 2 mL of 1x ammonium chloride solution (Beckman Coulter Stem-kit). Flow cytometry studies on thawed samples were conducted as per a fresh CB sample except cell lysing was replaced by adding 2 mL of Hank's Balanced Salt Solution (HBSS; BioWhitteker, Lonza cat: 10-547F) to ensure viability and accuracy of the cell count.

Colony forming cell Assay

The enumeration of colony forming cells (CFC) was done on CB samples post volume-reduction with added DMSO/Dextran. Using a 3 mL syringe and a 16G 1.5'' needle, 0.5 mL sample of buffy coat was transferred into a 5 mL Falcon sterile tube (Becton Dickinson Cat: 53300-421) containing 3.5 mL of a mix of HBSS with 2% Fetal Bovine Serum (FBS)(Bio Whittaker cat: 14501F). The 4 mL mixed buffy coat / HBSS / 2% FBS was slowly added over a 4 mL Ficoll Paque Plus (GE Healthcare Life Science cat: 71-7167-00AG) in a 15 mL conical polypropylene tube (Becton Dickinson cat: 352099) and centrifuged at 400xg for 30 minutes at room temperature. The mononucleated layer was then collected with a sterile transfer pipette (Starsted cat: 86.1171.001) into a 13 mL polypropylene tube (Sarstedt cat: 62.515.006) and the mixed solution of HBSS / 2% FBS was added for a total of 10 mL. After a centrifugation at 600xg for 10 minutes at room temperature, the supernatant was suctioned and the pellet reconstituted with another 10 mL of HBSS with 2% FBS for a second identical centrifugation. The final pellet was reconstituted in 1 mL of HBSS, a 0.3 mL sample was

taken for CBC and a 10 µL of cells was mixed with 10 µL of Trypan blue for viability count using a haemocytometer. Cells were resuspended to a final concentration of 1.5 x 10^5 and 0.75 x 10^5 NC/mL. Of this final concentration of cells a 200 µL sample was mixed with 2 mL of Methocult 4034 media each (Stem Cell Technologies, Vancouver, Canada), vortexed for 3 to 4 seconds and allowed to sit for approximately 5 minutes. Using a 3 mL syringe with a 16 gauge blunt-end needles (Stem Cell Technologies cat: 28110), the media was aspirated and equally plated in three of the four 15 mm wells in a plate (VWR Nunclon multidishes cat: 62407-068) with sterile water (Baxter, cat: JF7623) in the last well. Both plates of Methocult 4034 media contains stem cell factors (SCF), GM-CSF, IL-3, G-CSF and erythropoietin (EPO). Both plates were incubated at 37ºC in a 4% CO_2 incubator for 14 days. Using an inverted microscope, colony forming units (CFU) were scored for granulocyte-macrophage (CFU-GM), multipotiential granulocyte-erythrocyte-macrophage-megakaryocyte (CFU-GEMM) and burst-forming unit erythrocyte (BFU-E) colonies. A CFU-GM colony is defined as a colony containing at least 40 macrophages and granulocytes. A large CFU-GEMM colony contains more than 500 multi-potential progenitor cells. A BFU-E colony contains more than 200 erythroblasts in a single or multiple clusters. The performance of the analysis was based on the calculation of the percentage of $CD34^+$ cells becoming colony-forming cells which should vary between 10-20% of the $CD34^+$ incubated.

Short Tandem Repeats (STR)

The identity of each cell source (CBU and transplant recipient) was determined molecularly by STR patterns. This technique is the method of choice for identity testing in forensic medicine, genealogy analysis, etc. STRs (microsatellites in genetic terms) are short tri- or tetra-nucleotide sequences repeated in tandem at various sites on all chromosomes, and most STR loci have been mapped and given a specific address. Each STR locus has a variable length depending on the number of tandem repeats. DNA sequences flanking the STR loci are conserved in the general population, thus common PCR primers for each STR locus (address) can be used in order to determine the length of the STR in any individual. Since the number of STR tandem repeats, although highly polymorphic, is limited, each individual cell source was tested at 9 different STR loci (see below). The probability that two separate samples would have all 9 identical STR patterns is extremely remote. Thus, this method was used to provide a unique molecular “fingerprint” to each cell sample source. The identity and relative quantification of all cell sources (pure – pre-transplant, and mixed – post-transplant) was determined using a semi-quantitative PCR assay.

The identity of each CBU pooled, of the HLA matched CBU and of the transplant recipient was determined by STR. The STR analysis was done on DNA extracted from the cord blood leuko-platelet layer cells and on the recipient leucocytes collected from a peripheral blood sample. The progression and identification of the HSC engrafting the recipient was verified and measured every 7 days post-infusion starting at day +14 post

transplantation until neutrophil engraftment followed by the regular unrelated donor assessment.

DNA Extraction

A 200 - 400µL 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 RBCs, and then centrifuged at 720xg for 2 minutes to pellet the nucleated cells. The reddish supernatant, consisting of lysed RBCs was discarded, and the whitish pellet (containing the nucleated cells) was gently disrupted by slow votex mixing, and 3 mL of cell lysis solution was added to it. All cells were lysed and their DNA content was released into the suspension. The tube was placed on ice for 2 minutes, after which 1 mL Protein Precipitation Solution was added, vortexed at a high speed for 20-30 seconds and left on ice for 5 minutes. The Protein Precipitation solution precipitates protein while leaving the DNA in suspension. It was then centrifuged at 1440xg at 4ºC for 15 minutes and the supernatant was transferred into a clean 15 mL conical tube. Three millilitres of 100% isopropanol was added to the pellet and visual verification of a DNA precipitate was done. The precipitated DNA was transferred using a sterile transfer pipette into a clean 15 mL conical tube. 3 mL of 70% ethanol was added and the tube was inverted 5 times to wash the DNA. It was centrifuged at 1440xg at 4ºC for 3 minutes. The supernatant was gently decanted and the tubes 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). DNA

concentration and purity were assessed by measuring UV absorption of the sample at 260 nM and 280 nM using 2 µL of the DNA solution on a NanoDrop ND-1000 (NanoDrop, Wilmington, DE, USA) spectrophotometer. DNA was then diluted to a final concentration of 0.1 µg/µL using DNA Hydration Solution.

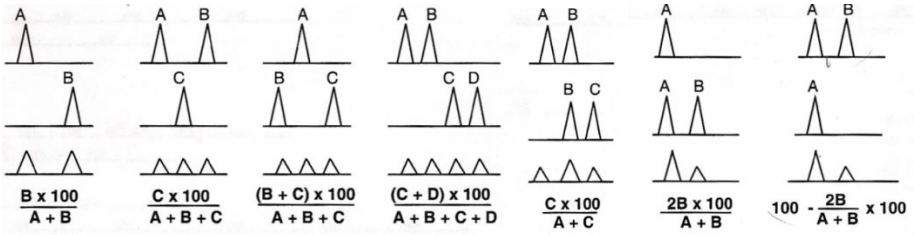
PCR Amplification of STR loci

Nine STR loci were amplified under identical Polymerase Chain Reaction (PCR) condition. The loci used were D1S1660, D3S2387, D5S1456, D5S818, D7S820, D8S1179, D13S317, D16S539, and D20S481. In a 200 µL PCR tube, the selected fluorescent primer pair (4nM) with 0.1µL of the DNA sample, 0.1U Taq DNA polymerase, 1.5 mM $MgCl_2$, 4 nM dNTPs and PCR buffer (Perkin-Elmer 10X buffer) were mixed according to the manufacturer's instructions. After an initial incubation at 94ºC for 5 minutes to denature the genomic DNA , 35 cycles were performed using the following profile: denaturation at 95ºC for 30 seconds, annealing at 57ºC for 30 seconds, and a final elongation step at 72ºC for 45 seconds. After completion, the PCR fragments were analysed on the ABI PRISM 3100 Genetic Analyzer ("automated capillary electrophoresis sequencer"). A Performance Optimized Polymer (POP7) was added to a 2 µL of the PCR amplicon with 8 µL of de-ionized water, 10 µL of de-ionized formamide and 0.5 µL of Marker-Genescan-500Liz size standard. Double-strand denaturation was done using the thermal cycler at 95ºC for 5 minutes. The samples were then loaded on the ABI sequencer for fragment length analysis. The fragment lengths were determined by the number of STR present at each locus for each individual sample as compared to the in-

run molecular size marker. In a mixture of two or more samples (chimerism) in a post-transplantation event, each CBU would contribute a PCR fragment of unique length.

DNA Chimerism Interpretation

Calculation of the percentage of DNA chimerism was done using the area under the curve of the CBU's STR amplification peaks. These were preferably chosen to be 4-8 or up to 12 base pairs apart in order to facilitate analysis. Different calculation formulas were used, depending on peak patterns, which may vary in 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 Qantification Kit was used to identify the presence of any Y chromosome in the peripheral blood sample of the recipient at day +14 and day +21 post-transplantation. A 0.1, 0.01, 0.001 and 0.0001 µg/µL DNA concentration of those samples were used to verify the sensitivity of the analysis.

A dilution of 1:10 was performed using 1 µL of 0.1 µg/µL Male DNA, Day +14 DNA or Day +21 DNA with 9 µL of unknown female DNA providing a final concentration of 0.01 µg/µL. The later was used and diluted to a final concentration 0.001 µg/µL by using

1 µL of 0.01 µg/µL DNA with 9 µL of unknown female DNA. This last step was performed a last time to obtain a final DNA concentration of 0.0001 µg/µL of the Male DNA, the Day +14 DNA and Day +21 DNA samples.

In a 25 µL capillary PCR tube, 2 µL of the each DNA samples concentration and of sterile water as negative control and female DNA as negative DNA control was mixed with 10.5 µL of the Quantifiler Y Human Male Primer Mix, containing target-specific primers and FAM dye-labeled probe, and with 12.5 µL of the Quantifiler PCR Reaction Mix, containing dNTPs, buffer, AmpliTaq Gold DNA polymerase (Applied Biosystems ©) according to the manufacturer's instructions. Samples were centrifuge at 735xg on the LightCycler ® Centrifuge Adapter for less than a minute and then placed in the LightCycler ® 2.0 Instrument of Roche. After an initial incubation at 95ºC for 10 minutes to denature the genomic DNA, 40 cycles were performed using the following profile: denaturation at 95ºC for 15 seconds, amplification at 60ºC for 1 minute. After completion, an absolute Quantification analysis based on the concentration of standard samples was performed using the LightCycler ® Software 4.05 (Roche).

Elution and indirect coombs

Evaluation of the presence of immunoglobulin G (IgG) anti-A and anti-B on the leukocytes was done by elution and indirect coombs. Leukocytes from the pooled CBUs were washed up to six times with 5 mL of HBSS in a 5 mL glass tube and centrifuged at 1500xg for 1 minute. Supernatant from the final wash was preserved as IgG negative control and the supernatant of the first wash was preserved to determine the presence of

IgG anti-A and anti-B. The pellet was diluted with approximately 1 mL of saline solution (NERL Blood Bank cat: 8505) and incubated at 56ºC for 10 minutes. Post incubation, the mix was centrifuged at 1760xg for 2 minutes. The eluate was transferred into an identified glass tube and prepared with 3 ± 1% A, B or O red blood cells (Ortho Clinical Diagnostic®) and incubated at 37ºC for 15 minutes. The eluate and the negative control final wash were washed three times and centrifuged for 18 seconds. Two drops of anti-IgG were added to each tube and centrifuged for 18 seconds. The final pellet was verified for agglutination in presence of anti-A IgG and/or anti-B IgG and documented. One drop of IgG was added on all negative agglutination tubes to verify if agglutination was possible.

Rosette technique

A rosette technique was employed using pooled blood group A cord blood buffy coat samples and negative control using pooled blood group O cord blood buffy coat samples. The buffy coat sedimentation was done on Ficoll gradient to isolate the mono-nucleated cells, followed by two washes with HBSS/2% FCS as rinsing solution and a centrifugation at 600xg for 10 minutes at room temperature. The pellet was resuspended with 50 µL of Phosphate buffered saline/Ethylene-diamine-tetra-acetic acid (PBS/EDTA) and 5% human albumin in a 5 mL glass tube. A drop of 50 µL of murine mono-clonal anti-A antibody (Ortho Clinical Diagnostic ®) was added to the resuspended pellet and incubated at 37°C for 20 minutes. A single wash was done after adding 6 mL of FCS 2% solution followed by a centrifugation at 1760xg for 2 minutes. The maximum amount of

supernatant was retrieved leaving approximately 200 µL of solution which was tapped lightly for re-suspension of the cells into the solution. The pellet was put on ice for 20 minutes after adding 3 ± 1% group A red blood cells at concentration (Ortho Clinical Diagnostic®) and incubated at 37ºC for 10 minutes. The final product was resuspended with 3 mL of HBSS/2% FCS and centrifuged at 289xg for 3 minutes. The pellet was then resuspended with 50 µL of FCS solution and one drop was placed on a microscope slide and stained with Wright-Giemsa for rosette identification. The stained nucleated cell linked to one or more red cells was defined as a Rosette using a microscope at 40X.

Mag Sepharose

Verification of the IgG Fab-region binding on leukocytes after pooling CBUs was done using the Mag Sepharose technique. Pooled blood group O mothers and CBUs were used as negative controls and pooled ABO incompatible CBU mothers and CBUs used as positive controls. After pooling and washing three CBUs, the pellet was resuspended with 50 µL of PBS/EDTA and 5% human albumin. A drop of 100 µL of Protein A or Protein B Mag Sepharose beads (GE HealthCare®) was dispensed into an Eppendorf tube (Abbott Laboratory X Systems cat: 9527-40). Using the magnetic rack, the Mag Sepharose storage solution was removed and a 250 µL of the pooled CBUs pellet was added. An incubation of 60 minutes was done with a slow end-over-end mixing. In the first method, two washes with PBS/EDTA and 5% human albumin were conducted followed by the use of a magnet to separate the Mag Sepharose beads from the liquid. A second method consisted of using directly the magnet without any previous

wash. The content retained on the magnet was spread on a microscope slide and stained with Wright-Giemsa. Detection of cells bound on the beads was observed using a microscope at 40X.

Pooling of Adult Units

A group of four cryopreserved units were pooled in the first attempt of the pooling method development. The dilution solution used was an equal mixture of Gentran 40 and 5% human albumin prepared in 60 mL syringes (BD, cat: 309653) with 18G 1.5G needles to ensure a 1-in-10 dilution. The first syringe contained exactly the same volume as the unit to be diluted. The other three syringes contained the left over volume of the mixed solution ensuring the 1-in-10 dilution. (Example: for a 20 mL unit, one syringe contained 20 mL of dilution solution and three syringes each containing equally divided 160 mL of dilution solution). The unit was inserted into a sterile plastic bag and thawed in a 37ºC water-bath for 2 minutes or until content was in a liquid phase. A sampling site coupler was inserted into the unit. The first syringe was inserted and the solution infused slowly while rocking the unit bag. The unit was then kept at 4°C ± 2°C (on ice) to ensure osmotic exchange for a period of 5 minutes. The syringe was used to retract the content of the unit and then transferred into an empty 600 mL transfer bag (Fenwal, cat: 4R2023). Every unit bag was rinsed two to three times and the content was transferred into the same 600 mL transfer bag. The pooled unit was then mixed slowly to ensure homogeneity in the content of the bag. A 1 mL sample was taken for TNC counts. Four units totaling 80 mL were diluted with 300 mL of the dilution solution to ensure a final

DMSO concentration of approximately 2%. A second assay was done using three adult cryopreserved units totaling no more than 60 mL diluted with 540 mL of the same mixing solution to ensure a 1% DMSO concentration post pooling. Post dilution and pooling, all pooled units were centrifuged at 600xg for 9 minutes at 4ºC. A 600 mL transfer bag was spiked into the pooled 600 mL bag without disturbance of the pellet. The pooled supernatant was expelled using the Optipress leaving the pellet with approximately 26 mL of supernatant. This pellet was then mixed for 15 minutes to ensure homogeneity and a sample was taken for TNC count.

CB pooling

The number of CBUs was based on the sums of as many CBUs as required to pool a minimum of 1 x 10^6 $CD34^+$ cells/kg pre-thaw of recipient weight. For example, a recipient weighing 80 kg requires as many CBUs to ensure a total of ≥ 80 x 10^6 $CD34^+$ cells pre-thawing. The first pooling method consisted of a semi-closed system using a stop-cock, a 60 mL syringe and two 600 mL transfer bags (MacoPharma cat:VSE4110XA), one containing an equal mix of Gentran 40 and 5% human albumin and 3 in 1 tubing (MacoPharma, cat:EPE1017A) (Figure 1). The first cryopreserved CBU was inserted into a sterile plastic bag and thawed in a 37°C water bath until content was in liquid phase which takes approximately 2 minutes. The unit was spiked in one of the three tubing attached to the stop-cock. An equal volume of the thaw CBU was inserted into a 60 Ml syringe with the dilution solution. The solution was slowly infused into the thaw CBU and kept at 4°C ± 2°C (on ice) for a period of at least 5 minutes. The second

and third units were thawed and diluted using the same method. The diluted units were transferred, using the stop-cock and the 60 mL syringe, into the 600 mL transfer bag identified as pooled unit bag (VES4110XA, MacoPharma; 4C5628, Fenwal). Using the same technique, each unit bag was rinsed up to three times with the mixed solution of 5% human albumin/Dextran 40 and transferred into the same 600 mL pooling bag. The 600 mL transfer bag containing the pooled CBUs was centrifuged at 600xg at 4°C for 9 minutes. Post centrifugation, a 600 mL bag was spiked into the pooling bag for the supernatant to be expelled using the Optipress to a final volume varying between 20 mL to 30 mL.

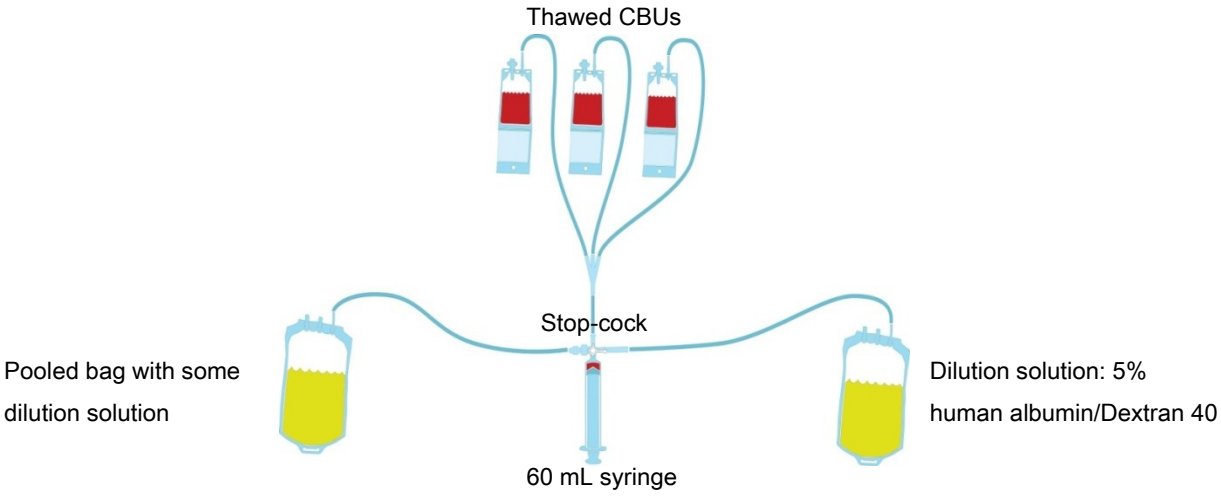


Figure 1: First Pooling Method

This first pooling method required simplification to ensure the pooling of a maximum number of CBUs within a limited number of times. The stop-cock and the syringe were discarded and the transfer system was replaced by larger unique tubing

ensuring more rapid transfer into the 600 mL bag (Figure 2). The dilution solution content and volume were not changed. However, instead of thawing the three units of the same pool one after the other, one unit per pool was thawed and three pools were done at the same time, diluted and rinsed before being replaced by the other unit.

Each pool bag of three CBUs was then re-pooled using a 10-in-1 tubing (MacoPharma, EPE 10177). Forty (40) to 60 mL of PBS/EDTA (Miltenyi Biotech, cat: 700-25) with human albumin added for a final concentration of 0.5% was used to rinse all pooled bags ensuring full transfer in a final 600 mL bag. A final centrifugation at 600xg at 4ºC for 9 minutes was performed followed by the expression of the supernatant using the Optipress to reduce the final pool CBUs bag to a volume of 95 mL ± 5 mL.

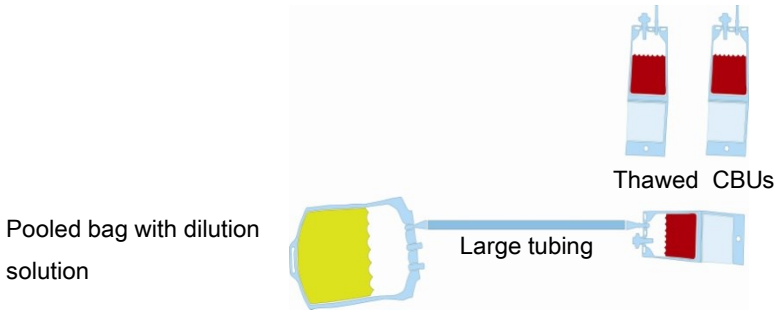


Figure 2: Simplified Pooling Method

CD34 Selection

The final pooled CBUs bag was injected with 100 mL of air. It was followed by the slow addition of the 7.5 mL CliniMACS CD34 Reagent (Miltenyl Biotec cat: 171-01) draw from the vials with a 10 mL syringe with a 16G needle. The bag was placed on a rocking

platform for a period of 45 minutes at room temperature and the bag was 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 done after adding approximately 400 mL of PBS/EDTA with 0.5% human albumin. The supernatant was expelled on the Optipress for a final volume of ≤ 300mL. A Cell Separation Column placed in the CliniMACS Magnetic Separation Unit was used according to the manufacture to isolate the magnetic-antibody bounded CD34 expressing cells contained in the 300mL bag (figure 3). The positive fraction volume is always 40 mL and is transferred by the CliniMACS into a 150 mL transfer bag. At the end of the selection the 150 mL transfer bag containing the $CD34^+$ selection is taken from the equipment and transferred under the biological safety cabinet where a sample of 0.5 mL is taken for CFU assay, CBC and CD34, CD45 and CD3 cell counts and 7AAD viability.

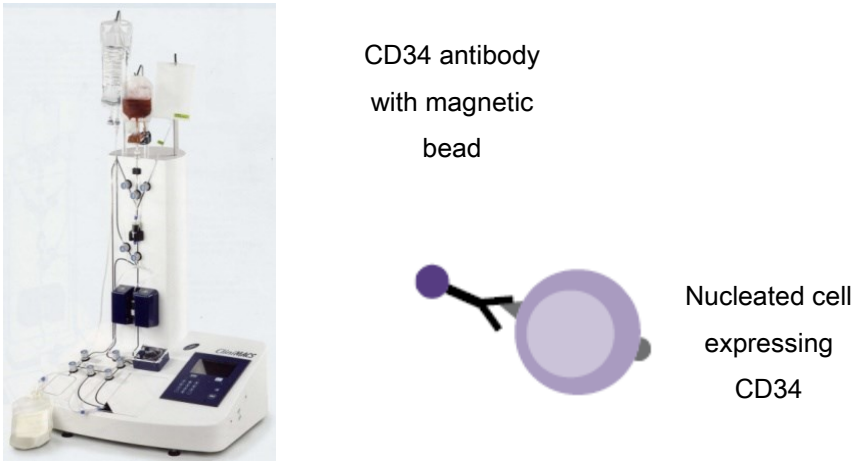


Figure 3: CD34 Selection with CliniMACS

The negative fraction was verified for its content in CD34+ cells by the retrieval of a 2 mL sample after the insertion under the biological safety cabinet of a sampling site coupler. The negative fraction bag was then centrifuged at 600xg at 4ºC for 9 minutes followed by the expression of the supernatant using the Optipress to concentrate the leucocytes in a volume varying between 30 mL to 40 mL. A 10 mL sample was used for bacterial and fungal analysis, a 5 mL sample was used for DNA extraction (for STR baseline analysis) and the rest was preserved as two 10-13 mL samples in 15 mL polystyrene tube at -80ºC freezer.

Effect of plasma volume on Pooled Cryopreserved CBU Samples

Thawing CBU was done with a dilution solution composed of an equal mix of Dextran and human albumin. CBU samples were thawed and diluted using three different dilution solutions in order to determine the effect of each solution on the NC recovery. The first dilution was with a solution containing equal volumes of Dextran 40 and 5% human albumin. The second was composed of equal volumes of Dextran 40 and the pooled CBU's plasma. The third dilution was composed of one quarter 5% human albumin, one quarter of the pooled CBUs plasma and one half of Dextran 40. Three 5 mL polypropylene tubes were prepared with 300 µL of the different dilution solution. Three different CBU samples, the volume of which varied from 300 µL to 1000 µL, were thawed in a 37°C water bath and 100 µL of each CBUs sample was pooled in the three different tubes. The diluted samples were put on ice for a period of

5 minutes to ensure osmotic exchange, as for pooled CBUs. A final 2400 µL of the same dilution solution was added to ensure final DMSO concentration of 1%. Pooled samples tubes were reversed a couple of time to ensure adequate mixing. TNC count was done on each tubes and the percentage of nucleated cells recuperated was calculated with the total NC count of the three pre-cryopreserved CBUs.

Effect of red blood cells on Pooled Cryopreserved CBUs

CBUs were thawed and diluted using additional red blood cells in the dilution solution in order to determine the effect on the NC recovery. Cryopreserved CBUs were inserted in a sterile plastic bag and thawed in a 37°C water bath for 2 minutes. After inserting a sampling site coupler, each cord blood unit was first diluted with an equal mixed solution of 5% human albumin and Dextran 40 using a 60 mL syringe with a 16G needle and left on ice for a period of at least 5 minutes. Using the same syringe, the content was aspirated and equally transferred into two 600 mL transfer bag (VESM 401Q, MacoPharma; 4C5628, Fenwal Inc), both containing an equal mixed solution of 5% human albumin and Dextran 40. One of the pooled bags also contained an additional volume of 5 mL of cord blood red blood cells corresponding to the blood group of the pooled CBU. Each cryopreserved unit was rinsed twice with the mixed solution and equally transferred into the 600 mL bag using a 60 mL syringe to ensure a final DMSO concentration of 1% in the pooled bag. The 600 mL transfer bags were centrifuged at 600xg at 4°C for 9 minutes. A 600 mL bag was spiked into the pooled bag to

expel the supernatant using the Optipress for a final volume of 20 mL ± 5 mL. The pellet was mixed gently ensuring homogeneous content. Samples were taken in the pooled transfer bags and in the pellet bags for TNC count.

Statistics

Descriptive statistics were used to compare TNC and $CD34^+$ cells counts, viability and cell counts recovery. All statistical analyses were conducted with 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 statistically significant.

Descriptive statistics were used to compare TNC and $CD34^+$ cell counts, viability, and colony forming units (CFUs) pre and post-processing. Results are given as mean and standard deviation (SD). Analyses of the variables following normal *t* test distribution were carried out a one-way analysis of variance (ANOVA). In the presence of asymmetric distribution, non-parametric analyses and correlation were done. Two independent samples *t*-Test ensured the comparison of two groups of cases on one variable with the Mann-Whitney U test. If the ANOVA was not applicable, an independent sample t-Test was used between variables or tests for categorical and Mann-Whitney U-tests for continuous variables. The power and the analysis of the sample size were done using the software Power and Precision 4.1 (2011).

Ethics

The research protocol was approved by the Ethical Research Committees of the McGill University Health Center (GEN#06-018). Informed consent for the Clinical Research Cord Blood Bank was given by the mother before the child's birth and before active labor. Consent included that the CBU will not be reserved for family use, the completion of a medical questionnaire, that serology testing, such as HIV and hepatitis, will be done on the mother within 7 days post-partum and information on the mother and newborn, such as type of delivery, sex and weight of newborn will be collected in their chart.

Consent was also signed by the adult donor, including a phlebotomy. The consent included the confirmation of the anonymity of the donation which was made ensured by the unit nurse.

Consent form and explanations to recipient was provided by the transplant physician. A period minimal of two weeks was given to the recipient before consent signature.

CHAPTER 3: RESULTS

Cord Blood Banking

Collection and Qualification

The McGill University Health Center (MUHC) – Clinical Research Cord Blood Bank (CRCBB) opened while beginning its collaboration with the Public Bank of Héma-Québec in October 2007. Since the opening, 2,701 mothers registered for the Public Cord Blood Bank (CBB) and/or the Clinical Research Cord Blood Bank (CRCBB) at an average of 33.5 ± 5.8 weeks of gestation. Some future mothers refused (11%) to participate to the CRCBB. The obstetricians and nurses working in the MUHC Birthing Center were trained on in utero collection method, decontamination, labeling and packaging with respect to Health Canada Cells, Tissues and Organs (CTO) for Transplantation Regulations and to the Foundation for the Accreditation of Cellular Therapy Standards. The integration of these new tasks increased their load of work and caused the absence of collection of many CBUs in the first 6 months (31.3%). In the following years, CBUs were not collected for the most part due to *Abruptio placentae* (11.2%), emergency cesarean (C-section) (10.1%) and newborn distress (10.1%). Of a total of 2313 collected CBUs, 895 (38.7%) CBUs were sent to the public bank (Héma-Quebec) following qualification or due to consent only obtained for public donation. Of the remaining 1404 units destined for the CRCBB, 922 (66%) qualified for clinical application while the others were kept for validation (20%) or were disqualified (14%)

mainly due to a volume of less than 10 mL of blood (Figure 4). The majority of the CBUs were collected after vaginal delivery (74.6%) with no evidence of gender bias (i.e. 51.3% male and 48.7% female). Only five (5) CBUs out of the 1418 analyzed by the CRCBB were contaminated with *Staphylococcus aureus* or *Escherichia coli,* representing less than 0.35% of the collected units. The diverse ethnic background of collected CBUs including 33% of non-Caucasian origin (Figure 5) reflects the multiethnic nature of the population served by the MUHC.

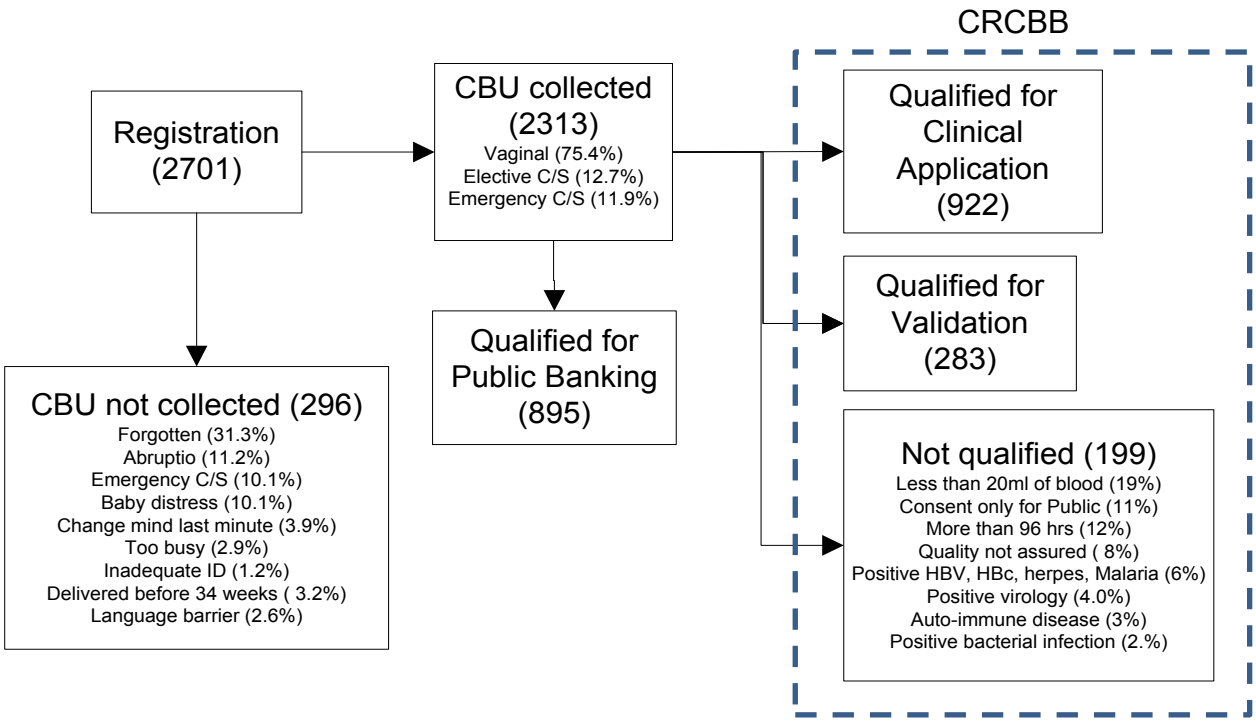


Figure 4: Number of Registered Mothers, number of collected and qualified CBUs, and CBU allocation from November 2007 to June 2012

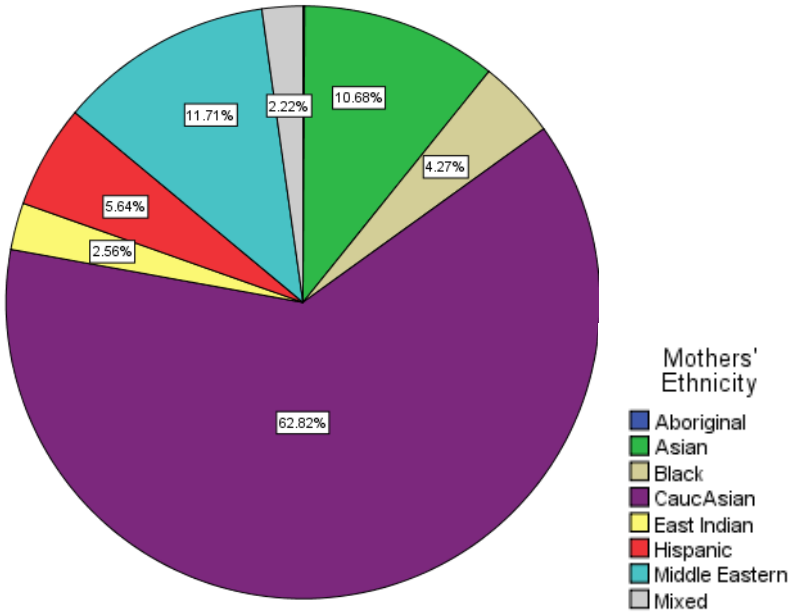


Figure 5: Ethnicity of mothers

Processing and cryopreservation

In collaboration with the Public CBB, all CBUs were collected using the *in utero* method, which is a collection done while the placenta is still in the uterus. Identical processing and cryopreservation methods were used for CBUs kept for validation, as well as for clinical application. The processing evaluation was done by measuring the Total Nucleated Cell (TNC) and the hematopoietic stem cell ($CD34^+$) recovery. TNC count included nucleated red blood cell (nRBC) to standardize with most of the literature. Even if the mean of nRBC is 5.3% (S.D. = 5.2; n = 1168), more than 5% of the CBUs contained more than 15% of nRBC (maximum 55%). Comparison between the results of

the units kept for clinical application and those kept for validation showed no difference for collected volume, TNC pre and post-processing (Table 2). The volume frozen after volume reduction for units kept for clinical application seems to be larger compare to those used for validation ($p = 0.001$), as for CD34 counts pre and post-processing ($p = 0.001$ and $p = 0.023$).

Table 2: Comparison of Cord blood volumes, TNC and CD34 counts and yields pre and post-processing for CBUs kept for validation versus CBUs qualified for clinical application

Mean	Collected Volume (mL)	Frozen Volume (mL)	TNC Pre (x 10^9/bag)	TNC Post (x 10^9/bag)	CD34 Pre (X 10^6/bag)	CD34 Post (X 10^6/bag)
Clinical Application (n = 921)	98.6 (SD=27.4)	20.1 (SD=3.2)	1.03 (SD=0.59)	0.57 (SD=0.31)	3.29 (SD=3.3)	2.32 (SD=2.4)
Validation only (n = 249)	95.3 (SD=26.3)	19.3 (SD=3.9)	0.98 (SD=.51)	0.60 (SD=0.36)	2.48 (SD=2.2)	1.92 (SD=1.97)
Significance between both groups (p-value)	0.082	0.001	0.244	0.302	0.001	0.023

When comparing the proportion of $CD34^+$ cells per TNC cells present in the initial collection bag, I can observe a positive linear regression ($R^2 = 0.414$, $p \leq 0.001$) with a significant correlation (Pearson = 0.668, $p \leq 0.001$) (Figure 6). Although the mean of $CD34^+$ cells count per unit is 3.1 x 10^6, more than 10% of the units contain more than twice the number of $CD34^+$ cells and 2% contained ≥15 x 10^6 $CD34^+$ cells. However, 9% of the collected CBUs contained less than 0.5 x 10^6 $CD34^+$ cells which would disqualified them for clinical application.

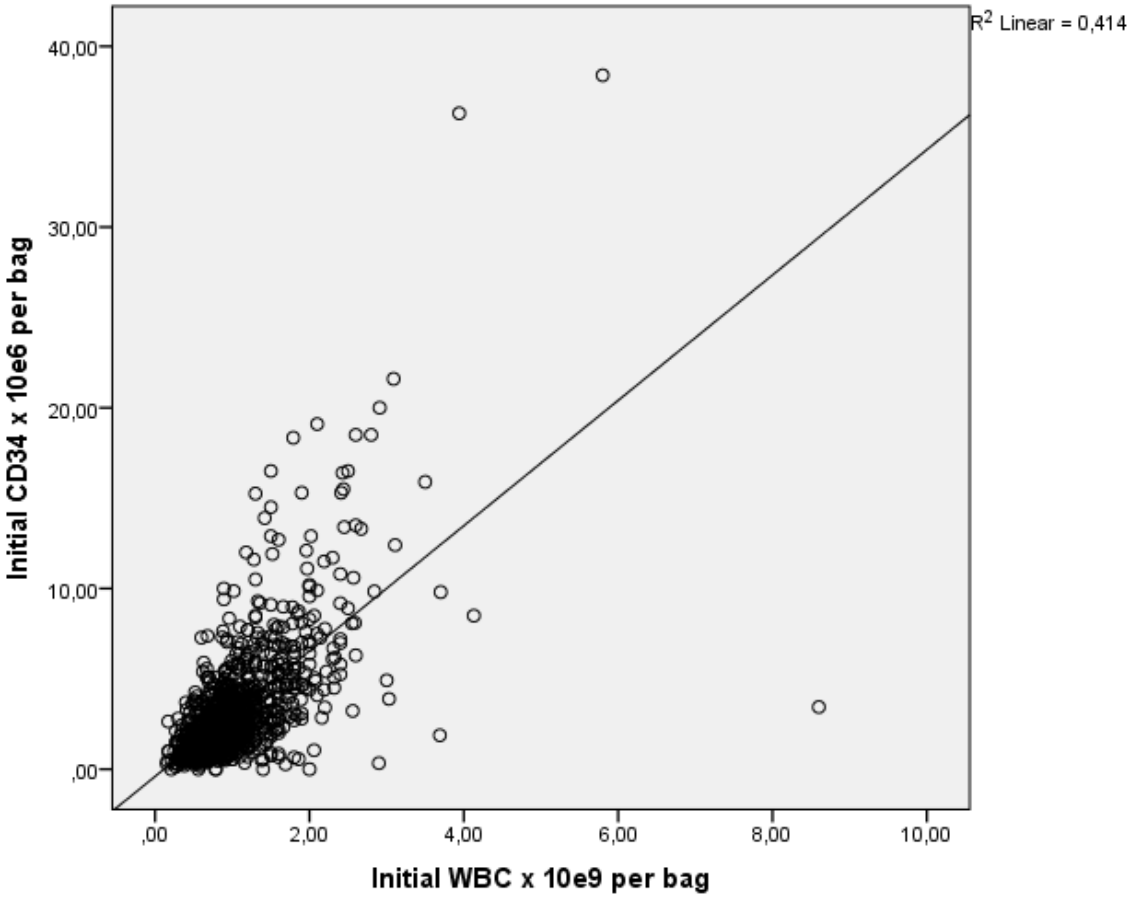
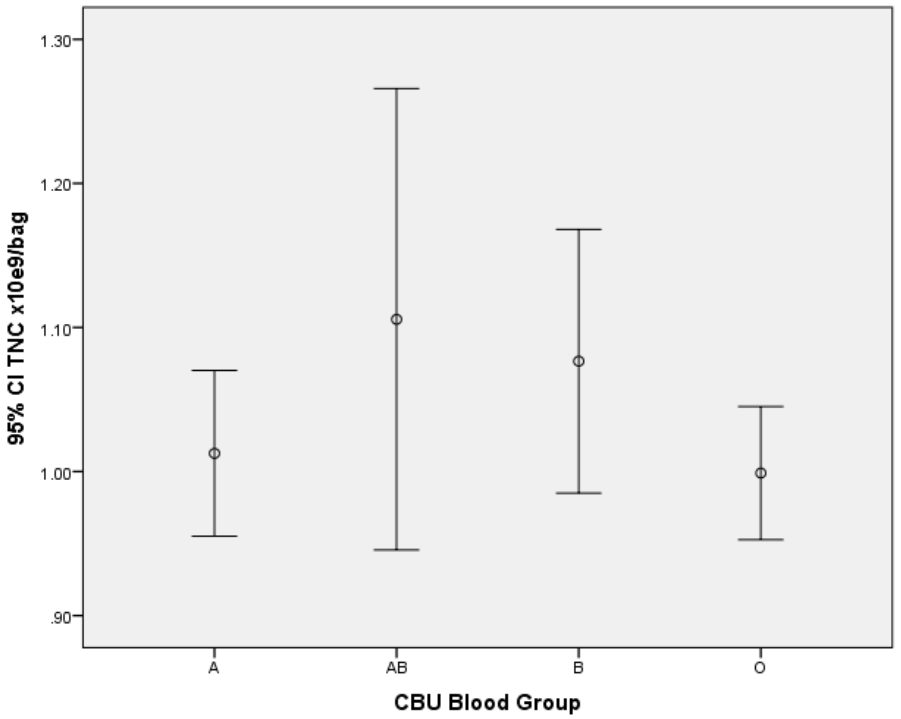


Figure 6: Correlation of TNC versus $CD34^+$ cell counts on collected CBUs (n = 1170, Pearson 0.668, $p \leq 0.001$)

Different factors were analyzed for their impact on the initial TNC and the CD34 counts and observed that gender, presence of meconium, antibiotics given to the mother during delivery, group B streptococcus (GBS), oxytocin infusion during labor or type of delivery were not influencing the counts. In contrast, the presence of maternal fever during labor significantly increased mean TNC counts of the collected CBU to 1.14 x 10^9 cells/bag from 0.98 x 10^9/bag ($p = 0.019$), but did not significantly affect the mean $CD34^+$ count (3.4 x 10^6/bag versus 2.8 x 10^6/bag, $p = 0.134$) (Table 3). However, the ratio of

$CD34^+$ cells versus TNC cells per initial collection bag decreases when mothers were receiving oxytocin during labor ($p = 0.037$) and when newborns gender were a girl ($p = 0.003$) (Table 4). CBU blood group seems to impact differently TNC and $CD34^+$ counts at collection. The units with blood group AB showed the highest number of TNC and the lowest number of $CD34^+$ cells, however the highest number of $CD34^+$ cells is CBUs of blood group B (Figure 7).



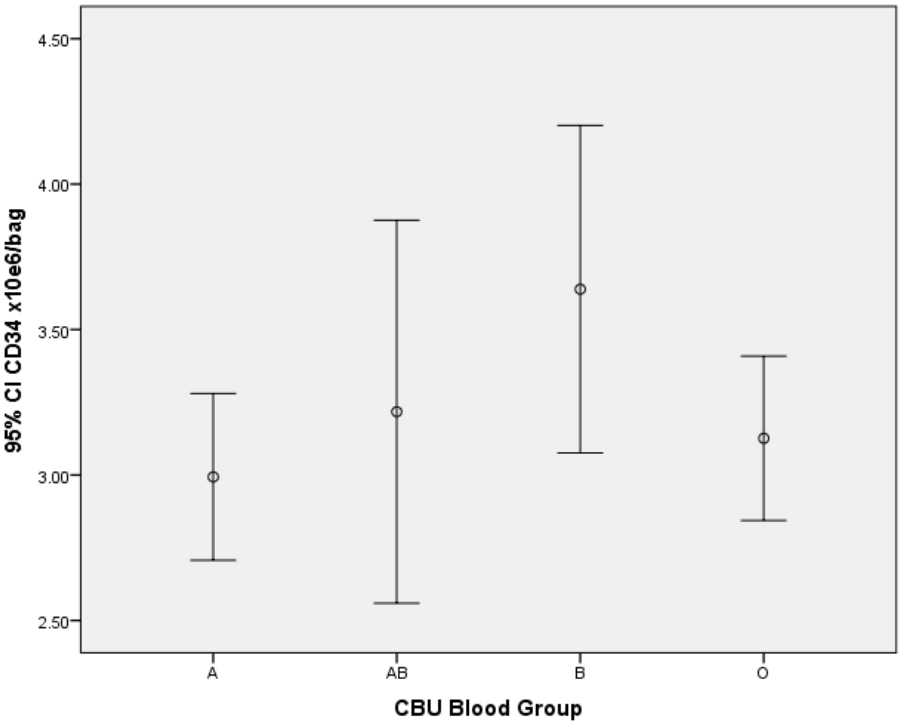


Figure 7: Mean of TNC and CD34⁺ cells according to CBU blood group with a 95% confidence limits

Table 3: Effect of different variables on TNC and CD34 counts per initial collection bag

Variables		TNC ($x 10^9$)	p-value	$CD34^+$ ($x 10^6$)	p-value
Maternal Fever during labor(n=638)	Yes	1.14	0.019	3.39	0.134
	No	0.98		2.84	
Antibiotic during labor(n=639)	Yes	1.03	0.381	3.00	0.706
	No	0.99		2.86	
Oxytocin during labor (n=607)	With	01.05	0.395	2.67	0.424
	Without	0.99		2.98	
Meconium at birth (n=593)	Yes	1.04	0.493	2.57	0.126
	No	1.00		2.99	
Group B Streptococcus (n=471)	Positive	1.07	0.093	2.98	0.436
	Negative	1.00		2.89	
Delivery Mode (n=609)	Vaginal	1.02		3.09	
	C/S	0.92		2.68	
	Emergency C/S	0.99		3.28	
	Vaginal vs C/S		0.104		0.462
	Vaginal vs Emergency C/S		0.084		0.619
	C/S vs Emergency C/S		0.975		0.837
Gender (n=608)	Male	1.00	0.52	3.14	0.098
	Female	1.02		3.00	

Table 4: Effect of different variables on $CD34^+$ (10^6)/TNC (10^9) ratios per initial collection bag

Variables		CD34/TNC	p
Maternal Fever during labor	Yes	2.92	0.541
	No	3.05	
Antibiotic during labor	Yes	3.06	0.867
	No	3.03	
Oxytocin during labor	Yes	2.5	0.037
	No	2.92	
Meconium at birth	Yes	2.82	0.968
	No	2.81	
Group B Streptoccocus	Positive	2.29	0.467
	Negative	2.19	
Delivery Mode	Vaginal	3.04	0.903
	C/S	2.95	
	Emer. C/S	3.13	
Gender	Male	3.25	0.003
	Female	2.83	

Following the initial qualification, each unit was volume reduced to approximately 20 mL ± 2m using one of the five different Top-bottom programs configured on the Optipress (Table 1). The programs C, D and E were the most frequently used for CBU volume varying at collection from 61 mL to 140 mL. When larger CBUs (> 141 mL) were processed on program D and E, I can observe similar recovery, however the number of CBUs processed being so limited prevents us from confirming the significance of the results (Table 5). A comparison of all programs of CBUs brought us to observe that program C ensures the best recovery when the initial volume is less than 160 mL.

CB collection bags contain 35 mL of CPD, which was mixed with blood volume varying between 10 mL to a maximum of 250 mL in a single bag. The proportion of anticoagulant versus the volume of the blood contained in the collection bag seems to

significantly improve the yield of $CD34^+$ and TNC. On the other hand, I observed a better $CD34^+$ cell yield (73.5%) in presence of low blood volume compared to a larger blood volume (70.0%) (Table 6). The TNC yield is going in the same direction as $CD34^+$ cells, however even if significant, the difference was less than 1% between large and small blood volume.

Table 5: Effect of Processing Program on TNC Yield from different CBU initial volumes

				Initial Volume (mL)								
				< 60	61-80	81-100	101-120	121-140	141-160	161-180	181-200	> 201
TNC Yield (%)	Processing Program	B	Mean	70%	20%	77%	54%	43%	-	-	-	-
			Count	2	1	3	1	1	0	0	0	0
		C	Mean	84%	86%	81%	85%	79%	83%	65%	70%	-
			Count	30	110	158	53	35	13	6	3	0
		D	Mean	81%	87%	79%	78%	73%	70%	68%	99%	-
			Count	4	53	118	53	25	15	7	1	0
		E	Mean	82%	85%	80%	77%	76%	75%	74%	71%	75%
			Count	10	64	153	105	51	17	14	6	2
		F	Mean	-	-	95%	74%	53%	-	-	-	-
			Count	0	0	2	1	2	0	0	0	0

Table 6: Effect of Anticoagulant and blood ratio on TNC and $CD34^+$ cell yields

	Ratio of Anticoagulant versus initial cord blood volume											
	0 to 1 (< 70 mL)		1.01 to 2 (71- 105 mL)		2.01 to 3 (106-140 mL)		3.01 to 4 (141-175 mL)		4.01 to 5 (176-210 mL)		5.01 to 6 (> 211 mL)	
	Mean	Total N [1]	Mean	Total N	Mean	Total N	Mean	Total N	Mean	Total N	Mean	Total N
TNC Yield (%)	70.0	1	70.8_a	149	70.6_a	682	70.1_a	247	70.4_a	70	70.0_a	21
CD34 Yield (%)	70.0	1	73.5_a	149	$71.9_{a,b}$	682	71.3_b	247	$70.9_{a,b}$	70	$70.0_{a,b}$	21

Note: Values in the same row and sub-table not sharing the same subscript are significantly different at $p < .05$ in the two-sided test of equality for column means. Cells with no subscript are not included in the test. Tests assume equal variances. Tests are adjusted for all pairwise comparisons within a row of each innermost sub-table using the Bonferroni correction.

1. This category is not used in comparisons because the sum of case weights is less than two.

The TNC and $CD34^+$ cell recovery is a determinant of the quality of any processing method. I demonstrated that using the different Optipress program I was able to conserve a similar correlation between $CD34^+$ cells and TNC in the buffy coat bag (N = 1167, Pearson = 0.705, p = 0.0001) (Figure 8). Even if the mean of $CD34^+$ cells was lower post-processing with 2.24 x 10^6 per bag, more than 10% of the units contained more than 4.4 x 10^6 $CD34^+$ cells and 1% contained more than 11.2 x 10^6 $CD34^+$ cells.

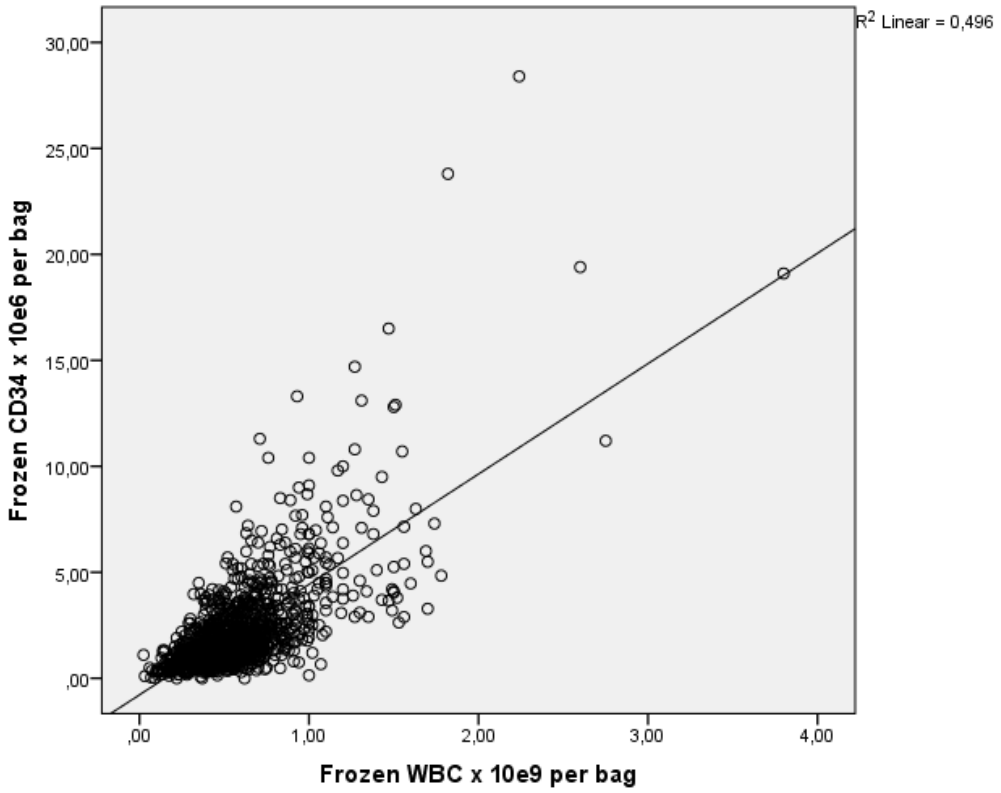


Figure 8: Correlation of TNC versus $CD34^+$ cell counts on post-processing CBUs

The time between collection and processing has been analyzed to evaluate its possible role on cell counts, viability, as well as cells recovery. Observation on pre-

processing samples collected at different time between collection and processing, when the unit is preserved at room temperature, demonstrated that TNC ($p = 0.317$) and $CD34^+$ ($p = 0.253$) cell counts are not affected by the delay. On the other hand, a longer delay has a great effect on the mean of leukocytes ($CD45^+$) cells viability with a reduction from 85.9% at ≤ 48 hours to 54.6% when CBUs is between 72 to 96 hours (Table 7). This reduction is also observed with the loss of more than 4×10^8 $CD45^+$ cell counts between those same delay, however this difference is not significant ($p = 0.317$). The TNC data at > 96 hours cannot be analyzed due to small number of units. When looking at the mean viability of $CD34^+$ cells in the same period, I observed that these cells preserve a greater viability compare to TNC with ≥ 80% when the processing is done within 96 hours after collection. This stable viability was also confirmed by colony-forming-cells (CFC) assay, which demonstrate that $CD34^+$ cells proliferation and differentiation potential is not affected by a delay of up to 96 hours after collection, the small variability not being significant ($p = 0.353$) (Table 7).

Table 7: Effect of time on TNC and $CD34^+$ counts and viability

Delay between collection and processing	TNC per bag X 10^9	CD34 per bag X 10^6	CD45 per bag x 10^8	CD34 viability %	CD45 viability %	CFU-GM per CD34 %
≤ 48 hours	1.03 (n = 1037; SD = 0.59)	3.27 (n = 1010; SD = 3.9)	7.29 (n = 998; SD = 5.6)	94.6 (n = 977; SD = 16.4)	85.9 (n = 992; SD = 29.8)	15.3 (n = 738; SD = 9.4)
After 48 to 72 hours	0.96 (n = 116; SD = 0.52)	2.72 (n = 110; SD = 2.2)	5.08 (n =108; SD = 3.45)	91.8 (n =102; SD = 5.5)	66.6 (n =107; SD = 20.1)	14.1 (n = 95; SD = 11.8)
After 72 to 96 hours	0.81 (n = 14; SD = 0.23)	1.83 (n = 14; SD = 1.40)	3.22 (n =14; SD =1.44)	83.9 (n =14; SD = 27.2)	54.6 (n =14; SD = 18.6)	18.7 (n = 13; SD = 14.8)
> 96 hours	0.81 (n = 2; SD = 0.37)	2.23 (n = 2; SD = 2.4)	6.2 (n = 2; SD = 3.98)	99.9 (n = 2; SD =0.14)	88.0 (n = 2; SD = n/a)	21 (n = 1; SD = n/a)
p Value	0.317	.253	0.0001	0.04	.0001	0.353

Another analysed factor was the sedimentation of the cord blood, defined as the rate by which red blood cells sediment in a period of one hour. I regrouped all CBUs per 6 hours post-collection and verified if the delay would ensure a better TNC and/or $CD34^+$ cells yield. I observed that the TNC yield is increasing from 75% to almost 95% when the delay between collection and processing is closer to 42 hours ($p = 0.0001$) (Figure 9). A boxplot graft of TNC cells yield also show that the yield will not increase furthermore, it will even start reducing at hour 66 after collection and after. In contrast, this increasing yield is not observed for $CD34^+$ cells in the same periods ($p = 0.158$; $n = 1180$) (Figure 10).

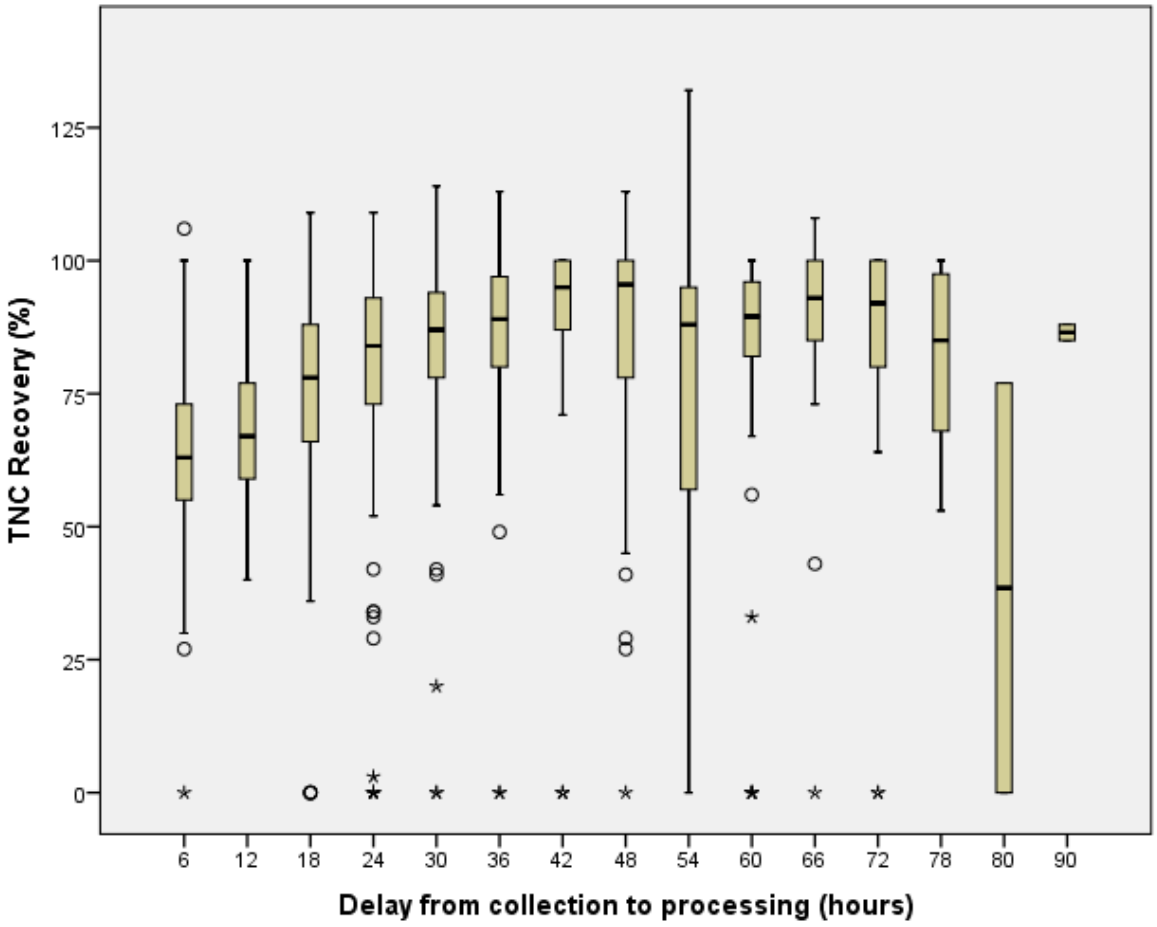


Figure 9: Effect of time between collection and processing on TNC recovery ($p \leq 0.0001$)

(n=1170)

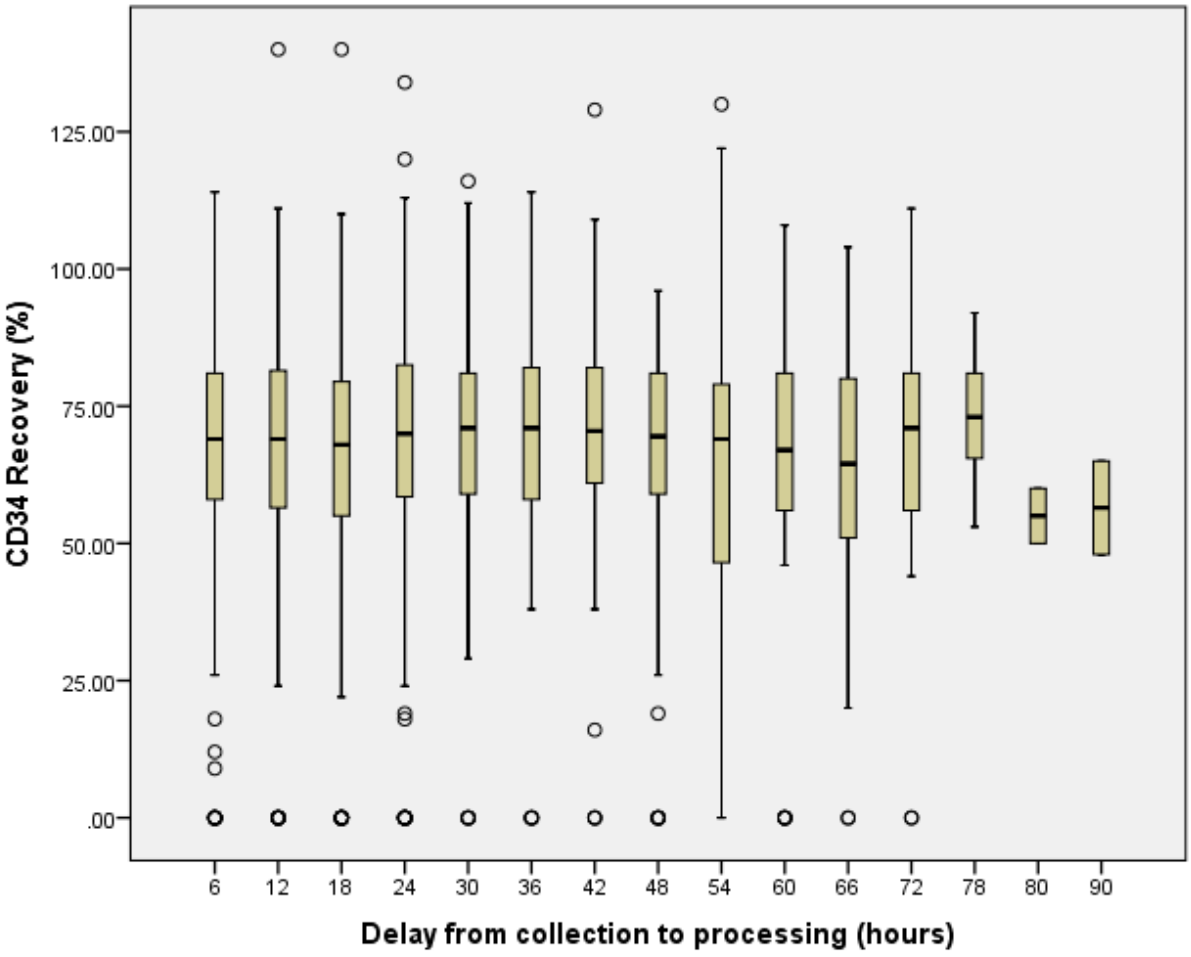


Figure 10: Effect of time between collection and processing on $CD34^+$ cell recovery ($p = 0.158$) ($n = 1170$)

Cord blood pooling

Development of Pooling Method

The first objective of this phase 1 pilot study was to develop a CB pooling method containing the sufficient viable $CD34^+$ cell after performing a positive CD34 cell selection. Twenty CBUs were estimated to be sufficient to obtain 1 x 10^6 $CD34^+$/kg of recipient weight based on the $CD34^+$ cell count pre-cryopreservation. Adult peripheral blood

donors were initially used in early trials of pooling. Two different final DMSO concentrations were verified. When pooling 4 adult donors a final DMSO concentration of 2% was obtained and when pooling 3 adult donors the final concentration was 1%. Based on samples taken after pooling and on the pellet post-washing, TNC yields could be optimized from 70% to 92% post-polling and from 70% to 82% post-washing, when decreasing the final DMSO concentration to ≤ 1% (Table 8).

Table 8: Effect of final DMSO concentration on the TNC yield and viability of pooled adult donor units.

2% DMSO	Pre-Processing		Post-Thawing			Post-Centrifugation on Pellets			
# Unit[&]	Volume (mL)	TNC $\times 10^7$	TNC $\times 10^7$	Yield %	Viability %	Volume (mL)	TNC $\times 10^7$	Yield %	Viability %
4	83	0.45	0.517	89%	89%	40	0.30	58%	88%
4	79	0.93	0.34	37%	51%	19	0.17	45%	45%
4	75	0.69	0.58	84%	87%	40	0.47	81%	85%
Mean	79	0.69	0.48	70%	76%	33	0.31	70%	87%
1% DMSO	Pre-Processing		Post-Thawing			Post-Centrifugation on Pellets			
# Unit[&]	Volume (mL)	TNC $\times 10^7$	TNC $\times 10^7$	Yield %	Viability %	Volume (mL)	TNC $\times 10^7$	Yield %	Viability %
3	59	0.75	0.69	91%	75%	19	0.59	86%	72%
3	58	0.69	0.59	86%	n/a	16	0.57	97%	n/a
3	53	0.46	0.45	98%	n/a	25	0.28	62%	97%
Mean	57	0.63	0.58	92%	75%	20	0.48	82%	85%

Following the adult peripheral blood trials, to ensure a DMSO final concentration (f.c.) of ≤ 1% post pooling, the combined frozen volume of pooled CBUs were limited to

less than 60 mL given that after a 10 fold dilution, the maximum volume that could be accommodated in the pooling bags was 600 mL. Aliquots were taken from the pooled products and on the pellets after centrifugation showed that the percentage of TNC yield was 81.3% post-pooling and 76% post-centrifugation (Table 9), which is similar to the 3 adult donor pools done with a DMSO 1% f.c. (Table 8).

Table 9: Effect of 3 CBUs Pooling on TNC Yield and Viability at a DMSO (f.c.) of 1%.

Pre-Processing			Post-Thawing		Post-Centrifugation on Pellets		
# of CBUs Pooled	Pooled Total Volume (mL)	Pooled Total TNC ($X 10^9$)	TNC ($X 10^9$)	Yield (%)	Volume (mL)	TNC ($X 10^9$)	Yield (%)
3	38	1.08	0.78	72	27	0.79	97
3	49	1.61	1.40	88	24	1.10	79
3	50	2.76	n/d	n/d	33	1.70	61
3	50	1.39	0.92	66	22	0.70	76
3	44	2.37	2.35	99	12	1.58	67
	Mean	**2.03**	**1.36**	**81.3**	**23.6**	**1.17**	**76**

Other pooling assay were performed to verify the viability of the TNC when pooling from six (2 x 3 CBUs followed by creating a larger pool of the two pools) to 12 CBUs (4 x 3 CBUs followed by the pool of the 4 pooled CBUs) (Table 10). Those assays were all technically done within two hours of the first thaw CBU. Three pools of six cryopreserved CBUs and two pools of twelve cryopreserved CBUs obtained an average TNC yield varying from 101% to 84% (SD 6.9%) (Table 10). Those results confirmed that the pooling methodology ensured adequate viability when pooling up to 12 CBUs within

2 hours. In contrast, pooling 45 thawed CBUs, which took 6 hours to do, resulted in a low TNC yield of 45.8% (Table 11). It was concluded from the above that the pooling process needed to be completed within 2 hours in order to optimize TNC yields. In order to achieve this, the central tubing, the stop cock and the syringe were replaced by a single larger tubing (Hospira, Secondary Irrigation Set) to ensure an easier and more rapid transfer of the diluted buffy coat to the 600 mL pool bag (Figure 2).

Table 10: Results of TNC counts and TNC yields on pools of 6 and 12 CBUs

	# of Units Pooled	TNC of pooled CBUs pre-cryopreservation ($X 10^9$)	TNC in pool ($x 10^6$)	TNC Yield (%)
6A	3	2.27	2.4	106
6B	3	2.47	2.4	97
6AB	6		Mean	101
7A	3	3.72	2.75	74
7B	3	2.29	2.14	93
7AB	6		Mean	84
8A	3	1.83	1.7	93
8B	3	2.6	1.93	74
8AB	6		Mean	84
9A	3	2.24	2.08	93
9B	3	1.95	1.94	99
9C	3	2.15	1.94	90
9D	3	1.22	1.2	98
9ABCD	12		Mean	95
10A	3	1.64	1.44	88
10B	3	2.21	1.99	90
10C	3	1.09	0.83	76
10D	3	1.72	1.6	93
10ABCD	12		Mean	87

Table 11: Effect of Time on Pooled CBUs TNC Recovery

Total of 45 CBUs	TNC Yield(%)	Hrs post pooling
Pool A of 18 CBUs	45,8%	6 hours
Pool B of 12 CBUs	57,8%	4 hours
Pool C of 15 CBUs	82,7%	2 hours

Pools varying from 30 to 42 CBUs were created using this simplified technique and were completed within two hours with a mean TNC recovery of 69.5% (58.9% to 95.9%) confirming the positive results of the simplified pooling method (Table 12).

Table 12: TNC yields in large pools of CBUs using the simplified pooling method

Number of Pooled CBUs	TNC recovery (%)
39	95.9
42	58.9
42	63.6
30	59.7
Mean	69.5

CD34 Selection of Pooled Cord Blood Units

After pooling and washing, the CBUs pellet was resuspended to be enriched for $CD34^+$ cells. Compiled results showed an improved positive selection is obtained when the number of pre-selection $CD34^+$ cells was greater than 1.5×10^6. The selection of the appropriate tubing would also impact on the yield. In presence of $CD34^+$ cell counts of 76×10^6 larger tubing was used with a final $CD34^+$ selection of 22×10^6 $CD34^+$, reducing the yield to 30% (Table 13). Still, a mean $CD34^+$ cells selection of 14.2×10^6 with a recovery of 46.9% was obtained. Positive selection ensures the extraction from the product of a maximum of leukocytes, expressed by the purity of the product with a mean

of 80.7%. T cells (CD3) depletion was also verified and a mean reduction of CD3+ cells to 23.2 x 10^4 per pooling was obtained with a minimum of $CD34^+$ cells loss of 2.8 x 10^6 in the negative fraction bag (Table 13). The large $CD34^+$ cell counts difference between the pre and post- selection, is believed to be, in most cases, due to the trapping of cells in one or in both filters of the CliniMACS column. During one selection, a clot in the lower filter necessitate to reverse the flow to wash out the filters and the change of the column to pursue the positive selection with the leftover of the pellet bag and also allowed for the recovery of the majority of trapped cells.

The negative fraction of all $CD34^+$ cell selection were analysed for their potential bacterial and fungal contamination. The fist $CD34^+$ selection was contaminated with Escherichia coli, which was also present in one of the CBU pooled and none of the following selections were contaminated.

Table 13: $CD34^+$ immunoselection yields of pooled CBUs

Number of CBUs pooled	Post $CD34^+$ Selection				Negative Fraction
	$CD34^+$ Counts (x 10^6)	$CD34^+$ Yield (%)	Purity (%)	CD3 Counts (x 10^4)	$CD34^+$ Counts (x 10^6)
12	0.23	15%	N/A	N/A	N/A
45	11.2	23%	82.7%	69.7	15
30	21.9	70%	99%	7.7	1
42	22	30%	75.7%	1.76	1
39	13.6	85%	75.2%	25.9	0.82
42	13.7 (8.76 + 4.24)	43%	75.1%	18 (12 + 6)	0 (0.3 + 0)
30	13	71%	73%	0.12	1
33	18.2	38%	84%	39.3	1
Mean	**14.2**	**46.9%**	**80.7%**	**23.2**	**2.8**

Pooled Cord Blood Units

TNC recovery contained in Table 10 and 11, show large differences between the TNC yields of the different pooled CBUs ranging from 61% to 97% in Table 10 and from 74% to 106% in Table 11. Analysis of the data suggested that ABO blood group incompatibility between the mothers and the pooled CBUs could explain this variability in TNC yield. To explore this possibility further, additional pools containing 3 CBUs were prepared where there was 0, 1, 2 or 3 CBUs in the pool that were ABO incompatible with one or more mothers. There was a trend toward reduced % TNC yield with increasing number of CBUs with maternal-fetal ABO incompatibilities in the pool that reached statistical significance when all (3) units were incompatible (98.2% versus 76.8%, p = 0.052) (Figure 11).

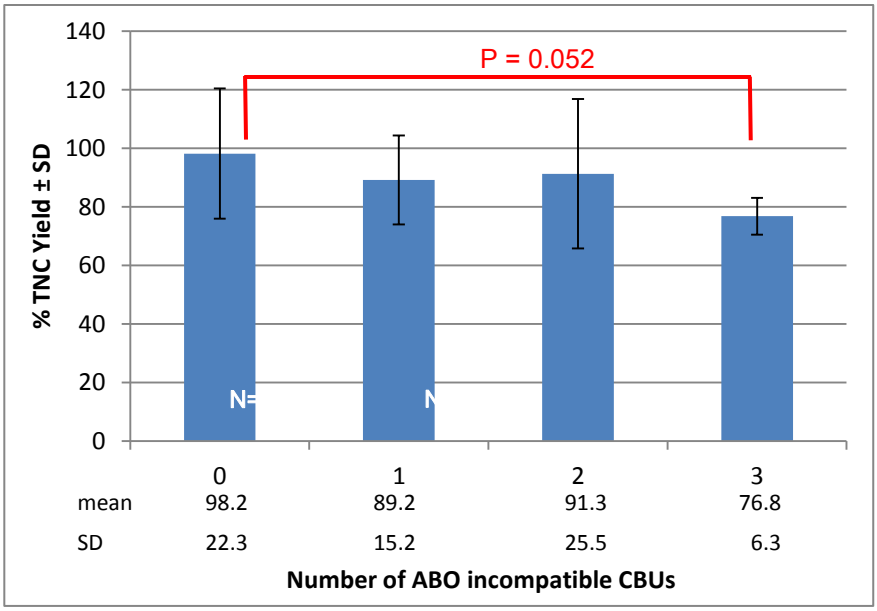


Figure 11: Relationship between the number of CBUs with maternal-fetal ABO incompatibilities and % TNC yields in pools of three units.

Screening for the Presence of Maternal anti-A and anti-B IgG in Pooled CBUs Plasma or Bound to CBU Leukocytes

To determine if passively transferred maternal anti-A or anti-B IgG are binding pooled leukocytes which could account for the decrease of TNC yields in pools of CBUs with maternal-fetal ABO incompatibilities, plasma from CBU pools and eluates from CBU pooled leukocytes were screened for the presence of antibody by indirect Coombs testing. As shown in Table 14, variable amounts of anti-A and or anti-B IgG was detected in the plasma of pooled CBUs. Interestingly, IgG anti-A and anti-B were also eluted from leukocytes of blood group incompatible pooled CBUs (Table 14).

Table 14: Presence of IgG anti-A and anti-B in Pooled CBUs Plasma and Eluates of CBU Leukocytes (Indirect Coombs Test).

Mother ABO	CBUs ABO	Maternal-Pool ABO Compatibility	TNC Yield	Screen for IgG anti-A and anti-B in plasma of pooled CBUs		Screen for IgG anti-A and anti-B on eluate of pooled CBUs leukocytes	
				IgG anti-B	IgG anti-A	IgG anti-B	IgG anti-A
O, A, O	O, O, O	Compatible	108%	4+	2+	0	0
A, A, A,	O, O, O,	Compatible	68%	3+	1+	1+	0
O, O, O,	O, O, O,	Compatible	100%	4+	1+	0	0
A, A, O	O, O, O,	Compatible	88%	4+	3+	0	0
O, O, O,	O, O, O,	Compatible	90%	4+	4+	0	0
A, A, O	A, A, A	Incompatible	72%	1+	0	0	0
A, O, O	A, O, O	Incompatible	76%	4+	4+	2+	1+
A, B, A	A, A, O	Incompatible	93%	1+	1+	1+	0
O, A, O	B, O, O	Incompatible	83%	0	3+	0	1+
O, A, O	B, A, A	Incompatible	72%	1+	1+	2+	1+

Effect of CBU Plasma Volume on TNC Yields

After observing that maternal anti-A and anti-B IgG could be eluted from pooled leukocytes, new pools were prepared by replacing the 5% human albumin of the dilution solution by the plasma of the pooled CBUs. This thawing method aimed to increase the presence of maternal anti-A/B IgG by using the CBU's plasma. As shown in Figure 12, in the presence of maternal-fetal ABO incompatibility the TNC yields were lower when pooled CBUs were resuspended in their own plasma compared to when the dilution solution is composed instead of 5% albumin (5% to 16% less TNC yield). These findings are consistent with maternal anti-A/B IgGs being present in the CBUs plasma which in larger volume increases the amount of IgG anti-A and/or anti-B in the ABO incompatible pools causing lower TNC yields.

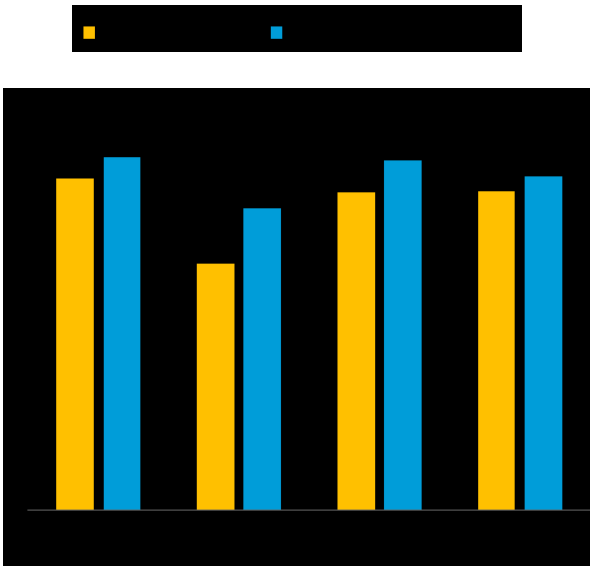


Figure 12: Comparison of % TNC yields of pools reconstituted with CBU plasma versus 5% albumin.

Effect of CBU Red Blood Cell Volume on TNC Yields

To determine if an increase of anti-A and anti-B IgG would reduce the TNC, I verified the reverse hypothesis, by increasing the number of cord blood red blood cells (RBC) when pooling CBUs. The aim was to determine if increased absorption of ABO-incompatible maternal antibodies by RBCs could improve TNC yields. Selection of the pooled CBUs was based on the previous incompatibility level classification between pooled CBUs and mother's CBUs' blood group. The diluted products were equally divided in two pooling bags, one of which contained added cord blood red blood cells (RBC) of the same blood group as the pooled units. After compiling the final results for both groups, differences between the normally pooled CBUs and those pooled with additional RBC were calculated. As shown in Figure 13, there was a trend towards higher TNC yields (≥ 5%) when an increased volume of RBCs were present in all pooled CBUs. The difference was greater in the 3 incompatible CBU group varying from 9.2% up to 16.9% difference in TNC recovery, though this did not reach statistical significance. One important confounding factor is the number of nRBC, which may affected results of the pooled TNC recovery, the number of nRBC in banked CBUs varying between 0% and 55% (Mean = 5.3; S.D. = 5.2).

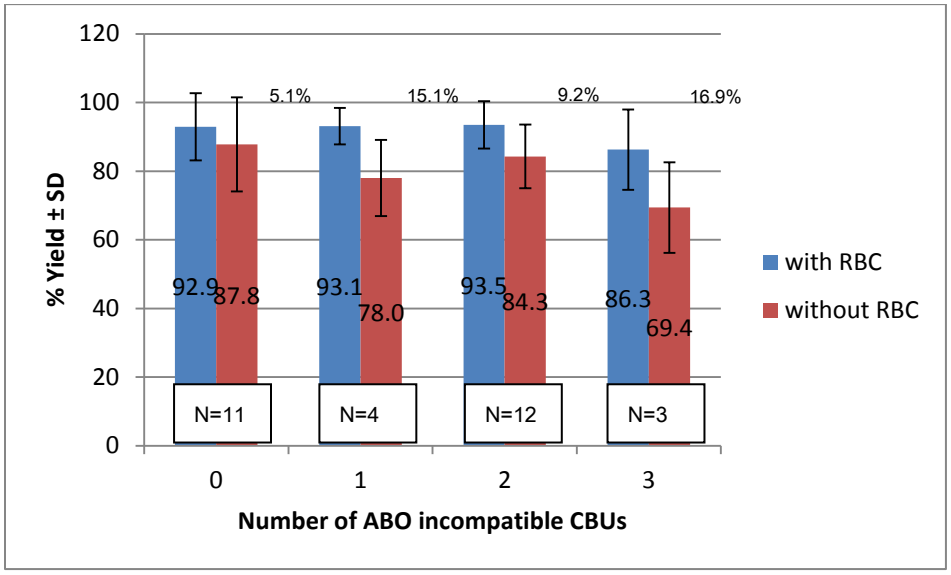


Figure 13: Effect of RBC volume on TNC yield post-thawing and pooling

Measurement of Pooled CBUs composition by DNA STR Analysis

To determine whether pooled CBUs were losing TNCs equally or selectively depending on maternal-fetal ABO incompatibilities, the post-pooling chimeric composition of pooled CBUs was determined by STR PCR assay. CBUs were pooled with and without RBCs to confirm if RBCs could selectively affect the dominance of one unit in presence of maternal-fetal ABO incompatibility. The cord blood RBC added to a group of pooled CBUs contained a small number of nucleated cells but, as mentioned earlier, this remained a potentially confounding factor. The measure was based on the ratio in percentage of TNCs from each CBU as a pooled unit without any cell loss (determined by automated cell counting), which was compared to the percentage of the individual units post-pooling (determined by STR analysis). Observation of the prevalence of a unit is observed but not consistent with the number of ABO incompatibility between maternal-

fetal units (Table 15). A strong variability in the prevalence is observed in presence of all compatible units, ranging from 5.2% up to 11.6%. As observed in previous assay, a stronger predominance is observed in presence of 2 and 3 ABO incompatibilities with 28.5% (pool A) and 19.6% (pool 12) respectively.

Table 15: Relative composition of CBU pools pre and post-pooling

Compatible	Mother CBU ABO	Calculated proportion Pre-Pool	Mean proportion of Pool	Difference
pool C	O/O	24.4%	30.0%	5.6%
	O/O	44.3%	40.9%	-3.4%
	O/O	31.2%	29.1%	-2.1%
pool #3	O/O	6.8%	18.4%	11.6%
	O/O	66.0%	51.2%	-14.8%
	O/O	27.2%	30.3%	3.1%
pool #6	O/O	39.9%	36.7%	-3.2%
	O/O	22.3%	27.5%	5.2%
	O/O	37.8%	35.8%	-2.0%
pool #7	O/O	42.2%	41.5%	-0.7%
	O/O	22.2%	31.2%	9.0%
	O/O	35.6%	27.3%	-8.2%
pool B	O/O	62.0%	51.9%	-10.2%
	A/O	16.9%	23.0%	6.2%
	O/O	21.1%	25.2%	4.1%

Incompatible	Mother CBU ABO	Calculated proportion Pre-Pool	Mean proportion of Pool	Difference
pool A	A/A	29.3%	12.0%	-17.3%
	A/A	40.2%	68.7%	28.5%
	A/B	30.5%	19.3%	-11.2%
pool #1	A/A	50.8%	53.7%	2.9%
	O/O	38.1%	36.0%	-2.1%
	A/A	11.1%	10.2%	-0.9%
pool #2	AB/A	31.8%	36.4%	4.6%
	AB/A	29.2%	28.4%	-0.8%
	B/O	39.1%	35.3%	-3.8%
pool #4	A/A	30.1%	30.2%	0.1%
	O/O	37.0%	34.8%	-2.2%
	B/B	32.9%	35.1%	2.1%
pool #8	B/A	42.0%	40.8%	-1.2%
	B/B	36.4%	34.6%	-1.8%
	A/A	21.7%	24.7%	3.0%
pool #9	O/A	52.8%	47.1%	-5.7%
	A/A	36.6%	39.2%	2.6%
	A/O	10.6%	13.8%	3.1%
pool #10	O/O	51.1%	52.8%	1.7%
	O/B	17.4%	11.8%	-5.6%
	A/A	31.6%	35.4%	3.8%
pool #11	B/O	20.2%	26.6%	6.3%
	B/B	39.3%	36.0%	-3.2%
	A/A	40.5%	37.4%	-3.1%
pool #12	B/A	28.8%	47.7%	18.9%
	O/A	21.8%	41.4%	19.6%
	B/O	49.4%	10.9%	-38.5%
pool #13	B/AB	45.5%	41.5%	-4.0%
	O/O	20.9%	22.8%	1.9%
	A/A	33.6%	35.8%	2.1%

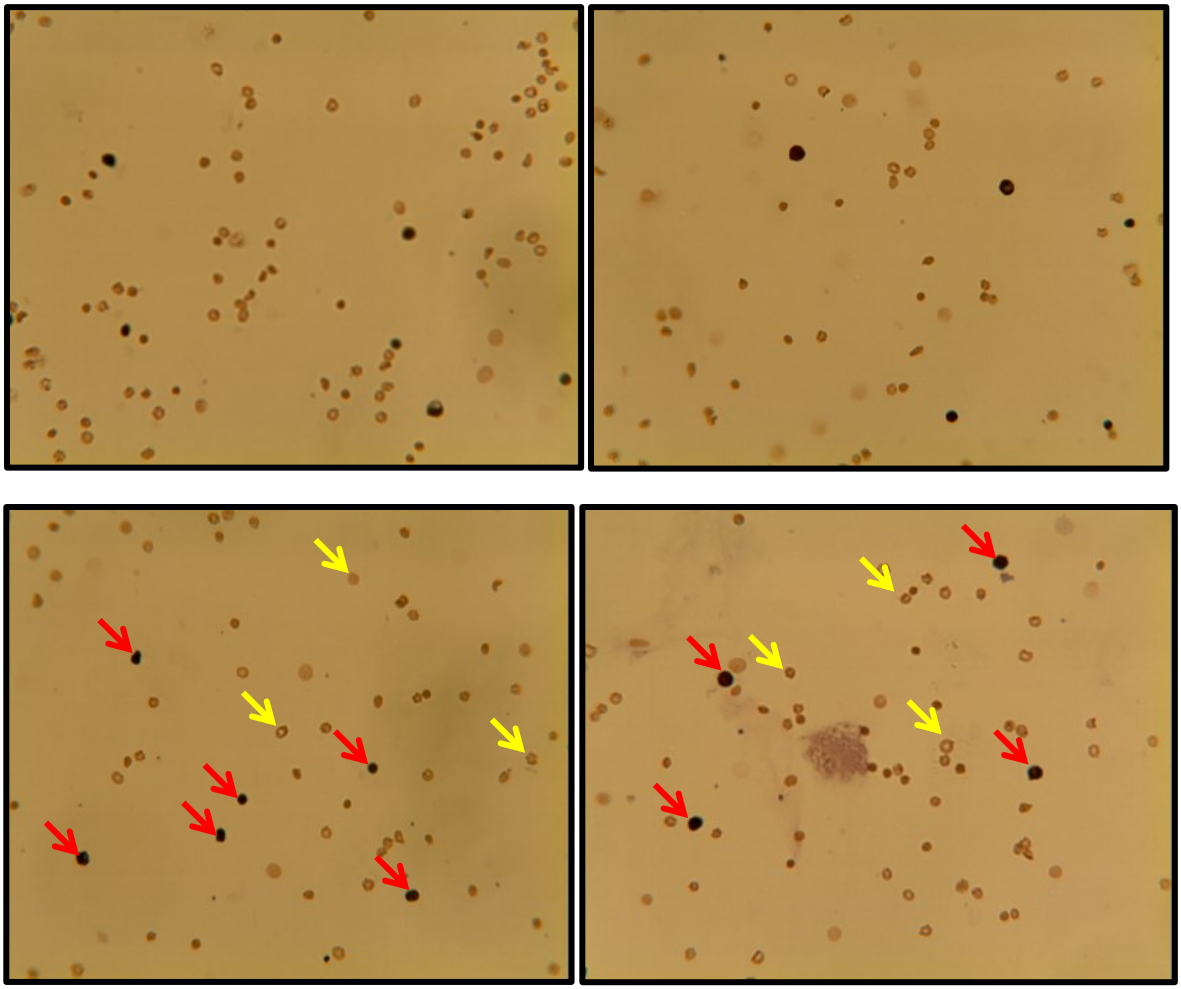
However, a prevalence of only 2.1% is observed in the presence of three ABO incompatibilities between maternal-fetal CBUs. Insufficient information and limited

number of pooled CBUs did not allow me to identify if ABO incompatibility or another factor such as HLA, the IgG anti-A/B counts or complement activation may influence the prevalence of a CBU. Further analysis is required.

Demonstration of Maternal Anti-A/B IgG Binding to CBU Leukocytes by Rosette technique

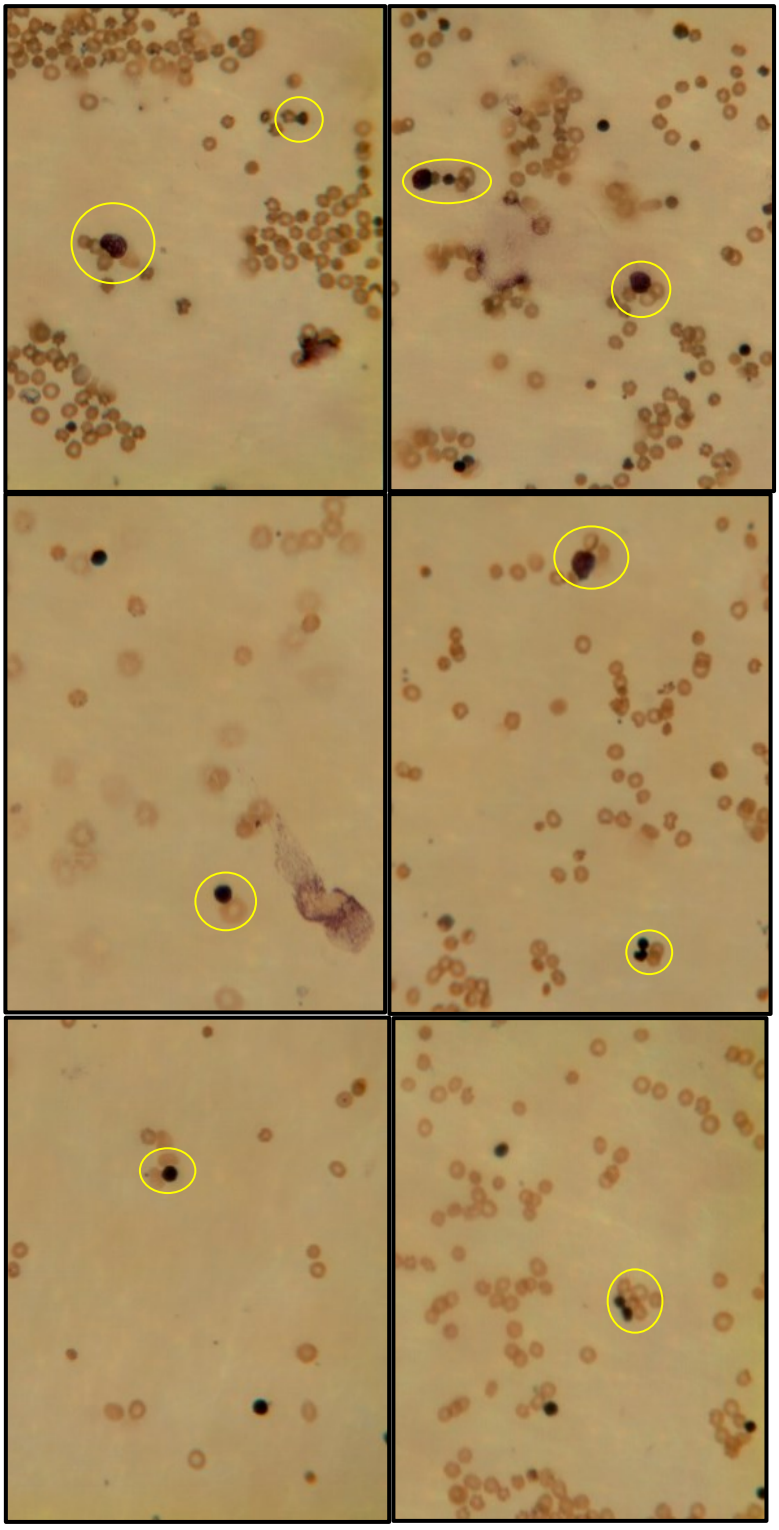
The elution of IgG anti-A and anti-B documented in Table 14 confirmed the IgG binding on pooled leukocytes. Possible modes of binding include non-specific physical absorption, specific binding to expressed ABO antigens via the $F(ab)^2$ region, or attachment of IgG anti-A or anti-B antibodies to leukocyte Fc receptors (FcR). A Rosette assay was used to determine if IgG anti-A and/or anti-B bound to CBU leukocytes could still bind A and B antigens via FcR. Figure 14 shows that RBC rosetting of CBU leukocytes did not occur when there were no maternal-fetal ABO incompatibilities, all CBUs and maternal blood group selected being of blood group O (control). In contrast, from one to five RBCs could be seen to bind CBU leukocytes when maternal-fetal ABO incompatibilities were present (Figure 15).

These results confirm that maternal anti-A and anti-B IgGs can bind to CBU leukocytes and show that some of these antibodies have exposed $F(ab)^2$ regions capable of antigen-specific binding to ABO A and B antigens. Binding of anti-A and anti-B to CBU leukocytes FcR is most likely.



Blood group O CBUs were pooled and stained with Wright-Giemsa. All four slides show nucleated cells (red arrows) surrounded and not attached to red blood cells (yellow arrows).

Figure 14: Rosette on Pooled CBUs Leukocytes – Negative Control (10x)

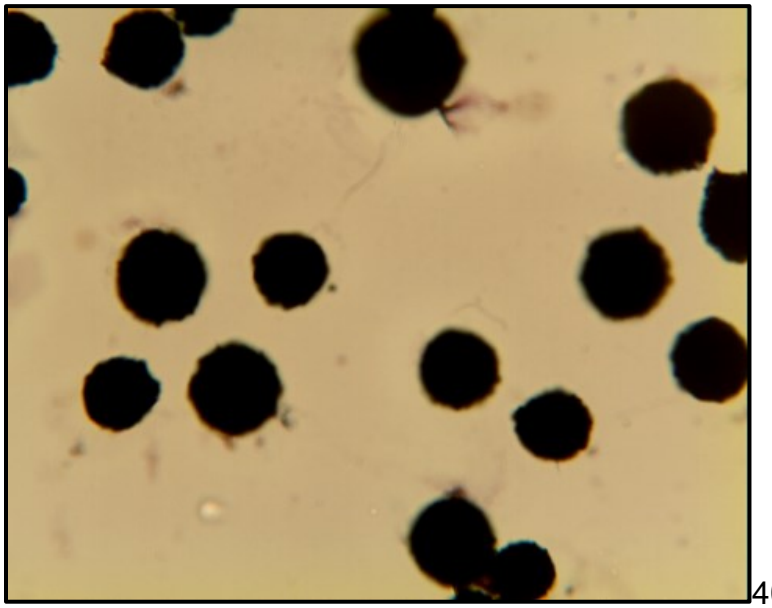


Blood group A CBUs were pooled and stained with Wright-Giemsa. The yellow circles show nucleated cells surface-bound to one or more red blood cells to form rosettes.

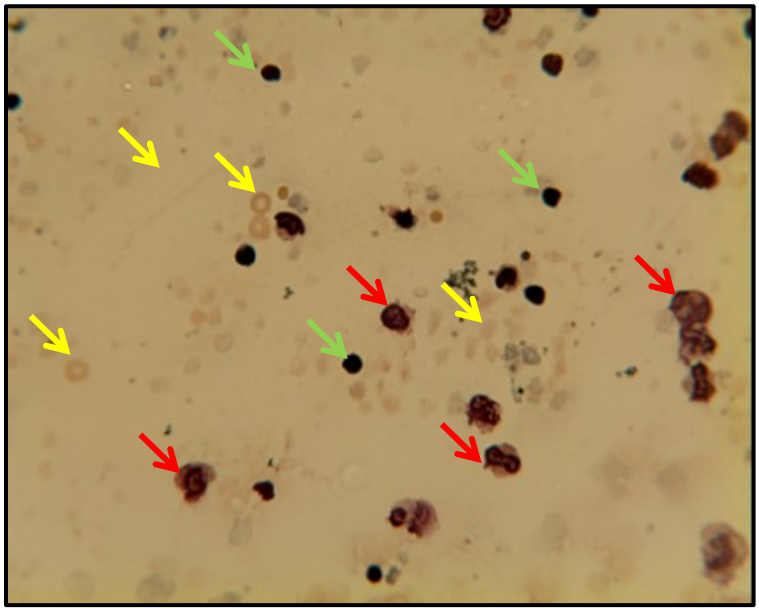
Figure 15: Rosette Technique on Pooled CBUs Leukocytes – Blood Group A (10x)

Mag Sepharose Assay

The rosetting technique showed that some of the maternal anti-A and/or anti-B IgG bound to CBU leukocytes, probably via leukocyte Fc receptors, could also bind RBCs in an ABO A or B antigen-specific manner. It is also possible that maternal IgG can bind ABO-incompatible antigens expressed on CBU leukocytes. Staphylococcal Protein A and the Streptococcal Protein G are known to bind the IgG Fc region. These proteins bound to magnetic beads are commercially available (Protein A Mag Sepharose and Protein G Mag Sepharose). The presence of cells bound IgG with accessible Fc regions can be detected by incubating with Mag A/B beads and looking for the adherence of cells to the beads. No leukocyte rosettes were observed using O-type pooled CBUs as negative control (Figure 16). In contrast, leukocyte rosettes were readily observed using CBUs with maternal-fetal ABO incompatibility (Figure 14 and 15). Rosetting was less marked when post incubation magnetically separated beads were washed twice before examination (Figure 17) than when examined directly after magnetic separation (Figure 18), demonstrating the fragility of cellular binding to the beads. These results provide evidence that maternal IgGs can also bind CBU leukocytes in an antigen-specific manner via their $F(ab)^2$ region.



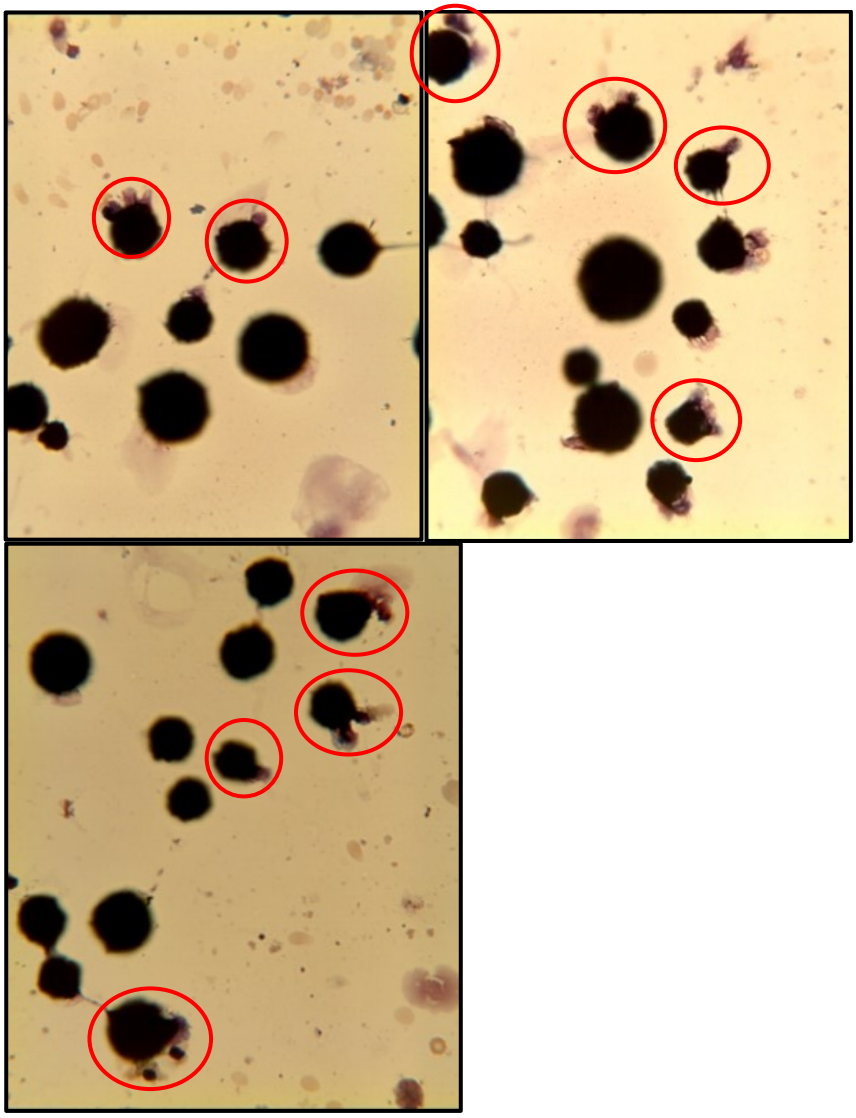
40x



10x

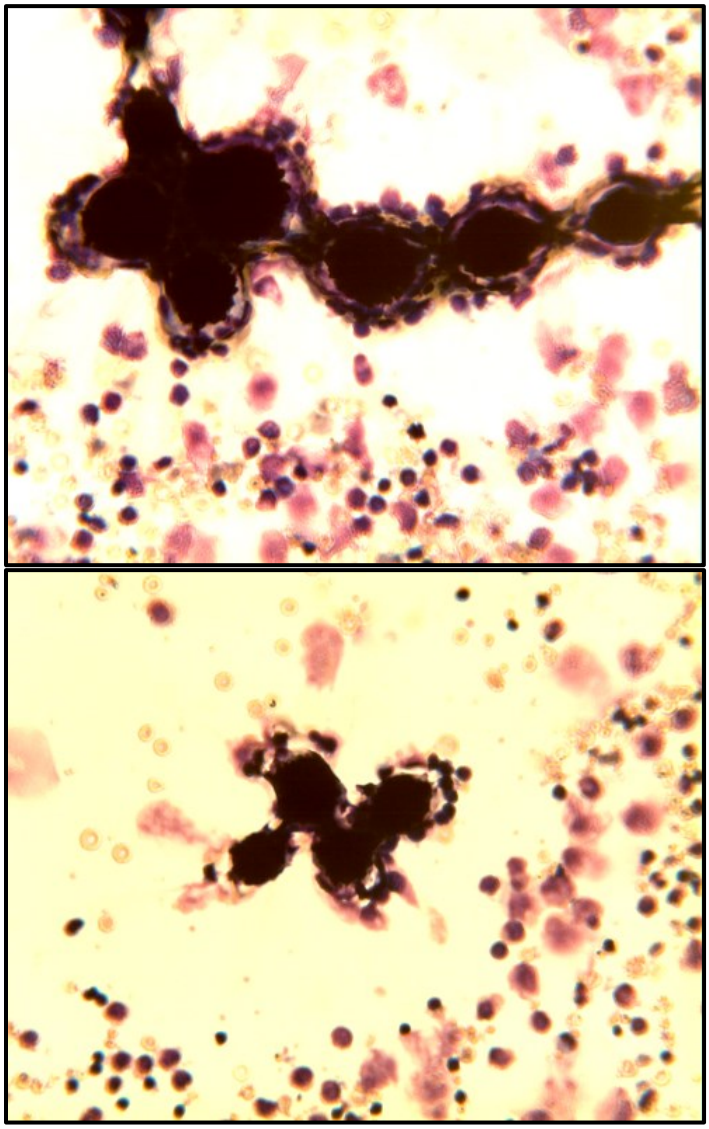
Blood group O CBUs were pooled and stained with Wright-Giemsa. No nucleated cells (red arrows) could be seen to bind to the Mag G Sepharose beads (green arrows). Some red blood cells (yellow arrows) are also present.

Figure 16: Mag G Sepharose Negative Control



Incompatible Pooled CBUs were washed twice and stained with Wright-Giemsa. The red circles show one or more nucleated cells bound to Mag G and Mag A Sepharose beads.

Figure 17: Incompatible Pooled CBUs washed twice before incubation with Mag A and Mag G Sepharose (40x)



Incompatible Pooled CBUs were stained with Wright-Giemsa. Enhanced binding of leukocytes to Mag A and Mag G Sepharose can be seen when examined directly without prior washing.

Figure 18: Unwashed incompatible pooled CBUs before incubation with Mag A et Mag G Sepharose (40x).

Outcome of Allogeneic Stem Cell Transplantation using Third Party Support from Mixed Cord Grafts

Transplant Recipients

Seven patients received 4/6 to 5/6 HLA matched CBU, where HLA Class I, A and B loci were analysed using low resolution molecular typing method, and Class II HLA at high resolution molecular typing for DRβ1. The HLA typing was done 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 to be transplanted as a single CBU.

Patient #1 was a 41 year old CMV-positive male with blood group A weighing 81 Kg. He was diagnosed in 2007 with an unclassifiable CD5-negative B-cell lympho-proliferative disorder that initially was morphologically similar to CLL. His course was one of successive partial remissions (PR) of short duration following multiple treatment regimens including rituximab with cyclophosphamide, vincristine and prednisolone (R-CVP), Fludarabine, Cyclophosphamide 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 group B who weighed 56 Kg. He was diagnosed in 2009 with Philadelphia-chromosome positive chronic myeloid leukemia (CML) in myeloid blastic crisis presenting with extramedullary

chloromas. He was initially treated with imatinib mesylate, a tyrosine kinase inhibitor (TKI), but then switched to nilotinib due to imatinib intolerance. He was induced with the "7+3" regimen and combined TKI therapy and six months later required salvage treatment with FLAG-Ida because of disease progression on nilotinib without evidence of Abl kinase domain mutations. Each course of chemotherapy was followed by over two months of pancytopenia complicated by aspergillus infection. At the time of transplantation, the patient was in complete cytogenetic remission with no evidence of extramedullary disease but increasing BCR/ABL expression by PCR.

Patient #3 was a 30 year old CMV-positive female with blood group O and weighed 88 Kg. She was initially diagnosed with acute promyelocytic leukemia (APL) in 2007 but relapsed two years later with secondary acute myelogenous leukemia (AML FAB M4). She was transplanted with a hypoplastic bone marrow following treatment with HiDAC x 3 and VP16/arsenic.

Patient #4 was a 57 year old CMV-positive female with blood group O and weighed 72 Kg. This patient was diagnosed with high risk Ph-negative B-acute lymphoblastic leukemia (ALL) in 2010 (high WBC, high risk cytogenetics). The patient was transplanted in first complete remission (CR1) after induction, consolidation, and CNS irradiation as per the Dana Farber Institute (DFI) ALL 4 protocol.

Patient #5 is a 46 year old CMV-positive male with blood group O who weighed 76 kg. The patient was diagnosed with B-cell small cell lymphoma (SCL) in 2007 and achieved a first complete remission (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 is a 37 year old CMV-positive female with blood group B who weighed 48 kg. The patient was diagnosed with T-cell acute lymphoblastic leukemia in 2011. She was Philadelphia-chromosome negative. Twenty- eight (28) days post-induction, she had 8% persistent lymphoblast. She achieved a first complete remission (CR1) after treatment with 6-MP and methotrexate. A search was launched for unrelated donor for more than 6 months before cord blood pooling protocol participation.

Patient #7 was a 53 year old CMV-positive male with blood group B who weighed 143 Kg. He was diagnosed in 2011 with Philadelphia-chromosome positive chronic myeloid leukemia (CML). He was initially treated with imatinib mesylate then switched to nilotinib after developing resistance with cytogenetic relapse and transformation to CML blast crisis. The recipient also suffered from many morbidity factors, such as ischemic heart disease, respiratory syncytial virus and obesity. A search was launched for unrelated donor and the registry advised us of the unlikeliness of finding a compatible adult donor.

Clinical Grafts

The selection of the pooled CBUs was based on the ABO blood group which required to be identical to the matched CBU and also that the pooled CBUs would also

be ABO maternal-fetal compatible. The number of CBUs pooled was based on the $CD34^+$ cell counts per CBU pre-cryopreservation ensuring a pool of 1 x 10^6 $CD34^+$ cells per kilogram of the recipient. The CBUs, being the Public Cord Blood Bank rejected units due to small volume collected, contained between 0.20 x 10^6 to 28.4 x 10^6 $CD34^+$ cells. The weight of the recipients varied between 48 to 143 Kg requesting the pool of up to 45 cryopreserved CBUs.

In order to be able to later track the origin of the cells that engrafted in each patient, STR DNA analysis was done on pre-transplant recipient blood leukocytes and all sources of cells infused (Table 16). I can account for many CBUs containing the same length of repeats for a STR locus such as in patient #1 where in locus D1S1660 with an STR length of 237 base pairs (bp) is observed in 16 of the 45 CBUs, or on locus D5S818 of the same patient where the STR length of 155bp was observed in 24 CBUs. Even if many lengths of repeats are found to be identical in many CBUs pooled, when comparing all pooled CBUs, the matched CBU and the recipient with all nine loci, none of them were found to be identical.

Table 16: STR analysis of recipients, matched CBU and third party CBUs.

	D1S1660	D3S2387	D5S1456	D5S818 (5a)	D7S820	D8S1179	D13S317	D16S539	D20S481
Patient #1	229+241	182+190	190+202	151+155	218+222	183	176	155+159	219+231
Match CBU	233+237	190+198	194+206	151	202+222	179	188+196	155+163	227
Pooled CBUs									
1	233 + 247	198 + 202	189 + 205	151 + 155	214+222	183 + 187	187+195	n/av.	n/av.
2	245	186+194	201+205	155	n/av.	175+183	188+200	159	231+243
3	233+241	174+194	189+201	151+155	206	187	176+192	163	231+239
4	233+237	198+206	197+201	151+155	206+210	183+187	188+196	n/av.	223+235

5	237 + 245	182 + 194	201 + 209	155 + 159	218	183	195	141+155	n/av.
6	237	182+194	197+201	137+147	210+222	187+195	188+192	159	235
7	241	178 + 186	197 + 201	155+159	206+214	175	191	151+163	n/av.
8	229+245	174+190	n/av.	n/av.	214+218	171+179	176	155+159	235+239
9	249	182+202	n/av.	147+159	214	187+191	176+188	159+163	219+247
10	231+235	186+198	193+197	155	214+218	179+187	192	163	231+239
11	233+241	186+194	197+201	159	206+214	183+187	188+192	163	n/av.
12	241+245	182+194	201+205	151+155	210+214	183	188	155	231
13	233	194+202	201	n/av.	210+222	179+183	188+192	155+159	227+235
14	230 + 238	193 + 201	191 + 195	153	220	169 + 181	182 + 190	143 + 155	231 + 243
15	237+241	98+20	193+205	155+159	210+214	171+187	188+192	159+163	223+239
16	241	198+202	193+201	151+155	210+214	179+183	176+188	147+159	223+231
17	229+233	174+202	193+197	155	n/av.	175+179	176+192	155+159	239
18	225+245	190 + 194	201	133+151	210+222	171+175	176+196	147+159	231+239
19	230 + 234	174+186	197+201	151+155	214+218	171 + 183	183 +191	151 + 155	239
20	237+245	194+198	197	155	210+218	171+183	188	141+157	223+239
21	237+245	194+202	205	155+159	222+226	163+195	180+192	159	215+243
22	229+245	174+202	n/av.	151	210+214	n/av.	176	159	235+243
23	237+241	198	193+201	151+155	418+226	187	184+200	155	239+243
24	233+237	174+188	209	155	214	167+179	188+200	147+151	239
25	233+241	194	189+201	151+155	217	187	176+196	159+163	235
26	237+241	190+194	189+201	141+151	206+210	191+195	176+192	159+163	235
27	245	174+198	n/av.	151+155	218	179+183	188	155+159	235+243
28	237+241	182+194	193+205	159	n/av.	175+191	188	147+159	231+239
29	230 + 242	173 + 193	199	149 + 157	224	181 + 189	186	155+159	231 + 235
30	237+245	194	193+201	147+155	222	175+183	180+192	159	223+235
31	229+233	190+198	189+201	137+147	218+226	183+187	188+196	155+163	243
32	n/av.	178+182	189+209	151	214	171+179	176+188	155	239+243
33	242 + 246	202+210	205	147+159	210+218	175 + 179	175+191	147+155	223+247
34	263+241	194+198	205+209	147+159	206+222	179+187	176	163+167	219+239
35	230 + 238	182+190	193+201	151+155	210+214	167 + 171	183+191	147+155	227+243
36	230 + 242	173 + 193	199	149 + 157	224	181 + 189	186	147 + 151	239 + 243
37	241 + 247	182+194	201	155	214	175 + 183	191	155+163	231+247
38	241	186+198	205	151+155	206+218	174+187	188	n/av.	219+223
39	229 + 237	193 + 201	191 + 195	153	220	169 + 181	182 + 190	155+163	231 + 239
40	237	198+202	197+201	n/av.	206+210	187+191	192	155+159	239+243
41	233+245	183+202	n/av.	147+15	206+214	175+179	180+188	147+155	235+239
42	233	178+202	189+205	151+155	218+222	178+191	188+196	151+155	227+231
43	233+237	186+198	n/av.	155+159	218+222	n/av.	188	155+159	235+243
44	237+241	n/av.	193+201	151+159	210+214	183+195	188+192	155+159	223
45	233+237	n/av.	189+201	n/av.	206+218	171	176	147+159	231+235
	229 = 05	174 = 08	189 = 08	133 = 01	206 = 10	169 = 02	176 = 13	141 = 02	215 = 01

	230 = 02 233 = 14 237 = 16 238 = 01 241 = 14 242 = 02 245 = 11 249 = 01	178 = 04 182 = 07 186 = 06 190 = 05 194 = 17 198 = 13 202 = 13 206 = 01	193 = 09 197 = 09 201 = 22 205 = 10 209 = 04	137 = 02 141 = 01 147 = 07 149 = 02 151 = 17 153 = 02 155 = 24 157 = 02 159 = 11	210 = 14 214 = 17 218 = 12 222 = 09 226 = 03	171 = 08 175 = 09 179 = 11 181 = 04 183 = 15 187 = 14 191 = 05 195 = 04	180 = 03 184 = 02 188 = 21 192 = 17 196 = 07 200 = 03	143 = 01 147 = 09 151 = 05 155 = 21 157 = 01 159 = 21 163 = 12 167 = 01	219 = 03 223 = 08 227 = 03 231 = 13 235 = 13 239 = 17 243 = 12 247 = 03

Each column indicates an STR locus verified for its length of repeats for example "D1S1660" represent locus 1660 on chromosome 1. Each chart cell indicates the PCR fragment length (in base pairs). Two fragment lengths indicate a heterozygous state, whereas one fragment length indicates homozygosity. The most frequent PCR fragment length is indicated in yellow.

	D1S1660	**D3S2387**	**D5S1456**	**D5S818 (5a)**	**D7S820**	**D8S1179**	**D13S317**	**D16S539**	**D20S481**
Patient #2	229+245	194	205	151+155	206+222	171+191	180+192	151+155	243
Matched CBU	225+237	174+186	201	141+155	214+222	175+187	176+192	151+159	239
Pooled CBUs									
1	234 + 242	194+202	197 + 205	147+155	206+210	179 + 187	176 + 192	139	239
2	242	198	199 + 203	149	212 + 216	181 + 189	178 + 190	147 + 155	219 + 231
3	233+245	182+206	189+205	151	218	191+199	188+192	147+155	231+243
4	230 + 242	194 + 202	187 + 191	149 + 153	220	177 + 189	186 + 190	167 + 171	219+223
5	226 + 238	198 + 202	195 + 199	153	204 + 216	189	178 + 186	155 + 159	223
6	226+241	194	197+201	141+151	206+214	171+187	192	147+163	239
7	234 + 246	194+206	189	142+159	214+222	183 + 191	191	151 + 160	239
8	233+241	186+198	197	155+159	206	179+187	188+196	147+155	231
9	229+233	182+198	193	147+155	214	183+187	176+188	151+159	227+243
10	237+241	178+186	189+197	155+159	206+214	183+191	188	155+159	239+243
11	233+237	194+198	189+201	155+159	214	171+175	188+192	151+163	239
12	233+241	n/av.	189+193	155	210+218	171+183	180+186	155+159	235+239
13	229+237	186+190	189+201	151+155	206	183+187	176	151+163	239+243
14	233+237	178+198	n/av.	155+167	206	n/av.	188	155+159	n/av.
15	237	190+198	n/av.	151+159	210	175+191	184+192	155+147	215+239
16	233	182+190	193+205	151+155	206+218	187+191	196	159+163	231+239
17	237+241	178+190	189+201	159	218+222	n/av.	180+188	155+163	239+247
18	241	190+194	n/av.	151+155	214+218	175+183	192+200	151+155	239
19	233+241	174+206	201+205	151+159	210+214	183	184+196	155+159	235+239
20	241+245	200+206	205	151+155	214+222	n/av.	184+192	n/av.	231+235
21	241	186+190	189+205	151+155	218+222	179+183	188+192	159+167	231+243
22	233+241	194+198	189+201	151+155	214	183+187	188+192	155+163	231
23	233	190+198	89+197	155	n/av.	171+191	188+196	159+163	223+231
24	233+241	174+182	197+201	155	206+214	171+187	n/av.	141+163	227+239
25	233+237	178+186	193+197	151+155	214+218	183+191	180	159	239
26	241+245	186+198	201	151	214+218	183	176+188	155+159	219+239

27	233	198	189+201	147+155	206+210	171+187	180+192	163	227
28	237+245	194	201	155+159	218+222	175+183	188+192	147+155	231+235
29	234 + 246	190+206	189+201	151	214	183 + 187	187 + 195	159 + 163	231+243
30	233+237	198+202	205+209	151+159	206+222	163+183	188+192	147+163	231+239
			187 = 01			163 = 01		139 = 01	
		178 = 04	189 = 11			171 = 06		141 = 01	
	226 = 02	182 = 04	191 = 01	147 = 03		175 = 04		147 = 07	219 = 03
	229 = 02	186 = 06	193 = 04	149 = 02	204 = 01	179 = 03	176 = 04	151 = 05	227 = 03
	230 = 01	190 = 08	197 = 07	151 = 14	206 = 10	181 = 01	180 = 04	155 = 14	231 = 11
	234 = 03	194 = 07	199 = 02	153 = 02	210 = 05	183 = 14	184 = 03	159 = 12	235 = 04
	237 = 09	198 = 10	201 = 11	155 = 18	214 = 13	187 = 10	188 = 13	163 = 11	239 = 16
	241 = 12	202 = 02	203 = 01	159 = 09	218 = 09	189 = 03	192 = 12	167 = 02	243 = 06
	245 = 04	206 = 05	205 = 07	167 = 01	222 = 06	191 = 07	196 = 04	171 = 01	247 = 01

Each column indicates an STR locus verified for its length of repeats for example "D1S1660" represent locus 1660 on chromosome 1. Each chart cell indicates the PCR fragment length (in base pairs). Two fragment lengths indicate a heterozygous state, whereas one fragment length indicates homozygosity. The most frequent PCR fragment length is indicated in yellow.

	D1S1660	**D3S2387**	**D5S1456**	**D5S818 (5a)**	**D7S820**	**D8S1179**	**D13S317**	**D16S539**	**D20S481**
Patient # 3	n/av.	n/av.	n/av.	n/av.	n/av.	n/av.	n/av.	n/av.	235+239
Matched CBU	233+237	186	197+205	151+155	214+222	183+187	188+192	148+160	231+247
Pooled CBUs									
1	n/av.	178+192	197+201	155+159	214	183	188+196	155+159	219+231
2	241+245	194+198	201	155+159	202+214	183	188+196	155+159	223+235
3	241	194+210	197+201	143+155	214	187+191	176+192	155+159	231+239
4	237+241	194+202	189+197	155+159	n/av.	183	188	155+159	239
5	233+245	174+178	n/av.	151+155	210+214	171+187	180+192	155	231+243
6	233+249	182+198	201+205	155+159	n/av.	179+187	184+188	163+167	231+239
7	225+233	182+206	205	151+155	n/av.	183+187	196+200	155+163	223+243
8	237+245	198+210	189+193	142	206+218	179+187	188+192	159+163	235
9	233	174+206	201	151+159	n/av.	171+183	176+188	147+155	221
10	229+241	174+194	193+205	137+155	206+210	171+195	192+196	159+171	n/av.
11	229+233	178+190	n/av.	147+151	218	178+187	180+188	155+159	239
12	233+249	174+186	201	147+155	n/av.	183	188	151	235+239
13	237	182+202	n/av.	151	218+222	187	192+196	147+155	239+243
14	n/av.	194+198	193+201	151+159	206+210	175+191	190+188	163	239
15	233+237	190+194	189+209	147+155	214	175	188	155+163	235+239
16	229+233	186+190	193+201	155+159	218+226	183+199	188+192	159	239
17	237+241	194+202	189+197	155	210+214	183	188+192	159+167	231+239
18	233	178+202	201	151	210+214	183+191	188+196	155	231+243
19	245	182+198	n/av.	151+155	210+214	n/av.	192+196	n/av.	239
20	237+241	174186	205	155	214+222	183	180+192	159	n/av.
21	239+243	184+198	197+205	151+155	214	183+195	n/av.	155+163	231+239
22	241+245	190+194	201+205	151+155	214+218	179+183	n/av.	n/av.	231+243

23	237+241	186+198	189+201	147+155	206+218	n/av.	192+196	147+163	235+243
24	237+245	174+206	n/av.	155	222	179+183	196+200	151+159	239
25	225+233	186+190	n/av.	159	214+222	167+187	176+192	n/av.	223+227
26	237	190+198	n/av.	151+159	210	175+191	184+192	155+147	215+239
27	241	174+178	200	151+159	214+218	n/av.	n/av.	151+155	n/av.
28	241	198+202	197+201	151	214+218	183	n/av.	147+159	219+231
29	233	190+194	205	151+155	206+214	163+175	188	155+159	231+243
30	233+237	194+198	201+209	155+159	218+222	175+191	184+188	159+163	227+235
31	229+233	202+206	189+193	155	206+210	171+183	192	159+163	231+243
32	229+233	178+190	201	155	210+222	183+187	192	151+159	235
33	237+241	190	201+205	147+155	214	183+187	184+192	147+155	235+239
34	237	178+194	189+197	159	214+218	175+183	184+188	n/av.	231+243
35	233+241	190+198	189+201	151+155	n/av.	179+187	188+200	155+159	231
36	237	182+194	201+205	151+155	n/av.	183+187	188+192	147+159	235+247
37	237+245	202	201+205	155+159	218	183	188+192	n/av.	231+235
38	n/av.	182+198	194+206	n/av.	202+214	179+183	184+188	147	223+235
39	233+241	174+194	201+205	151	218	183+187	188+192	n/av.	239
40	241+245	194+202	194+202	n/av.	214+218	183	176	147+155	223+243
41	229+241	190+198	189+201	147+151	210+226	183+187	192+200	155+159	235+243
42	241	190	201+205	147+151	218	187+191	176+192	155+163	235+239
	229 = 06 233 = 16 237 = 14 241 = 16 245 = 08 249 = 02	174 = 08 178 = 07 182 = 06 186 = 05 190 = 12 194 = 14 198 = 13 202 = 08 206 = 04 210 = 02	189 = 09 193 = 05 197 = 07 201 = 21 205 = 12 209 = 02	143 = 01 147 = 07 151 = 19 155 = 26 159 = 13	206 = 06 210 = 10 214 = 19 218 = 14 222 = 06 226 = 02	163 = 01 167 = 01 171 = 04 175 = 06 179 = 06 183 = 24 187 = 15 191 = 06 195 = 02	176 = 05 180 = 03 184 = 06 188 = 21 192 = 20 196 = 09 200 = 04	147 = 09 151 = 04 155 = 20 159 = 19 163 = 10 167 = 02 171 = 01	215 = 01 219 = 02 223 = 05 227 = 02 231 = 13 235 = 13 239 = 16 243 = 11 247 = 01

Each column indicates an STR locus verified for its length of repeats for example "D1S1660" represent locus 1660 on chromosome 1. Each chart cell indicates the PCR fragment length (in base pairs). Two fragment lengths indicate a heterozygous state, whereas one fragment length indicates homozygosity. The most frequent PCR fragment length is indicated in yellow.

	D1S1660	**D3S2387**	**D5S1456**	**D5S818 (5a)**	**D7S820**	**D8S1179**	**D13S317**	**D16S539**	**D20S481**
Patient #4	237+245	178+206	201+197	147+155	210+218	183+187	192+196	143+159	223+239
Matched CBU	233+237	190+194	193+197	151+155	210+226	179+183	188+192	159+163	239
Pooled CBUs									
1	237+241	174+194	201+205	142+151	210+214	187+191	176+192	151+163	n/av.
2	229+241	190+194	193+197	151+155	214+222	171+183	188+196	155+159	231+239
3	237+241	198	185+197	155+159	214+218	171+187	192	159+163	235+239
4	241+245	198	201+209	137+151	218	183+191	188+192	147+155	235+239
5	237	182+188	201	159	218	175+195	192	147+155	239+251
6	245	188+194	189+201	155+159	206+214	183+187	188	163	223+243

7	229+237	190+198	189+205	155	214	171+183	180	159	235+239
8	235+239	182+194	189+201	151+155	206+21	171+183	188+192	155+163	239
9	233+241	182+198	189+193	151+155	214+222	183	192	147+163	231+235
10	241+245	190+202	193+205	155+159	214+218	178+183	192+196	155+159	227+235
11	229+233	190	201+205	151+155	210+222	179+187	188+200	143+155	239+243
12	229+245	190+198	193+197	159+163	214+218	179+199	192+200	155+163	231+235
13	233+245	186+198	201	151+155	214	187+191	180+188	151+155	231+235
14	233+237	174+198	201+205	151+159	214+218	171+183	188	155	235
15	241+245	182+190	193+205	155+159	210+222	183+187	188+192	147+159	235+239
16	229+245	178+186	197+201	147+159	218+222	175+191	194	155+159	227+243
17	237+241	186+194	201	151+155	210+222	175+183	192+196	147+159	223+235
18	237+245	182+188	197	155+159	206	171+190	188+192	147+155	231+235
19	233+237	190+198	189+201	151+155	210+214	183+191	180+192	143+161	223+243
20	229+241	198	193+201	151+155	210+214	175+183	188	151+159	243
21	237+245	182+202	209	151+159	206+218	175+187	192+196	155+163	239+243
22	225+229	174+186	189+193	155+159	214	171+187	176+188	155+163	227+235
23	237+245	186+202	201+205	151+159	210+214	183+187	188+196	151	239
24	233-245	174+182	197+205	151+159	218+222	191	196	147+159	239+243
25	241	186+206	189+205	147	218	183	188	147+159	231+243
26	229+237	198+202	197+201	155+159	214	183+191	196	147+159	231+235
27	233+237	182+206	189+205	151	206+218	191+199	188+192	155	231+239
28	229+233	190	201	147+151	222+226	183+187	180+188	155	219+231
29	229+237	198+202	205	155	214+222	187	192	151+163	227+239
30	241	186+202	197+205	155	218+222	175+183	188+192	151+163	231+243
31	233+237	198	205	155+159	210+222	183+191	188+196	155	
32	245	182+190	197+201	151+159	214+222	179+187	188+200	143+167	231+243
33	237+241	190	201+205	155+159	218+226	179+183	184+200	151+155	235+239
34	241	194+198	193+201	151+159	210+214	183+187	184+188	147+163	239
35	233+241	194+202	201+205	155	206+214	183+187	192+200	159+167	231+239
36	237+241	182+198	197+213	151+155	214	171+183	192+196	155+159	223+227
37	245	178+198	189+201	151	210+214	n/av.	192	n/av.	239+243
38	233+241	178+194	189+205	147+155	214	175+191	176+180	159+169	239+247
39	229+233	190+206	201	151+155	206+218	179+183	180	n/av.	235+239
	225 = 01 229 = 11 233 = 12 237 = 16 241 = 16 245 = 13	174 = 04 178 = 03 182 = 10 186 = 08 190 = 11 194 = 08 198 = 15 202 = 07 206 = 3	185 = 01 189 = 10 193 = 08 197 = 10 201 = 21 205 = 16 209 = 02 213 = 01	143 = 01 147 = 04 151 = 21 155 = 24 159 = 18 163 = 01	206 = 07 210 = 10 214 = 22 218 = 14 222 = 12 226 = 02	171 = 08 175 = 07 179 = 05 183 = 23 187 = 14 191 = 11 195 = 01 199 = 02	176 = 03 180 = 06 164 = 02 188 = 19 192 = 19 196 = 09 200 = 05	143 = 03 147 = 10 151 = 07 155 = 18 159 = 14 163 = 11 167 = 02	219 = 01 223 = 04 227 = 05 231 = 12 235 = 15 239 = 19 243 = 11 247 = 01 251 = 01

Each column indicates an STR locus verified for its length of repeats for example “D1S1660” represent locus 1660 on chromosome 1. Each chart cell indicates the PCR fragment length (in base pairs). Two

fragment lengths indicate a heterozygous state, whereas one fragment length indicates homozygosity. The most frequent PCR fragment length is indicated in yellow.

	D1S1660	D3S2387	D5S1456	D5S818 (5a)	D7S820	D8S1179	D13S317	D16S539	D20S481
Patient #5	229+237	178+186	197+201	147+155	214	191+195	188	147+109	235+239
Matched CBU	233	182	201	151	222	187	180+188	151+155	235+239
Pooled CBUs									
1	241	178+194	189+201	151	206+226	159+171	180+188	147+167	239
2	233	182+190	193+205	151+159	214+218	175+179	184+188	155+159	235+243
3	237	174+178	189	142+151	210	183+191	176+196	155+159	219+235
4	233	174+198	189	155	214	179+183	188	155+163	227+235
5	229+241	186+190	189	142+151	214	183+195	188+192	155+159	231+243
6	233+241	186+194	201+205	151	206	187+191	180+188	159+163	231+247
7	233+245	190+198	197+205	155	214+218	187+191	188+192	147+163	235+243
8	233+237	190+198	197+209	151	206+230	183+187	188+192	147+163	231
9	229+237	178+190	189+197	151	214	175+191	192	151+155	231+239
10	229+233	194+198	201	159	218	179+183	176+188	155+167	227+235
11	229+233	178+190	201+205	151	218	151	176+192	146	239
12	233	198+202	201	155+159	210+218	171+179	192	155+159	231+243
13	233+237	170+174	193+197	155	214	183+191	188+192	155+159	219+243
14	237+245	194+202	189+193	151+155	218	187+199	188+196	155	235
15	237+245	194+202	201	151+155	214+218	183+187	192+196	147+159	235+239
16	237+241	182+190	205	151+155	206+210	187	192+200	147+159	239+243
17	229+241	198	197+201	159	206+218	179+183	188+196	155+159	223+243
18	241	190+194	197+205	155	218+222	187+191	184+192	159	239
19	245	194+202	201	151+155	206+214	183	192	147+159	231+239
20	233+241	178+186	197+201	141+155	213	175+187	184+188	151+155	239+247
21	245	194+210	189+209	147+155	210+214	175+187	188+192	151+163	231+239
22	225	178+194	201	151+155	214	183+195	176+188	147+159	n/av.
23	241	194+202	189+209	151+167	222+226	183+187	192	159+163	215+235
24	233+241	198+206	189+201	151	214+218	179+187	184+192	155+159	227+231
25	233+241	186+194	197+201	151+159	202+214	187	188	155	231+247
26	233+241	194+202	193	151+159	214+218	175+195	188+192	163+167	223+243
27	237+241	190+202	201+205	155+159	218+222	175+187	188+196	159+167	239+247
28	229+241	202	189+201	155	210+222	183+187	188	165	235+247
29	245	174+194	201+205	151	218	187+191	176+180	161+165	239+247
30	233+245	174+194	189+209	141+159	214+226	183+191	184+188	155+163	231
31	229+241	194+202	201	151+155	206+214	167+187	180+188	151+155	239+247
32	233+245	182+190	197+201	155+159	218+222	171+183	180+188	159+163	231+239
33	233+241	187+205	201	151+159	214+218	171+183	188+200	155	223
34	241+245	178+190	189+197	151+155	202+222	179+187	188+192	163	223+239

35	233+241	188+202	197+205	137+159	210+218	175+187	188+192	155+159	218+222
36	241+245	198	197+201	151+155	214+218	175+187	192	157+161	239+243
37	237+241	189	193+205	151+155	206+214	179+183	188	155+163	231+235
38	233+241	190+194	189+197	147+155	214+218	171+187	176+192	159+163	231+235
39	241+245	194	197+201	133+155	214+218	187+191	196+200	155	235+239
40	229+237	194+210	193+205	151+155	206+218	180+184	188+196	147+157	215+235
41	233+241	186+190	189+197	151	214+222	183	176+192	155+159	231+235
42	229+233	178+198	197+201	155+159	214	183+187	188+192	155	n/av.
	229 = 09 233 = 20 237 = 10 241 = 22 245 = 11	178 = 08 182 = 03 186 = 05 190 = 13 194 = 18 198 = 09 202 = 10	189 = 14 193 = 06 197 = 16 201 = 21 205 = 11 209 = 04	147 = 02 151 = 25 155 = 23 159 = 12	202 = 02 206 = 09 210 = 06 214 = 22 218 = 20 222 = 07 226 = 03	159 = 01 171 = 05 174 = 08 179 = 09 183 = 18 187 = 22 191 = 09 195 = 03 199 = 01	176 = 07 180 = 05 184 = 05 188 = 27 192 = 21 196 = 07 200 = 03	147 = 08 151 = 04 155 = 21 159 = 19 163 = 12 167 = 04	219 = 02 223 = 04 227 = 03 231 = 14 235 = 14 239 = 16 243 = 09 247 = 07

Each column indicates an STR locus verified for its length of repeats for example "D1S1660" represent locus 1660 on chromosome 1. Each chart cell indicates the PCR fragment length (in base pairs). Two fragment lengths indicate a heterozygous state, whereas one fragment length indicates homozygosity. The most frequent PCR fragment length is indicated in yellow.

	D1S1660	**D3S2387**	**D5S1456**	**D5S818 (5a)**	**D7S820**	**D8S1179**	**D13S317**	**D16S539**	**D20S481**
Patient #6	241	198	193+205	151+155	214+222	179+191	184+192	147+159	235+243
Matched CBU	229+233	182+190	201	155	218	183+191	188+192	151+155	223+239
Pooled CBUs									
1	237+241	182+190	189+205	155+159	210+222	187+195	176+184	147 + 155	243
2	229+245	174+198	201+205	151+163	214	175+183	184+192	159	239+247
3	241+245	190+194	193+205	151+155	210+218	183+187	192	159	239
4	245	174+190	197+205	147+151	218+222	183+195	176+184	155+159	239+243
5	233	182+194	201	151+159	222	167+183	188+196	159+163	227
6	237+245	174+190	197+201	147+151	218+226	187+191	192	155+159	235+239
7	233+245	166+186	189+201	155	206+210	179+191	188+196	143+147	239+243
8	233+241	178+182	197+201	151+155	210+214	171+179	192	151+155	227+243
9	233+241	178+182	197+201	151+155	210+214	171+179	192	151+155	227+243
10	241+245	182+194	197+201	151+159	218+230	171+183	192	159	219+239
11	237+241	182+186	201+205	155	214+222	187	180+192	163	223+239
12	233+245	198+202	205	147+155	210+218	183+187	180+192	147+155	231+239
13	229+233	186+194	201+205	155+159	214	179+187	188	147+155	235+243
14	241+245	182+198	193+197	137+159	214+222	179+183	180+188	143+155	231
15	233+245	186+190	193+201	151+163	210+218	183+187	184+188	155+163	219+235
16	233+241	194+198	193+205	151+155	210+218	171+184	176+192	155+159	235+239
17	245	194	189+201	159	210+214	183+187	188+192	151+165	231+239
18	229+241	202	193+201	143+155	214+218	175+183	192	155+159	235+247
19	241	190+194	197+205	147+151	214+218	175+187	192+196	141+167	219+235
20	237+241	198+202	189+205	147+151	220+228	175	180+188	141+159	223+235
21	241+245	178+186	193+205	151+155	214+222	179+187	188+192	155+163	235
22	233+245	182+186	201+205	151	214	179+187	192	151+163	231+239

23	237+241	190+194	201+205	143+155	214+222	183+191	184+188	143+155	231+239
24	233+241	196	193+197	151	206+222	175+187	176+188	155+169	231+239
25	241+245	190+194	193+201	151+155	218	183+187	176+180	165+169	239+243
26	233+241	178+198	201	143+151	214+218	179+183	188		
27	237	188+194	201+205	155+163	206+222	179+187	188+196	155+161	231+243
28	241+245	180+192	193+209	143+151	214+218	179+183	192	161+165	223+243
29	241	174+192	193+201	151+155	222	179+183	176+180		
30	241	178+190	189+201	159	202+226	187+195	180+188	155	239
	229=3	174=4	189=5	137=1	202=1	167=1	176=6	143=3	219=3
	233=11	178=5	193=10	143=4	206=3	171=4	180=7	147=4	223=3
	237=6	182=8	197=8	147=5	210=9	175=5	184=5	151=4	227=3
	241=19	186=6	201=19	151=19	214=14	179=11	188=13	155=16	231=7
	245=14	190=9	205=14	155=15	218=12	183=15	192=15		235=8
		194=10	209=1	159=7	222=10	187=15	196=4	159=9	239=15
		198=6		163=3	226=2	191=3		163=5	243=9
		202=3			230=1			167=1	247=2

Each column indicates an STR locus verified for its length of repeats for example “D1S1660” represent locus 1660 on chromosome 1. Each chart cell indicates the PCR fragment length (in base pairs). Two fragment lengths indicate a heterozygous state, whereas one fragment length indicates homozygosity. The most frequent PCR fragment length is indicated in yellow.

	D1S1660	D3S2387	D5S1456	D5S818 (5a)	D7S820	D8S1179	D13S317	D16S539	D20S481
Patient #7	245	190+194	190+198	147+155	206+222	180	176+190	155+163	231+239
Matched CBU	241	194+202	193-201	155	214+222	180+192	190+196	159+163	223+23
Pooled CBUs									
1	233+237	194+202	201	143+159	210+222	187+195	176+188	147+155	235+239
2	233+245	178+202	201+205	151+159	214+218	171+183	176+190	151+167	223+235
3	237+241	190+198	193+201	147+151	210+222	183	184+196	143+159	231+239
4	233	178	197	151+159	210+214	183	192+196	163	243
5	245	178+202	189+201	155	214	183	188	159	227+239
6	241+245	190+202	197	133+159	218	179+183	188+192	163+167	223+235
7	229+233	186+202	189+201	151	214+218	171+183	188+192	155+159	227+235
8	233+245	182+186	189	151+155	214	175+187	192	147	239+243
9	229+245	182+198	189+201	151+155	206+218	191	176+188	147+159	235+239
10	241	182+198	201	159	214+218	179+183	188+192	159+163	235+239
11	249	194	189+197	151+159	210+218	171	192	147+167	231+243
12	229+241	182+186	201	151+155	222+226	187+191	180+184	147+151	227+247
13	233+237	186+198	193+201	151+159	206+214	187+191	188+192	151+163	239+243
14	237+241	178+194	201+205	151+155	206+218	183+187	192+196	147+159	243
15	229+245	194+198	201+205	151+159	214+218	179+183	192	147+155	223+243
16	241	186+194	197+209	155	206+218	171+183	192+196	147+155	239+247
17	237+241	190+198	197+201	151+155	206+214	179	188+192	155+163	235
18	233+237	182+186	197	151+159	214	175+179	184+188	147+159	239+243
19	241+245	178+194	201+205	155+159	218+222	183+191	184+192	155	223+235
20	237+241	194+202	189+209	151	206+214	171+175	176+184	155	231+239
21	237+245	194+198	201	147+155	218+222	179+191	188+192	163+165	239+243
22	245	190+194	197	151+155	210+226	183	188+192	147+165	231+243
23	241+245	178+182	201+205	151+155	218	185+195	188	163	223+239
24	237	182+186	197	151+155	222+230	179+195	188+192	163	223+227
25	237+241	198+206	189+201	155+159	218+226	185+191	188+192	143+167	231+239
26	229+233	198	189-201	147+151	206+218	187+191	192	155+163	223+239
27	237+245	182+190	197+209	151+155		179+187	184+192	143+167	239+243
28	241	178+194	197+201	151+155	206+214	175+179	184+188	163+167	239+243
29	241	186+190	193+205	151	214+218	179+187	180+188	163	227+235
30	229+241	194+198	193+201	137+151	206+222	195	176+188	163+165	239+243

31	37+245	194+202	201+205	147+155		167+171	184+188	155+163	239+243
32	237+241	182+198	193+205	155+159	210+214	175+191	196	159	231+239
33	237+241	182+198	193+205	155+159	210+214	175+191	196	159	231+239
	229=4	174=7	189=8	137=1	206=9	167=1	176=5	143=3	223=7
	233=8	178=7	193=6	143=5	210=7	171=6	180=2	147=10	227=5
	237=14	182=9	197=10	147=4	214=15	175=6	184=8	151=3	231=7
	241=16	186=9	201=20	151=22	218=15	179=10	188=18	155=9	235=9
	245=12	190=6	205=9	155=18	222=7	183=12	192=18	159=9	239=20
	249=1	194=11	209=11	159=13	226=3	187=8	196=6	163=13	243=13
		198=12			230=1	191=9		167=6	247=2
		202=7				195=4			
		206=1							

Each column indicates an STR locus verified for its length of repeats for example “D1S1660” represent locus 1660 on chromosome 1. Each chart cell indicates the PCR fragment length (in base pairs). Two fragment lengths indicate a heterozygous state, whereas one fragment length indicates homozygosity. The most frequent PCR fragment length is indicated in yellow.

The proportion of each pooled CBUs $CD34^+$ cell counts versus the $CD34^+$ cell counts of the ≥ 4/6 HLA matched CBUs was also evaluated and visually represented into pie charts (Figure 19 to 25). The proportion of the matched CBUs varied between 25.4% to 53.7% of the $CD34^+$ cells infused to the recipients. The percentage of each CBU within the pool represents between 0.01% to a maximum of 8% of all $CD34^+$ cells infused to the recipient. The number of CBUs pooled directly impact on the proportion of the pooled CBUs with the matched CBU.

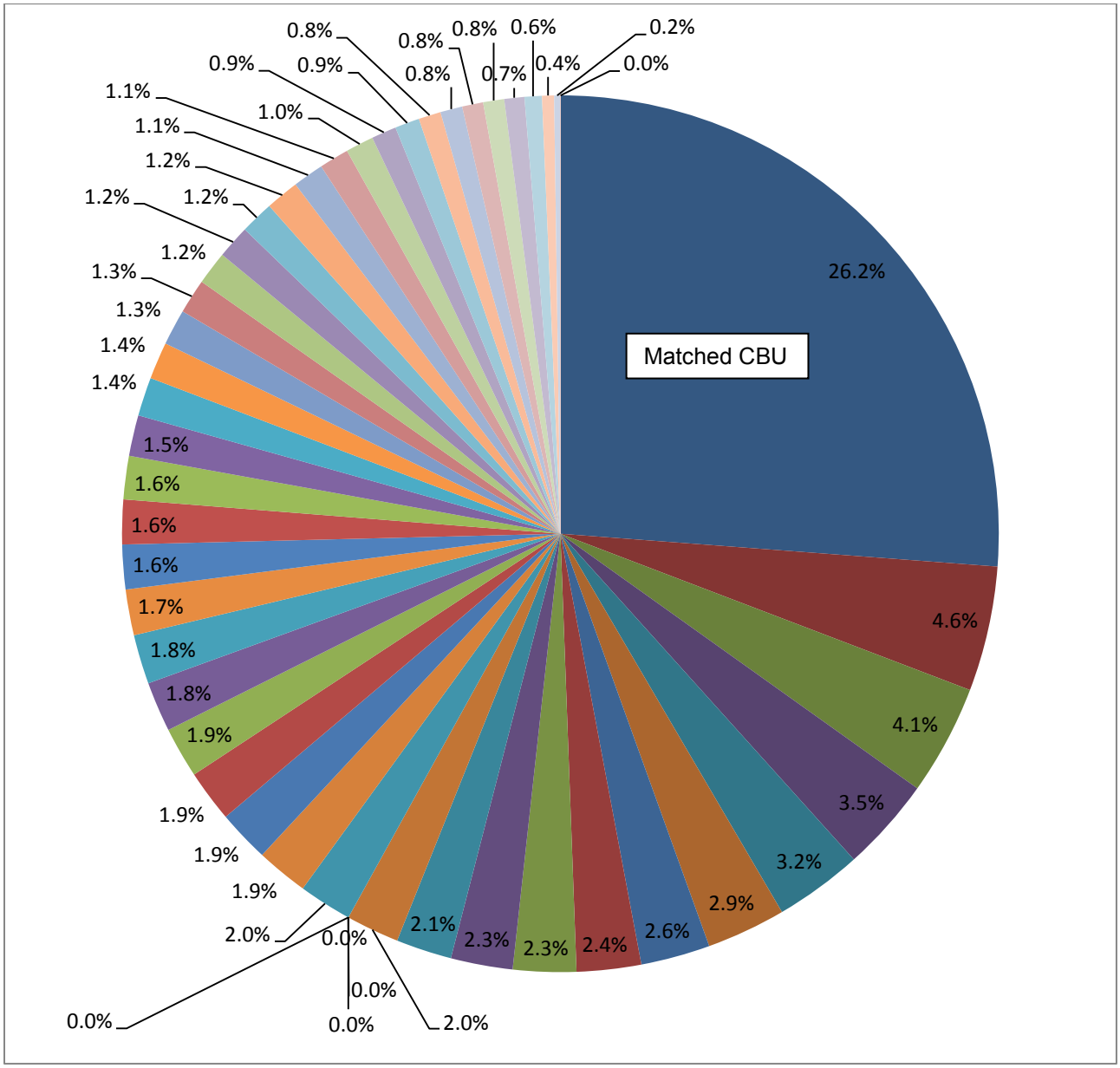


Figure 19: Percentage of the 4.1 x 10^5/kg $CD34^+$ Cells Transplanted for Patient #1

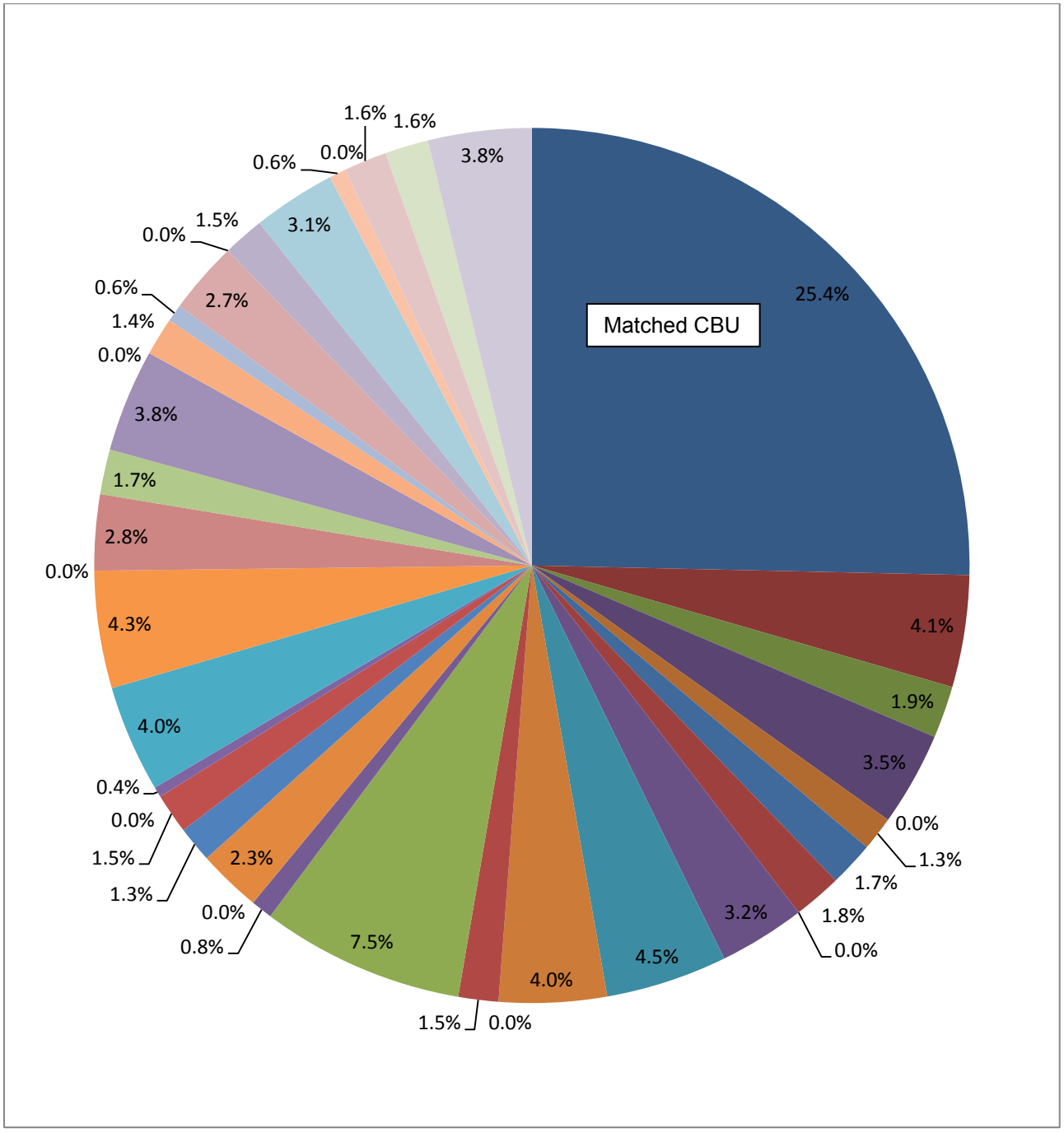


Figure 20: Percentage of the 2.67 x 10^5/Kg $CD34^+$ Cells Transplanted for Patient #2

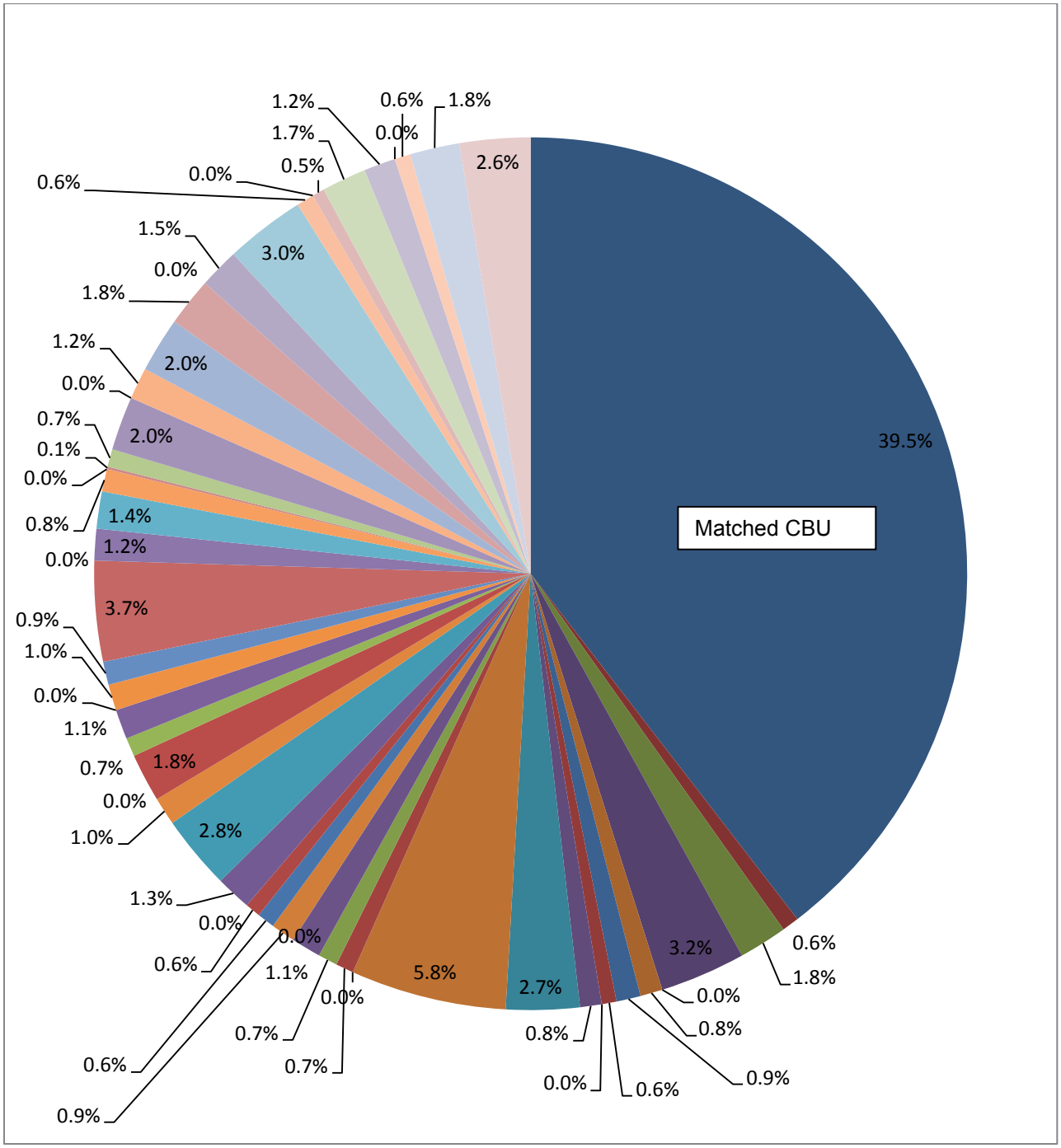


Figure 21: Percentage of the 4.13 x 10^5/kg $CD34^+$ Cells Transplanted for Patient #3

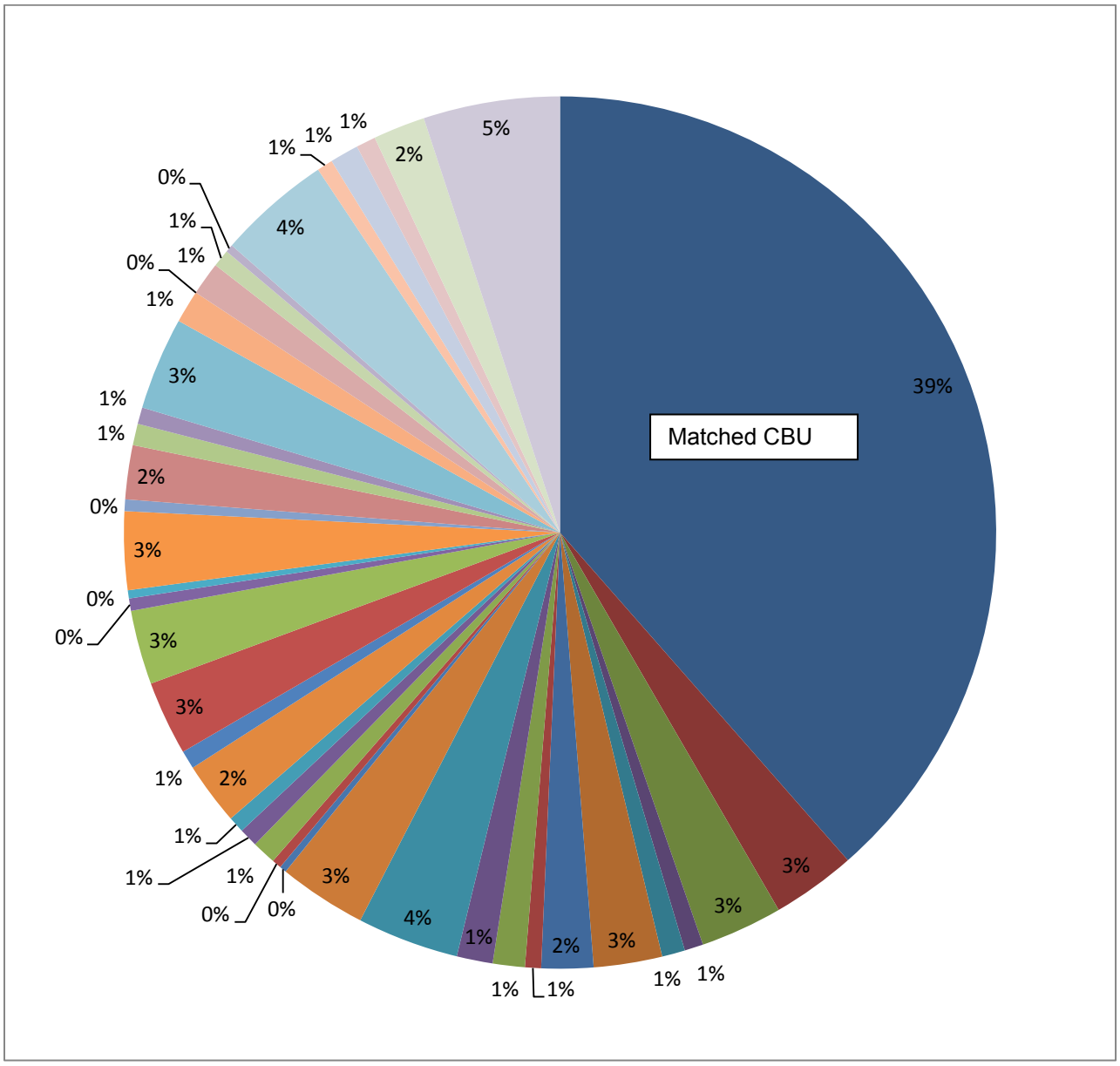


Figure 22: Percentage of the 3.6 x 10^5/Kg $CD34^+$ Cells Transplanted for Patient #4

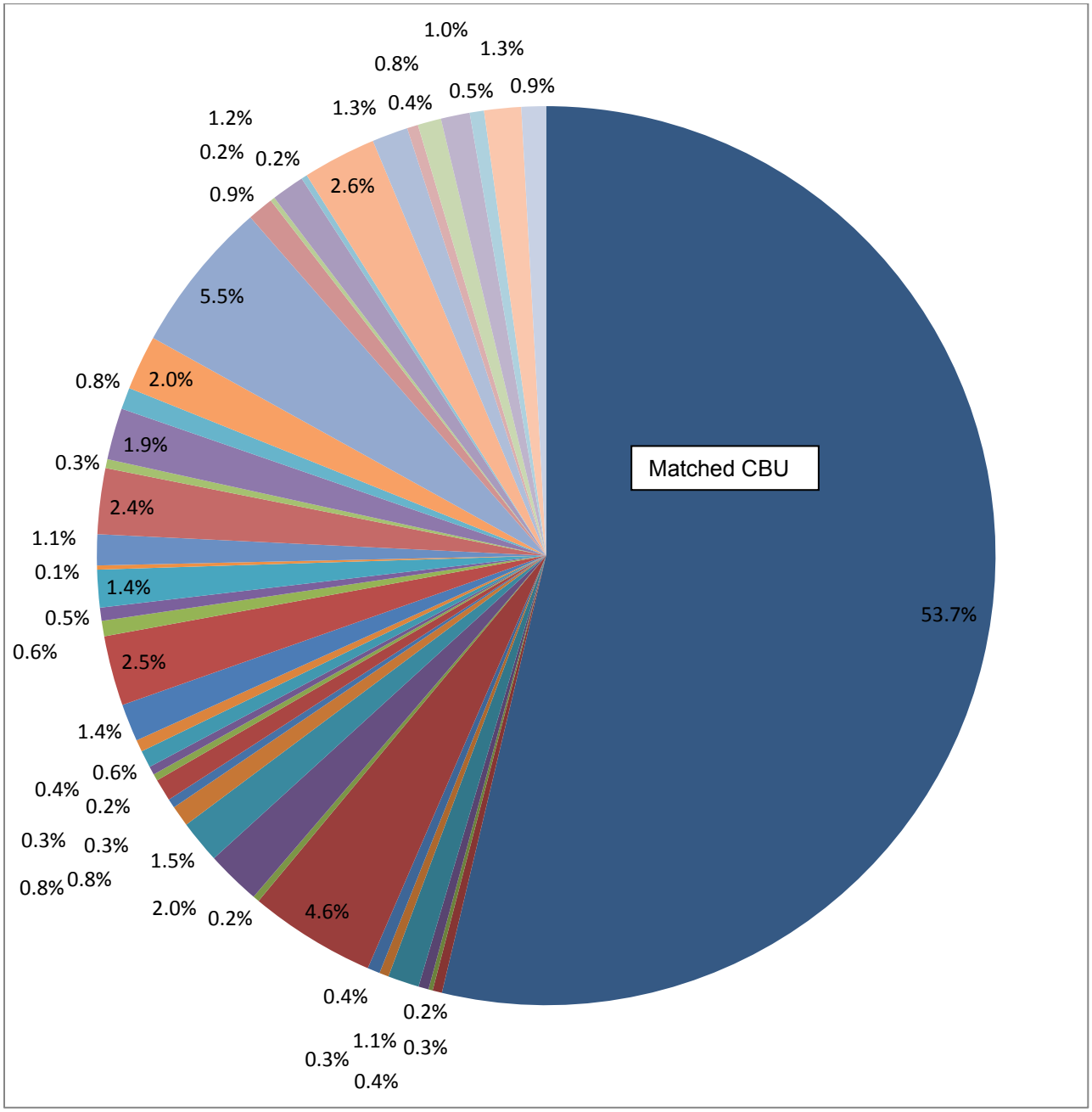


Figure 23: Percentage of the 3.8 x 10^5/Kg $CD34^+$ Cells Transplanted for Patient #5

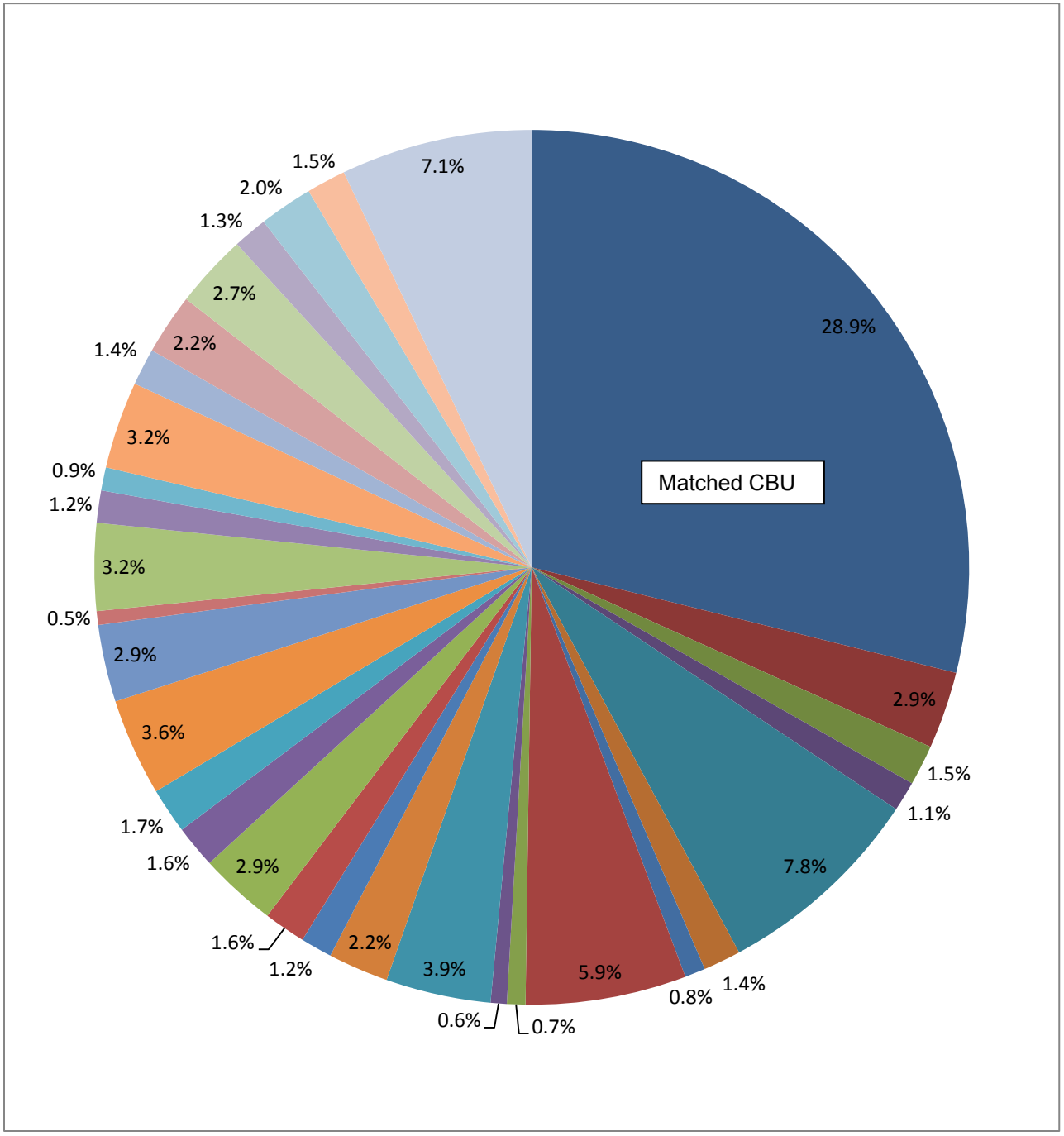


Figure 24: Percentage of the 4.9 x 10^5/Kg $CD34^+$ Cells Transplanted for Patient #6

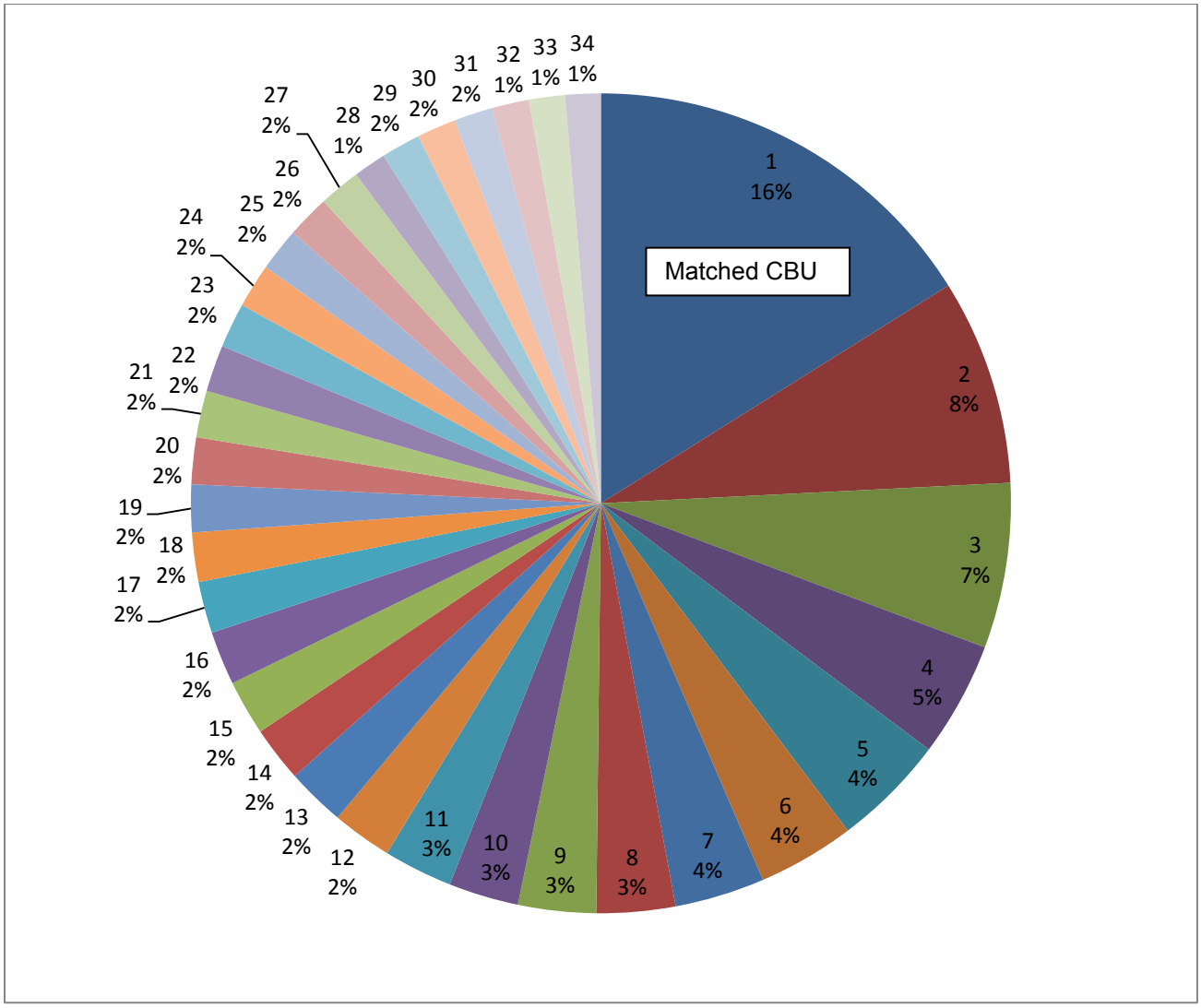


Figure 25: Percentage of the 3.02 x 10^5/Kg $CD34^+$ Cells Transplanted for Patient #7

Day of Transplantation

Seven recipients consented to be transplanted in this Phase 1 Pilot study. The recipients were 30-57 years of age with a median weight of 76 (48-143) kg. Fifty-seven percent of the recipients were transplanted with a ≥ 4/6 HLA matched CBU and as many were ABO blood group incompatible with the recipients. The matched CBUs were infused first followed by the infusion of the pooled CBUs within an average of within 66.6 minutes. Up to 45 CBUs of different volumes and of different TNC and $CD34^+$ counts were pooled to create the "third party unit" containing ≥ 1 x 10^6 $CD34^+$ cells/kg of recipient weight pre-thawing. As shown in Table 17, the average number of $CD34^+$ cells contained in the ≥ 4/6 HLA matched CBU and infused was 1.2 x 10^5/Kg, with the "third party unit" containing a mean of 2.5 x 10^5/Kg from the pooled CBUs for a total of 3.7 x 10^5 $CD34^+$ cells/kg. Three of the recipients had a major blood group incompatibility with the matched CBU and another one had a minor blood group incompatibility; all others were compatible. All chosen matched CBUs had a minimum of one HLA mismatched but never more than two. Only one of the patients had an HLA class II DR mismatch, all other had one HLA class I A locus mismatch and four recipients had an additional class I HLA B locus mismatch.

Engraftment

The median time to neutrophil recovery (ANC ≥ 500/mm^3) was 19.5 (15-29) days post-infusion for all recipients except one who had primary graft failure (Figure 27). The

first DNA chimerism assay was performed on day +14 by DNA STR analysis. It showed a 100% $CD3^+$ and $CD33^+$ cells matched CBU donor chimerism, including for the one patient who had primary graft failure (Table 18). Only two recipients had a reduced matched CBU donor chimerism in the first 100 days post-transplant. The first transplanted recipient had a mixed matched CBU/recipient chimerism at day +100 of 90%, which returned to the normal 100% the following weeks. On the other hand, patient #5 never achieved 100% matched CBU chimerism. On day +21, the chimerism was of only 35.5% of the matched CBU indicating a graft rejection, which was confirmed at day +40. The median time to platelet recovery ($\geq 20 \times 10^9$) was 53 days (29-175) (Table 17). In Table 17, I observed that one patient had platelet engraftment at only day +175, probably secondary to concurrent CMV infection and treatment with gancyclovir. The median of platelet recovery of the other four recipients was day +48.

The verification of the absence of bridging in recipients peripheral blood at day +14 and day +21, was verified by real time-PCR (RT-PCR) on the Y chromosome. To ensure accuracy of the results, the recipient had to be a female and transplanted with a matched CBU that was also female. Only one recipient complied with those two criteria (i.e. recipient #4). The pooled CBUs infused to this recipient was equally composed of 21 male and female donors. I observed the absence of any Y chromosome, thereby reducing significantly the chance of having any CBUs that were pooled being transient in the blood stream of the recipient two weeks post-transplantation (Figure 26). Many

dilutions were done to confirming the negative presence of Y chromosome at day +14 and day +21.

Table 17: Absolute Quantification of Y Chromosome on Day +14 and Day +21 Recipient #4 chimerism

Position	Name	CP*	Position	Name	CP*
1	Neg Control Water	26.91	15	Day +21 0.01	27.15
2	Neg Control Water	26.89	16	Day +21 0.01	27.09
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	27.16
6	Pos Control Male 0.1	24.53	20	Day +14 0.001	27.25
7	Day +14 0.1	25.93	21	Day +21 0.001	27.3
8	Day +14 0.1	25.92	22	Day +21 0.001	27.27
9	Day +21 0.1	26.58	23	Pos Control Male 0.0001	> 35
10	Day +21 0.1	26.53	24	Pos Control Male 0.0001	> 35
11	Pos Control Male 0.01	27.41	25	Day +14 0.0001	27.11
12	Pos Control Male 0.01	27.21	26	Day +14 0.0001	27.02
13	Day +14 0.01	26.9	27	Day +21 0.0001	27.01
14	Day +14 0.01	26.79	28	Day +21 0.0001	27.07

*CP: crossing point

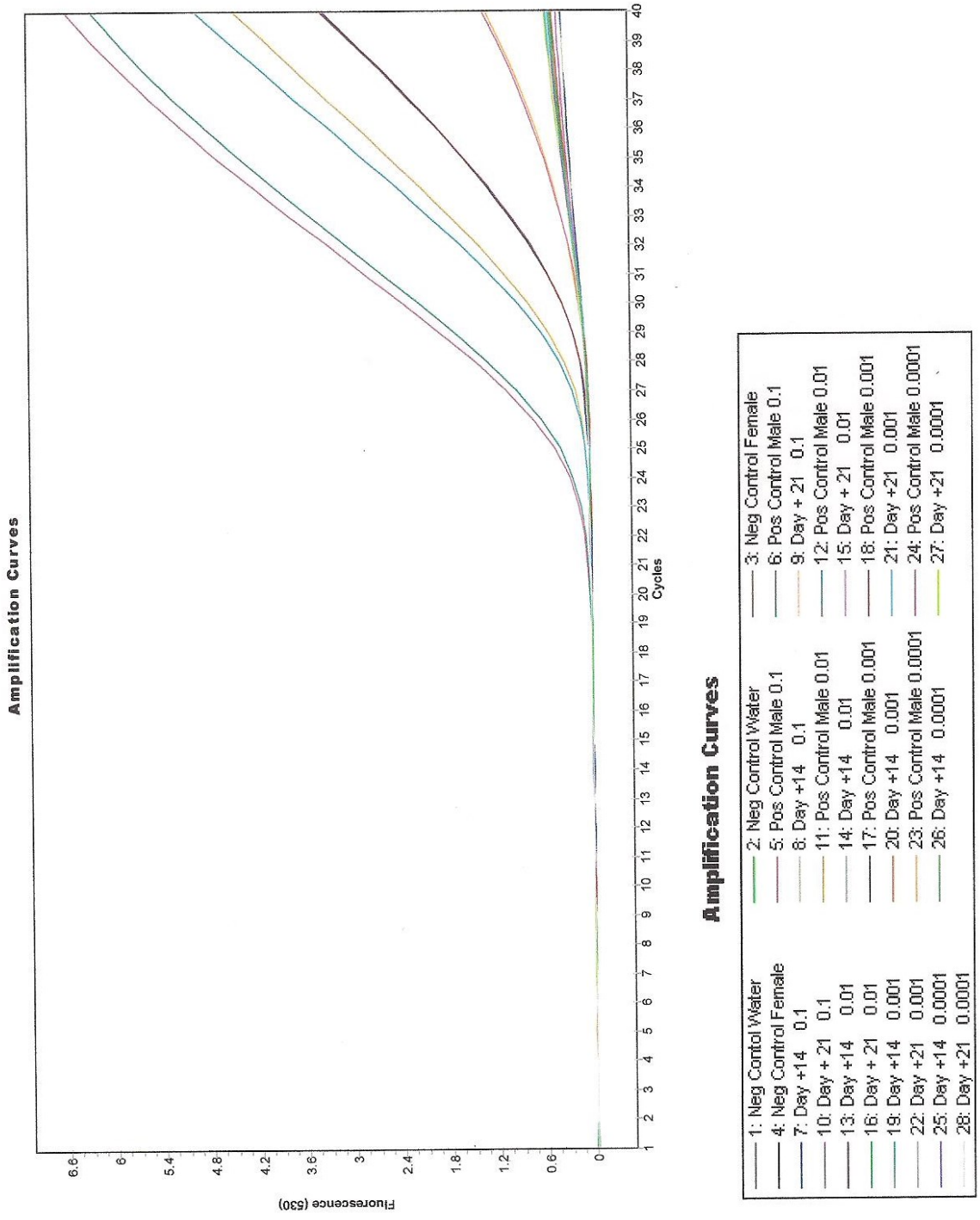


Figure 26: Amplification Curves of Y Chromosome testing done at day +14 and Day +21 on recipient #4 at different concentrations.

Patient Outcomes

Five of the seven patients transplanted are alive and four are off immunosuppression at the time of writing and only one patient 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 with refractory 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 with double matched cord blood transplantation and remains in complete remission. Six patients developed transient cutaneous grade I/II acute graft-versus host disease (AGVHD) while one (patient #1) developed grade III AGVHD (skin and gut) that responded promptly to steroid therapy. To date, none of the patients developed chronic GVHD. There were no transplant-related complications or negative patient outcomes that raised issues about the feasibility and safety of the third party cells infused in this group of very high risk patients.

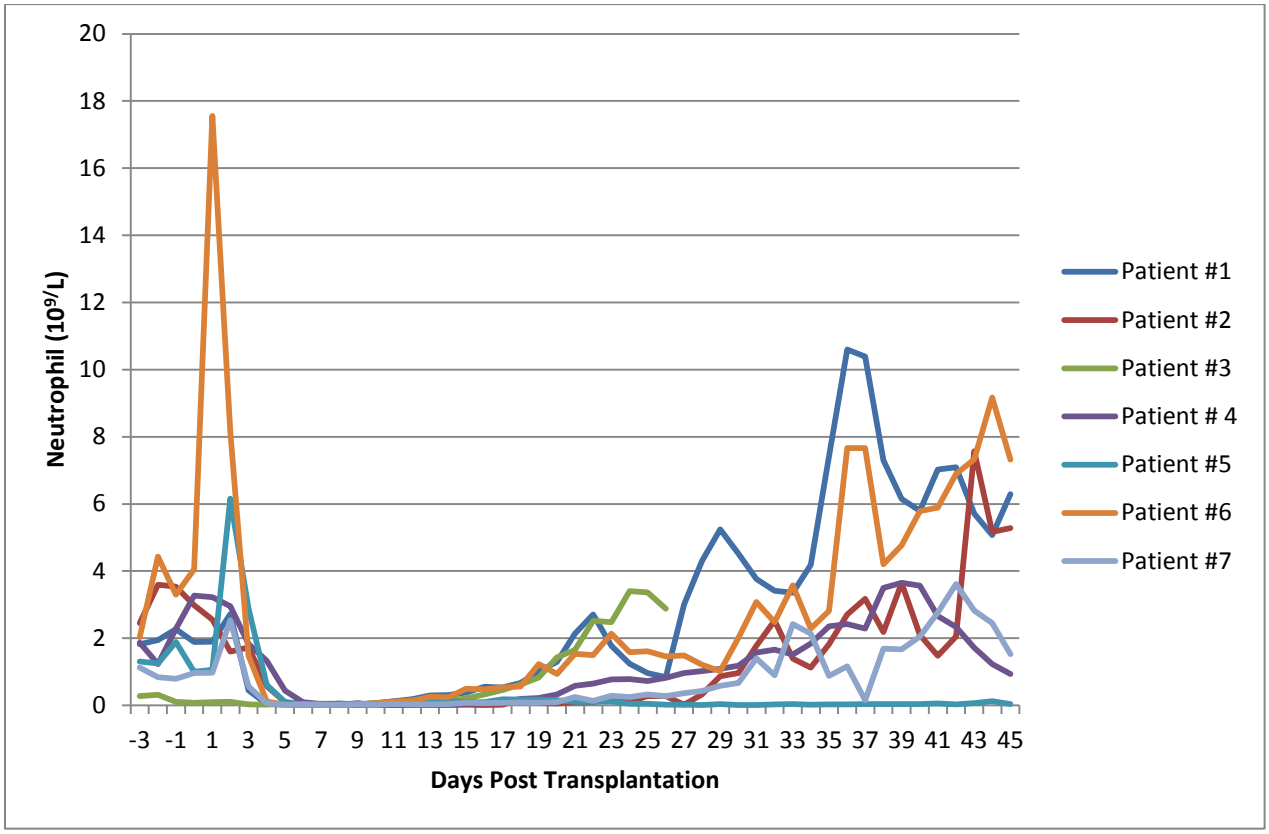


Figure 27: Recipients Neutrophil Recovery Post Transplantation

Table 18: Summary of Grafts

Pt #	Diagnostic	Age (Sex)	Weight (kg)	CMV status		Patient ABO/Match CBU ABO	Ethnicity	HLA Locus Mismatch	TNC $x10^7$/ kg Matched CBU	CD34 ^{5} (×10 /kg) Matched + rd 3 Party	Total CD34 ^{5} infused (×10 /kg)	3rd Party CD3 ^{3} (×10 /kg)	# HLA MM	# Pooled CBUs	Ethnicity	Infusion Delay between Matched and Pooled CBUs	Neutrophil Engr. ^{9} (ANC ≥ 0.5 × 10 /L)	Platelet Engraft (≥ 20 000)
				Recipient	Matched CBU													
1	B-LPD	41 (M)	81	pos	pos	A/O	Arabic	A and B	2.9	1.1 + 3.0	4.10	8.6	2	45	33 cauc; 2 arabic; 10 other	66 min	Day +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	Day +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	Day +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	Day +21	53
5	B-SCL	45 (M)	76	pos	pos	O/O	Black	A and B	3.0	2.1 + 1.7	3.80	2.4	2	42	25 cauc; 1 black; 16 other	74 min	n/a	ND
6	ALL	37 (F)	48	pos	pos	B/A	Pakistan	A and B	4.0	1.1 + 3.8	4.90	0.03	2	30	18 cauc; 6 asian; 4 arabic; 2 other	18 min	Day +18	29
7	CML-BC	53 (M)	143	Pos	Pos	B/A	Polish	Dr*B*1	1.7	0.3 + 2.7	3.02	0.27	1	30	22 cauc; 4 arabic; 3 asian; 1 black	80	Day +29	53
Mean		42	81	-	-	-	-	-	2.8	1.2 + 2.5	3.69	2.39	-	37	-	66.6 min	Day +21.2	70
Median		41	76	-	-	-	-	-	2.7	1.1 + 2.5	3.8	1.4		39	-	72	19.5	53

Table 19: Summary of Transplant Outcomes

Pt #	Diagnostic	Age (Sex)	Weight (kg)	DNA Chimerism (CD3 and CD33 subpopulations) % of Matched CBU							GVHD	Length of stay since Transplant	Overall Survival		
					+14	+21	+28	+56	+100	At death			+100	2013/05/23	
1	B-LPD	41 (M)	81	*CD3*	*100%*	*100%*	*100%*	*100%*	*90%*	*100%*	Grade II/III (S/GI)	94	Alive	Died +360	
				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		Alive +1141
				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	
				CD33	*100%*	*100%*	*100%*	N/A	N/A	*100%*					
4	B-ALL	57 (F)	72	CD3	*100%*	*100%*	*100%*	*100%*	*100%*		Grade II/III (S/GI)	57	Alive		Alive +687
				CD33	*100%*	*100%*	*100%*	*100%*	*100%*						
5	B-SCL	45 (M)	76	CD3	*94%*	*35.5%*	*0%*	N/A	N/A		N/A	58	N/A	N/A	Alive + 510
				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 +264
				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 +68
				CD33	*100%*	*100%*	*100%*	*100%*	N/A						
Mean		42	81	-	-	-	-	-	-	-	-	64	-	-	-
Median		41	76	-	-	-	-	-	-	-	-	58	-	-	-

S = Skin; GI = gastro-intestinal; L = Liver

CHAPTER 4: DISCUSSION

Hematopoietic stem cell (HSC) transplantation is an accepted treatment for life-threatening hematological diseases. The principal HSC sources for adult population are bone marrow and peripheral blood. However, only 25% to 30% of recipients will have a compatible related donor and approximately 65% will find an unrelated adult donor through the international registry based on the required HLA-matched donor [244]. In the absence of such donors, the alternative source considered by numerous transplant physicians is cord blood stem cells. Cord blood offers a rapid accessibility and may accept reduced HLA compatibility. However one constraining factor persists, namely the limited number of nucleated cells present in the CBU to ensure engraftment in larger size recipients even if more than 25, 000 CB transplants have been performed worldwide from approximately 40 public banks, [245,246].

To overcome this limitation, the most frequently used method is double CBUs (dCBUs), which consists of infusing two units, each containing a minimum of 1.5 x 10^7 NC/kg of recipient weight [180,185]. 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 single CBU transplantation [247]. The selected unit requiring a smaller number of nucleated cells per Kg of recipient weight increases the chances of finding a ≥ 4/6 HLA match CBU in 75% of the searches.

In spite of this increase, 25% of the searches launched will remain without any donor due to the difficulty of finding two CBUs containing the minimum of TNC required that are HLA 4-6/6 compatible to each other and with the recipient [247]. The cost of such method can also be considered a limitation in many countries, bringing it from 35, 000$ as a single CBU to 70, 000$ for dCBUs.

Instead of using a second CBU to increase the number of $CD34^+$ cells and to ensure engraftment, it has been reported that co-transplantation of a ≥ 4/6 HLA matched CBU with a third party unit created by the $CD34^+$ selection from an HLA haplo-identical apheresis related donor, can be used to increase the cell dose and enhance engraftment. Similar to dCBUs, the matched CBU has to contain a minimum TNC count of 1.5×10^7/kg of recipient weight. Fernandez, being the first transplant physician using this method, selected matched CBUs containing a mean TNC of 2.39×10^7/kg and 1.1×10^5 $CD34^+$/kg of recipient weight [215] and infused a third party unit containing a mean $CD34^+$ cells varying between 24 to 105×10^5/kg $CD34^+$ cells [213,248]. Neutrophil engraftment time and overall survival (OS) obtained was similar to dCBUs, but the availability of a related haplotype donor remains an issue. According to the 2011 Canadian Census, the number of siblings per family has been evaluated to 1.5 [249]. Parents are also haplo-identical with their children identifying them as potential donors. However, the aging population reduces the chance of finding a parent in good

health of less than 60 years old, making this method less accessible for adult recipients in future years.

A new strategy recently studied is intra-bone infusion of cord blood units. There are two existing approaches. The first consists of the intra-bone infusion of a CBU followed by the intravenous infusion of a second CBU. A second approach is the intra-bone (intra-osseous) infusion at different sites of one CBU chosen based on the single CBU transplantation criteria. This latter requires the thawing of the CBU at bedside with the infusion of approximately 6 mL of the CBU followed by the infusion of 1 mL saline after local anesthesia until infusion of the thaw CBU total volume. Both methods were successful with a ≥ 80% neutrophil engraftment even when reduced-intensity conditioning was required [233,250]. Lymphoid cell chimerism was achieved at a mean of 15 days and a full myeloid chimerism at a mean of 20 days [233] making both methods equivalent to the single CBU transplantation method [251,252].

CB HSC expansion is another studied method which increased the level of understanding in homing, cell-cell interaction and hematopoiesis [253]. Some Phase 1 studies are promising, but the growth of the culture, sometimes requiring months of treatment, increases the cost and makes these procedures prohibitive [254].

The common factor stated by all these methods is the recommended number TNC going from 2.5 to 5 x 10^7/kg with some researchers suggesting an increase TNC count in presence of a lower HLA compatibility and a need of ≥ 2 x 10^5 $CD34^+$ cells/kg to ensure engraftment of a single CBU. To increase the number of these cells when having access to a single ≥ 4/6 HLA match CBU can be achieved with a source that has never been considered: the rejected units of public cord blood banks that represent approximately 70% of all collected CBUs [255-257]. This Phase 1 pilot study was conducted to evaluate the safety and feasibility of developing an alternative approach with the aim of increasing the number of $CD34^+$ cells/kg by creating a third-party unit using the rejected CBUs. To perform this research project, the preservation of small CBUs was necessitating the creation of a clinical grade research CBB.

Cord Blood Banking of Small CBUs

Superior Quality of small units

The number of rejected units has increased since the opening of the first cord blood bank in New York, where the minimum qualification volume was 40 mL with no minimum nucleated cell count [256]. The transplant population being more challenging and the increased use of CBUs as stem cell source for adult population brought many CBB to increase their cut off volume to ≥ 75 mL and the number of TNC count at collection to ≥ 1.25 x 10^9 [258]. It has been reported by

Balabanova of the National Marrow Donor Program, that units containing ≥ 2 x 10^9 TNC will be transplanted within the first 2 – 2 ½ years of long-term banking compare to more than 10 years for units of ≤ 2 x 10^9 TNC [259]. The MUHC-CRCBB collaborated with the Québec Public Cord Blood Bank with the purpose of maximising cord blood donation by processing the units rejected by them given that mothers consented to the research project before labor. The public bank requires that mothers register before week 36 and requires that collected CBU be of ≥ 65 mL with a minimum of 1.2 x 10^9 TNC to be processed and banked. Based on these qualification criteria, the MUHC-CRCBB processed 61.3% of the collected units that had to also be compliant to Health Canada CTO regulations and to the Foundation for Accreditation of Cellular Therapy (FACT) standards.

Even in the presence of low volume and TNC cell count, these units were found to have a positive correlation between TNC and $CD34^+$ cell counts (figure 8). This relationship was first observed between the volume and the total nucleated cell counts of larger units followed by a relationship with $CD34^+$ cells [72,108,112,118,120,260,261]. The data compilation of the first five years of activities surprised us by the large number of $CD34^+$ cells count contained in small CBUs even in the presence of low TNC. More than 8% of collected units of < 65 mL of cord blood were containing from 3 to 7 x 10^6 $CD34^+$ cells and 13% contained

from 2 to 2.9 x 10^6 $CD34^+$ cells. This observation demonstrates that volume or TNC count should not determine the quality of a unit nor the number of $CD34^+$ cells post-processing. Other factors corroborate my observation, such as maternal fever during labour, which impacted positively only on the number of TNC but not on $CD34^+$ cells, leading Public banks to long-term cryopreserve some CBUs containing less CD34 than expected. I also verified the impact of induction of stress on the mother and on the future newborn by the use of Oxytocin during labor, and observed a negative impact of the number of $CD34^+$ cells in the CBU (table 3). Another factor that has been presented by various studies as having a positive impact on the number of TNC and $CD34^+$ cells is the presence of meconium [72,262]. My data, on the other hand, did not demonstrate any significant increase in the TNC or $CD34^+$ cells counts or their ratio in the presence of meconium. Another contrasting difference between publications is in the presence of blood group O CBUs, where Lee (2011) observes an increased number of TNCs, $CD34^+$ cells, and $CD34^+$ cells/TNCs [75]. My data show an increase in the number of TNCs when CBUs blood group is AB and was at its lowest in blood group O, and $CD34^+$ cells counts were at the highest in CBUs of blood group B and at his lowest in blood group A (Figure 7). Some other factors are corresponding to publications, such as the observation of an increase in the $CD34^+$/TNC ratio counts during delivery of a boy compared to a delivery of a girl

(3.25 versus 2.83 $CD34^+$ per 100 TNCs (p=0.003)). These favourable factors brought us to process some rejected CBUs containing a mean of 3.1 x 10^6 $CD34^+$ cells (SD 4.2 x 10^6), which could have been banked by public banks if cut-off values were not based on volume and TNC, but on CD34 counts instead. A bank that has responded to this challenge is the CBB in France were the qualification of a collected unit is based on a count of ≥ 2 x 10^6 $CD34^+$ cell/bag and cryopreserved CBU must contains ≥ 1.6 x 10^6 $CD34^+$ cell/bag ensuring a maximum of $CD34^+$ cells during a transplantation [263].

After qualification, CBUs are processed by reducing the volume, which holds its own determining quality criteria based on the minimum loss of nucleated and $CD34^+$ cells. For this reason, some banks prefer not to process units, maximizing the number of cells infused to the recipients but this choice is reducing significantly the number of units that can be stored in the same cryofreezer. For those who chose to process collected units, it may be done using a large variety of methods. Some banks fractionate the unit by removing only red blood cells, other the plasma and some fractionate both red blood cells and plasma through manual processing method or partially or totally automated methods [258,260,264]. The CB processing method used in this research was the Top-bottom volume reduction, which fractionate plasma and red blood cells. I observed that not only the configuration of the OptiSystem has an impact on cell

recovery, but also two other important factors: the delay between collection and processing, and the ratio of anticoagulant and blood.

CBBs standards and regulations require cryopreserving CBUs within 48 hours of collection. To better understand the importance of this time limitation, I analysed the TNC and $CD34^+$ cells recovery and viability on 1169 CBUs at different time between collection and cryopreservation. I observed that TNC recovery significantly improves when the delay post-collection is getting higher when closer to 54 hours, followed by a drop to 30% at 80 hours post-collection ($p \leq 0.0001$). Oddly, I also observed a stable recovery of $CD34^+$ cells when collection times is under 78 hours with a reduction when over 80 hours. Contrasting results have been recently obtained by Meyer-Monard (2012) with a better recovery within 15 hours of the collection, however even if divergent, the recovery verified was only the TNC and the processing method was different [265]. Processing types such as Tob-bottom, with or without Hespan or Pentaspan or red cell depletion, plasma depletion and the use of different system such as OptiSystem, Sepax and AXP has been documented to give differences in the recovery of TNC and $CD34^+$ cells [115,128,260,266]. The growing number of processing types and equipment raise several questions about factors affecting cell recovery. The use of the Optipress with different configurations, which varied principally on the buffy coat level, demonstrated a TNC recovery over 80% when

the volume was ≤ 100mL. Higher volumes still ensured mean TNC recovery varying between 71% and 79%. The common point between delay after collection and increased TNC recovery in presence of low volume was possibly the ratio between blood and anticoagulant. In presence of one to two parts blood for one part CPD (Citrate, phosphate, dextrose) anticoagulant, $CD34^+$ cells recovery is significantly superior to TNC cells recovery. A much smaller difference was observed in presence of three and four parts of blood for one part anticoagulant (Table 6). Further analysis are required to verify if the use of anticoagulant such as heparin, AS-1 (sodium choride, adenine and mannitor), EDTA (ethylene-diamine-tetraacetic-acid) or ACD-A (anti-coagulant citrate, dextrose formula-A) would decrease cells adhesion and increase natural sedimentation more rapidly while enhancing long term TNC and $CD34^+$ cells viability.

Another quality determinant of an adequate CBU is its cell viability. I observed that $CD34^+$ cells maintained ≥ 91.8% viability at 72 hours post collection compared with ≥ 66.6% TNC viability within the same time delay when CBUs were kept at room temperature. I also verified $CD34^+$ cells viability at 96 hours, and even if the number of sample is limited to 14, the mean viability is 83.9%. Querol (2010) evaluated the clonogenic efficiency (CE) of $CD34^+$ cells and determined it to be 36 ± 25% of incubated cells [258]. The clonogenic

efficiency (CE) of CD34 in CFU is a determinant of the potential growth of the cells as much as their viability. $CD34^+$ viability was confirmed by the growth of CFU-GM to a proportion of 14.1% at ≤ 72 hours. Insufficient number of CBUs were analysed for their differentiation potential post-thawing to have adequate statistical power especially in older units (≥ 72 hours). These results indicate that CBUs rejected due to a delay of more than 48 hours after collection may unfortunately be eliminating an otherwise adequate source of hematopoietic stem cells. The 72 hours delay should be further studied by using older CBUs and measuring the same qualification criteria after cryopreservation to verify the impact of cryopreservation of older cells. All these facts causing significant differences reinforced the importance of validating the procedures chosen by a cord blood bank from the collection bag to long term cryopreservation methods and equipment.

Third party unit

The main goal of processing and cryopreserving rejected small CBUs was to develop a functional third party unit. Despite the identification of a longer viability of $CD34^+$ cells over TNC, it is impossible to kept at room temperature or at 4ºC until several CBUs until sufficient number of the same blood group would be available to be cryopreserved as a larger pooled CBU, entailing that all units be cryopreserved as a single unit with a final concentration of 10% DMSO within

the regulatory 48 hours. Even if DMSO is known to have toxic side effects, the infusion rate and temperature are very important, to ensure a slow osmotic exchange and to reduce cell damage ensuring long term preservation [267-270]. A new product called the trehalose, a nontoxic disaccharide of glucose, demonstrated similar potential as DMSO, but until further research are ensuring quality and safety, DMSO will still be the product of choice constraining us to take into consideration its toxicity [271-273]. More important compare to the time of infusion, DMSO removal can be more damaging to the cell. As in DMSO injected into the product, the dilution solution must be slowly infused. Upon infusion, the cell will swell above their initial volume and then shrink as the cryoprotectant moves out until osmotic equilibrium is established [274,275]. My thawing method was inspired by the traditional cord blood thawing method developed by Rubinstein (1995), which consisted of partial removal of DMSO by adding an equal volume dextran/human albumin[124]. This method has been used in many studies and is adequate for the thawing and infusion of a single CBU in the following 20-30 minutes. However this method attains only 58% TNC viability after two hours post-thawing and 38% after four hours [124,142,154,276]. Since the possible number of CBUs to be pooled was estimated to approximately 20 units, it was necessary to reduce the DMSO concentration to 1% or 2%. I observed after some assay on adult buffy coat and confirmed when using CBUs, that TNC

recovery post-thawing and post-washing was increased at a 1% DMSO concentration with 92% and 82% respectively (Table 8) ($p = 0.46$; $p = 0.03$) possibly due to the DMSO toxicity however no specific analyses were done to address this hypothesis. This convinced us to pool 3 CBUs instead of one, thus ensuring the dilution of DMSO to 1%. The first CB pools of 6 and 12 CBUs performed within 2 hours, demonstrated TNC recovery of ≥ 80% (Table 10). However, when more than 45 CBUs were pooled, three uninterrupted series of 18 CBUs, 12 CBUs and 15 CBUs were thawed, pooled and washed. The recovery measured after the last pool showed that the longer the delay between pooling and the following procedure the lower the recovery with 45.8% after 6 hours versus 82.7% two hours after pooled CBUs. A few researchers developed thawing and washing devices such as Cytomate, however TNC and $CD34^+$ cell recovery still varied between 63.3% to 90% and 70.2% to 95% respectively and the device was taking 15 minutes for a single CBU which was taking much too long in the event of the pooling of 45 CBUs [277]. A modification of the technique was required in order to be able to pool a maximum number of CBUs in 2 hours (table 12). Even if the length of time has to be limited, my pooling method ensured a superior recovery two hours post-thawing which was better than any other accepted thawing method [124,275,277,278].

The selection criteria of the CBUs composing the new third party unit was solely based on identical or compatible ABO blood group as the ≥ 4/6 HLA matched CBU selected for the recipient. The number of units to be pooled was corresponding to ≥ 1 x10^6 $CD34^+$ cells/kg of recipient weight before thawing. The number of CD34+ cells/unit cryopreserved varying between 0.01 to 28.4 x 10^6 (average of 2.32 x 10^6) and the average weight of the adult population to be transplanted being approximately 80 kg, I was expecting to pool an average of 35 different CBUs. The infusion of 2 and even 3 CBUs have been performed without observing a significant higher rate of acute graft versus host disease (GVHD) or chronic (GVHD) [247]. Lister pushed further the boundaries with the infusion of up to 7 CBUs and observed grade 2 aGVHD of the skin and no cGVHD [237]. However, the infusion of an average of 35 CBUs with presumed different HLA increases significantly the risk of an allogeneic reaction mediated by cord blood T cells. Based on this hypothesis, T-cell depletion was done by positively selecting $CD34^+$ cells from the thawed, washed and pooled CBUs.

Positive selection of cryopreserved cord blood has been documented as early as 1995 with a recovery of only 26.7% [279]. It has been practiced most often on bone marrow containing from 1 to 4% of cells expressing CD34 antigen and on mobilised peripheral blood. Most recovery on fresh peripheral blood or fresh bone marrow obtained a mean of 65.2% ± 11.3% [147,159,280,281]. However my

average recovery varied between 15% and 85% (mean = 46.9%) recovery from initial counts up to 22 x 10^6 $CD34^+$ cells. Overload of the CliniMACS column was eliminated as a possible error by observing recovery of 70% and 30% on similar initial counts 0f 21.9 x 10^6 and 22 x 10^6 respectively. The latter was done using a larger CliniMACS column to reduce the overload risk but instead it reduced the recovery. Even if not measured, another factor that may have impacted on the CD34 recovery was the platelet count. Bruno (2002) compared $CD34^+$ selections and observed a borderline recovery in presence of lower platelet count before selection ($p = 0.053$) [160]. Activation of a large number of platelets post-thawing increases the chance of clumping and trapping nucleated cells, thereby reducing recovery [282]. Other source of clumping may be the repetitive centrifugations increasing the rupture of cell membrane from the lysing of granulocytes and platelets causing the release of DNA in the solution, creating DNA clumps of different sizes [283]. Some researchers use DNase to reduce the risk of DNA clumping [283,284], however it has not been approved for clinical applications, limiting its application in laboratory research. Another possibility verified was the use of different clinically approved cell sorting equipment which is very limited. The CliniMACS choice was supported by McNiece who evaluated both equipment and demonstrated that recovery and purity was superior with

CliniMACS [155]. Contrary to my methodology where the selection is done on cryopreserved cells, this latter was done on a fresh product.

CD34 is a transmembrane glycophosphoprotein expressed at the surface of cell through three classes of epitopes identified as class I to III [285]. A recent study measured the impact of the binding by antibodies using only one epitope and found that it would increases the risk of $CD34^+$ cell loss [286]. Additionally, thaw products containing DMSO reduce the expression of class I CD34 epitope on AML blasts cells by altering the glycoprotein [287]. The CliniMACS antibody binding class II epitopes would not offer an additional explanation for the variability in the recovery and purity. However, there is no data on the effect of cryopreservation on the binding capacity of any of the epitopes classes post thawing. Low recovery can also be explained by the reduction of $CD34^+$ expression by more than half on stem cells post-thaw [287]. Therefore, to increase the binding of the $CD34^+$ MoAb, I increased the recommended incubation time from 30 minutes to 45 minutes at room temperature, which I suggest may improve recovery. The purity obtained from the pooled CBUs has been very satisfactory with a mean of 80.7%. The lower purity (75%) could be explained by the presence of lower $CD34^+$ initial count (13 to 18 x 10^6) and the inverse (96%-99%; 21.9-22 x 10^6) [160,288]. Another important measure of the purity would be the count of $CD3^+$ expressed by T cells. Unlike the relationship found between the

concentration of CD34 and purity, $CD3^+$ count was much lower in the more recent pools compared to the first one. The higher $CD3^+$ count of the last selection can be due to the large number of nucleated cell processed even if the concentration was within the recommended range of the manufacture. Further analysis should be done to verify the impact of platelets, cell counts or other factors influencing the purity, CD34 recovery and CD3 depletion on thawed and pooled CBUs.

IgG anti-A and anti-B binding leukocytes

The development of such new methods resulted in pooling multiple cryopreserved CBUs together before their infusion into recipients. To date, the expression "multiple infusions" was used to describe the direct infusion of one unit after another post-thawing into the recipient [161,237]. The only study in an animal model using pooled CBUs from multiple donors was published in 2000, where fresh cord blood mononucleated cells (MNC) isolated with Ficoll were pooled and infused into NOD/SCID mice [289]. Unlike this publication, my research provides the first pooling of thaw CBUs methodology used in human. The only technique similar to my technique was the Rubinstein thawing method due to the washing step post thawing. According to Laroche (2005), the recovery of TNC post-thaw of a single CBU using Rubinstein method should be around 89% and post-wash at 82% [275]. My first pool of three CBUs obtained a TNC recovery that

varied between 61% and 97% and between 74% and 100% when pooling 6 to 12 CBUs. Looking for the source of this wide range of TNC recovery, I observed some possible link with the blood group compatibility between pooled CBUs. The number of pools being limited, it was impossible to determine if a specific incompatibility between certain blood groups was reducing significantly the TNC recovery. Interestingly, when pooled CBUs were regrouped based on the number of incompatibility between maternal and fetal ABO, the TNC recovery was significantly lower in presence of 3 blood group maternal-fetal incompatibility compare to pooled maternal-fetal compatible CBUs (76.8% vs 98.2%, $p = 0.052$).

The blood group immunoglobulin classes, anti-A and anti-B even if predominantly IgM component are also IgG and IgA [290]. The maternal-fetal crossing of IgG through the placental Fc receptor such as blood group (ABO), Kidd (JK), anti-s, Rh, Kell (Kp, K, Js) Lewis has already been demonstrated [77]. It is also known that in the last week of pregnancy, the fetus receives a large volume of IgG from the mother, which level increases also with the number of pregnancies [291]. The IgM is unable to be transferred and even if it is produced at birth by the newborn, its levels are very low with 5-15 mg/dL compare to the IgG levels being 598-1672 mg/dL [77]. In presence of blood group incompatibility, this maternal-fetal IgG crossing may cause hemolytic disease in the newborn [292,293]. This rare disease is induced by the maternal IgG anti-A and anti-B binding and

lysing the newborn red blood cells. I hypothesised that this situation can be transferable where IgG would bind onto the leukocytes. An elution was made on samples of pooled CBUs and confirmed the binding of IgG on pooled leukocytes, which may explain the reduced TNC recovery in presence of maternal-fetal ABO incompatibility (Table 14). The IgG anti-A/B binding leucocytes may be greater than before due to the type of processing used at the CRCBB.

During CBU Top-bottom volume reduction, the proportion of the cord blood content is altered by removing most of the red blood cells and plasma. The newborn hematocrit (HTC) normal range is 43.5% ± 4.2%, on the other hand when mixed with the 35 mL of CPD contained in the cord blood collection bag this range is reduced to an average of 32.4% ± 0.06 % [77]. When volume reducing the cord blood volume from 95 mL (SD = 24.6 mL) to 21 mL (SD = 3.3 mL), the final HTC varies from 5% to 89.3% (mean = 54.2%; SD = 11.6%) and a platelet count increases from 204.2 x 10^9/L (SD = 61.4x10^9/L) to 744.3 x 10^9/L (SD = 337.6 x 10^9/L). This proportions modification of each blood component may account for the variability in TNC recovery post-pooling in presence of maternal-fetal ABO incompatibility.

Although the existence of Fc receptors on monocytes, granulocytes and lymphocytes is well known, the binding of blood group IgG on pooled leukocytes in presence of maternal-fetal blood group incompatibility has never been

observed [294]. The rosette assay done on pooled CBUs leukocytes confirmed that IgG anti-A and anti-B bind leukocytes through their $F_c\gamma$ receptors. The mechanism of activation of complement in presence of IgG, led us to believe that pooled CBUs TNC would be lysed in presence of blood group incompatibility. However, my observation did not confirm any IgG binding on $CD34^+$ cells. The expression of $F_c\gamma$ receptors on early hematopoietic progenitor cells has been shown by Wu in 1996. His data suggested that of the three classes of $F_c\gamma$ receptor ($F_c\gamma R$), only $F_c\gamma$ RII is expressed at the surface of the cell. This finding was also observed with platelets and megakaryocytic cells $F_c\gamma R$ [295]. These finding let us to believe that IgG anti-A and anti-B in presence of maternal-fetal incompatibility may bind HSC, therefore increasing cell death when pooled. The presence or absence of IgG binding through the F_c region does not preclude the possibility of its possible binding through the F_{ab} region. It was suggested by Mölne (2008) to avoid unnecessary rejection caused by ABO incompatibility when transplanting HSC, the A and B antigens being expressed on HSC as well as on human embryonic stem cells [296]. Using Mag-Sepharose assay, I demonstrated that some IgG are binding through the F_{ab} region even if the IgG were not specificly IgG anti-A and anti-B. Other IgGs, such as anti-CMV, (the most common congenitally transmitted infection) cross the placenta and could

also bind some leukocytes. Further analysis would be required to determine the types and impacts of IgG binding HSC.

Such new findings brought us to look for the effect of a larger volume of red blood cells or larger volume of plasma when volume- reducing CBUs. Even if volume reduction was initiated to maximise freezer space, red cell depletion was principally done to reduce the risk of transfusion reactions when transplanting a blood group incompatible CBU to a recipient [129,137]. Red cell depletion is preserving all plasma volume but the gelatin separation technique was increasing loss of TNC [297]. It was tested by adding hydroxyethylstarch (HES), reducing the loss of TNC from a range of around 25% to less than 10% [128,129]. Nonetheless, some CBBs preferred performing plasma volume reduction preserving all red blood cells to ensure absence of TNC loss. Those two scenarios were tested when pooling three CBUS to observe the effect of an increase volume of plasma or red blood cells on TNC recovery. The first assay increased plasma volume on a very limited number of pooled CBUs, yet I observed a lower TNC recovery when the proportion of plasma is increased in the presence of maternal-fetal blood group incompatibility compared to compatible blood group (Figure 12). A single adult transplantation with CBUs depleted only of RBC was published and showed a longer neutrophil engraftment at day +31 with no other unexpected adverse events [298].

In the second assay, I tested the opposite situation by adding cord red blood cells to the dilution/rinsing solution before pooling of CBUs. Using the same CBUs divided into two before poolings, I compared pools in presence of 0 to 3 maternal-fetal blood group incompatibilities. Once again, the number of assays is limited but I observed a better TNC recovery in presence of a higher concentration of red blood cells in all groups (Figure 13). Nonetheless, when more than 3 maternal-fetal blood groups are incompatible, the difference in the TNC recovery is 10% better compared to compatible maternal-fetal CBU pools. The differences can be explained by the IgG anti-A and Anti-B of the mother having a greater affinity to the blood group antigens present on red blood cells compared to the one expressed on leukocytes. Blood group expression on CB red blood cells being about 12% less compared to adult reduces the binding and lysing of red blood cells instead of IgG binding to leukocytes [299,300]. The difference between TNC recovery of incompatible and compatible maternal-fetal blood group being less important with plasma depleted technique compared to red cell depleted technique probably had no impact on cord blood pooling, however it has been shown that this type of volume reduction will ensure twice as much recovery of TNC compared to red cell and plasma depleted technique [125,128]. In a research done by Rosenthal from 2001 to 2007, patients with malignant diseases were transplanted with plasma-depleted (PD) cord blood

and others with red cell (RD) depleted cord blood. The time to neutrophil engraftment was similar (day +20) for red cell-depleted compared to plasma-depleted (day +25) ($p = 0.12$) however the number of TNC infused to the recipients was much higher in PD CB with a median of 6.8×10^7/kg versus a media of 4.6×10^7/kg for RD CB [301]. This observation is supporting the need to increase the number of nucleated cells per Kg of recipient weight when RD is performed on the infused CBU. The proportion of nucleated cells of each CBU before and after pooling in presence of 0 to 3 blood groups with maternal-fetal incompatibility when pooling three CBUs was measured (Table 15). I observed in certain incompatible pools the predominance of blood group O CBUs (pool #4, #10 and #12) when maternal blood group was also group O. When comparing compatible CBUs (pool C, #3, #6, #7 and B) with some incompatible ones (pool #1, #2, #3 and #8), I observed less variability in the proportions before and after pooling in the latter compared to the compatible pooled CBUs. These observations confirm that blood group compatibility is not the only factor affecting the TNC recovery when pooling CBUs. My results confirmed the binding of IgG on leukocytes, and several factors should be investigated. One factor affecting the cryopreservation of CBU red cell is the fragility of the RBC membrane. Upon thawing adult red cell membranes normally rupture because the membrane can expand by only 2-3%. However, the elasticity of cord RBC membrane is

decreased by 21%, thus increasing the probability of lysis post-thaw and reducing possible binding with any type of IgG [300]. Another factor is maternal-fetal transfer of IgG which is at an equal amount between the mother and the fetus at week 33 of the pregnancy [77]. In the last few weeks of pregnancy, the fetal IgG level increases from 20% up to twice the maternal IgG concentration [302], increasing the chances of the number of them binding to leukocytes. The variability may also be associated with mothers' blood group O and B, where more anti-A and anti-B would cross the barrier of the placenta, and where IgG anti-A was more lytic than IgG anti-B [303,304]. Finally, a last factor is the agglutination of red cells when a certain minimum number of bound antibody molecules, and complement activation is more likely to happen in the presence of an IgG anti-A even if they were ABO identical with their mothers [79,80].

With respect to the actual finding and possible impact on TNC and $CD34^+$ recovery in the presence of pooled maternal-fetal incompatible blood groups, and knowing that ABO incompatibility between the transplanted stem cells and the recipient may increase adverse events, I decided to ensure that pooled CBUs would be ABO compatible between mothers and CBUs.

Transplantation of Seven Patients

Engraftment of neutrophil

The goal of this Phase 1 pilot study was to develop and to demonstrate the feasibility and safety of performing a $CD34^+$ selection on multiple CBUs pooled post-thawing to create a support third party unit. Seven patients with a mean of 42 years with different hematological malignancies were transplanted from 2009 to 2013. The first product thawed and directly infused was the ≥ 4/6 HLA matched CBU containing a minimum of ≥ 1.5×10^7 TNC/kg of recipient. The selection of this CBU was based on two important factors: the number of TNC it contained post-processing and the HLA compatibility with the recipient, both being closely related. When a CBU is transplanted as a single unit with a lower HLA compatibility (5/6 or 4/6) it was suggested by Stanevsky to increase the minimum of TNC per kilogram of recipient weight to 4×10^7 or ≥ 5×10^7 TNC/kg respectively [247,305]. This suggestion has been recently supported in a study comparing the impact of TNC dose between pediatric and adult population[306]. Based on those recommendations, none of the matched CBUs selected for my recipients were suitable to be transplanted alone, the biggest one containing 4×10^7/kg with a 2 HLA mismatch between CBU and the recipient. Unlike TNC, there is no $CD34^+$ cell scale in the presence of an increased HLA incompatibility but only the recommended dose of ≥ 2×10^5/kg pre-thawing [161,162].

To ensure infusion of sufficient $CD34^+$ cells, a median of 39 (30-45) CBUs were thawed and pooled to ensure a $CD34^+$ cell count pre-thawing of $\geq 1 \times 10^6$/kg of recipient weight. Even if approximately 75% of the cells were lost in the thaw-pool-wash and $CD34^+$ selection process, recipients received from the third party pooled units nearly twice the amount of $CD34^+$ (mean of 2.5×10^6 $CD34^+$ cells/Kg) as compared to the number of $CD34^+$ cells contained in the matched CBU (mean of 1.2×10^6 $CD34^+$ cells/kg). The infusion of such an amount of cells was to ensure a faster neutrophil recovery. According to Haspel (2006) by infusing the matched CBU before the pooled third party CBUs, I also increased its chance of engraftment by 76% [207]. Another factor that probably influenced the engraftment of the matched CBU, was the infusion of the third party unit within a mean delay of 66.6 minutes (18-96 minutes), increasing directly the number of $CD34^+$ cells transplanted, as if it was part of the same unit but further away to ensure greater HSC homing from the first infused unit. Depending on the cell cycle of the infused cells, it has been estimated that homing will start in the first 5 minutes post-infusion and will continue for up to 3 hours [39,42]. The migration into the bone marrow endothelium being 20 to 40 minutes after the crawling process begins, increases the chances for the first infused CBU to engraft.

The blood group incompatibility is not problematic for stem cell transplantation, but it can create problems related to the transfusion of blood products containing plasma such as platelets, and fresh-frozen plasma such as erythrocyte hemolysis [290,307]. In the event that recipients will require many red cell transfusion and if the recipient is immuno-compromised or has mixed chimerism, the chances of a transfusion reaction is increased [308,309]. By selecting blood group compatible pooled CBUs, I lessen the risk of any reaction post-infusion. My previous observation that the mother's CBU blood group should be compatible with the pooled CBUs blood group, also respected this criterion.

The methods frequently used by transplant physicians to increase the number of TNC when using cord blood unit as stem cell source, are the co-transplantation of a second CBU or of a third party composed of purified $CD34^+$ cells from haplo-related peripheral blood collection. Based on the reported neutrophil recovery time estimated among adults or large children undergoing myeloablative conditioning transplantation those methods are similar to single CBU [310]. However, compared to single CBU transplantation they offer a more efficient immune and graft-versus-leukemia effect [211,310,311]. The intra-bone single CBU and dCBUs transplantation after myeloablative conditioning regimen were also compared and demonstrated neutrophil engraftment with a median time of 23 and 28 days respectively [312]. Comparing these results with my Phase 1 pilot

study obtained with only 7 recipients, I observed comparable neutrophil engraftment with a median time of +19.5 days (15-29 days). Recently, a study evaluated the influence of cell dose on neutrophil engraftment and observed that increasing the $CD34^+$ cells count to ≥ 1.8 x 10^5 per kg of recipient weight, would shorten engraftment time by almost one week [313]. Rocha (2011) made a distinction between pre and post-thaw $CD34^+$ counts to be infused, recommending 1.2 x 10^5/kg of pre-thaw count and 1.7 x 10^5/kg post-thaw. This recommendation is almost half the $CD34^+$ cells I infused using the match and pooled CBUs, which offers an explanation for my shorter engraftment time. Only one patient did not show evidence of myeloid or lymphoid engraftment on day +40, who was salvage 71 days after the first transplantation using dCBUs and engrafted at day +35. The platelets engrafted in a median of 53 (29-175) days, which compared to the third party haplo related unit with 32 (13-98) days can be considered much longer [204,215]. On the other hand, one patient had a longer platelet engraftment with day +175. The cause of such delay could be explained by a deficient bone marrow micro-environment, which was also observed in long neutrophil recovery after each induction.

The strength of my pilot study was the pooling of CBUs being HLA blind with the recipient and with the HLA matched CBU to support the identified unique CBU which was ≥ 4/6 HLA compatible with the recipient. The possibility of finding

one or several units within the pooled CBUs with a ≥ 4/6 HLA compatibility with the recipient is almost impossible. This assumption is based on the number of alleles in HLA class I A, B and C varying from 1788 to 2934 per class, class II Dr β1 with 1418 alleles, DQ with 50 to 323 alleles (HLA Nomenclature website, 2013). A study done on 1899 unrelated American-European individuals showed the sharing of the same DrB1-DQA1-DQB1 haplotypes in less than 1% of these individual [314]. This lower chance also translates into finding an unrelated donor which is stable to 40%, even if there are increasingly more available donors in the International registry [315]. Beatty demonstrated in 1995 that for some ethnic groups will never find a compatible 6/6 matched unrelated BM[316]. The diverse ethnic background of my 5 recipients out of the 7 transplanted (Table 18) is an example of this difficulty of finding an HLA compatible donor. This reduced chance of compatibility is also increased by the low presence of ethnic CBUs in the pooled CBUs reducing even more the probability of finding any HLA allele compatible within the pooled third party with the recipient and the matched unit. According to Lister (2007), this lack of relationship between all CBUs and the recipient do not determine a reduced chance for any pooled CBU to engraft, after obtaining a long-term engraftment in the presence of only one compatible locus (HLA B). Nonetheless, the lower compatibility and the very low dose of each pooled cell unit bring the possibility of engraftment to almost nil.

When transplanting dCBUs or haplo-related third party unit, the identification of the engrafted unit is provided by STR PCR measurement of relevant donor DNA in the recipient peripheral blood which most often correlates with the time of neutrophil engraftment. In my Phase 1 study, using CD3 cell as lymphoid lineage and CD33 cell as myeloid lineage cells I obtained a 100% ≥ 4/6 HLA matched CBU chimerism in 6 out of the 7 recipients at day +14 with a median engraftment time of 19.5 days. The only recipient obtaining less than 94% match CBU chimerism at day +14 and with 35.5% at day +21 never engrafted. This relationship was also demonstrated by Avery (2011) in determining the engrafting unit when transplanting double CBUs. The unit engrafting with a median neutrophil engraftment time at day +16 was determined when donor chimerism at day +14 post-transplantation where higher than 90% compared to an engraftment at day +24 when donor chimerism was lower than 90% [313]. This implies the presence of a mixed chimerism in at least 10% of recipients at day +14. The transplantation of more than one stem cell product increases the chance of long term mixed chimerism which may impact negatively on neutrophil and especially on platelets [243]. The transplantation of dCBUs or a third party unit demonstrates that one unit is predominant and most often the second unit or third party unit is transient [209,317]. Newell (2012) was also able to predict the unit that would eventually engraft by the one having the highest CD3

chimerism at day +7 [317]. My transplantation method demonstrated a stronger advantage by the presence of only the ≥ 4/6 HLA compatible unit at day +14 from a peripheral blood chimerism for all the recipients.

The hematopoietic mixed chimerism is defined as the presence of at least two different DNA sources in the same individual. In my study, the DNA sources originate from 31 to 46 different individuals in the same recipient. The precision of chimerism identification using the PCR STR assay is highly sensitive, in order to ensure identification of the engrafted product, 9 different primer pairs were used and STR length was determined for each cell source in all pooled units, for the matched CBU and for the recipient before the transplantation (Table 16). The presence of any of the pooled CBU was detectable at day +14 and in the following days post transplantation. To demonstrate the possible presence of any CBU from the pool, I compiled the number of CBUs with the same PCR fragment length and measured its proportion to the pooled CBUs. I observed that 33% to 73% of the units had the same PCR fragment length for a unique primer, increasing the chance of detection by PCR (Table 20). The probability of observing a PRC fragment length from DNA is also influence by the number of cells contained from each CBU in the pool. The proportion of $CD34^+$ cells infused in the recipients originating from all products was also verified for each recipient (Figure 19 to 25). Some of the CBUs representing less than 1% of all infused

products were not detected by PCR, since the method's lowest threshold of detection is 2%.

Table 20: Proportion of PCR Fragment Length per Pooled CBUs

Primer	Patient #1	Patient #2	Patient #3	Patient #4	Patient #5	Patient #6	Patient #7
1	36%	40%	38%	41%	48%	63%	53%
2	38%	33%	33%	38%	43%	33%	40%
3	49%	37%	50%	54%	50%	63%	67%
4	53%	60%	62%	62%	60%	63%	73%
5	49%	37%	50%	54%	50%	63%	67%
6	53%	60%	62%	62%	60%	63%	73%
7	38%	43%	45%	56%	52%	47%	50%
8	47%	47%	48%	46%	50%	53%	43%
9	38%	37%	38%	49%	38%	50%	67%
# CBUs pooled	**45**	**30**	**42**	**39**	**42**	**30**	**30**

The percentage was the number of CBUs containing the same PCR fragment length on the number of pooled CBUs.

The percentages of cells containing the same PRC fragment length were also evaluated to ensure a sufficient volume of detectable DNA. Using Day +14 and +21 chimerism of Patient #4, I identified 8 different polymorphic lengths on D3S2387 and of the 6 polymorphic lengths on STR locus D7S820 (Table 16 Patient #4). This patient received $CD34^+$ cells originating from a pool of 42 CBUs with the additional ≥ 4/6 HLA matched CBU (Figure 29). The proportion of the matched CBU versus all infused $CD34^+$ cells was 39% and the proportion of each CBU pooled varied between 0.3% and 4.4% (Figure 22). STR loci are

widely considered to be effective in identity applications due to their variability between individuals [318]. However, for a given set of STR loci the variability is much less polymorphic compared to HLA. I measured the proportion of all CBUs having the most PCR frequent length on D3S2387 (198 bp) and D7S820 (214 bp) and observed an increased ratio of $CD34^+$ cells from the pool versus the matched unit at 24% and 37% respectively (Figure 32 and 33). I concluded that even if the limit of detection for a cell population may vary from 2% to 5% [319,320], there was no trace of any of the pooled CBUs on day +14 and +21 in Patient #4 (Figure 30 and 31). Since this recipient was the only female transplanted with a female ≥ 4/6 HLA-compatible CBU, I was able to confirm the absence of any Y chromosomes on day +14 and day +21. This result can bring us to assume that none of the recipients had any pooled CBUs in the bone marrow or peripheral blood two weeks post-transplantation.

Most DNA chimerism studies post-transplantation are done using STR-PCR technique on DNA extracted from peripheral blood mononucleated cells at different times after transplantation [321,322]. The enrichment of the cell fraction into CD3 and CD33 for my STR-PCR assay not only increased the sensitivity of the test but also documented the presence of CD3 (T cell) and CD33 (neutrophil) on day +14 for all recipients. When transplanting dCBUs, the dominant unit with higher CD3 chimerism at day +7 predict its engraftment in most transplantations

[317]. Since I observed the presence of only the ≥ 4/6 HLA CBU on day +14 suggested a possible graft versus graft immune interaction and lysis of all other small units [323,324].

Despite the presence of a single CBU at day + 14, +21 and +28, patient #3 had an acute relapse and died at day +28. Graft rejection is also a severe complication after allogeneic transplantation, with a mortality rate of 50% [325]. Of my transplanted population, only one patient rejected the infused CBU. This recipient increased his neutrophil count up to 0.38 x 10^9/L on day +20 and had no infection or neutropenia problems in 45 days. The recipient was able to survive 71 days until being salvaged with the infusion of two ≥ 4/6 HLA matched CBUs and sustained an engraftment on day +35 of a single unit. Another known post-transplant complication in HSC transplantation is the acute graft versus host disease (aGVHD). It is less likely to happen after transplantation of mismatched cord blood than after mismatched bone marrow. However, the rate of aGVHD was higher among recipients receiving dCBUs and haplo-related third party versus recipient receiving a single unit or the co-transplantation of a CBU [205,215,247]. All 6 of my recipients who engrafted had aGVHD grade I-III. The T cells contained in the ≥ 4/6 HLA matched CBU may have eliminated the residual host T cells and their depletion in the pooled unit may have contributed in limiting aGVHD to a low grade and graft rejection [326-328]. I also observed that

all of the recipients were at high risk of relapse and for some of them a few leukemia cells were still present at admission. As for the transplantation of two cord blood units where both units potentially increases the graft-versus-leukemia (GVL) effect [329], on day +100, five of my patients were still alive with no signs of relapse, possibly due to a graft versus leukemia (GVL) effect.

Cytomegalovirus (CMV) is frequently responsible for morbidity and mortality post transplantation [330]. Brown (2010) demonstrated that naïve cord blood T cells transplanted to adult recipient could initiate immune responses to CMV as early as 8 weeks after transplantation. However, some patients are unable to clear the CMV infection. In the present study, all recipients had positive CMV serology before transplantation, and four experienced CMV reactivation, one recipient died on day +28 and another never engrafted. Only one patient developed long term CMV infection 5 months post transplantation (Figure 28).

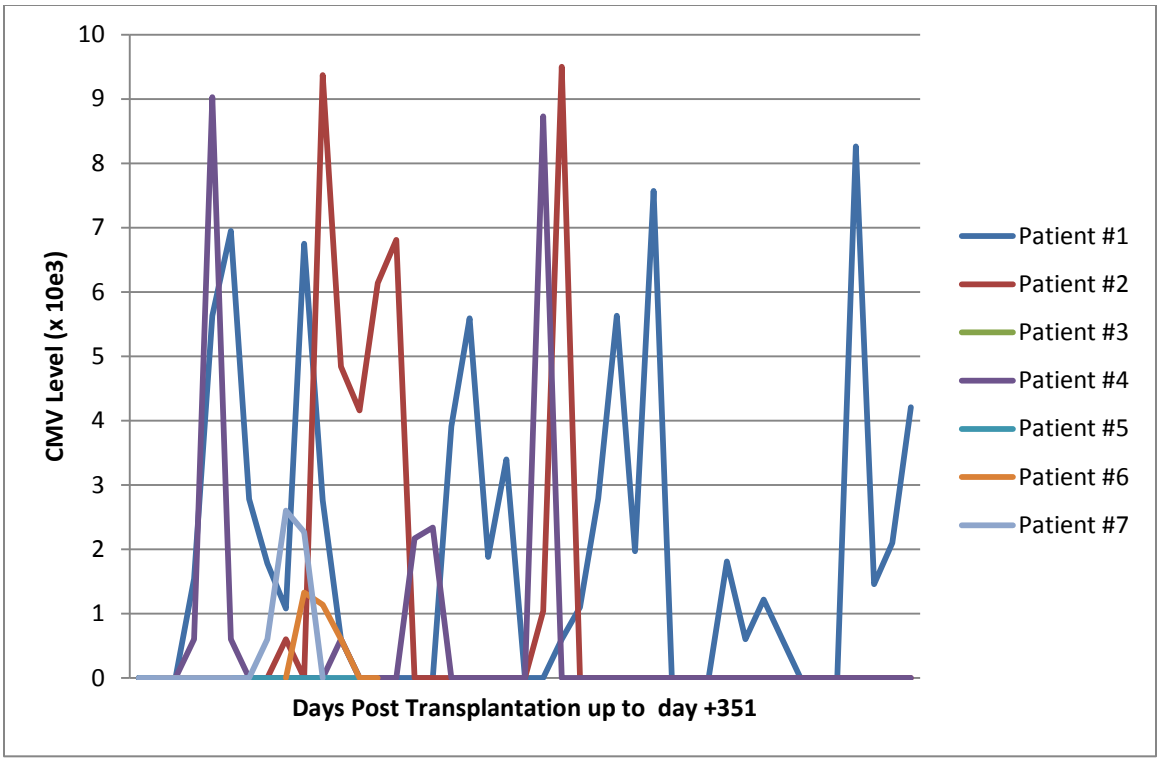


Figure 28: CMV Level Post-Transplantation

The overall survival on day +100 was 83% of all engrafted patients and 86% for all infused recipients. Even if these percentages are lower than dCBUs or haplo-related third party unit, it has been demonstrated that an advanced status of the leukemia has significant negative impact on overall survival [60]. My project included only patients with advanced disease, but nevertheless the outcomes demonstrated a positive avenue in offering such treatment to this population.

Mesenchymal stem cells (MSC) can also influence the process of engraftment. MSC do not express CD34 on their cell surface, thereby the selection of $CD34^{+}$ cells from the pooled CBUs ensure depletion of not only T,

NK and B cells but also of MSC. This depletion could reduce the chances of the pooled CD34+ cells to home by reducing cell-cell interaction [190,333,334]. Le Blanc (2007) demonstrated that the infusion of a product that is HLA compatible with the infusion of MSC enhance neutrophil engraftment and lead to a unique DNA chimerism [335]. Also, by thawing and directly infusing ≥ 4/6 HLA matched CBU containing MSC, I increased cell-cell interaction, thereby increasing the chances of homing into the bone marrow [123,334]. Homing of the first infused CBU may increase level of stromal-derived factor-1 (SDF-1) in the bone marrow, creating a stronger chemo-attractant for the infused CD34+ cells [336,337]. The use an infusion of MSC after the infusion of the matched and the pooled CBU would probably reduce neutrophil and platelet engraftment time. This will have to be conducted in a future independent research.

In a time of cuts and costs in the health care system, the decision to perform dCBUs transplantation, CD34+ selection on third party haplo-related transplantation or this new Phase 1 study is partially based on cost-effectiveness. Nonetheless only two studies clearly measure the cost-effectiveness between myeloablative conditioning unrelated CB and bone marrow/peripheral blood stem cell (BM/PBSC) transplantation. Costa (2007) estimated the cost of a CB transplantation to be 34 360$ US based on an overall survival of 27.9% the first year compare to 16 346$ US for BM/PBSC with a one year overall survival of

47% [331]. Another study document the cost per day survived post-transplantation with a cost of 1 016$ for matched unrelated bone marrow donor with a length of stay of less than 48 days and 2 082$ for CB transplantation with the highest hospital stay [332]. None of them clearly compared the cost-effectiveness between different types of CB transplantations. According to the outcomes observed in the pilot study, the engraftment time, the aGVHD and a median length of stay of 58 days are considered to be similar to dCBUs. However, dCBUs requires the purchase of two CBUs at a cost of 35 000$US for a total of 70 000$US. The CBUs pooled are normally discarded by the public bank after collection. The processing, freezing, serology, mycology and microbiology testing bring the cost of a single unit to 350$. The average number of units required for a pooled CBU transplantation is 40 which bring the cost of the purchase of those unit to 14 000$ to which a 10 000$ cost for the pooling and $CD34^+$ selection must be added. Even if the cost of the $CD34^+$ selected pooled CBUs cost is 24 000$, there is an economy of more than 11 000$. In addition to a cost are the maximisation of all donated CBUs, which may bring down the cost of a single CBU in the future, and the increased availability of stem cell transplantation to a larger transplant population having no limit in recipient' weight.

The safety of the procedure was demonstrated by the absence of any contamination, by the engraftment of the infused $CD34^+$ cells and by the absence

of any adverse events when transplanted into seven adults following a myeloablative conditioning. Other factors may be involved in the homing of the matched unit instead of the third party pooled CBUs, and unknown allo-reactions could impact on neutrophil and platelets recovery. This would require further research.

Figure 29: Baseline STR of Patient #4

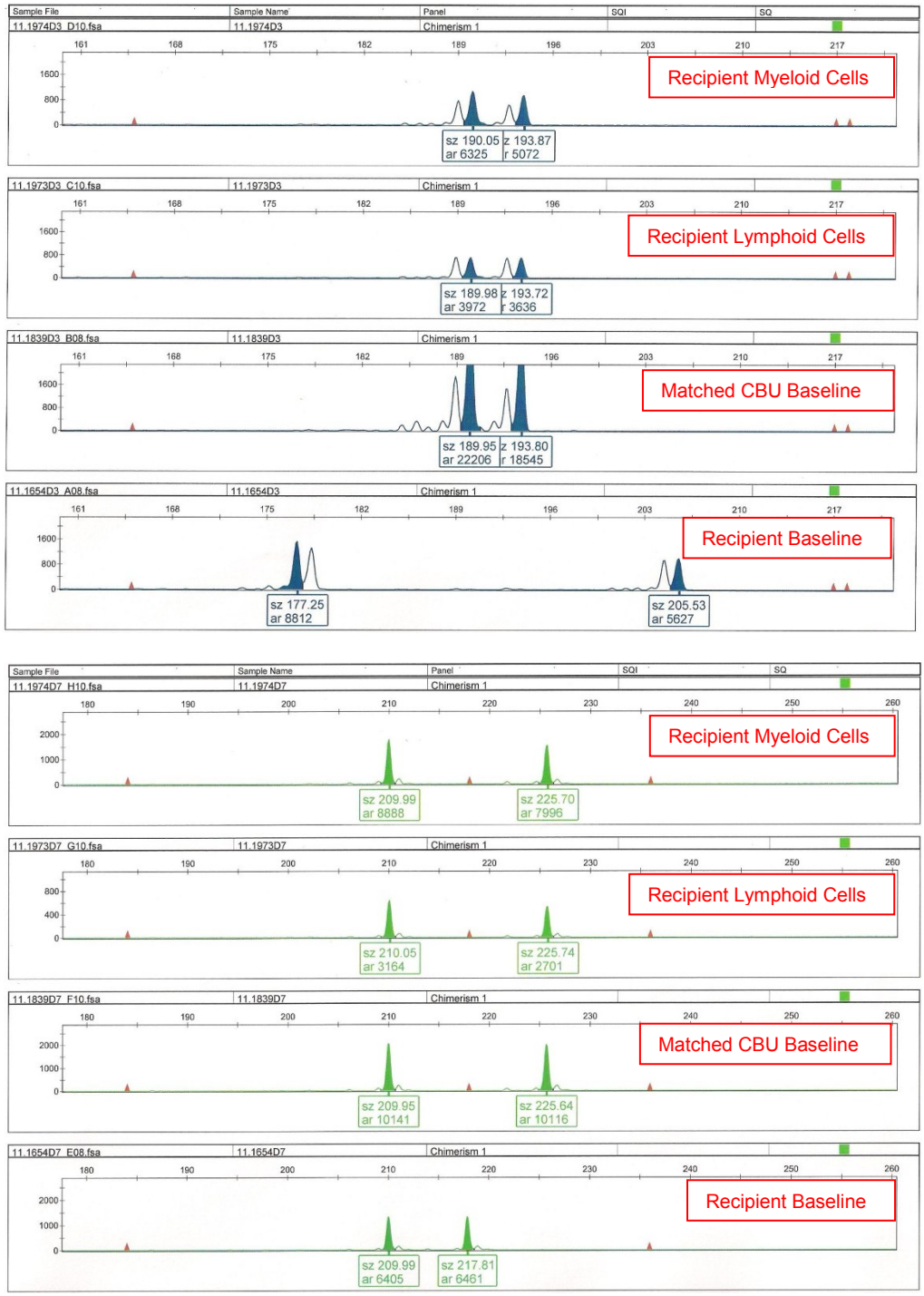


Figure 30: Day +14 Post Transplantation Chimerism of Patient #4

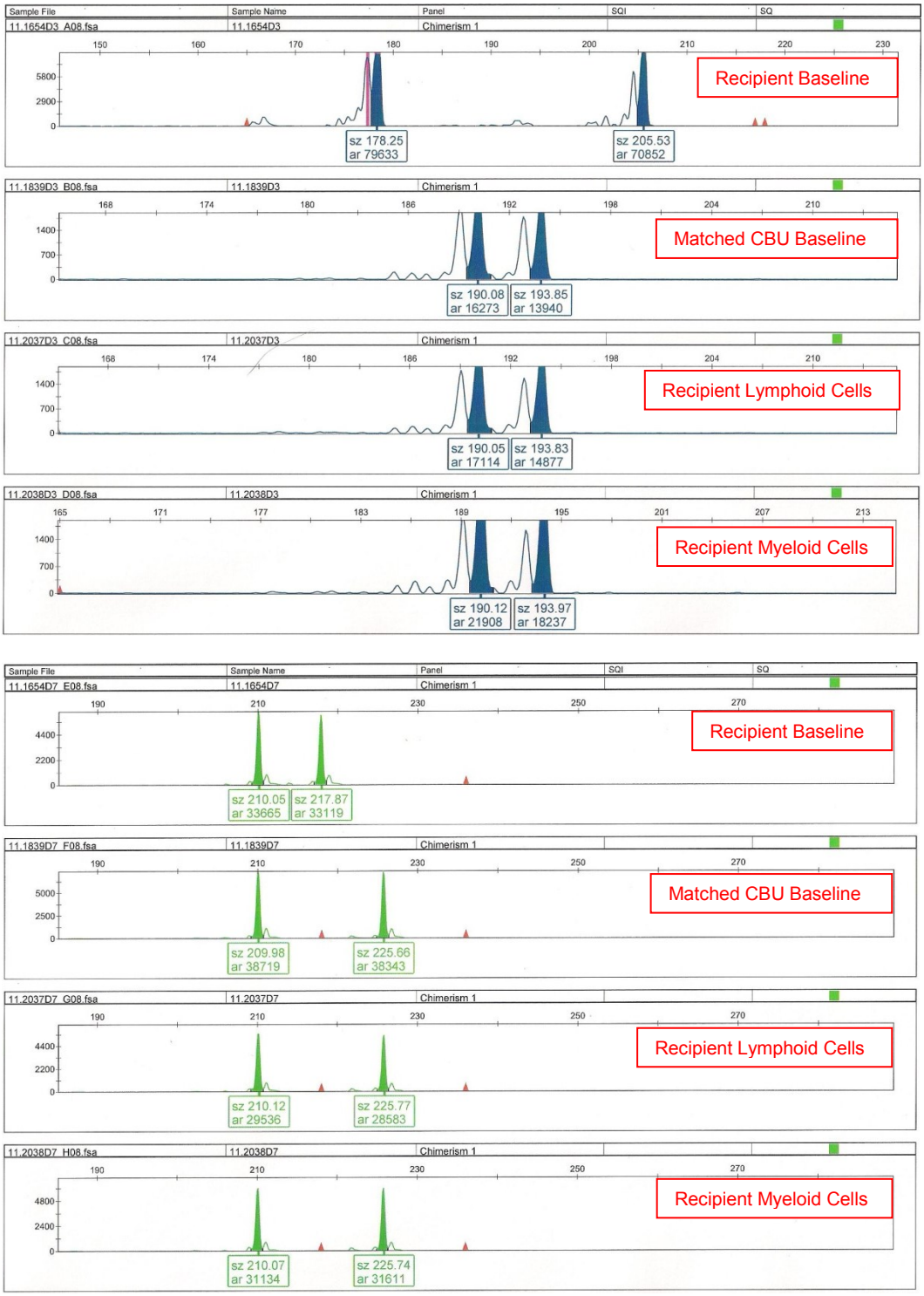


Figure 31: Day +21 Post Transplantation Chimerism of Patient #4

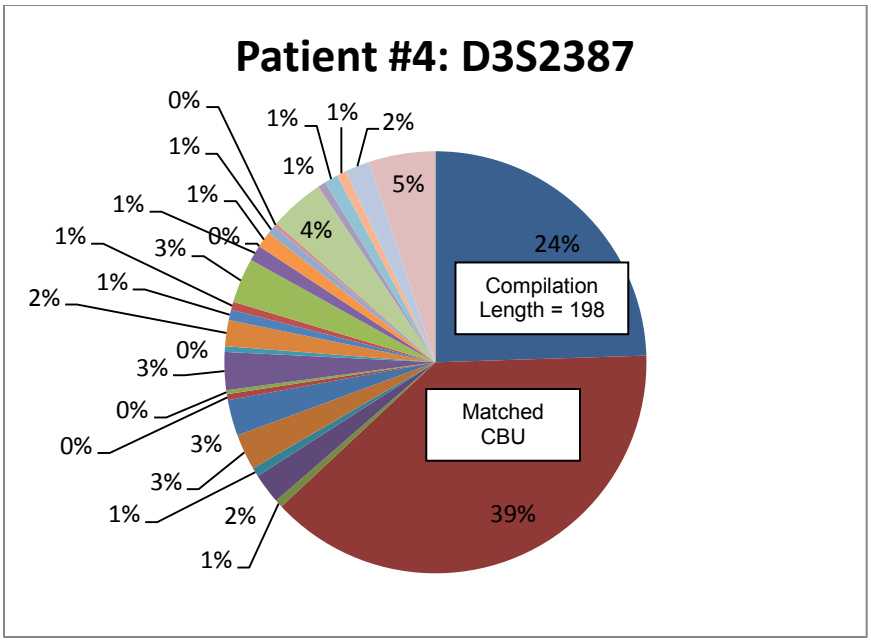


Figure 32: Percentage of each CBU after CD34⁺ Selection Based on STR-PCR compilation length on D3S2387

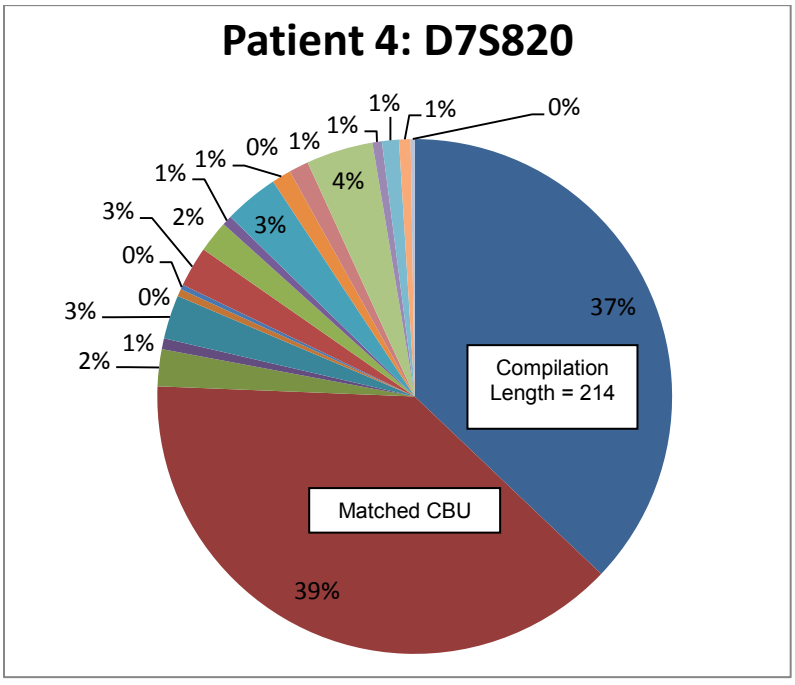


Figure 33: Percentage of each CBU after CD34⁺ Selection Based on STR length on D7S820

CONCLUSION

Cord blood transplantation provides an alternative source of hematopoietic stem cells for recipients requiring allogeneic stem cell transplantation. However, the majority of recipient who may benefit from this source is small-weight recipients. The main goal of this thesis was to develop a method that would allow for cord blood transplantation in larger-weight adult populations. For this purpose, a third party unit of enriched $CD34^+$ cells from cryopreserved cord blood units (CBUs), rejected by the public cord blood bank, was to be co-transplanted with a ≥ 4/6 HLA matched CBU in larger adult recipients. In order to proceed with this type of transplantation, I had to devise a method of pooling several cord bloods with adequate TNC counts. I demonstrated that pooling cryopreserved CBUs was feasible when respecting some criteria. Although Dimethyl sulfoxide (DMSO) is a necessary component for cryopreservation, it is toxic, and requires dilution to a ≤ 1% concentration to ensure adequate cell viability upon thawing. However, nucleated cell viability was stable only for a short time, which established the second criterion, namely, that the pooling technique should be completed within 2 hours.

Following many pooling assays, I observed binding of immunoglobulin-gamma (IgG) anti-A and anti-B to the pooled leukocytes. This observation occurred more frequently in the presence of blood group incompatibility between mother's cord

blood and pooled CBUs. The impact of such an observation on the recovery of pooled nucleated cells cannot be confirmed, but can be a source for future studies. Still, with respect to the possible impact on the recovery of nucleated cells, the third criterion is the blood group compatibility between mother's cord blood and cord blood.

The safety of this new product has been demonstrated by the transplantation of seven recipients without any serious unexpected adverse events. This new transplantation method obtained similar results as double CBU transplantation and co-transplantation with a haplo-related third party $CD34^+$ product. Surprisingly, the engraftment of the single ≥ 4/6 HLA matched CBU was not only observed by the DNA chimerism at day +14 but also by the absence of any of the pooled CBUs at the same time. With respect to the high risk status of the recipients, the overall survival at 100 days post-transplantation was 83% for all engrafted recipients and 86% for all transplanted recipients. The level of graft-versus-host-disease was similar to any cord blood transplantation method. More than a year after transplantation, three recipients survived and are living a normal active life.

These results demonstrate that increasing the number of HSCs by using third-party cells from pooled HLA-blind CBUs to support a ≥ 4/6 HLA compatible CBU is safe, feasible, and results in rapid engraftment. The advantage of this

approach is that it may be possible to obtain rapid neutrophil engraftment without having to find more than one compatible CBU, while salvaging otherwise discarded CBUs.

My research study provided hematopoietic stem cell transplantation to a large adult population; however an increased number of transplantations would result in significant long term outcomes such as relapse risk, treatment-related mortality and overall survival. Future studies related to this new transplant methodology are warranted. By first infusing the third party composed of enriched $CD34^+$ cells, it will be possible to better understand its role. This leads to new questions about factors involved in the homing of stem cells and their niching in the bone marrow. Efforts should also be made to further simplify the pooling method and ensure an easier access to other transplant centers. Recently, discussions on tightening the cut-off in CBU qualification increases the chances of bigger CBUs containing a larger amount of $CD34^+$ cells being discarded. Public banks are concerned about the impact of cost increases if nucleated cell number requirements and volume cut-offs are increased. My research offers a solution that would not only maximise the use of all collected cord blood units, but also provides a solution for possible quality cut-off changes in public banking.

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