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Flow cytometric analysis of stem cells derived from umbilical cord blood

By

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SUMMARY

Successful engraftment is highly dependent on the quality and quantity of stem cells and nucleated cells in cord blood. Storage of umbilical cord blood is expensive and it will be very useful if factors that influence cell count and viability could be identified to aid in the decision to process and store cord blood collections for the ultimate aim of successful engraftment. This study determined the standards for laboratory parameters of haematopoietic potential, such as collection volume, post processing volume, CD34+/45+ cell counts and viability of stem cells and leukocytes and cell ratios for the cord blood bank. In this research we determined whether maternal age and infant gender have an effect on laboratory parameters. We studied the effect of 4°C and room temperature (RT) as well as the period of storage on the same laboratory parameters. The quality and recovery of stem cells and leukocytes after laboratory manipulation was determined**.** Established standards for leukocyte count, stem cell count and collection volume compare well with other international UCB banks. Maternal age and infant gender have no influence on laboratory parameters and could therefore not be used as a measure of cell quantity and quality prior to processing. Cell count and cell viability of stem cells is not significantly influenced by 4°C or RT temperature or 24h and 48h storage. Leukocyte cell count and viability is not affected by storage temperature, but increased storage periods showed high levels of leukocyte cell count deterioration and decreased leukocyte cell viability.

Processing of UCB causes significant cell loss in stem cells and leukocytes. Processing or no processing of UCB has showed no affect on the viability of stem cells stored at different storage periods and temperature. Temperature has no affect on leukocyte cell counts and viability of either processed or unprocessed leukocytes but increased storage periods dramatically decrease leukocyte count and viability. The information generated by this study will assist in the process of optimizing the storage of UCB.

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LIST OF ABBREVIATIONS AND SYMBOLS

7-AAD	7-Amino-Actinomycin D
ADCC	Antibody dependent cell cytotoxicity
AGM	Aorta-gonodal-mesonephros
B cells	Bone marrow produced cells
BFU	Burst forming unit
BFU-E	Erythroid burst forming unit
Bmi1	BMI1 polycomb ring finger oncogene
BMSC	Bone marrow stromal cells
°C	Degrees centigrade
CAL	Calibration
CDKI's	Cyclin dependent kinase inhibitors
CFC-B	Bone marrow colony forming cells
CFC-T	Thymus colony forming cells
CFU	Colony forming units
CFU-GM	Granulocyteactophage progenitor cell
CFU-MEG	Megakaryocyte progenitors
CI	Confidence interval
C-Kit ligand	CD117 cytokine receptor
CPDA-1	Citrate phosphate dextrose adenine
CSCs	Cancer stem cells
CSFs	Colony stimulating factors
CXP	Cytomics extreme programming
dH_2O	Distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

E7.5	Embryonic day 7.5
EDTA	Ethylenediaminetetraacetic acid
EG	Endoglycan
EGF	Epidermal growth factor
EPC	Endothelial progenitor cells
ES	Embryonic stem cell
FC	Flow cytometer
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocuanate
FL	Fluorescence
FS	Forward scatter
GATA	Cell growth transcription factor
G-CSF	Growth stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GvHD	Graft versus host
GvL	Graft versus leukemia
h	hour
hASC	Human adipose tissue-derived stromal cells
HES	Hydroxethyl starch
hfMSC	Human fetal mesenchymal stem cells
HLA	Human leukocyte antigen
HLA-DR	Human leukocyte antigen DR
HLDA	Human leukocyte differentiation antigen
HPCs	Haematopoietic progenitor cells
HSCs	Haematopoietic stem cells
hUCMSCs	Human umbilical cord mesenchymal stem cells
IL-8	Interleukin - 8

ISHAGE	International society of haematherapy and graft engineering
Jagged1	Member of the delta/serrate/lag-2 (DSL) family
kD	kiloDalton
KO	Knockout
LCA	Leukocyte common antigen
Ln	Natural logarithm
Log	Logarithm
µl	Microliter
MCL	Multi-carousel loader
MIP-1α	Macrophage inflammatory protein 1α
MMP-9	Metalloproteinases-9
MNC	Mononuclear cells
MSCs	Mesenchymal stem cells
MVE TEC	Mid valley engineering thermal electronic controller
NH_4Cl	Ammonium chloride
NK	Natural killer
NMDP	National marrow donor program
Notch	*Drosophila melanogaster* with notches apparent in their wingblades.
NSCs	Neural stem cells
PCLP	Podocalyxin-like protein
PCR	Polynucleic acid chain reaction
PE	Phycoerythrin
QC	Quality Control

QS	Quadstat regions
%	Percentage
RCPA	Royal College of Pathologist of Australia
RNA	Ribonucleic acid
RT	Room temperature
SCF-C	Stem cell factor - C
SD	Standard deviation
SDF-1	Stromal derived factor 1
Shh	Sonic hedgehog
SOP	Standard operating procedure
Sox2	Sry-related HMG box 2
SS	Side scatter
STATA	Statistical analysis
T cells	Thymus cells
TGF alpha	Transforming growth factor
Thy-1	Thymocyte-1
TLX	Human homologue of the Drosophila **(tl)** tailless gene
TNC	Total nucleated cell count
UCB	Umbilical cord blood
USA	United States of America
Wnt	Wn (wingless) and Int (Internal)

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

For over a century, stem cells have fascinated both scientists and clinicians. The origin of the term “stem cell” can be traced back to the late 19th century. The term stem cell was used to describe cells committed to give rise to the germline while others used it to describe a proposed progenitor of the blood system (Ramalho-Santos and Willenbring, 2007). In 1961, stem cells were first identified by Ernest McCulloch and James Till in the haematopoietic system in experiments showing that single cells that derived from mouse bone marrow could proliferate and give rise to multiple differentiated types of blood cells (Till et al., 1961).

Most fully differentiated cells in adult organisms are no longer capable of cell division but they can be replaced by the proliferation of a subpopulation of less differentiated self renewing cells called stem cells. These stem cells persist throughout life in diverse tissues and therefore play a critical role in the maintenance of most tissues and organs.

1.2 MORPHOLOGY AND CHARACTERISTICS OF STEM CELLS

Morphologically stem cells resemble small mature lymphocytes and normally a large fraction of stem cells are quiescent. The main characteristic properties of stem cells are:

- There is no limitation on cell division.
- With each division the daughter cell could either remain a stem cell or commit to terminal differentiation.
- The remaining stem cell itself is not terminally differentiated.

Stem cells have the ability to differentiate into any tissue type (pluripotentiality) and have self-renewal capacity (the ability to divide indefinitely, without cell differentiation), while progenitors cells (descendents from stem cells) have limited multipotentiality and a lesser degree of self renewal capacity (Serafini and Verfaillie, 2006).

1.3 STEM CELLS TYPES

There are multiple types of stem cells, each defined by their differential potential:

- *Totipotent* stem cells contain all the genetic information to generate any type of body cell including extra-embryonic membranes such as the placenta to construct a complete viable organism for example the cells produced by the fusion of a sperm and egg cell.
- *Pluripotent* stem cells generate any body cell, except cells of the extra embryonic membranes. These cells could differentiate into all three germ layers: ectoderm, mesoderm and endoderm cells and include embryonic stem cells, embryonic germ cells and embryonic carcinoma cells. Under specialized conditions that prevent differentiation pluripotent cells could be maintained indefinitely e.g. embryonic stem cells (Simard and Rivest, 2004).
- *Multipotent* stem cells occur in haematopoietic stem cells, neural stem cells, mesenchymal stem cells and are antecedents of specialized cells in particular tissues that are permanently committed to a specific function. They replace dead or damaged tissue for example haematopoietic stem cells that could differentiate into several different types of blood cells but cannot develop into other tissues like brain cells.
- *Unipotent stem cells* can only differentiate along one lineage to produce one cell type e.g: erythroid progenitor cells only differentiate into red blood cells (De Kretser, 2007).

1.4 STEM CELL DIVISION

Stem cell division is required where there is a recurring need to generate and replace terminally differentiated cells that are incapable of cell division in a great variety of tissues like blood, skin, muscles, intestine and nerve cells. Fifty percent of the daughter stem cells from each generation must remain stem cells in order to maintain a constant stem cell population (Potten and Loeffler, 1990). Stem cells therefore divide at a relatively slow rate. Stem cell division is accomplished by:

- Environmental asymmetry division – the daughter stem cells are initially similar but environmental influences redirect the cells into different pathways. The stem cell number could be reduced or increased according to the niche demand.
- Divisional asymmetry division – the stem cell has an internal asymmetry and at division results in a daughter cell with programmed stem cell characteristics and the other daughter cell with differentiation properties (Ho and Wagner, 2007).

1.5 MOLECULAR REGULATION OF STEM CELLS

Haematopoietic stem cell (HSC) production and proliferation kinetics is controlled and regulated by intrinsic and extrinsic factors. Studies on genetic loss of function and gain of function were used to determine the molecular nature of stem cell regulatory pathways (intrinsic factors). Signal integration depends on large signaling complexes and spatial proximity of molecules to facilitate interaction (extrinsic factors). When stem cells divide, they can generate progeny with the same developmental potential as the original cell, a process referred to as self-renewal. Self-renewal is driven intrinsically by gene expression, transcription factors, epigenetic control, and microRNA in a cell-type-specific manner (Cheng et al., 2005). This process is modulated through interactions with extrinsic cues such as growth factors from the microenvironment in which stem cells reside.

1.5.1 Cell intrinsic regulators

Single cell reverse transcriptase PCR has indicated the presence of elevated levels of cyclin dependent kinase inhibitors (CDKI's) in HSC cells, that function as intrinsic regulators by inhibition of the HSC cycle (Bertet and Kaldis, 2007). The low metabolic activity and the quiescent nature of HSC cells were confirmed by staining HSC cells with DNA and RNA nucleic acid dyes (Shen et al., 2008). As expected, the cells showed low staining capacity.

Haematopoiesis (blood formation) is a complex process regulated by nuclear proteins like transcription factors that coordinate lineage differentiation of stem cells. Targeted mutagenesis of these genes has revealed critical roles for the X-linked transcription factor GATA-1 in haematopoiesis. Mutations in GATA-1 result in abnormal erythrocyte and megakaryocyte differentiation (Del Vecchio et al., 2007). Other cell-intrinsic regulators include orphan nuclear receptor (TLX), polycomb transcriptional repressor Bmi1, high-mobility-group DNA binding protein Sox2, basic helix-loop-helix Hes genes, histone modifying enzymes and chromatin remodeling proteins (Shi et al., 2008).

De Haan et al. (2002) have reported that the expression levels of a large number of genes may be responsible for controlling stem cell behavior. Results obtained form murine experiments on inbred mouse strains, where stem cells vary widely in number and activity have led to the hypothesis that these genes may be analogous to those genes responsible for the inter-individual behavior of human haematopoietic stem cells.

1.5.2 Cell extrinsic regulators

Extrinsic regulation involves control of self renewal and differentiation by external factors like cell to cell interaction. The extrinsic state of stem cells depends on their spatial and temporal history and affects their responsiveness to extrinsic signals from the microenvironment. Cell-extrinsic signaling molecules, such as Wnt, Notch, Sonic hedgehog (Shh), TGFalpha, EGF, and FGF are involved in mechanisms that allow stem cells and specific neural stem cells to renew themselves (Shi et al., 2008).

Akala and Clarke (2005) discovered that the Polycomb protein Bmi1 is responsible for the maintenance of both adult HSCs and neural stem cells. Studies on murine and human embryonic stem cells indicated that Polycomb group proteins play a dynamic role in collaboration with transcriptional regulators in actively maintaining

stem cells in an undifferentiated state. This report suggests that this mechanism might apply to multiple types of stem cell regulation and maintenance systems.

1.6 STEM CELL MICROENVIRONMENT

The development of an in-vitro microenvironment by using human mesenchymal stromal cells has provided a “controlled laboratory environment” in which the relative significance of chemokines and adhesion molecules on stem cells have been studied. The identification of the molecular interactions between stem cells and their niche has led to an understanding of the mechanisms that control the self-renewal and pluripotency of stem cells (Can, 2007).

The haematopoietic system is defined as a specialized, tissue niche localized in bone-marrow colonized by migrating stem cells. Several groups of cells like mesodermal cells, adipocytes, fibroblastic cells and endothelial cells as well as extracellular matrix elements interact with HSCs to promote or inhibit their self renewal, differentiation and migration (Sujata and Chaudhurithes, 2007). The microenvironment is also regulated by intrinsic and extrinsic factors.

1.7 STEM CELL TRAFFICKING

1.7.1 Migration

Stem cell migration requires multifactorial processes that involve cytokines, adhesion molecules and matrix degrading enzymes such as metalloproteinases (Allport et al., 2002). In vitro experiments performed by Charbord (2001) concluded that stem cell movement across the endothelium is induced by stromal cell derived chemokine gradients across an endothelial barrier. Chemokines are cytokines with direct chemotactic (cell movement) effects on receptor expressing target cells. Stromal derived factor 1 (SDF-1) produced by bone marrow stromal cells is the only chemokine that acts on haematopoietic stem and progenitor cells and enhances migration of these cells. Bone marrow CD34+ HSC cells migrate more avidly in response to SDF-1 than mobilized progenitors (Marquez-Curtis et al., 2008).

The ability of stem cells to traffic around the body to suitable sites for haematopoiesis is demonstrated by:

- Homing/engraftment i.e. the transendothelial migration of stem cells from the bloodstream into the marrow microenvironment. Homing is recognized by the ability of stem cells to reside at the microenvironment (retention) and to engraft i.e. the ability of cells to divide and form functional progeny in the given microenvironment (Chavakis et al., 2008).
- Mobilization involves the release of stem cells from the marrow microenvironment through transendothelial migration into the bloodstream (Mohle et al., 1999).

1.7.2 Engraftment

Experiments on transplants have demonstrated two phases of bone marrow engraftment (Jones et al., 1989):

- Initial but transient engraftment produced by committed progenitor cells.
- Delayed but long term haematopoietic reconstitution as a result of HSCs.

Infused HSCs first locate in the microvascular system of the lung and liver. They then pass through marrow sinusoids, migrating through extracellular bone marrow spaces into stem cell niches of the bone marrow (Fukuda and Pelus, 2008). Passage through endothelial barriers requires tethering through endothelium expressed addressins that bind haematopoietic cell selectins followed by attachment mediated by integrins (Liu et al., 2003). Selectins are receptors expressed on haematopoietic cells and endothelium. Ligands for selectins are present on endothelium and are termed addressins. Tethering by selectins allows integrin mediated adhesion to the endothelium. Integrins allows cell extracellular matrix and cell-cell adhesion to provide firm attachment and migration of HSCs through the endothelium and bone marrow extracellular space (Mazo et al., 1998). Successful engraftment and the rate of stem cell migration depends on changes in the strength of cell-ligand interactions formed by the number of receptors and their

affinity state and the strength of the adhesion receptor interactions (Prosper and Verfaillie et al., 2001).

1.7.3 Mobilization

Mobilization of progenitors is initiated by the down regulation of adhesion molecules (e.g. integrins) that results in a decrease in avidity to bone marrow stromal and endothelial cells, which express the corresponding ligands (Mohle et al., 1999). In the extravascular spaces of the bone marrow, stromal cells provide potential sites for recognition by stem cell surface markers including cell surface and extracellular matrix ligands and adhesion molecules (Lee et al., 2004).

Extracellular signals from haematopoietic growth factors are mediated via cell surface haematopoietic growth factor receptors. Most growth factors are members of the Type I cytokine receptor family. Cytokines are very important in stem cell release from the marrow environment and are administered to mobilize stem cells into the peripheral blood (Lonial, 2004). Examples of cytokines are growth stimulating factors (G-CSF), GM-CSF and chemokines like IL-8, MIP-1α (Levesque and Winkler, 2008). There are several mechanisms by which G-CSF mobilizes HSCs from the bone marrow, for example the activation of neutrophils causes the release of neutrophil elastases capable of cleaving CXCR4 on HSCs, thereby reducing HSC-bone marrow interaction (Gieryng and Bogunia-Kubi, 2007). Another mobilization strategy with G-CSF is by CD26 present on primitive HSCs that is able to cleave SDF-1 to an inactive form causing mobilization of the stem cell (Campbell and Broxmeyer, 2008).

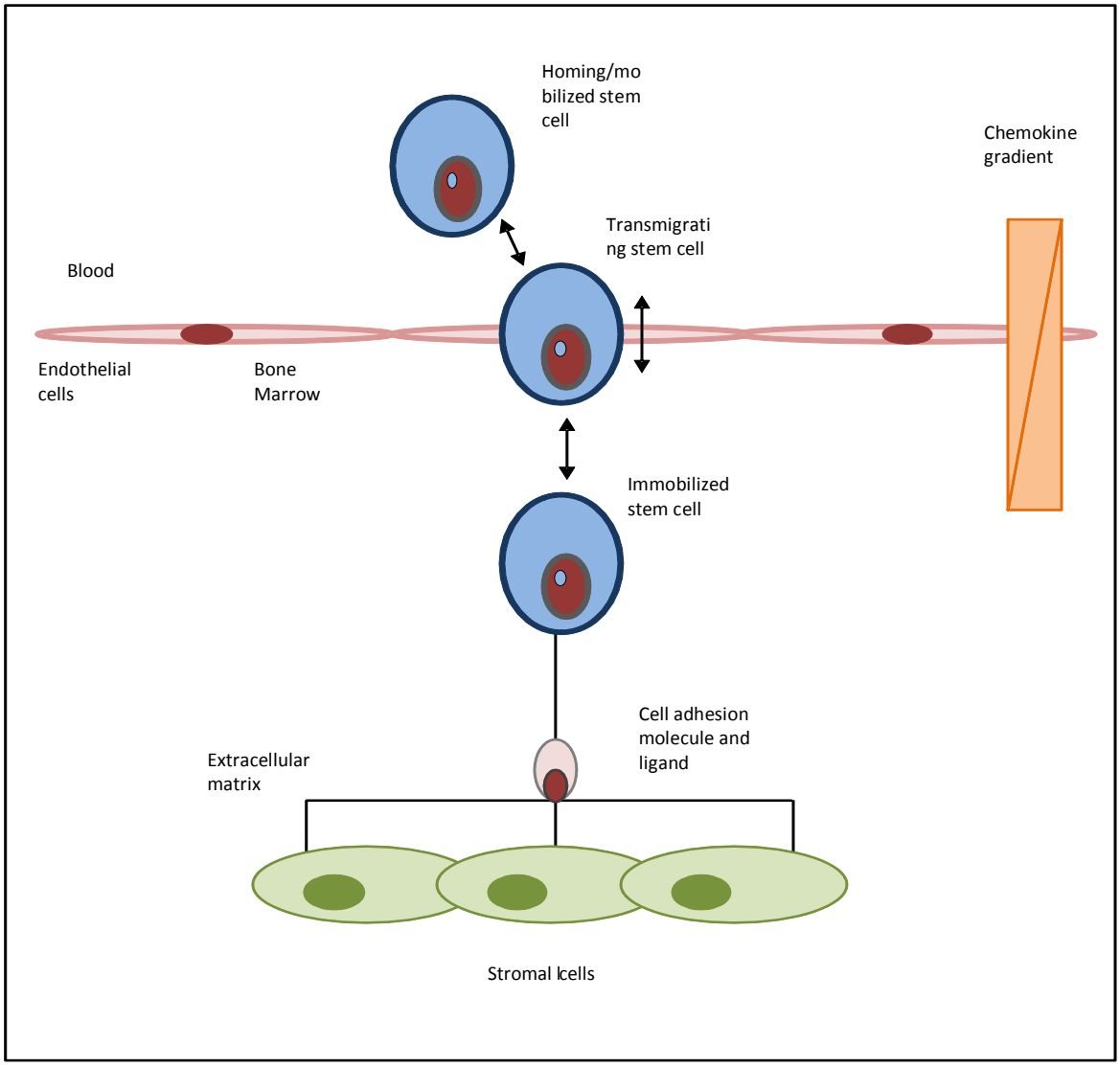

Figure 1: Mobilization and homing of stem cells under the influence of adhesion molecules, ligands and chemokines. (Drawn by D Thompson).

1.8 STEM CELL PLASTICITY AND TRANS-DIFFERENTIATION

It is also possible that stem cells from one tissue might be able to differentiate into cells giving rise to other tissues, a phenomenon called developmental plasticity (Herzog et al., 2003). It was reported that haematopoietic stem cells derived from bone marrow cells gave rise to non-haematopoietic tissue such as epithelial cells of the skin and lung as well as neural cells (Wagers and Weissman, 2004). The possibility that HSC can undergo trans-differentiation or fusion of HSC and somatic cells, may explain this phenomenon but this remains highly controversial. An

alternative explanation is that mesenchymal stem cell (MSCs) reside within the bone marrow and have the ability to produce bone, muscle or adipose cells upon proper stimulation (Clarke and Frisen, 2001).

Research by Hsieh and Gage (2005) indicated that plasticity is determined by both extrinsic and intrinsic factors such as chromatin remodeling and epigenetic gene regulation that regulate gene complexes to control cell destiny and function during plasticity. The therapeutic applications of haematopoietic stem cell plasticity would possibly provide an accessible source of cells that could be redirected to the repair of various damaged tissues and tissue replacement therapies.

1.9 CD34+ HSC ANTIGEN

CD34+ HSC antigen is part of the differentially glycosylated Type 1 transmembrane single chain sialomucin family of glycoproteins (Simmons et al., 1992). CD34 epitopes are classified into 3 classes based on enzyme sensitivity (Sutherland et al., 1996 and Gratama et al., 1999). The CD34 monoclonal antibody 581 is used in Stem-Kit reagents and specifically recognizes the Class III epitope of the CD34 molecule as determined by enzymatic cleavage patterns (Greaves et al., 1995 and Sutherland et al., 1992). The 581 monoclonal antibody was assigned to CD34 during the 5th HLDA Workshop on Human Leukocyte differentiation Antigens (HLDA) in Boston U.S.A. in 1993.

Non haematopoietic cells like, fribroblasts and endothelial progenitors are also CD34+ of which some like mesenchymal cells circulate in the umbilical cord blood. Early progenitor cells of the immune system are also CD34+. The immunophenotype of haematopoietic stem cells is therefore best characterized by the markers expressed by stem cells: CD34+,CD38- Thy-1 (Terrace et al., 2007), c-Kit (Ozer et al., 2008) and markers that are absent on haematopoietic stem cells: CD33 (Knaän-Shanzer et al., 2008), CD38 (Majeti et al., 2007). Lineage markers and HLA-DR CD34 antigen is expressed on all haematopoietic precursor cells including multipotent stem cells. For almost 30 years the cell-surface protein,

CD34 antigen, has been regarded as a marker in the isolation, identification and enumeration of haematopoietic stem cell populations and progenitors. CD34+ HSC antigen has increasingly been used as a marker for the identification of other tissue-specific stem cells, including thyroid cells and pancreatic precursors (Fierabracci et al., 2008 and Puglisi et al., 2008). Vast amounts of stem cells and progenitor cells may or may not express CD34+ HSC antigen but CD34+ HSC expression has been shown to distinguish activated progenitors from quiescent cells. Recently CD133 marker has been shown to be expressed on primitive human haematopoietic cells (Giebel et al., 2006).

Like CD34+ HSC, podocalyxin, and endoglycan (EG) are also members of the single-pass transmembrane protein sialomucin family that show distinct expression on early haematopoietic precursors and vascular-associated tissue (Furness and McNagny, 2006). Podocalyxin and endoglycan might contribute to progenitor function and behavior (Kerr et al., 2008). Expression of podocalyxin-like protein (PCLP) decreases during differentiation of haematopoietic cells. Endoglycan is present on human B cells, T cells and peripheral blood monocytes. Upon activation of B cells, EG is increased with a concurrent decrease in PCLP (Kerosuo et al., 2004).

The CD34+ HSC antigen is subjected to a range of tissue-specific post-transcriptional and post-translational modifications and this might alter the function of the antigen. To date, the complete function of CD34 remains elusive (Nielsen and McNagny, 2008).

1.10 CD45+ ANTIGEN

The CD45+ antigen is also known as the leukocyte common antigen (LCA). The CD45 molecule is expressed on the surface of all human leucocytes (Serra-Pages et al., 1995). The CD45 molecule comprises of four different isoforms generated by alternative splicing of three exons, with molecular weights of 180, 190, 210 and 220 kD (Newman et al., 1984 and Fabre et al., 1977). The CD45 antigen is

expressed on every type of haematopoietic cell except mature erythrocytes and immediate progenitor cells to this lineage (Coffman et al., 1981 and Dalchau, et al., 1981). The CD45 clone used in the Stem-Kit reagents is known as J33. It binds to all the CD45 isoforms present on human leucocytes (Cobbold et al., 1987) and was assigned to CD45 during the 3rd HLDA workshop in Oxford, England in 1986. CD45 expression on bone marrow leukocytes is very important in cell motility as CD45 regulates progenitor movement and retention by influencing both the haematopoietic and non-haematopoietic compartments. Experiments on CD45 knockout (KO) cells showed defective motility and reduced homing of haematopoietic progenitor cells (Shivtiel et al., 2008).

1.11 SOURCES OF STEM CELLS

There are two main sources of mammalian stem cells: embryonic stem cells found in blastocysts, and adult stem cells found in adult tissues. In a developing embryo, stem cells can differentiate into all of the specialized embryonic tissues. In adult organisms, stem cells and progenitor cells act as a repair system for the body, replenishing specialized cells, but also maintain the normal turnover of regenerative organs, such as blood, skin or intestinal tissues.

1.12 EMBRYONIC STEM CELLS

1.12.1 Characteristics of embryonic stem cells

Embryonic stem (ES) cells are characterized by the fact that the cells are isolated from blastocysts which are early embryos and have the ability to diversify into an array of cell types in culture. The blastocyst consists of an outer layer, the trophoectoderm, the inner layer and the inner cell mass with a spacious cavity, the blastocoel, which consists of (ES) cells that eventually form the fetus (Foulk, 2001).

In 1999 James Thomson and colleagues at the University of Wisconsin were the first to isolate human ES cells and grow them for extended periods in cell culture (Thomson et al., 1999). ES cells can proliferate indefinitely in culture and yet retain an unrestricted developmental potential. ES cells have the potential to be used for

treatment of a large number of diseases but to date no successes in humans have been reported. Culturing of ES cells may cause the development of malignant teratocarcinomas but this depends on embryonic stage, species-specificity and immunological competence of the host (Bulić-Jakus et al., 2006). Following implantation of the ES, the cells will differentiate and lose the multipotentiality.

1.12.2 ES cells and ethical issues

ES cell research is an area that has given rise to much debate internationally, within science, law and politics as well as within philosophy and ethics. The controversy on when life begins influences the debate on ES cells for research and application. Whether life begins at the moment of fertilisation, at organ development, at the moment of perceived consciousness or from the moment the foetus is able to survive outside the uterus is a personal and religious opinion. The use of ES cells could therefore be claimed to destroy life. As a result of this opinion, U.S. government funding for research using ES cells was banned, until 2009.

1.12.3 ES cell research

The culturing of human ES cells renders possible research that was previously only available in animal models. ES cells could be used for studies on self-renewal, stem cell commitment, differentiation, maturation and cell-cell interaction. There is currently considerable hope that studies on ES cells will lead to new therapies (Borge and Evers, 2003). The ultimate goal for stem cell research is the ability to differentiate ES cells into desired cells that could be used for transplants. Only differentiated ES cells could be used for transplants as undifferentiated cells ES cells have a high potential of becoming malignant (Yang et al., 2008)..

1.12.3.1 Tissue and organ engineering

There is a great future for tissue and organ engineering by using ES cells. It was found that ES cells in culture differentiate into nerve cells after addition of retinoic acid (Wichterle and Peljto, 2008). At Harvard University, organs such as a bladder

have been engineered in a laboratory. A scaffold of polymers was formed and seeded with muscle cells and placed in a culture medium where cell proliferation transformed the “scaffold” into in an organ with similar properties to a bladder. These bladders were successfully transplanted into recipient animals (Atala et al., 1993).

1.12.3.2 Therapeutic cloning

ES cells could be used for therapeutic cloning by modifying the cells to consist of the same genetic makeup as the recipient who is being treated. The nucleus of an unfertilized egg of an unrelated donor is removed and replaced by the nucleus of a somatic cell of the donor. The egg will be allowed to develop to embryonic stage and the ES cells removed, cultured and induced to produce the required cells for the recipient followed by transplantation (Hwang et al., 2005).

1.12.3.3 Gene therapy

Gene therapy consists of the isolation of ES cells from blastocytes and culturing in vitro to grow and proliferate. This is followed by transfection of the ES cells with a DNA fragment containing a non functional mutant allele of the gene to be knocked out as well as antibiotic resistance genes to be used for selection of transformed cells. A process of homologous recombination occurs in which transfected DNA replaces the homologous DNA sequence. ES cells are heterozygous for the gene and are selected by antibiotic resistance. These cells are injected into the blastocoel of a recipient embryo and allowed to grow to birth (Saric et al., 2008). Possible future use of gene therapy treatment includes glioblastomas and sarcomas (Germano et al., 2008), heart failure (Yamada et al., 2008), diabetes and liver failure (Jiang et al., 2008).

1.13 ADULT STEM CELLS

Adult stem cells have been identified in many tissues including the brain, retina, muscle, heart, lung, kidney, liver and pancreas. It is likely that most tissues contain stem cells. Adult stem cells differ from ES cells in that adult stem cells are less plastic than ES cells in that adult stem cells are tissue specific and their

differentiation potential is restricted to the cells of the tissue in which they reside (Graf et al., 2008). To date the different types of adult stem cells which have been isolated are: haematopoietic, muscle, neural, skin, endothelial, intestinal and mesenchymal stem cells.

1.13.1 Skin and hair stem cells

Stem cells responsible for the renewal of skin and hair are well characterized (Terskikh, 2005). These cells are exposed to harsh environmental conditions and are continually renewed by division of stem cells to give rise to a population of transit amplifying cells i.e. daughter cells committed to differentiation following a limited number of rapid cycles of division to terminal differentiation. This process will replace the damaged cells (Kamstrup et al., 2008).

Epidermal stem cells are clinically used in skin grafts taken from undamaged skin on a badly burned patient. These cells are rapidly grown in large numbers in culture and grafted back to reconstruct an epidermis to cover the burns. As this is an autologous procedure, complication of graft rejection by the immune system is eliminated (Schulz et al., 2000).

1.13.2 Stem cells of the intestine

The intestine is lined by a single layer of epithelial cells responsible for digestion of food and absorption of nutrients. In the digestive system, potential damaging chemical processes occur and cells have a life span of a few days before dying by apoptosis. Epithelial cells are repaired by constant cell renewal governed by the Wnt signaling pathway (Fevr et al., 2007) and the stem cell population is maintained by the Notch pathway (Stappenbeck et al., 1998). Stem cells in the crypts of the small intestine proliferate rapidly to produce absorptive epithelial cells, goblet, enteroendocrine cells and Panneth cells.

1.13.3 Neural stem cells (NSCs)

Neural stem cells (NSCs) persist in a few regions of the adult mammalian brain, and when grafted into a developing or damaged brain, can generate new neurons and glia appropriate to the site of grafting (Suhonen et al., 1996). The ability of bone marrow stromal cells (BMSC) to differentiate towards a neuronal phenotype followed by transplantation into injured spinal cord tissue open the possibility for the treatment of paraplegia and neurological degenerative diseases like Alzheimer's (Vaquero and Zurita, 2008). NSCs are the most immature progenitor cells in the nervous system and have the ability to extensively migrate to areas of pathology in the central nervous system. Their inherent tumor-trophic properties and their capacity to differentiate into all neural phenotypes has led to the development of novel cell-based therapies for brain malignancies by which engineered NSCs are used as cell-based therapeutic agents to eliminate malignant cells in the brain (yip and Shah, 2008).

1.13.4 Mesenchymal stem cells

The adult bone marrow contains a population of multipotent mesenchymal stromal cells (MSCs), characterized by expression of stromal cell surface markers (CD133+) and differentiation into mesenchymal lineages (Hunt et al., 2008). MSCs are able to differentiate into bone, cartilage, muscle, marrow stroma, tendon-ligament, fat and other connective tissues. MSCs may be an attractive therapeutic tool for regenerative medicine and tissue engineering because of their multipotency, easy isolation and culture, highly expansive potential and immunosuppression properties (Fu and Li, 2008).

MSCs are naturally found as perivascular cells, referred to as pericytes, which are released at sites of injury, where they secrete large quantities of bioactive factors that are both immunomodulatory and trophic (Schabort et al., 2008). The trophic activity inhibits ischaemia-caused apoptosis and scarring while stimulating angiogenesis and the mitosis of tissue intrinsic progenitor cells. Autoimmunity is inhibited by immunomodulation of the injured site (Caplan, 2008).

1.13.5 Muscle stem cells

Satellite cells are considered to be adult skeletal muscle stem cells. These cells are highly dependent on their specific niche for satellite cell activation, proliferation and differentiation (Boonen et al., 2008). Skeletal muscle injuries account for up to 35%-55% of all sports injuries. Activation of muscle stem and progenitor cells causes differentiation into myotubes and facilitates skeletal muscle repair or growth (Li et al., 2008). The transforming growth factor-beta (TGF-beta) super family members are elevated post-injury and they function to regulate myogenesis and wound healing.

1.13.6 Adipose stem cells (ASCs)

Human adipose tissue-derived stromal cells (hASC) are present in white fat (Iwashina, 2008). Autologous adipose-derived mesenchymal stem cell therapy involves harvesting of fat from the patient, isolating the stem and regenerative cells followed by administering the cells back to the patient. It is difficult to culture and stimulate cells in vitro for repair of damaged tissue. Adipose stem cells are becoming a popular and readily available source of adult cells for stem cell applications, due to ease of harvesting and the abundance of adipose-derived mesenchymal cells. Progress in stem cell research has led to the utilization of stem cells for orthopedic tissue regeneration and is a promising candidate for therapy in myocardial infarction (D'Andea et al., 2008). However, the frequency of human ASC cells that differentiate towards cardiomyocytes is low (Van Dijk et al., 2008). Since 2003, autologous adipose mesenchymal cell therapy for chronic osteoarthritis has been commercially available in veterinary regenerative medicine (Black et al., 2008).

1.13.7 Haematopoietic stem cells (HSCs)

Bone marrow harvested peripheral blood and cord blood are important sources of haematopoietic stem cells (HSCs). In adults, haematopoietic stem cells are found mainly in bone marrow, and they depend on contact-mediated signals from the marrow stromal cells to maintain their character (Kronenwett and Haas, 2008). In

fetuses, blood stem cells are mainly found in the liver (Howitz, 2007). More than 100 billion blood cells are lost every day in humans and must be continually produced from haematopoietic stem cells. This limited life span of mature cells necessitates a high cell turnover to replace blood cells at a rapid rate.

Studies of haematopoiesis have been greatly aided by in vitro assays in which stem cells or committed progenitor cells form clonal colonies when cultured in a semisolid matrix. Stem cells are the most important cells in haematopoietic cell production and the estimated stem cell frequency in bone marrow is about one stem cell per 20 million nucleated cells (Conteras, 2005). Stem cells circulate at low cell concentrations in the peripheral blood system of normal adults. Exercise, stress and infection result in a physiological increase in circulating peripheral stem cells (Gelders, 2006).

1.13.7.1 The influence of colony stimulating factors on haematopoiesis

Colony stimulating factors (CSFs) are glycoproteins that support haematopoiesis at low concentrations (10^{-12}M) by binding to specific cell surface receptors like transmembrane tyrosine kinases but mostly to the cytokine receptor family (Olsson et al., 1992). CSFs are synthesized by various cell types like endothelial cells, fibroblasts, macrophages, lymphocytes and their concentration in the blood increases rapidly in response to bacterial infection in tissue, thereby increasing the number of phagocytic cells released from the bone marrow into the bloodstream (Yu et al., 2008). CSF function to control the rate of cell division or the number of division cycles, it facilitates cell differentiation, influences commitment of cells for differentiation and increases the probability of cell survival. The pharmacological administration of growth stimulating factors or cytotoxic chemotherapy results in increased circulating stem cell numbers, which are collected by leucapheresis for stem cell transplants (Arslan and Moog, 2007).

1.13.7.2 The process of blood haematopoiesis

Multipotent haematopoietic (blood forming) stem and progenitor cells are the rare but life supporting cells from which circulating blood cells derive as well as

osteoclasts in bone (Broxmeyer et al., 1993). The stem cell proliferation and differentiation follows a stochastic process and divide to produce more stem cells and various committed progenitor cells (transit amplifying cells), each able to give rise to only one or few types of blood cells. The committed progenitor cells divide extensively until the cells terminally differentiate into mature blood cells, which usually die after several days or weeks (Metcalf, 1999).

Haematopoietic stem cells divide infrequently to give rise to two different progenitor cells: a myeloid progenitor cell that can differentiate into most of the mature terminally differentiated functional blood cells like erythrocytes, white blood cells, platelets, basophils, neutrophils, macrophages and dendritic cells or a lymphoid progenitor cell that can differentiate into any of the various types of lymphocytes (NK cells, T cells or B cells) (DiJulio,1991). T cell precursor cells migrate to the thymus where they undergo differentiation into T cells while B cells undergo differentiation in the bone marrow (Ikuta et al., 1992).

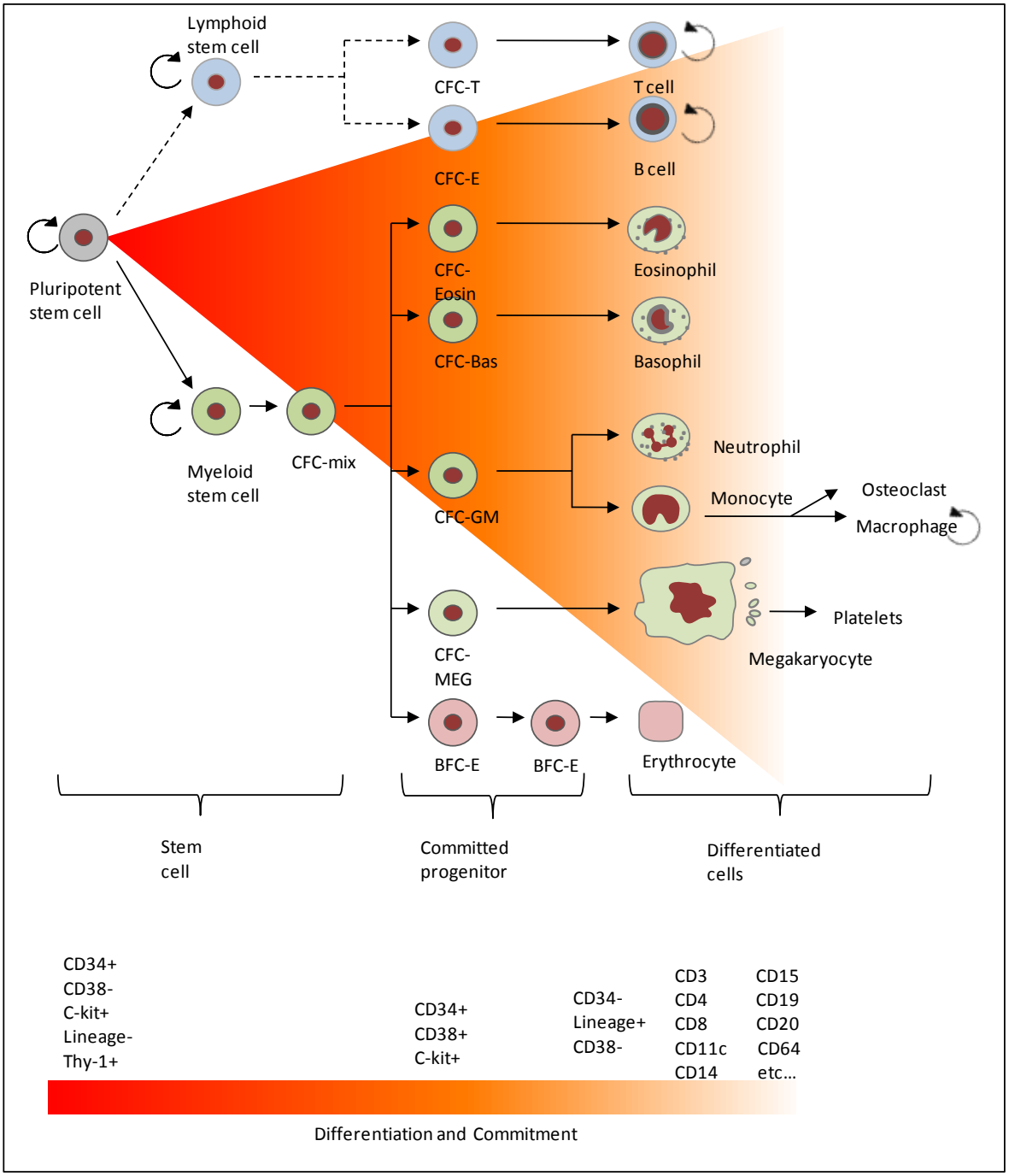

Figure 2: Schematic presentation of haematopoiesis. Indicated are the different developmental stages of haematopoiesis. Illustration of the phenotypic differentiation gradiënt during the metabolic state of the stem cell to progenitor cells. (Drawn by D Thompson).

Stem cells depend on contact signals from stromal cells for division and differentiation. The cell surface receptor Notch1 is expressed on CD34+ HSC haematopoietic precursors, whereas one of its ligands, Jagged1, is expressed on bone marrow stromal cells (mesenchymal stem cells). In early haematopoiesis, Notch signaling modulates a growth factor signal and in the absence of growth factor stimulation, the Jagged1-Notch pathway preserves CD34+ HSC cells in an immature and uncommitted state. Stem cells are confined to a particular niche and when they lose contact with this niche they tend to lose their stem cell potential and become committed differentiated cells (Walker et al., 1999). Similarly, stem cell factor C (SCF-C) is expressed on the stromal cell surface and the receptor C-Kit ligand is expressed on stem cells. Metalloproteinases (MMP-9) release KitL ligand that enables bone marrow repopulating cells to translocate to a permissive vascular niche favoring differentiation and reconstitution of the stem/progenitor cell pool (Heissig et al., 2002).

Descendants of the haematopoietic stem cells continue to proliferate and undergo several rounds of division as the cells become committed to specific differentiation pathways that are determined by growth factors. These factors channel precursor cells along specific pathways of blood cell differentiation. Once they become fully differentiated blood cells cease to proliferate.

A Study by Thoma et al. (1994) to phenotype the rare populations of haematopoietic cells in UCB expressing the cell-surface marker CD34 was studied with flow cytometric and mRNA-phenotype determined by cDNA-polymerase chain reaction (cDNA-PCR) analysis. Results indicated that vast majority of CD34+ HSC UCB cells coexpressed CD38, CD18, HLA-DR, and CD33. Rare subpopulations of CD34+ HSCCD38-, CD34+ HSCCD18-, CD34+ HSCHLA-DR-, and CD34+ HSCCD33- were also identified. A large proportion of CD34+ HSC UCB cells expressed CD13, CD45R, and to a lesser extent CD71. The CD36, CD51, and CD61 antigens were identified on a small number of CD34+ HSC cells. The CD34+ HSC cells stained with antibodies to CD61 and CD36 or CD51 can be divided into

subsets that may represent progenitor cells committed to the erythroid and/or megakaryocytic lineage. A variety of other lineage-specific cell-surface antigens including pre-T-cell marker CD7 and markers of early B cells, ie, CD10 and CD19, were not coexpressed with CD34+ HSC. Rare subpopulations of haematopoietic cells persist in UCB with different phenotypic markers as well as different levels of expression. As stem cells differentiate, the cells may express different phenotypes as a result of the metabolic state of the cells, a process called phenotypic gradiënt migration. CD34+ HSC detection levels may decrease as expression decrease, resulting in that these progenitors could not be quantified at the time of analysis. For this reason the utilization of total nucleated cell number remains the preferable method for quantification of cell dosage in UCB grafts.

1.13.7.3 HSC death

Haematopoietic stem cell death by apoptosis controlled by the availability of CSFs also plays a central part in regulating the numbers of mature differentiated blood cells. The capacity for unlimited self renewal is dangerous for any cell, as many cases of leukemia arise through mutations that confer this capacity on committed haematopoietic precursor cells that normally differentiate and die after a limited number of division cycles (Orkin 2000). The maintenance of differentiated blood cell populations is dependent on continual division of the self renewing haematopoietic stem cell.

1.13.7.4 Medical applications of adult bone marrow stem cells

Bone marrow transplantation is a well established and routinely applied clinical procedure using adult stem cells for the treatment of haematological cancers, marrow-failure disorders, genetic disorders, immunodeficiency syndromes, and certain metabolic diseases. Following high dose chemotherapy, haematopoietic stem cells obtained from the bone marrow or peripheral blood are transplanted into the patient in order to reconstitute new blood cells. Unfortunately, only 25 to 30 percent of potential recipients have HLA-identical siblings who can act as donors in allogeneic transplants (Thomas, 1983).

Bone marrow-derived endothelial progenitor cells (EPCs) have been studied as a strategy in regenerative medicine as a potential repair mechanism for cardiovascular disease morbidity and mortality, including ischemic heart disease and in-stent restenosis (Marsboom and Janssens, 2008). The discovery of adult human stem cells from the bone marrow, capable of generating angiogenic or contractile cells might offer new treatment options for patients suffering myocardial infarction and cardiomyopathy (Engelmann and Franz, 2006).

1.14 STEM CELL THERAPY

The limited identification of stem cells in adult tissue as well as the limitation on transplant organs with possible risk of organ rejection could be overcome if scientists could differentiate, undifferentiated autologous stem cells to directed cells types like heart and neural cells. This will overcome organ shortage, organ rejection and ethical concerns (Schulz, 2006).

The identification of stem cells by immunophenotyping as well as cell separation technologies to remove immune incompatible cells facilitates improved graft engineering (Consolini et al., 2001). Cell separation or CD34+ HSC cell purification is used to deplete the graft of T lymphocytes to remove potential graft versus host disease (GvHD) (Aversa et al., 2005). HSCs are ideal vessels for gene therapy of genetic diseases in that the transduced genes will be expressed for long periods in the stem cell population and their differentiating and mature descendants (Eliopouloset et al., 1998).

1.15 CANCER STEM CELLS

The capacity of a tumor to grow and propagate is dependent on a small subset of cells within the tumor, termed cancer stem cells. Research on cancer stem cells (CSCs) suggests that a fraction of the cells have stem cell properties of self-renewal and differentiation but contain their own stem cell neoplastic clone. Cancer stem cells may originate due to self-renewal and differentiation pathways occurring

in multi-potential stem cells, tissue-specific stem cells, progenitor cells and cancer cells (Galmozzi et al., 2006).

The following are highlights on the hypothesis of the origin of CSCs:

- Mutations and epigenetic changes may increase the long lifespan of stem cells allowing for increasing evolution towards malignancy.
- Asymmetric division of CSCs drives tumor growth and traits shared with normal stem cells, increase the occurrence of this phenomenon.

Identification of CSCs in human malignancies is therefore crucial in controlling and curing cancer. Identification and subsequent targeting of phenotypically defined cancer stem-cell populations will result in application of therapy that will prevent potential recurrence of cancer as well as achievement of increased survival rates for these patients. This has resulted in the increased identification of cancer stem cells in human cancer tissue such as colon cancer stem cells (Kreso and O'Brien, 2008), pulmonary cancer stem cells (Yagui-Beltrán et al., 2008). Ovarian cancer also contains stem cells or tumor-initiating cells (Ponnusamy et al., 2008).

CHAPTER 2

UMBILICAL CORD BLOOD

2.1 THE ROLE HSCS DURING HUMAN ONTOGENY

In mammalian development, four sites of blood cell formation are recognized, namely the yolk sac, bone marrow, spleen and thymus. Blood and endothelial progenitors emerge from the extra embryonic yolk sac blood islands at embryonic day 7.5 (E7.5). In early gestation the yolk sac supports the formation of primitive haematopoietic stem cells and erythrocyte genesis (Prereda abd Niimi, 2008), which are primarily composed of nucleated erythrocytes. The first site of haematopoietic stem cell development is identified as the (AGM) aorta-gonodal-mesonephros region (Pietilä and Vainio, 2005). Studies performed by Chen et al. (2006) indicated the supportive effect of stromal cells from human aorta-gonad-mesonephros (AGM) on the maintenance and expansion of umbilical cord blood CD34+ HSC cells in vitro. During the early weeks of human gestation, haematopoietic cells first emerge within the extraembryonic yolk sac (primitive haematopoiesis) and secondarily within the truncal arteries of the embryo (Zambidis et al., 2006). By the second trimester of gestation the HSC populates the bone marrow and takes over blood production in the spleen and thymus as the second and third site of haematopoiesis (Valsamo et al., 2007).

The transition in the location of haematopoiesis is associated with changes in HSC function. As formation of the organism becomes more complex, haematopoiesis occurs in the fetal liver, resulting in haematopoiesis of platelets and immune cell production. The fetal liver assumes the primary role of cell generation (Phan et al., 1998). Stem cell production and proliferation begins to decrease and eventually reaches a state of relative quiescence shortly after gestation. Stem cells have been isolated at all stages of development from the early developing embryo to the post-reproductive adult organism, but the fetal environment is unique as it is the only time in ontogeny that there is migration of stem cells in large numbers into different organ compartmen

Non-haematopoietic, non-endothelial cells with an immunophenotype similar to adult bone marrow-derived mesenchymal stem cells (MSCs) have been isolated from fetal blood (O' Donoghue and Chan, 2006). These cells have been identified and evaluated as human fetal mesenchymal stem cells (hfMSC) and are true multipotent stem cells with greater self-renewal and differentiation capacity than their adult MSC. hfMSC circulate in the fetal blood of the first trimester and are able to traffic into the maternal blood circulation, where they engraft into maternal bone marrow, and remain microchimeric for decades after pregnancy.

At birth, haematopoietic activity is distributed throughout the human skeleton but gradually recedes with time so that in normal adult life haematopoiesis is found mainly in the sternum and pelvis with small amounts in other bones like the ribs, skull and vertebrae (Lansdorp et al 1993).

2.2 UCB AS A SOURCE OF HAEMATOPOIETIC STEM CELLS

The umbilical cord represents the link between mother and fetus during pregnancy. UCB consist of red cells, plasma, platelets, white blood cells and differential components like neutrophils, eosinophils, basophils, monocytes, nucleated rel cells, lymphocyte subsets (CD19+ B cell lymphocytes, CD3+/CD16+/CD56+ NK cells and T cells and haematopoietic stem cells. This previously discarded tissue has recently been found to be a "store" of haematopoietic stem and progenitor cells. Annually, the global human birth rate is 100 million and therefore presents umbilical cord blood (UCB) as the largest non-controversial stem cell source. Since the first successful umbilical cord blood transplant by Gluckman in 1989, cord blood has become a reliable and rich source of haematopoietic progenitor cells (HPCs) for bone marrow transplantation in both pediatric and adult patients. HPCs are used for transplantation in patients with haematopoietic malignancies, inherited metabolic disorders, immune-deficiencies, neurodegenerative diseases and marrow failure like aplastic anemia (Kurzburg et al., 2001).

2.3 LEGAL AND ETHICAL ISSUES REGARDING UCB STEM CELLS

Since umbilical cord blood is no longer regarded as waste but clinically useful material in medical therapy, the legal, ethical and medical aspects of cord blood collection have become important (Ende, 1992). These aspects include:

- Immediate clamping of the human cord after birth before delivery of the placenta is shown to be harmful to the premature infant and can produce brain hemorrhage (Hofmeyer et al., 1988). To date there is no evidence that immediate cord clamping of full term infants in order to collect high volume cord blood is without risk to the donor. During UCB collection an infant looses 20% of the circulating stem cells, essential to premature babies.
- The right of the parents consent to donate or sell human tissue that doesn't belong to them.
- The legal right of possession by the donor. If the donor requires the blood at a later stage in life, the donor has potential for compensation if the cells no longer exist in the cord blood bank. This could be resolved by utilizing procedures in blood banking whereby the volunteer donor is promised a unit for themselves if medically needed.

2.4 CORD BLOOD BANK

Following the pioneering work of Gluckman, cord blood banks now operate in many countries with more than 8,000 cord blood transplants been performed worldwide (Ballen et al., 2006). Internationally, private and public cord blood banks have been established. In 1986, the National Marrow Donor Program (NMDP) was started in the United States to facilitate the recruitment and procurement of suitable marrow from unrelated donors for patients lacking related donors. NMDP currently operates the world's largest registry of unrelated adult donors and umbilical cord blood (UCB) units. To date the NMDP registry lists more than 6.7 million adult donors and 68,000 UCB units (Confer and Robinett et al., 2008).

These banks are based on the NMDP concepts of voluntary donation, viral screening and transplant of unfrozen donations, except that cord blood collections will be cryopresserved.

A careful history of genetic disease is recorded especially if the donation is to be used for a recipient with a known defect. Studies have indicated that freezing may have some negative effect on the reconstituting of the marrow in the host and that fresh cord blood is more desirable for transplants (Rubenstein et al., 1995). The donations are also tested for bacterial contamination and the frequency of contamination is relatively low as a result of collection of cord blood through a needle into a closed system (Ende et al., 1989).

2.5 ADVANTAGES AND DISADVANTAGES OF UCB

The use of UCB stem cells for bone marrow transplantation has several advantages over HSCs obtained from bone marrow or peripheral blood. One of the major advantages is that UCB stem cells have an immature T cell immunity (Qian et al., 1997) and therefore a less stringent HLA match is required for transplant (Laughlin et al., 2001). This fact also contributes to an additional benefit as a lower incidence of acute and chronic graft versus host disease (GvHD) occurs in recipients. Other advantages are the easy availability (Gluckman E., 2000), safe procurement (Cairo et al., 1997) and limitless supply of HSCs from UCB (Hows et al., 1992). As UCB is easy collected it results in increased ethnic representation of HLA matching for transplantations (Broxmeyer et al., 1989). It was also reported by Van de Ven et al. (1995) that UCB has a high cloning efficiency and self renewal capacity. As UCB is collected naturally and not by leucapheresis or bone marrow aspiration, there is the benefit of absence of donor attrition (Wadlow & Porter, 2002). There is also less linear viral transmission in UCB transplants and stem cells collected from UCB have higher differentiation frequency, self renewal capacity and a greater proportion of progenitor cells compared to bone marrow (Broxmeyer et al., 1992).

The major disadvantage of cord blood is insufficient cell dosage for transplantation in larger recipients due to limited cell number (Migliaccio et al., 2000). The tendency of delayed time to engraftment following transplantation results in delayed platelet and neutrophil renewal and delayed immune reconstitution (Abu Ghosh et al., 1999).

2.6 UCB VS. PERIPHERAL/BONE MARROW STEM CELLS

UCB in contrast to adult bone marrow for transplant purposes does not have any stromal cell content unless these are inadvertently collected by milking of the cord. It was concluded from studies by Hows (1992) that stromal cells form an essential part in the propagation of UCB stem cells and for identification of “self”. This absence of self expression by UCB donor cells may be responsible for the low occurrence of GvHD in successful transplants of UCB compared to HSCs from other sources. An advance to cord blood stem cells for transplantation is the circulating multipotent fetal stem cells that are so naïve with no significant recognition molecules.

Comparative immunophenotyping analysis was performed on cord and adult peripheral blood and showed similar values for CD3+ and CD8+ markers (Hagihara et al., 2003). CD4+ expression is higher in cord blood than in peripheral blood while the percentage of B cells (CD19+ marker) and Natural killer cells (CD56+ marker) activity was lower in UCB than peripheral blood. Antibody dependent cell cytotoxicity (ADCC) against red blood cells in UCB was similar to adult peripheral blood. Spontaneous antitumor activity in UCB correlated with adult peripheral blood and since antitumor activity could be induced, cord blood could be used for treatment of leukemic diseases (López et al., 2008).

2.7 ISOLATION OF HSC, MSC AND ENDOTHELIAL CELLS FROM UCB

Human umbilical cord mesenchymal stem cells (hUCMSCs) were isolated with HSC from the same umbilical cord. hUCMSCs tested positive for CD44, CD90, CD73, and CD117 and were found negative for CD33, CD34, CD45, and CD105

surface markers. Under the influence of lineage specific differentiation medium, the hUCMSCs showed multilineage differentiation potential and differentiated into adipogenic, chondrogenic, osteogenic, and neuronal lineages. Thus discovery has led to the understanding of the complexity of UCB. The isolating, identification and quantification of all these cells present in UCB cells from the same umbilical cord could lead to complete utilization of the available tissue for tissue engineering and cell therapy (Kadam et al., 2008).

2.8 CORD BLOOD PROGENITOR CELLS

Committed progenitors in UCB are detected with flow cytometery and clonal assays, colony forming units (CFU) and burst forming units (BFU). These cells have lost varying degrees of stem cell capacity and acquired progressive lineage restriction. High levels of erythroid BFU (BFU-E) (Wright et al., 1992 and Hann et al., 1982), megakaryocyte progenitors (CFU-MEG) (Zucker et al; 1992), and granulocyte-macrophage progenitor cells (CFU-GM) are found in cord blood (Broxmeyer 1992). Pre-term fetus cord blood contains two to four fold more BFU-E and CFU-GM cells than normal term neonates (Zucker–Franklin, 1992 and Christensen et al., 1986). CD34+ HSC marker antibody is used to discriminate haematopoietic CFU from more mature peripheral blood elements. CD34+ HSC antigen expression has been detected on progenitor cells and HSC cells capable of forming blast cell colonies, and early lymphoid progenitor cells but not on more mature cells (Brandt et al., 1988).

2.9 APPLICATIONS OF UBC STEM CELLS

Various haematological disorders such as Wiskott-Aldrich syndrome, juvenile chronic myelomonocytic leukemia, chronic myeloid leukemia, neuroblastoma, X-linked lymphoproliferative disease, aplastic anemia and Fanconi's anemia have been treated with cord blood transplants. In all successful transplants minimal GvHD and complete chimerism of the bone marrow was observed (Gluckman et al., 1992).

2.10 STEM CELL DOSE

The required dose for transplant depends on the stem cell source, the disease, patient health, HLA tissue matching and whether the donation is used for an autologous or allogeneic transplant. The dose (cells per kilogram) and viability of HSCs in a cord blood unit mainly determine engraftment success. UCB stored with Netcells are privately stored and if a "client" needs to be transplanted it will be considered as an autologous transplant. Internationally, very few autologous cord blood transplants have been performed and reported. It is therefore unclear what the minimum required nucleated cell and stem cell dose is to ensure successful engraftment for an autologous cord blood transplant. Most allogeneic umbilical cord blood (UCB) transplant studies indicate that the minimum nucleated cell dose needed for adequate engraftment is > 2 x 10^7 cells/kg in adults and children (Stiff et al., 2005). The number of CD34+ HSC cells remains the most important factor in stem cell transplantation to evaluate the haematopoietic recovery after engraftment (Gabús et al., 1993). A dose of only 0.3 x 10^6 CD34+ HSC cells per kg guarantees haematopoietic recovery within a reasonable number of days (Pérez-Simón et al., 1998). With this know dosage requirements for allogeneic transplants, factors like compatibility of HLA tissue matching might influence the minimum dosage requirement. For autologous cords stored at private cord banks the HLA tissue mismatch is of no importance and it's possible that a lower CD34+ HSC dosage might be required to ensure successful engraftment.

2.11 FACTORS THAT INFLUENCE STEM CELL DOSE

Stem cell count and viability is affected by physiological factor. Identification of these factors may help in collecting UCB more efficiently and lead to improvement of the UCB transplantation rate. Physiological factors that affect cell count and viability are:

2.11.1 Collection volume of UCB from umbilical cord

Collection volume and total nucleated (TNC) are influenced by several obstetric factors, such as infant and placental weight, where a greater weight predicts a

better UCB collection (Mancinelli et al., 2006). The method of collection also has a great influence on volume. UCB collections by cesarean section had more CD34+ HSC cells and higher collection volume, but lower TNCs than collections form natural delivery (Jan et al., 2007).

2.11.2 Cell loss during processing

Cell separation of HSC is of great interest to UCB banks as these banks want to ensure maximum UCB cell storage and successful engraftment but also to optimize storage space and costs of cord blood. Early methods of cord blood separation resulted in a significant loss of progenitor cells. Broxmeyer (1992) recorded a significant loss of fetal haematopoietic stem cells during cell separation techniques with Ficoll-Hypaque. Thierry (1992) also reported the fragility of UCB cells to “aggressors” related to cellular separation while Charboard (1996) observed a cellular yield of 80% using PERCOL cell separation technique. Recent advanced techniques have been developed that use a procedure where the donation is separated by centrifugation into a buffy coat fraction, a red cell fraction, and a plasma fraction. Stem cells are recovered in the buffy coat fraction. This technique showed increased recoveries of nucleated cell count, total progenitors and CD34+ HSC cells (Ademokun et al., 1997).

2.11.3 Cell loss after thawing

A high post-thawing CD34+ HSC count in UCB transplants correlates with event free survival in adult recipients and higher engraftment success in pediatric recipients (Gluckman et al., 1998). Colony forming evaluation studies used in the 1980s demonstrated that a reasonable amount of cord blood could be collected, cryopreserved and retrieved in a viable condition for successful transplantation of pediatric patients (Broxmeyer et al., 1989). Dimethylsulfoxide (DMSO) is used as a cryoprotectant during cryopreservation and it produces high cellular osmolarity. This causes osmotic shock in the thawed cells with a substantial loss in the number and viability of the leukocyte and CD34+ HSC stem cell populations (Lorache et al., 2005 and Rubenstein et al., 1995). It was reported by McKenna et

al. (2004) that cord blood units have a reduced CD34+ HSC number and viability after sample thawing.

2.11.4 Storage temperature of UCB

It was established that short term storage of HSCs at 4°C or room temperature for 24 hours in solutions with anticoagulant satisfactorily preserved the cells, but that cell count and viability decreased with prolonged storage time. The mononucleocide counts (MNC) counts were higher than CD34+ HSC cell counts when cells were stored at 4°C (Hubel et al., 2003).

2.11.5 Storage period of UCB

Studies performed by Hubel et al. (1999) have indicated that haematopoietic stem cells can be stored for up to 72 hours prior to preservation but that cell recovery and viability are significantly lower than in cord blood stored for 24 hours after collection. Higher levels of cell deterioration were detected in mononuclear cells than in CD34+ HSC cells with increased time after collection of UCB (Xiao and Dooley, 2003).

2.11.6 Maternal age

Younger maternal age, larger birth weight and shorter gestational age resulted in higher TNC and haematopoietic stem cell (CD45+/CD34+ HSC) content (McGuckin et al., 2007). Other predictors of optimal cord blood stem cell counts are mothers with fewer previous live births (Ballen et al., 2001). Maternal cell contamination of the UCB collection is low to absent but clinical studies indicated no or low risk of GvHD (Socie et al.,1994).

2.11.7 Infant gender

Research done by Nakagawa et al. (2004) revealed that cord blood from female donors has a higher TNC count than male donors while the opposite is true for CD34+ HSC stem cells (Thomas et al., 2006).

2.12 FACTORS THAT INFLUENCE ENGRAFTMENT

The outcome of UCB transplantation depends on many variables, mostly dependent on the recipient diagnosis, stage of disease, conditioning type (myeloablative, non-myeloablative, irradiation), nucleated cell and progenitor cell dose and HLA disparity. Myeloablative conditioning eradicates the disease and ensures engraftment of donor haematopoiesis in conventional allogeneic stem cell transplants. High morbidity and mortality remain the major post-transplantation complications resulting from treatment-related toxicity and GVHD (Walshe and Bishop, 2004). Non-myeloablative treatment or reduced-intensity allogeneic stem cell transplants (RIST) use reduced doses of chemotherapy and radiation as transplant conditioning. This treatment decrease early post-transplant complications, but higher incidences of mixed chimerism and graft rejection are observed compared with transplantation after myeloablative conditioning (Kreiter et al., 2001).

The increased use of DNA-based tissue typing has increased the accuracy and specificity of HLA typing allows for more precise HLA matching between donors and recipients. More precise HLA matching between donor and recipient has the following effects:

- Improves overall transplant survival (Bray et al., 2008)
- Improves engraftment rate (Petersdorf et al., 2001)
- Reduces acute and chronic GVHD incidence and severity (Morishima et al., 2002)

UCB is less restricted with regards to HLA matching requirements relative to bone marrow stem cells from adult donors (Hamza et al., 2004). Studies performed by

Kögler et al.(2005) reported that HLA matching was only predictive for survival after UCB transplantation if performed at the serological level for HLA-A and HLA-B, and high resolution allelic typing for HLA-DRB1.

HLA mismatching consequently increases the number of potential units available per patient, thereby improving the search for suitable units for patients with rare tissue types (Rubenstein et al., 1998). According to the New York Blood Center (NYBC) it was shown that patients had a 99% chance of finding a 4/6 HLA matched UCB unit, and a 70% chance of finding a well-matched UCB unit (defined as 5/6 or 6/6 HLA match, without mismatch in the graft-versus-host disease (Stevens et al, 2005).

Immaturity of the cord blood lymphocytes may contribute to this immune-tolerance, as suggested by the observation that:

- Activated UCB mononuclear cells (MNC) have a different mRNA and protein expression proper for several growth factors and interleukins important in immune cells as compared to normal adult peripheral blood MNC.
- UCB cells generate decreased T-helper-1(Th1)-type cytotoxic cellular responses to mitogenic stimulation.
- The phenotype of UCB B-cells is more immature than in normal adults.
- Lower CD4+/CD45+ T-cells number of in UCB.
- Lower activity of the UCB CD4+/CD45+ T-cells and natural killer-cells (NK-cells).
- UCB dendritic cells tend to have a lymphoid and immature phenotype, with a decreased capacity to produce IL-12 (thus further impairing Th1 response) (Bradley et al. 2005).

Recently Double-cord-blood transplantation (DCBT) was performed by Ballen et al.(2009) that offers an option for patients with mismatched grafts to receive reduced-intensity transplants. These unique transplants have two donors, both of

whom are usually HLA mismatched at one to two loci. High resolution HLA typing was performed. Successful engraftment and low incidence GVHD was observed.

The results of a study performed by Laughlin et al. (2001) demonstrated that HLA-mismatched umbilical-cord blood from unrelated donors can restore haematopoiesis in adults who receive myeloablative therapy. The incidence and severity of GVHD were low despite HLA mismatching. The following Kaplan Meier curve illustrates that transplant with increased HLA tissue match will have a shorter period to engraftment.

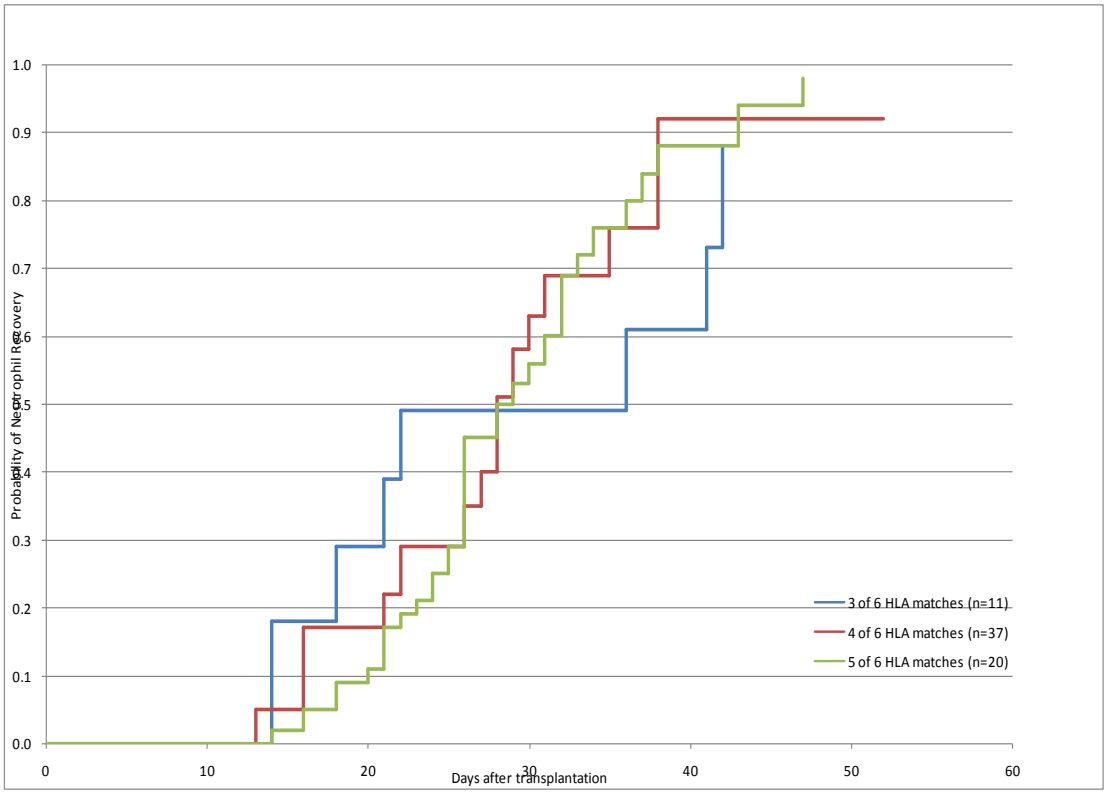

Figure 3: The effect of HLA tissue type matching on the rate of neutrophil engraftment. This Kaplan Meier curve indicates that an increase in the HLA matching 4/6HLA, 5/6HLA or 6/6 HLA will result in an increase in the rate of neutrophil recovery and successful engraftment. Adaptation from Laughlin et al. (2001) (Drawn by D Thompson).

Sufficient cell dose (total nucleated cells and haematopoietic progenitor cells) and quality of the cells is essential for successful engraftment. An increase in cell dose will result in a decrease in the number of days to engraftment. Delayed engraftment after cord-blood transplantation is thought to result from an inadequate population of progenitor cells in the graft. Delayed engraftment can lead to early transplant-related morbidity and mortality. The relationship between cell dosage at transplant and engraftment is clearly illustrated in the following Kaplan Meier Curve (Laughlin et al., 2001).

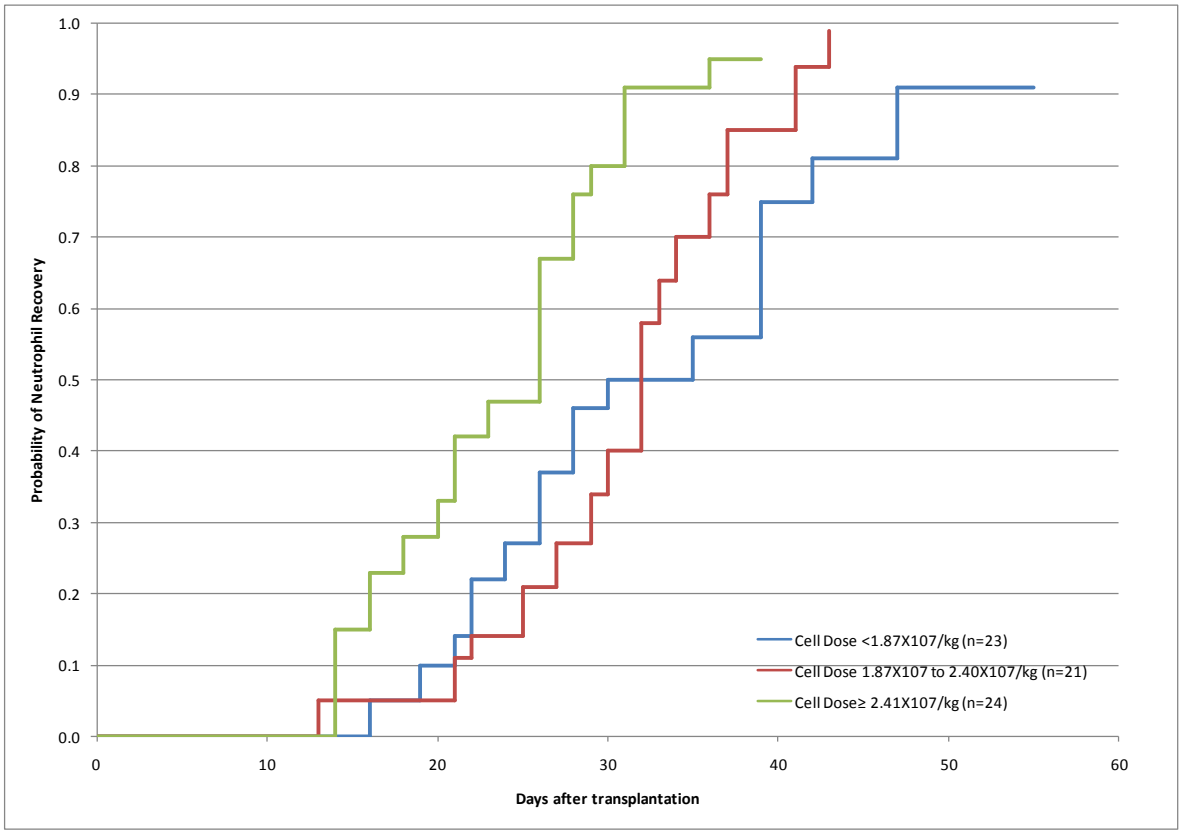

Figure 4: The speed of recovery and HLA tissue type matching on the rate of neutrophil engraftment. This Kaplan Meier curve indicates that earlier neutrophil engraftment was obtained with stem cell transplants that have higher cell transplant dosages. The days to successful engraftment is less in patients that received transplants with a cell dose of 2.4 x 10^7/kg compared to doses of 1.87 x 10^7/kg nucleated cells. Adaptation from Laughlin et al. (2001). (Drawn by D Thompson).

2.13 STEM CELL EXPANSION IN UCB

Ex vivo expansion of UCB stem cells before transplantation could potentially improve cell dose of UCB collections and the efficacy of UCB transplantations. An increase in UCB cell count will also open the field for gene therapy for several genetic diseases. There are two reliable ex vivo UCB expansion techniques known: (1) CD133+ cell selection followed by ex vivo liquid culture and (2) co-culture of unmanipulated UCB with bone-marrow-derived mesenchymal stem cells (MSCs). Successful cell expansion is followed by measuring total nucleated cell count (TNC), CD133+ and CD34+ HSC cell and colony-forming units (Robinson et al., 2006).

2.14 RELEVANCE AND MOTIVATION FOR THIS STUDY

It is hypothesized that infant gender and maternal age influence cell count and viability of CD34+ HSC stem cells and CD45+ leukocytes and that different storage temperatures and periods of storage also have an effect on these cells. Internationally cord blood banks operate with known standards for parameters like cell quality and quantity specific to the individual banks. Currently many Cord Blood banks are operating worldwide and Netcells Cryogenics is the major private cord blood bank in South Africa. This study is the first step in a program of investigation into the measurements of stem cell quality and quantity in order to establish standards against the rest of the cord blood industry.

Physiological and physical factors influence cell quality as well as the quantity of stem cells and leukocytes. The impact of these factors must be determined and outcomes from studies will be assessed, followed by implementation of these findings standard operating procedure for collection, transport and processing to improve results for successful engraftment.

2.15 AIM

The **primary aim** of this research project is to retrospectively analyze all data obtained anonymously from collection, processing and storage of UCB stem cells collected by Netcells Therapeutics. The collection volume, cell count, cell viability, ratio of stem cells: leukocytes, maternal age and infant gender will be analyzed in order to establish standard values and specifications for the cord blood bank

The **secondary aims** will be to study 25 cord blood collections, the effect of storage at temperatures of 4ºC and room temperature as well as different storage periods of 24h and 48h on cell quantity and viability.

CHAPTER 3
MATERIALS AND METHODS

The Faculty of Health Sciences Ethics Committee of the University of Pretoria approved this study as Protocol 118b in 2008.

3.1 PATIENTS

The primary investigation is a retrospective study of 913 UCB collections stored by Netcells Cryogenics on behalf of their South African clients, storing their infants cord blood, during the period January 2007- July 2008. Clients were recruited by means of leaflets, consultants, gynecologists and the Netcells web page. The study contained 492 male and 421 female infants. Informed consent was waived as non sensitive information was used. Netcells criteria for UCB processing are as follows:

- UCB units with collection volume <60ml (including 35ml CPD-A) were not processed.
- The final leukocyte total count must be > 2 x 10^8 total cells. Grafts that met the collection volume criteria prior to processing but not the total leukocyte cell count criteria were discarded with permission of the parents or a disclosure were signed by the parents to continue storage.

At the time of statistical analysis of the study a total of 918 UCB that passed the minimum volume criteria of 60ml, was processed by Netcells. Five units of the total 918 units were excluded from the study as post processing leukocyte results were < 2 x 10^8 total cells rendering a total sample size of 913 UCB units. The collection volumes of these samples ranged from 60 - 63 ml and were border line for the required minimum collection volume. Cell loss occurs during processing result in a decrease in cell number and therefore it could not be assumed that low collection volume was responsible for exclusion of these samples from the study. The

selection of the sample size was biased towards clinical acceptable samples but the exclusion of the 5 samples from the population would not have made a significance difference on the correlation between total leukocyte count and tested parameters for the retrospective study.

The maternal age of the 913 clients ranged from twenty years and 5 months to forty four years and 8 months with a mean age of thirty two years and seven months. Data was entered in a confidential manner into the Netcells Stem Cell Database and Netcells Infofinder.

The study design for the secondary investigation is a cross - sectional study of 25 (UCB) collections for the period 2007- 2008. Prior to delivery, informed was obtained from clients for analysis of stem cells for quality and quantity (Appendix 1). Before UCB CD45+/CD34+ HSC flow cytometric analysis, all client identifiers were removed to ensure anonymity of the samples.

3.2 CORD BLOOD COLLECTION

Cord blood was collected at birth by the attending obstetrician. The obstetricians were trained by Netcells Medical Director according to Netcells collection protocol supplied by Netcell Cryogenics (Appendix 2). The UCB collection kit (Danvers International Ltd, Colnbrook, United Kingdom) was supplied by Netcells. Cord blood collection occurred outside the uterus with the placenta above the foetus at the time of collection. After delivery of the baby the cord was clamped as soon as the mother and infant were stabilized. Deliveries occurred by Caesarean section and vaginal delivery, but complete data of the proportion of each delivery was not obtained for this study. The cord was sterilized with Tisept Sterets® (Medlock Medical, United Kingdom) and blood was collected from the umbilical vein by gravity into a Baxter 250ml collection bag (Baxter International Incorporated, Deerfield Illinois, USA) filled with 35ml CPDA-1 (Citrate Phosphate Dextrose Adenine) as anticoagulant. The UCB was packed in a temperate coolant kit (Inmark Incorparated, Birmingham, United Kingdom) and couriered by Skynet

Worldwide Express (Kempton Park, South Africa) to the processing laboratory. The cut of time from UCB collection to processing of the blood is 48 hours and UCB collections where delays extended 48h, were not processed. Time of UCB processing was recorded but information on time of birth was incomplete and inaccurate. Therefore the delay in collection and processing could not be determined and included in the study.

UCB from clients in the secondary study was transported to the laboratory within an hour of collection and processed immediately. Time of collection and processing of UCB units were accurately recorded in this study. A sample of each UCB collection prior to mechanical processing with the Sepax cell separator (Unprocessed UCB) was taken and stored at 4°C and RT for 24h and 48h. Following the cell separation procedure of the UCB (Processed UCB) a sample was taken and stored at 4°C and room temperature (RT) for analysis by flow cytometry at 24h and 48h after collection.

3.3 CORD BLOOD PROCESSING

The volume and temperature of the UCB was recorded on receipt at the processing laboratory and collections with a volume of less than 60ml (incl. CPDA-1) prior to processing were rejected according to Netcells specifications. Prior to processing of UCB for the secondary study, an additional aliquot of the unprocessed UCB was taken for flow cytometric analysis.

The UCB was processed at Room temperature (20°C - 24°C) in an environmentally monitored aseptic clean room with an automated and closed system, using the Sepax Cell Processing System in combination with a single use CS-530 separation kit (Biosafe, Eysins, Switzerland). The Sepax kit was connected to the UCB collection bag under sterile laminar airflow conditions (Bioflow II Labotec, South Africa). Six percent Hydroxethyl starch (HES) (Braun Medical, Germany) equal to 30% of the volume of the unprocessed UCB was added to the blood. During

sedimentation, HES facilitates the removal of red blood cells by sedimentation into different cell layers.

Blood separation occurred during processing in the Sepax Processor rotating syringe through centrifugation (Appendix 3). Component transfer was effected through displacement of the piston position of the syringe. The buffy coat containing the enriched CD34+ HSC stem cells and CD45+ nucleated cells was collected in a cryogenic bag (Pall, New York, USA) while the plasma and red blood cells were collected into separate blood bags. Prior to cryopreservation, an aliquot of the post processed UCB was taken for identification and enumeration of CD34+ HSC and CD45+ cells. Processed samples for the secondary study were stored at 4ºC and RT. These results were reported as the final processed cell count. Sterility testing of the processed UCB was performed by aliquoting plasma into the Bact/Alert® SN Culture bottles (Biomerieux, INC. Durham North Carolina, USA). Additional samples for viability testing after processing and cryopreservation were taken and frozen for future testing. Samples of plasma and red blood cells were frozen and stored for future sterility testing prior to transplantation.

The concentrated cells were cryopresserved according to Netcells standard procedure (Appendix 6) by addition of 10% Dimethyl Sulfoxide DMSO (Cryo-Sure, WAK-Chemie Medical GMBH, Steinbach, Germany) to the processed cord blood followed by freezing at a controlled rate to a temperature of -180°C according to a specific freezing protocol with the Kryo 560-16 Controlled Rate Freezer (Planer Plc, Middlesex, United Kingdom). See Appendix 5 for the freezing protocol. The cell concentration of the UCB unit was not determined after addition of DMSO. The cord blood was stored at -196°C in a vapor phase Nitrogen storage vessel MVE 1500 Series - 190 with MVE TEC 3000 (Mid Valley Engineering, Atlanta, USA) TEC - Thermal Electric Controller) nitrogen level and temperature display (Chart INC, Marrietta, Amerika). In this study, no viability testing or quantification of stem cells or leukocytes was performed on thawed UCB samples that were cryogenically stored.

3.4 CD34+ HSC/CD45+ IDENTIFICATION AND ENUMERATION

3.4.1 Specimen collection

All aliquots for flow cytometric analysis were collected aseptically in a sterile evacuated blood collection tube with 7.2mg K2EDTA anticoagulant (Beckton and Dickenson, Plymouth, United Kingdom). Prior to addition of DMSO, a sample form the concentrated buffy coat layer obtained from the Pall bag of the CS-530 processing kit was taken for flow cytometric analysis.

3.4.2 Specimen preparation

UCB cells were analyzed according to the Stem-Kit™ Reagents (Beckman and Coulter, Brea, Carolina, Amerika) product insert recommendations and Netcells Standard Operating Procedure (Appendix 4). Stem-Kit reagents consist of a two color fluorescent (CD45-FITC, CD34-PE) murine monoclonal antibody reagent, a two colour murine fluorescent (CD45-FITC, PE) isoclonic control, Stem Count (absolute count) reagent, a nucleic acid 7-AAD (7- Amino-Actinomycin D) viability dye and Lysing solution (NH_4Cl). The purpose of Stemkit™ reagents is to identify human progenitor cells (HPCs) that express CD34 antigen. The CD45 antigen has staining intensity characteristics of blast cells (detectable at lower levels than monocytes and lymphocytes), and CD34+ HSC cells exhibit low side angle and low to intermediate forward angle light scatter similar to blast cells. Briefly, the specimen reaction contained 100µl UCB incubated with 20µl CD45-FITC/CD34-PE monoclonal antibody and 20µl 7-AAD viability dye for 20 minutes at room temperature protected from light. The test was performed in duplicate, following the International Society of Hematherapy and Graft Engineering (ISHAGE) Guidelines for CD34+ HSC cell determination by flow cytometry. Twenty microlitres of the CD45+ FITC Isoclonic Control PE reagent and 7-AAD dye was incubated with UCB and processed like the duplicate test tubes to check for non-specific binding of CD34-PE monoclonal antibody.

The 7-AAD Viability Dye is a nucleic acid dye and non-permeable dye that is only able to cross the cell-membrane when not intact. It is absorbed by damaged and dead cells and binds to accessible DNA base pairs to cause fluorescence of the cells (bright events). It is used to distinguish between viable and non-viable cells for enumeration of the viable cells (negative events) of interest. After incubation, 2ml of freshly prepared 1 x NH_4Cl Lysing Solution 1ml (10x NH_4Cl) : 9ml (deonized H_2O) was added followed by 10 minutes incubation to ensure red blood cells lysis. RBCs could interfere during cell acquisition as nucleated red blood cells show CD34+ antigen expression. Stem Count with a known Fluorosphere concentration was added and the test tubes gently vortexed. The samples were analyzed within 1 hour of preparation.

3.4.3 Analysis protocol setup

Flow cytometric analysis was performed with the Beckman Coulter Cytomics FC500 Flow Cytometer with CXP System software version 2.2, Stem CXP software version 2.0 and Stemkit™ reagents (Beckman Coulter Inc., Fullerton Carolina, USA). The Flow Cytometer Argon laser was properly aligned for light scatter and the voltage and gain settings were standardized. The instrument was optimized for colour compensation for fluorescence intensity of the different fluorescence chambers. As part of Netcells Quality Assurance, a weekly internal Stem Trol QC was performed (Beckman Coulter) and Netcells participated in a quarterly External Quality Assurance Program of the RCPA (Royal College of Pathologist of Australia, Norhmead, Australia).

The UCB was analyzed with the flow cytometer and CXP software. A UCB panel was created consisting of 3 CXP stem cell protocols. Each protocol consisted of a series of 9 histograms, regions and gating strategies as recommended in the ISHAGE guideline for CD34+ HSC cell determination by flow cytometry (Sutherland et al., 1996) as indicated in Figure 5. This protocol is approved by the European Commission:

1. *Histogram 1 as FL1 CD45-FITC vs. Side Scatter (SS)* – is gated on all viable cells excluding the fluorospheres. The region includes all CD45+ leukocytes and excludes platelets, red blood cells and debris. The amorphous region includes all lymphocytes (bright CD45+ and low side scatter (SS)).
2. *Histogram 2 as FL2 CD34-PE vs. Side Scatter* - displays events of all viable leukocytes excluding fluorospheres. The region includes all viable CD34+ HSC events.
3. *Histogram 3 as FL1 CD45-FITC vs. Side Scatter* – displays viable CD34+ HSC and leukocyte events. The region includes al $CD45^{DIM}$ events.
4. *Histogram 4 as Forward Scatter (FS) vs. Side* Scatter – displays viable leukocytes and CD34+ HSC and $CD45^{DIM}$ events. The region includes all events with intermediate SS and intermediate to high FS i.e. CD34+ HSC.
5. *Histogram 5 as FL1 CD45-FITC vs. FL2 CD34-PE* – the amorphous region includes Stem Count fluorospheres and doublets. The listgate excludes negative events during acquisition to allow analysis of 75,000 CD45+ events. The histogram is ungated to include all events. The second quarter of the Quadstat regions includes all CD34+ HSC and CD45+ events.
6. *Histogram 6 as Forward vs. Side Scatter* - displays viable leukocyte events and the region includes all lymphocytes. This gating strategy verifies that the FS and SS gain parameters are properly set.
7. *Histogram 7 as Time vs. Side Scatter* – displays events from the Stem Count Fluorosphere singlets and determines that singlets accumulate homogenously and constantly over time. The CAL region includes the Stem Count Fluorospheres events.
8. *Histogram 8 as FL4 7-AAD viability dye vs. Side Scatter* – displays all viable cells and the region includes viable leukocytes (7-AAD viability dye negative events).
9. *Histogram 9 as CD34-PE vs. 7-AAD* – is gated on all CD34+ HSC events and the region includes viable CD34+ HSC cells.

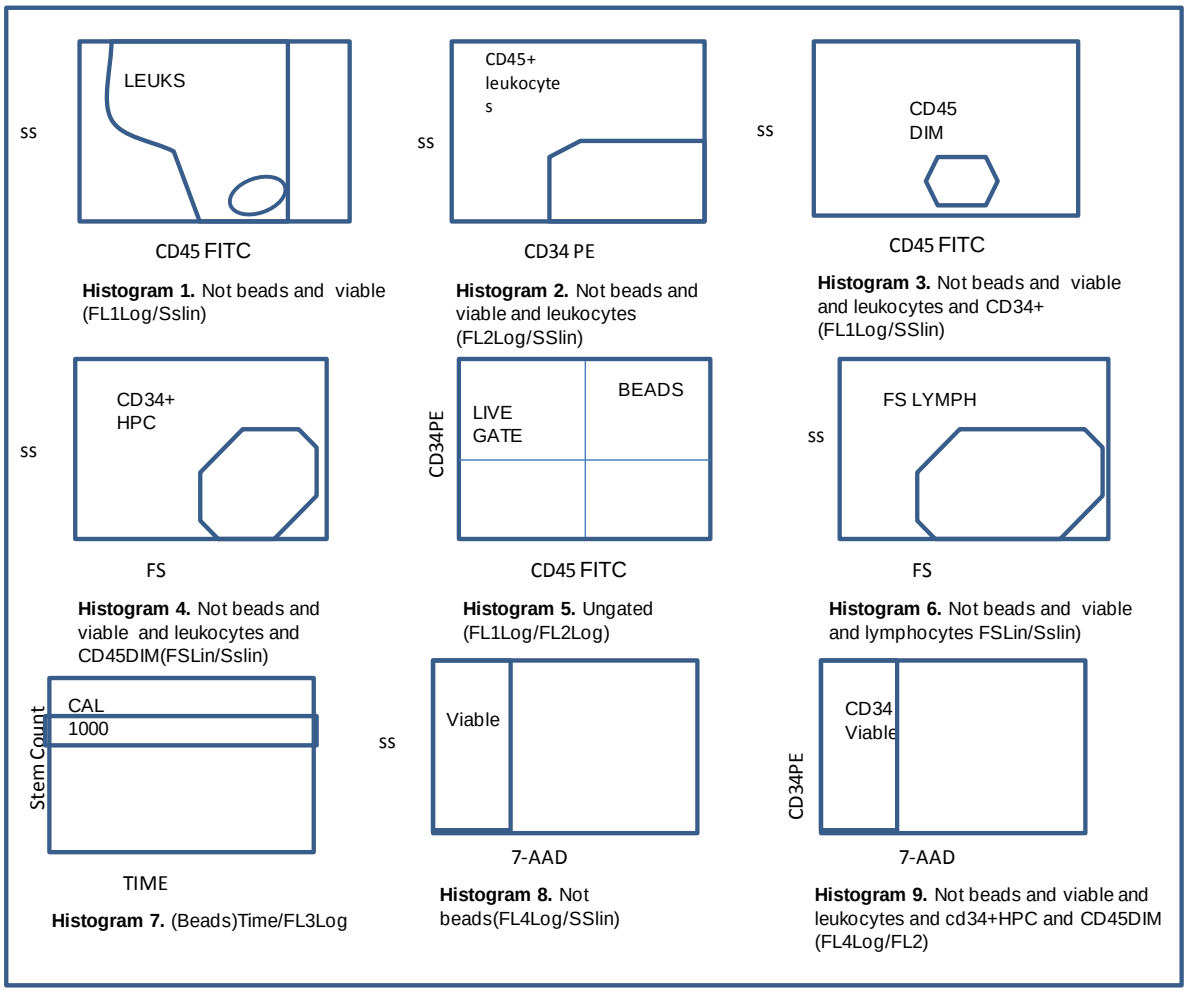

Figure 5: Histograms from the HPC protocol used for stem cell and leukocyte analysis, according to the ISHAGE standards. (Drawing by D Thompson).

3.4.4 Flow cytometer acquisition

Prior to acquisition, the specimens were vortexed and the single platform method was used for analysis. According to the ISHAGE Protocol, a minimum of 75,000 CD45+ events must be acquired for each tube with the Flow Cytometer in order for the analysis to be valid. The calibration factor as obtained from the fluorospheres reagent package insert was entered in the CAL region of the acquisition protocol for absolute count determination. A minimum of 1000 fluorospheres events must be acquired in order for the flow cytometer to calculate the absolute stem count.

3.4.5 Calculation of CD34+ HSC HPC and leukocyte count and viability

The absolute count of viable CD34+ HSC HPC is reported as the statistical result of the region of Histogram 4 as indicated in figure 3. The absolute CD34+ HSC count (cell/ml) is calculated as the average result of the duplicate tests minus the isoclonic control value. The count was acceptable if the result of each duplicate test was within 10% of the calculated mean value. The isoclonic control value must be less than 10% of the average CD34+ HSCHPC Count. CD34+ HSC HPC viability (%) was determined as the average value of the statistical results of the region in Histogram 9 from the duplicate test tubes.

The Absolute Count (cell/ml) of viable leukocytes is given as the statistical result of the rectangular region of histogram 1 and leukocyte viability as the statistical result of the region in histogram 8. The average value of the duplicate and isoclonic tests for the statistical results was reported as the leukocyte absolute count and viability.

3.5 STATISTICAL ANALYSIS

For all statistical analysis, STATA version 10 software was used. As some parameters have large numbers (like cell/ml and total cell count) it resulted in Standard deviation (SD) values over a very big range. The Geometric mean, SD and 95% Confidence Interval were therefore used to determine the findings for the CD34+ HSC and CD45+ cells/ml and total cell count, collection volume, processing volume, cell viability, CD45+: CD34+ HSC ratio, maternal age and gender. Descriptive statistics are presented for parameters influenced by maternal (age), neonatal (gender) and physical (time and temperature) factors.

For the retrospective study, data for these parameters were obtained for 913 UCB collections. Statistical Power calculation was performed to determine whether sufficient samples were included in the sample size for the post hoc comparative retrospective study. Statistical formula:

Power= $1 - \Phi(1.64 - \frac{T\sqrt{n}}{\sigma D})$

The power value for a two tail non directional hypothesis for the sample size of 913 units was 0.855. This concluded that the study was powered by a sufficient number of samples. The same parameters were tested for the temperature/time study.

The correlation coefficient is used to test the effect of infant gender, maternal age, storage temperature and period of storage on UCB collection volume, stem cell and leukocyte ratio, cell count and viability. Positive correlation $0 \leq r \leq +1$ indicates a positive linear increasing relationship between the two variables. Correlation > 0.8 indicates a strong correlation while r < 0.5 of a weak correlation. Negative correlation ($-1 \leq r \leq 0$) is the measurement of relationship when one variable increase the other variable will decrease. No correlation (r close to 0) occurs when there is a random nonlinear relationship between the two variables.

The null hypothesis that maternal age and infant gender, storage temperature and storage time has no affect on the parameters was tested by probability testing with a statistical significance level of 5% (two sided).

Variable	**Rationale**
Age	Increased maternal age results in lower cell count and viability.
Gender	Males are more likely to have lower cell counts than females.
Temperature	Storage at 4 °C temperature results in lower cell count and viability than room temperature storage.
Time	Increased storage time causes lower cell count and viability.

CHAPTER 4

RESULTS AND DISCUSSION

The engraftment outcome of UCB transplantation is highly dependent on cell number. It would be useful to predict UCB cell content using information on donor-related variables like maternal age, gender, the influence of storage temperature and time on cord blood before cell processing, thereby reducing costly processing and storage of less useful cord blood units.

SECTION A – RETROSPECTIVE STUDY

4.1 VARIABLES

4.1.1 Maternal age

In this study the maternal age of all 913 mothers at birth was assessed. The mean maternal age was 32.67 years (32.40 - 32.93, 95% Confidence Interval (CI)). The youngest mother was 20.51 years and the oldest 44.82 years (Table 1). The mean maternal age of mothers with female infants was 32.91 years (32.53 - 33.23, 95% CI) which is older than mothers with male offspring with a mean age of 32.53 (32.16 - 32.96, 95% CI).

4.1.2 Neonatal factors

Neonatal factors, such as delivery method, gestational age, infant gender and birth weight have been shown in previous studies to influence quantity and quality of stem cells and leukocytes (McGuckin et al., 2007). From the sample population of 913 collections, four-hundred-and ninety-two of collections were from male infants (53.9%) and 421 collections (46.1%) were from female infants.

4.1.3 Temperature

The mean temperature of 25 UCB collections used for the secondary temperature/time study was 23.4°C (23.2 – 23.6, 95% CI) on arrival at the laboratory.

4.1.4 Time

Time of infant birth and time of processing for all UCB units were recorded. for the secondary study was recorded. Samples were analyzed at exactly 24h and 48h from the time of collection in order to ensure that variability in time of testing did not influence the parameters.

4.2 PARAMETERS

The statistical results for the laboratory analysis on the influence of the variables on the parameters as well as calculated standards for these parameters are outlined in Tables 1 and 2.

4.2.1 UCB collection volume

Previous studies have indicated that maternal factors like maternal age at infant birth have an influence on the stem cell content and quality of cells as well as collection volume. In contrast, research by Jan et al., (2008) indicated that maternal age had no effect on CD45/CD34+ HSC cell counts of UCB collections.

The collection volume includes 35ml ACD-A anticoagulant. The mean volume of all 913 UCB collections at birth from the umbilical vein was 97.84ml ± 25.26 (96.20 – 99.48, 95% CI). In extensive studies done by other UCB laboratories (Roberedo et al., 2000) the mean collection volume was 84.6 ± 23.6 ml (including 35 ml anticoagulant). The collection volume of the UCB units in this study compare well with similar laboratories operating internationally. The correlation coefficient between maternal age and collection volume is r=0.02, indicating a random nonlinear relationship between the two variables as indicated in Figure 6. The p value=0.61 indicates statistically no significant evidence to reject the null

hypothesis. Therefore, in this study maternal age showed no direct positive or negative effect on the collection volume of UCB. This study is therefore consistent with similar research by Jan et al., (2008) and Ballen et al., (2001).

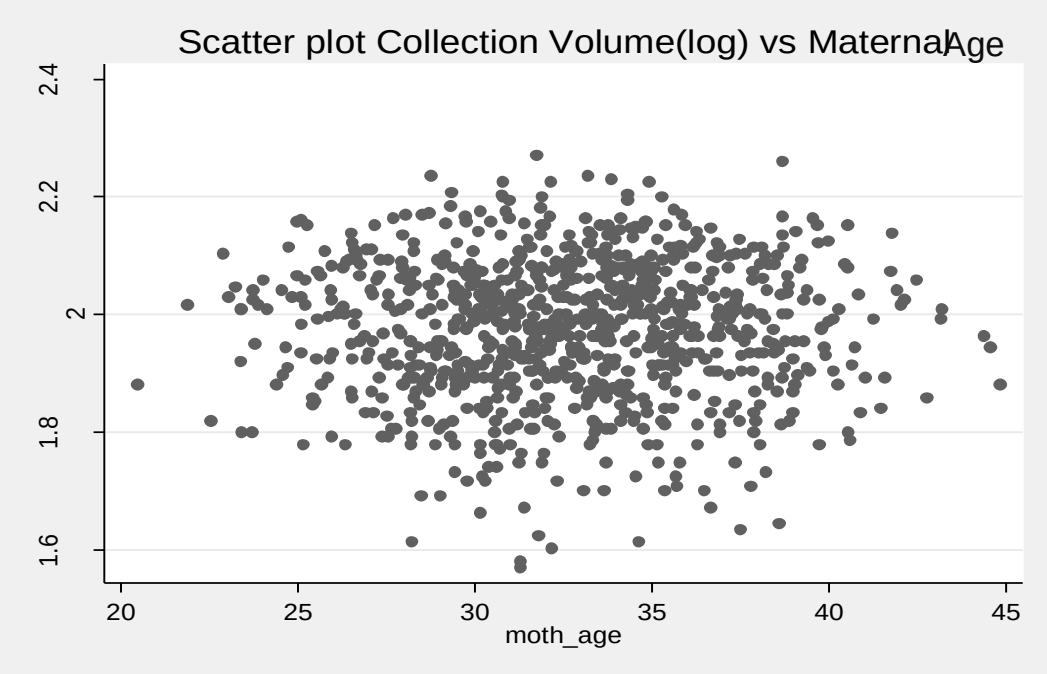

Figure 6: Scatter plot of the correlation between UCB collection volume (pre vol) and maternal age (moth age).

Interestingly, weak to strongly positive correlations between collection volume and leukocytes/ml (r=0.56), leukocyte total count (r=0.65), CD34+ HSC/ml (r=0.57) and CD34+ HSC total count (r=0.59) (Table 2) were observed. Volume, as expected, is clearly associated with cell count, as a larger collection volume will render a higher total cell count (CD34+ HSC/45+cells). As collection volume is used for calculation of total cell count, the increased volume will therefore result in higher total cell counts and therefore a stronger correlation between collection volume and total cell count is observed.

The mean collection volume is higher for females 98.06ml (95.64 – 100.48, 95% CI) compared to 97.64ml (95.39 – 99.89, 95% CI) (Table 1) in male offspring.

Probability testing rendered a p value=0.80, indicating that there is no statistical significant evidence to reject the null hypothesis that gender does not affect collection volume of male and female offspring.

Variable		n	Mean	SD	95% Confidence Interval	P
Maternal age	All	913	32.67		32.40 - 32.93	
	F	421	32.91		32.53 - 33.23	
	M	492	32.53		32.16 - 32.96	
	Minimum		20.51			
	Maximum		44.82			
Collection Volume	All	913	97.84	±25.26	Youngest	
	F	421	98.07	±25.17	95.64 - 100.48	0.80
	M	492	97.64	±25.35	92.10 - 96.6	
Post Volume	All	913	24.92	±0.37	24. 90 - 24.94	
	F	421	24.90	±0.39	24.86 - 24.94	0.12
	M	492	24.94	±0.35	24.91 - 24.97	
Leukocyte/ml	All	913	2.49×10^7		2.34×10^7 - 2.63×10^7	
	F	421	2.50×10^7		2.31×10^7 - 2.68×10^7	0.90
	M	492	2.48×10^7		2.25×10^7 - 2.70×10^7	
Leukocyte Total Count	All	913	6.69×10^8		6.33×10^8 - 7.05×10^8	
	F	421	6.78×10^8		6.34×10^8 - 7.22×10^8	0.64
	M	492	6.61×10^8		6.07×10^8 - 7.16×10^8	
CD34+/ml	All	913	94,557		88,450 - 100,664	
	F	421	91,224		82,110 - 100,339	0.32
	M	492	97,408		89,165 - 105,651	
CD34+Total Count	All	913	2.53×10^6		2.38×10^6 - 2.68×10^6	
	F	421	2.46×10^6		2.23×10^6 - 2.69×10^6	0.39
	M	492	2.59×10^6		2.39×10^6 - 2.79×10^6	
CD45+ Viability	All	913	86.58%	±10.03	85.93 - 87.23	
	F	421	86.24%	±10.19	85.26 - 87.22	0.34
	M	492	86.87%	±9.90	86.00 - 87.75	
CD45+ Viability	All	913	95.99%	±3.97	95.73 - 96.25	
	F	421	95.77%	±4.05	95.38 - 96.16	0.12
	M	492	96.18%	±3.97	95.84 - 96.53	
CD34+:CD45+ ratio	All	913	0.38%	±0.26	0.37 - 0.40	
	F	421	0.36%	±0.26	0.34 - 0.39	0.03
	M	492	0.40%	±0.27	0.37 - 0.42	

Table 1: Means, Standard Deviation (SD) and 95% Confidence Interval for parameters of the retrospective study. Probability testing for the influence of gender and maternal age on parameters. All =913, F=Female, M=Male.

	Maternal Age	Collection Volume	Processing Volume	Leukocyte /ml	Leukocyte Total	CD34+ /ml	CD34+ HSC Total
Maternal age	1.0000						
	913						
Collection Volume r	0.0172	1.0000					
p	0.6046						
	913	913					
Processing Volume r	-0.0235	0.2899	1.0000				
p	0.4780	0.0000					
	913	913	913				
Leukocyte /ml r	-0.0230	0.5960	0.5468	1.0000			
p	0.4877	0.0000	0.0000				
	913	913	913	913			
Leukocyte Total r	0.0052	0.6490	0.1227	0.8272	1.0000		
p	0.8751	0.0000	0.0002	0.0000			
	913	913	913	913	913		
CD34+/ml r	-0.0485	0.5663	0.4349	0.7611	0.6151	1.0000	
p	0.1431	0.0000	0.0000	0.0000	0.0000		
	913	913	913	913	913	913	
CD34+ Total r	-0.0332	0.5878	0.1228	0.5953	0.6865	0.9130	1.0000
p	0.3159	0.0000	0.0002	0.0000	0.0000	0.0000	
	913	913	913	913	913	913	913
CD45+ Viability r	0.0049	0.2773	0.2344	0.4351	0.3931	0.2936	0.2392
p	0.8834	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	913	913	913	913	913	913	913
CD34+ Viability r	-0.0593	-0.0203	0.1709	0.0835	-0.0261	0.1130	0.0395
p	0.0734	0.5404	0.0000	0.0116	0.4307	0.0006	0.2334
	913	913	913	913	913	913	913
CD34+ HSC/45+ Ratio r	∓0.0378	0.1914	0.0606	0.0971	0.0495	0.6298	0.6712
p	0.2538	0.0000	0.0671	0.0033	0.1353	0.0000	0.0000
	913	913	913	913	913	913	913

Table 2: Correlation (r) between maternal age and parameters. The probability (p) that maternal age has an influence on these parameters.

4.2.2 Post processing UCB volume

The post processing cell volume is very important for the calculation of final cell count of leukocytes and stem cells. Each UCB collection was processed to a final concentrated post processing mean volume of 24.92ml ± 0.37 (24.90 - 24.94, 95% CI). The final volume is independent of maternal age as it was processed by the Sepax machine and there was no correlation between collection and

processed volume. A weakly positive correlation r= 0.29 (Table 2) between collection and processed volume was observed, indicating that a change in collection volume will have a change in processing volume as well. The p value for this is <0.05 indicating that this association is not caused by the collection volume but by chance.

Female infants have a higher collection volume than males with resulting lower post processing volumes in females than male offspring. For post processing volume in female infants, the volume was 24.90ml (24.86 - 24.94, 95% CI) compared to a higher 24.94ml (24.91 - 24.97, 95% CI) in male infants. There is no significant evidence to reject the hypothesis that infant gender has no influence on the post processing UCB volume as the probability value=0.12. This observation is consistent with the fact that males have a higher cell to plasma ratio. Although a higher collection volume is obtained from female offspring, processing of the cord blood results in removal of plasma. In the case of female infants, a higher plasma volume is removed in females compared to male infants, with resulting lower post processing volume in female offspring.

4.2.3 Leukocyte count

Leukocyte count is determined by positioning of the region in the histogram containing all viable cells, to include all CD45+ events and excluding CD45- events (platelets, lysed red blood cells) as indicated in Figure 10 (histogram 1). A region is created around the lymphocyte population (bright CD45+ events). The absolute count of viable leukocytes is given in the statistic printout related to the CD45+ region. The absolute leukocyte mean count (leukocyte/ml) collected from 913 UCB collections was 2.49 x 10^7 cells/ml (2.34 x 10^7 – 2.63 x 10^7, 95% CI). The mean total leukocyte count collected for these collections was 6.69 x 10^8 (6.33 x 10^8 – 7.05 x 10^8, 95% CI). UCB units with a total leukocyte total count of less than 2 x 10^8 cells were excluded from this study and all 913 samples of this study met the minimum leukocyte cell count criteria. An infused nucleated cell count of (1.5 x 10^7/kg - 2.0 x 10^7/kg) (Barker et al, 2003 and Grewal et al., 2003)

is the minimum required cell count internationally for successful transplantation. The mean total nucleated cell count achieved in this study of included UCB units that met the minimum leukocyte criteria, is sufficient for successful transplantation of an infant to medium size adult according to international requirements for leukocyte count.

For the leukocyte/ml and leukocyte total count the correlation coefficients with maternal age were r =-0.02 and r=0.01, indicative of no correlation with maternal age, and that the possible relationships between the variables are random and non linear (Figure 2). The respective p value for leukocyte/ml p=0.49 and leukocyte total count p=0.88 is of no statistical significance to reject the null hypothesis that maternal age has no influence on leukocyte/ml or leukocyte total count. As expected, a strong positive correlation was observed between leukocyte/ml and leukocyte total cell count (r=0.83) but also between leukocyte/ml, CD34+ HSC/ml (r=0.78) and CD34+ HSC total count (r=0.60). These observations conclude that the presence of increased leukocyte cell content is an indication of increased leukocyte total count but also of the presence of an increased stem cell concentration in the UCB collection.

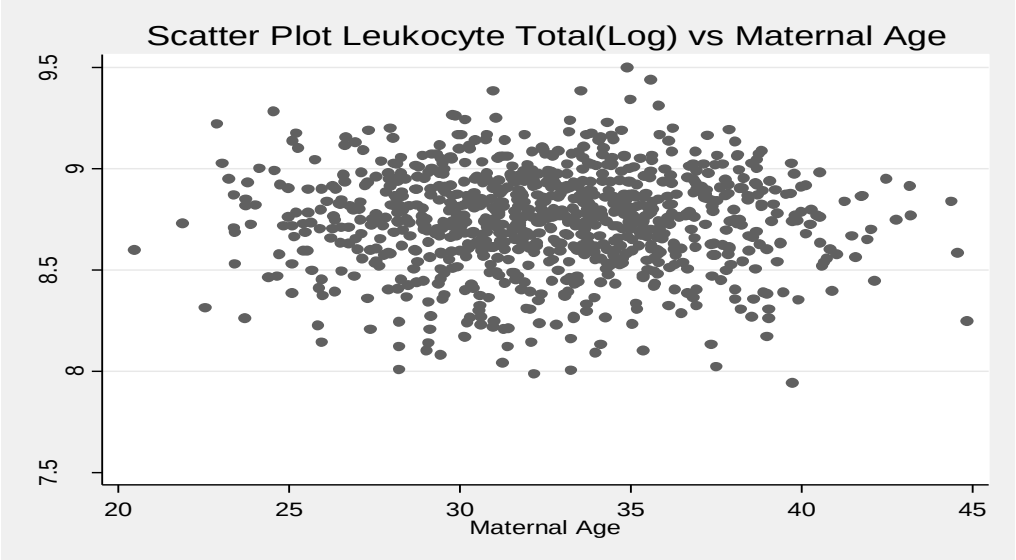

Figure 7: Scatter plot graph of the correlation between UCB total leukocyte (leuk tot) count and maternal age.

Female infants have a mean leukocyte/ml count of 2.50 x 10^7/ml (2.31 x 10^7 - 2.68 x 10^7, 95% CI) and leukocyte total mean count of 6.78 x 10^8 (6.34 x 10^8 - 7.22 x 10^8, 95% CI) (Table 1). The leukocyte/ml for male infants is 2.48 x 10^7 (2.25 x 10^7 - 2.70 x 10^7, 95% CI) with a mean leukocyte total of 6.61 x 10^8 (6.07 x 10^8 - 7.16 x 10^8, 95% CI). Whether gender cause a difference in leukocyte/ml and leukocyte total count was tested with probability testing. The respective p values for leukocyte/ml and leukocyte total count is p=0.90 and p=0.64. This is smaller than 5% significance level to reject the null hypothesis and therefore concludes that sex has no effect on leukocyte/ml and total leukocyte count.

4.2.4 CD34+ HSC count

From figure 10 (histogram 3 of each row) the viable absolute CD34+ HSC HPC count is given as a statistical printout. The events in this region are low side scatter and intermediate forward scatter with dim fluorescence intensity, meeting the ISHAGE criteria for CD34+ HSC stem cell quantification. For the 913 UCB collections, the mean CD34+ HSC/ml was 94,557/ml (88,450 - 100,664, 95% CI). The mean CD34+ HSC total count for all these collections was 2.53 x 10^6 (2.38 x 10^6 - 2.68 x 10^6, 95% CI). From studies is was indicated that to achieve successful engraftment suggests a minimum CD34+ HSC dosage count of 2 x 10^5/kg - 3 x 10^5/kg CD34+ HSC cells (Grewal et al., 2003 and Barker et al.,).

From these guidelines and calculated mean CD34+ HSC count obtained from the study consisting of 913 UCB collections that met the minimum requirements for processing, a child with an estimated weight of 13kg could be successfully transplanted based on CD34+ HSC dosage. It could be accepted that achieved CD34+ HSC cell counts in this study compares well with other international UCB stem cell banks.

There is no correlation between maternal age and CD34+ HSC/ml (r=-0.05) as well as with CD34+ HSC total cell count (r=-0.03). This is clearly illustrated in

the following Figure 8. The calculated p values for CD34+ HSC/ml (p=0.14) and CD34+ HSC total count (p=0.32) also significantly fail to reject the null hypothesis that maternal age does not affect CD34+ HSC/ml and total CD34+ HSC cell count.

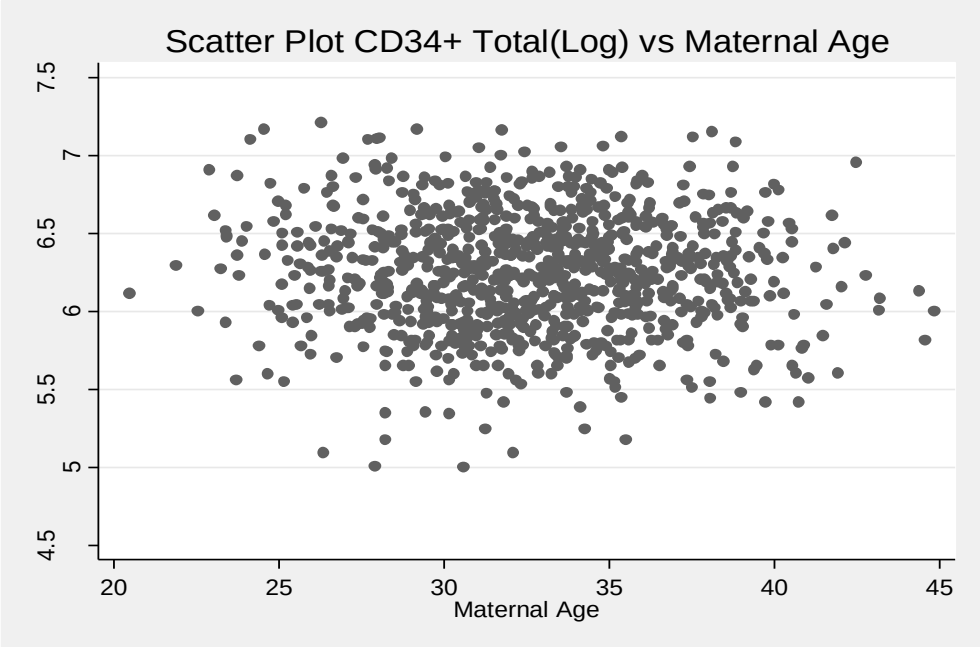

Figure 8: Scatter plot graph of the correlation between UCB CD34+ HSC total (CD34 tot) count and maternal age.

The mean CD34+ HSC/ml for the 421 female infants was 91,224/ml (82,110 – 100,339 95%, CI) compared to a higher CD34+ HSC/ml count in males at 97,408 (89,165 – 105,651, 95% CI) (Table 1). Male infants have a higher mean CD34+ HSC total count of 2.59 x 10^6 (2.39 x 10^6 – 2.79 x 10^6, 95% CI) compared to female infants with a mean CD34+ HSC total count of 2.46 x 10^6 (2.23 x 10^6 - 2.69 x 10^6, 95% CI). In order to determine whether the difference in CD34+ HSC/ml and CD34+ HSC count between males and females is of any significance, the p values respectively was determined, p=0.32 and p=0.39 (Table 1). These values are greater than the 5% significance level and there is no significant evidence to reject the hypothesis of “gender has no effect on stem cell counts” in favor of the alternative hypothesis.

Research by McGukin et al. (2007) concluded that female infants provide greater stem cell numbers than their male counterparts. The opposite trend was observed in this study; however the difference was of no significance.

4.2.5 CD45+ cell viability

The mean CD45+ (TNC) cell viability is 86.58% ± 10.03 (85.93 - 87.23, 95% CI) for the retrospective study collections. The correlation coefficient between CD45+ cell viability and maternal age was 0.01, which concludes that no correlation exists between maternal age and CD45+ cell viability, as illustrated in Figure 9. From the p=0.88 value it is evident that maternal age has no significant influence on CD45+ cell viability.

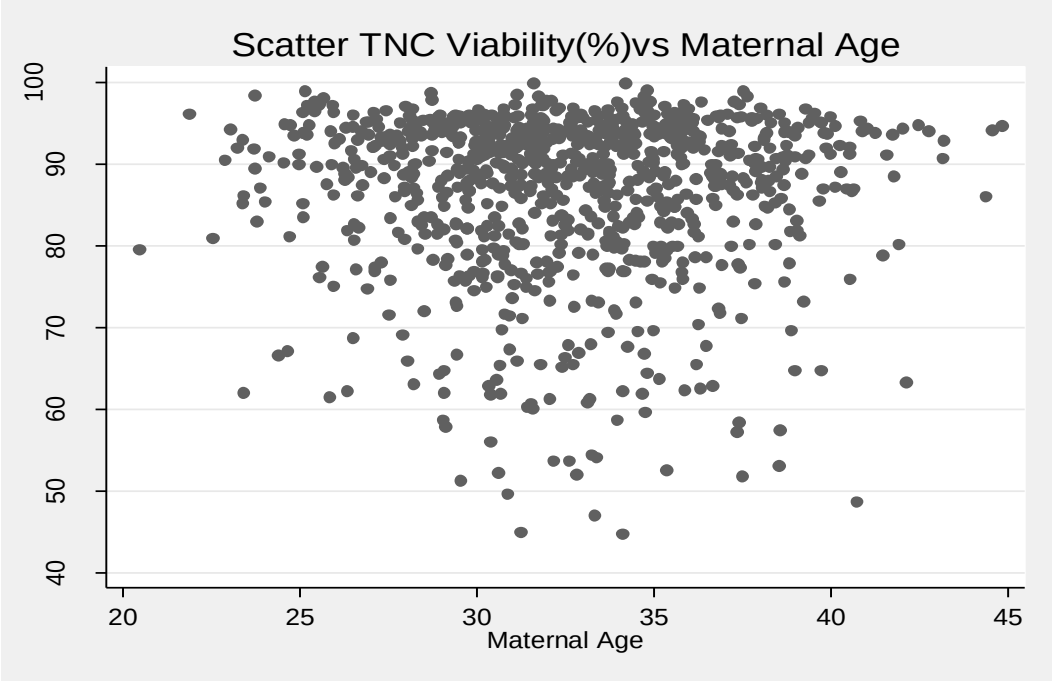

Figure 9: Scatter plot graph of the correlation between maternal age and TNC viability (Viab TNC).

There is a difference in CD45+ cell viability between infants of different genders, thus it is higher in male infants 86.87% ± 9.90 (86.00 – 87.75, 95% CI) than in female infants 86.24% ± 10.19 (85.26 - 87.22, 95% CI). The probability testing for

this parameter renders a p value=0.34, suggesting that the difference between CD45+ viability between female and male infants is of no statistical significance and that infant gender has no effect on leukocyte cell viability.

4.2.6 CD34+ HSC cell viability

The mean CD34+ HSC cell viability for the 913 collected UCB units is 95.99% ± 3.97 (95.73 - 96.25, 95% CI). For the 913 UCB collections, the correlation between CD34+ HSC cell viability and maternal age is r=-0.06, which is a random non linear relationship, that indicates no association between maternal age and CD34+ HSC viability (Figure 10). The p value=0.07 fails to reject the hypothesis that maternal age has no affect on CD34+ HSC viability. Difference in cell viability that may occur is caused by chance and not as a result of maternal age.

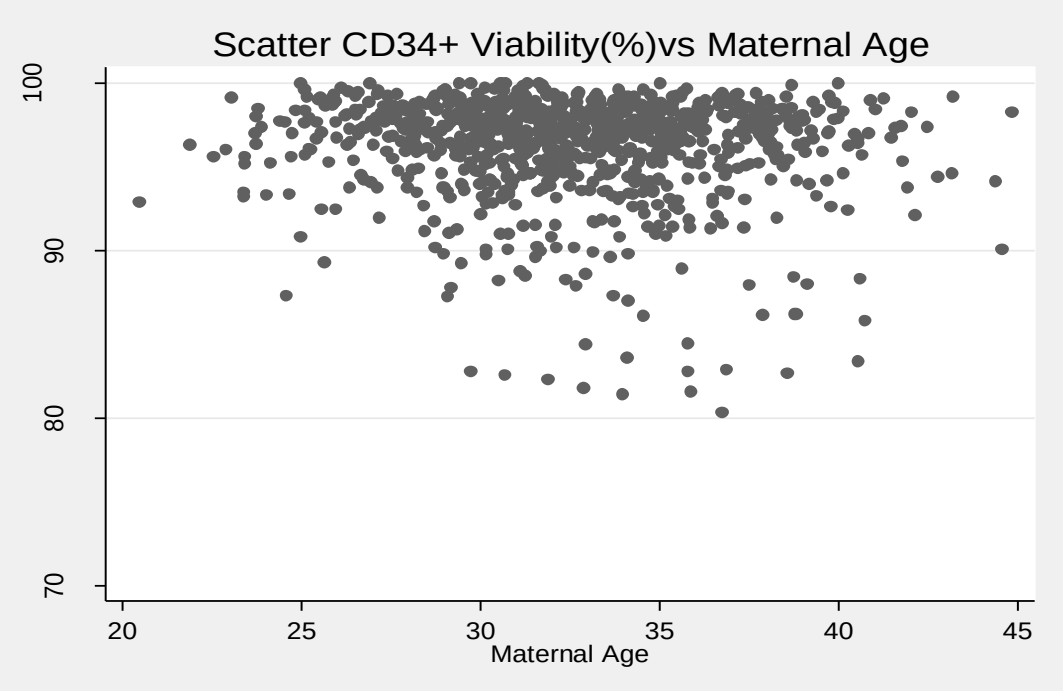

Figure 10: Scatter plot graph of the correlation between maternal age and CD34+ HSC cell viability (Viab CD34).

The mean CD34+ HSC cell viability for female infants is 95.77% ± 4.05 (95.38% – 96.16%, 95% CI). This is lower than the mean CD34+ HSC viability in males 96.18% ± 3.97 (95.84 - 96.53, 95% CI). By probability testing p=0.12, it was determined that this observed difference of higher CD34+ HSC viability in male infants, is not statistically significant and therefore fails to prove that gender influence CD34+ HSC cell viability.

4.2.7 CD34+ HSC:CD45+ ratio

The mean ratio of CD34+ HSC:CD45+ cell count for the 913 UCB collections is 0.38% ± 0.26 (0.37 - 0.40, 95% CI). There is no relationship r=-0.04 (Figure 11) between maternal age and CD45+/CD34+ HSC cell ratio. The hypothesis that maternal age does not influence cell ratios could not be rejected as the p value=0.25 which is significant higher than 5%. A positive correlation between cell ratio and CD34+ HSC/ml (r=0.63) and CD34+ HSC total cell count (r=0.67) was calculated. This is an obvious conclusion as an increase in CD34+ HSC/ml or CD34+ HSC total count will result in an increased ratio of CD34+ HSC:CD45+ cells.

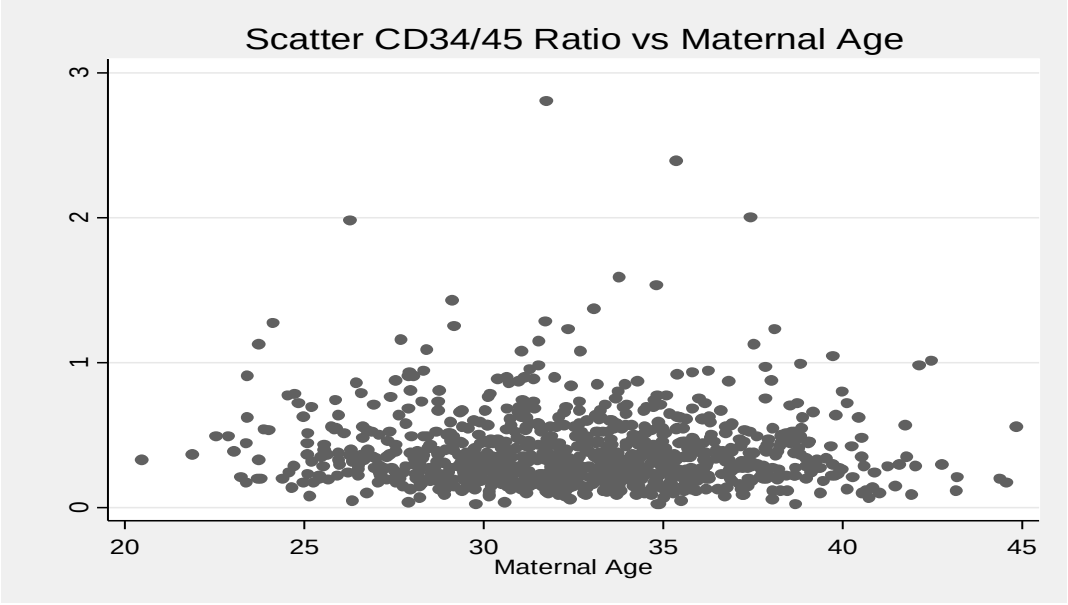

Figure 11: Scatter plot graph of the correlation between maternal age and CD34+ HSC/CD45+ cell ratio.

The ratio of CD34+ HSC cells to CD45+ cells in female infants has a mean value of 0.36% ± 0.26 (0.34 - 0.39, 95% CI). This ratio is higher in male infants with a mean ratio of 0.40% ± 0.26 (0.37 - 0.42, 95% CI). The calculated p value=0.03 indicates a significant difference between male and female CD34+ HSC:CD45+ cell ratios. Although it cannot be proven that male offspring have higher cell counts than females, the higher CD34+ HSC and lower leukocyte counts in males result in a higher CD34+ HSC to CD45+ ratio in males that was statistically proven. It is accepted that the difference in cell count is a result of gender influence on this parameter. This concludes that the ratio of CD34+ HSC:CD45+ cells are higher in male offspring than females.

SECTION B – TIME/TEMPERATURE STUDY

4.3 THE EFFECT OF TIME/TEMPERATURE ON UCB

Results for variable temperature and time influences on parameters obtained from this analysis are presented in Tables 3 to Tables 18.

4.3.1 CD34+ HSC cell count

The mean CD34+ HSC/ml and CD34+ HSC total cell count results obtained from the 25 processed and unprocessed UCB units studied in the time/temperature study, are illustrated in Table 3. The unprocessed UCB units have a higher mean CD34+ HSC/ml and CD34+ HSC total cell count when the samples are stored at RT compared to 4°C storage over 24h and 48h. It would therefore appear as if storage of unprocessed stem cells at RT is better than 4°C storage. UCB collections that were processed one hour after collection and stored for 24h, 48h at RT and 4°C showed higher mean CD34+ HSC cells/ml and total CD34+ HSC cell count stored at RT than at 4°C storage (Table 3).

UCB Sample Type	Storage Variable (Temperature (RT/°C)/Time (h))	Mean CD34+ cells/ml	Mean CD34+Total Cell Count
Unprocessed UCB	24h, 4°C	20920	
	24h, RT	21760	
	48h, 4°C	20960	
	48h, RT	21560	
Unprocessed UCB	24h, 4°C		2.23×10^6
	24h, RT		2.32×10^6
	48h, 4°C		2.24×10^6
	48h, RT		2.31×10^6
Processed UCB	24h, 4°C	79160	
	24h, RT	79960	
	48h, 4°C	75720	
	48h, RT	76400	
Processed UCB	24h, 4°C		1.98×10^6
	24h, RT		2.0×10^6
	48h, 4°C		1.89×10^6
	48h, RT		1.96×10^6

Table 3: mean CD34+ HSC cells/ml and mean CD34+ HSC total cell count for unprocessed and processed UCB stored at 4°C and RT (room temperature) for 24hour (h) and 48h.

4.3.1.1 The effect of variable storage temperature on CD34+ HSC cell count

The correlation factors between different storage temperature (4°C and RT) over 24h and 48h and CD34+ HSC/ml and CD34+ HSC total cell count of unprocessed UCB units is (r=0.98, r=0.99, r=0.99, r=0.99, (Table 4). The correlation parameter is highly positive and very close to 1. The effect of the 4°C temperature storage on the cells will almost be similar than the effect of RT storage on the cells. This indicates 4°C storage is not necessary better than RT storage. To determine whether differences that occur are due to variable temperature storage or due to chance, 5% significance levels probability testing was calculated. P values of p=0.09, p=0.15, p=0.08, p=0.11 (Table 4) were obtained. There is statistically insufficient evidence to prove that 4°C or RT storage influence CD34+ HSC/ml and CD34+ HSC total cell count.

UCB Sample Type	Storage Time (h)	Storage Variable (Temperature (RT/°C))	Correlation Parameter (r)	5% Significance Level Probability (p)
Unprocessed UCB (CD34+ cells/ml)	24h	4°C	0.98	0.09
	24h	RT		
	48h	4°C	0.99	0.15
	48h	RT		
Unprocessed UCB (CD34+ total cell count)	24h	4°C		
	24h	RT	0.99	0.08
	48h	4°C		
	48h	RT	0.99	0.11
Processed UCB Unprocessed UCB (CD34+ cells/ml	24h	4°C		
	24h	RT	1.00	0.40
	48h	4°C		
	48h	RT	1.00	0.42
Processed UCB (CD34+ total cell count)	24h	4°C		
	24h	RT	1.00	0.40
	48h	4°C		
	48h	RT	1.00	0.43

Table 4: Temperature is the storage variable and UCB is stored at 4°C and RT (room temperature). The correlation parameter (r) between the variable storage temperatures and CD34+ HSC cell/ml and CD34+ HSC total cell count is determined. The probability that influences caused by different storage temperature on CD34+ HSC cell/ml or CD34+ HSC total cell count is tested at 5% significance level.

There is perfect correlation (r=1) between CD34+ HSC/ml and CD34+ HSC total cell count of processed cells stored at variable temperature (RT and 4°C) for either 24h and 48h periods. The p values for the different combinations as shown in Table 4 (p=0.40, p=0.42, p=0.40, p= 0.43) is and greater than 5% significance. The null hypothesis that there is no difference between cell count of cells stored at 4°C or RT could not be failed.

4.3.1.2 The effect of variable storage time on CD34+ HSC cell count

The effect of different storage times (24h and 48 h) on CD34+ HSC/ml and total count were compared on processed and unprocessed UCB (Table 5). The correlations for all CD34+ HSC/ml and CD43+ total cell count of unprocessed

UCB with variable storage period were close to 1 (r=0.99, r=0.98) and (r=0.99, r=0.99), indicating a strong positive correlation. Probability testing rendered values of (p=0.91, p=0.66, p=0.79, p=0.85) p=0.07, p=0.06) and is therefore statistically insignificant.

UCB Sample Type	Storage Temperature (4°C/RT)	Storage Variable (Time 24h/48h)	Correlation Parameter (r)	5% Significance Level Probability (p)
Unprocessed UCB (CD34+ cells/ml)	4°C	24h	0.99	0.91
	4°C	48h		
	RT	24h	0.98	0.66
	RT	48h		
Unprocessed UCB (CD34+ total cell count)	4°C	24h		
	4°C	48h	0.99	0.79
	RT	24h		
	RT	48h	0.99	0.85
Processed UCB Unprocessed UCB (CD34+ cells/ml	4°C	24h		
	4°C	48h	0.98	0.07
	RT	24h		
	RT	48h	0.98	0.06
Processed UCB (CD34+ total cell count)	4°C	24h		
	4°C	48h	0.98	0.07
	RT	24h		
	RT	48h	0.98	0.06

Table 5: Storage time is the storage variable (24h and 48h) and UCB is stored at 4°C and RT (room temperature). The correlation parameter (r) between different storage periods (24h and 48h) and CD34+ HSC cell/ml and CD34+ HSC total cell count is determined. The probability that influences caused by different storage period on CD34+ HSC cell/ml or CD34+ HSC total cell count is tested at 5% significance level.

The correlation parameters between variable storage time (24h and 48h) and processed UCB CD34+ HSC/ml and CD34+ HSC total cell count are the same, r = 0.98. Probability testing for CD34+ HSC cells/ml and CD34+ HSC total cell count of processed cells are the same p=0.07 and 0.06. This observation concludes that variable storage period did not have an effect on processed CD34+ HSC cell count.

4.3.2 CD45+ cell count

An assessment of the mean CD45+cells/ml and CD45+ total cell count for unprocessed and processed UCB was performed. The mean count is higher when cells were stored at 4°C than RT storage, independent of the storage period (Table 6). In contrast, processed UCB showed higher mean CD45+/ml and CD45+ total cell counts when the cells were stored at RT for 24h and 48h periods.

UCB Sample Type	Storage Variable (Temperature (RT/°C)/Time (h))	Mean CD45+/ml	Mean CD45+Total Cell Count	Correlation Parameter (r)	5% Significance Level Probability (p)
Unprocessed UCB	24h, 4°C	7.15×10^6		0.87	0.68
	24h, RT	7.05×10^6			
	48h, 4°C	5.85×10^6		0.82	0.97
	48h, RT	5.84×10^6			
Unprocessed UCB	24h, 4°C		7.56×10^8	0.92	0.58
	24h, RT		7.41×10^8		
	48h, 4°C		6.22×10^8	0.80	0.50
	48h, RT		5.96×10^8		
Processed UCB	24h, 4°C	2.09×10^7		0.97	0.07
	24h, RT	2.15×10^7			
	48h, 4°C	1.69×10^7		0.74	0.24
	48h, RT	1.81×10^7			
Processed UCB	24h, 4°C		5.21×10^8	0.97	0.07
	24h, RT		5.38×10^8		
	48h, 4°C		4.22×10^8	0.74	0.24
	48h, RT		4.52×10^8		

Table 6: The mean CD45+cells/ml and mean CD45+ total cell count for unprocessed and processed UCB stored at 4°C and RT (room temperature) for 24hour (h) and 48h as obtained from the temperature/time study.

4.3.2.1 The effect of variable storage temperature on CD45+ cell count

The correlation between CD45+ cells/ml and CD45+ total cell count of unprocessed UCB and temperature is 87%, 82%, 92% and 80% similar (Table 7). The p values as illustrated in Table 7 indicate that variable temperature storage has no significant effect on CD45+ cells/ml and CD45+ total cell count.

Variable storage temperature showed strong correlation with CD45+/ml and CD45+ total count of processed UCB Table 7, (r=0.97, r=0.74). The calculated p values p=0.07, p=0.24, p=0.07, p=0.24 for CD45+/ml and CD45+ total count conclude that variable storage temperature does not significantly affect leukocyte cell count.

UCB Sample Type	Storage Time (h)	Storage Variable (Temperature (RT/°C))	Correlation Parameter (r)	5% Significance Level Probability (p)
Unprocessed UCB (CD45+ cells/ml)	24h	4°C		
	24h	RT	0.87	0.68
	48h	4°C		
	48h	RT	0.82	0.97
Unprocessed UCB (CD45+ total cell count)	24h	4°C		
	24h	RT	0.92	0.58
	48h	4°C		
	48h	RT	0.80	0.50
Processed UCB Unprocessed UCB (CD45+ cells/ml	24h	4°C		
	24h	RT	0.97	0.07
	48h	4°C		
	48h	RT	0.74	0.24
Processed UCB (CD45+ total cell count)	24h	4°C		
	24h	RT	0.97	0.07
	48h	4°C		
	48h	RT	0.74	0.24

Table 7: UCB is stored at variable temperature (4°C and RT) for 24h and 48h. The correlation parameter (r) between different storage temperature (4°C and RT) and CD45+ cell/ml and CD45+ total cell count is determined. Probability testing was calculated to determine whether influences caused by different storage period on CD45+ cell/ml or CD45+ total cell count was significant.

4.3.2.2 The effect of variable storage time on CD45+ cell count

The effect whether different storage periods, 24h and prolonged storage for another 24h influenced processed and unprocessed CD45+cells/ml and CD45+ total cell count was compared. As indicated in Table 8, r=0.91, r=0.83, r=0.66, r=0.91, r=0.91, r=0.91 there were good correlation (r>0.50) between variable storage period of CD45+ cells/ml and CD45+ total count of processed and

unprocessed UCB stored at RT or 4°C. Probability testing indicated that differences in CD45+ cells stored for 24h and 48h were statistically significant. Increased storage period has an effect on CD45+ cells/ml and total CD45+ cell leukocyte count of processed and processed UCB.

UCB Sample Type	Storage Temperature (4°C/RT)	Storage Variable (Time 24h/48h)	Correlation Parameter (r)	5% Significance Level Probability (p)
Unprocessed UCB (CD45+ cells/ml)	4°C	24h		
	4°C	48h	0.91	0.00
	RT	24h		
	RT	48h	0.83	0.00
Unprocessed UCB (CD45+ total cell count)	4°C	24h		
	4°C	48h	0.95	0.00
	RT	24h		
	RT	48h	0.81	0.00
Processed UCB Unprocessed UCB (CD45+ cells/ml	4°C	24h		
	4°C	48h	0.66	0.00
	RT	24h		
	RT	48h	0.91	0.00
Processed UCB (CD45+ total cell count)	4°C	24h		
	4°C	48h	0.91	0.00
	RT	24h		
	RT	48h	0.91	0.00

Table 8: CD45+ cell/ml and CD45+ total cell count of processed and unprocessed UCB stored at 4°C and RT (room temperature) are tested at variable storage period (24hour (h) and 48h).The correlation parameter (r) between different storage periods (24h and 48h) and CD34+ HSC cell/ml and CD34+ HSC total cell count was determined. Significance of influences caused by different storage periods on CD34+ HSC cell/ml or CD34+ HSC total cell count is tested with probability testing.

4.3.3 CD34+ HSC cell viability

The mean percentage viability of stem cells stored at RT and 4°C for 24h and 48h are listed in Table 9. The mean percentage CD34+ HSC cell viability is greater than 89% for all storage conditions (RT, 4°C, 24h, 48h). CD34+ HSC cells are very tolerant to factors that could influence cell viability. Unprocessed CD34+ HSC cells stored for 24h have higher mean cell viability (98.10% >

97.90%) when stored at RT than at 4°C. Interestingly, processed CD34+ HSC cells stored for 48h are more viable at 4°C storage than RT (98.00% > 97.20%). Processed CD34+ HSC cells are marginally more viable when stored at 4°C over 24h and 48h (96.40% > 96.20%) and (95.80% >95.60%) than at RT.

UCB Sample Type	Storage Time (h)	Storage Temperature (RT/°C))	Mean % Cell Viability
Unprocessed UCB (CD34+ cells/ml)	24h	4°C	97.90%
	24h	RT	98.10%
	48h	4°C	98.00%
	48h	RT	97.20%
Processed UCB (CD34+ total cell count)	24h	4°C	96.40%
	24h	RT	96.20%
	48h	4°C	89.50%
	48h	RT	91.10%

Table 9: The mean % cell viability of CD34+ HSC cells/ml and mean CD34+ HSC total cell count for unprocessed and processed UCB stored at 4°C and RT (room temperature) for 24hour (h) and 48h.

7AAD is a membrane permeable viability dye, meaning that as the cells become necrotic the membrane becomes more permeable for 7AAD. The dye intercalates with the cell DNA and the cells will show fluorescence. Figure 12, histogram 4 of each row, show the stem cell viability. The first quadrant of the histogram is 7AAD negative. Cell populations in this quadrant did not absorbed 7AAD and are therefore viable. As indicated in the histogram the more right the CD34+ HSC cell population moves on the 7AAD axis of the histogram the more fluorescence is observed, which indicates cell mortality. Dead cells will be observed in the most right quadrant. Cell populations in the second and third quadrants shows increased fluorescence and are cells in process of dying. The effect of different storage temperature and period of storage can clearly be seen from figure 12 (histogram 4 in all rows). The shift towards the right side on the 7AAD axis is very small, indicating that CD34+ HSC cell viability did not decreased much. This observation confirms the tolerance of stem cells to different storage conditions.

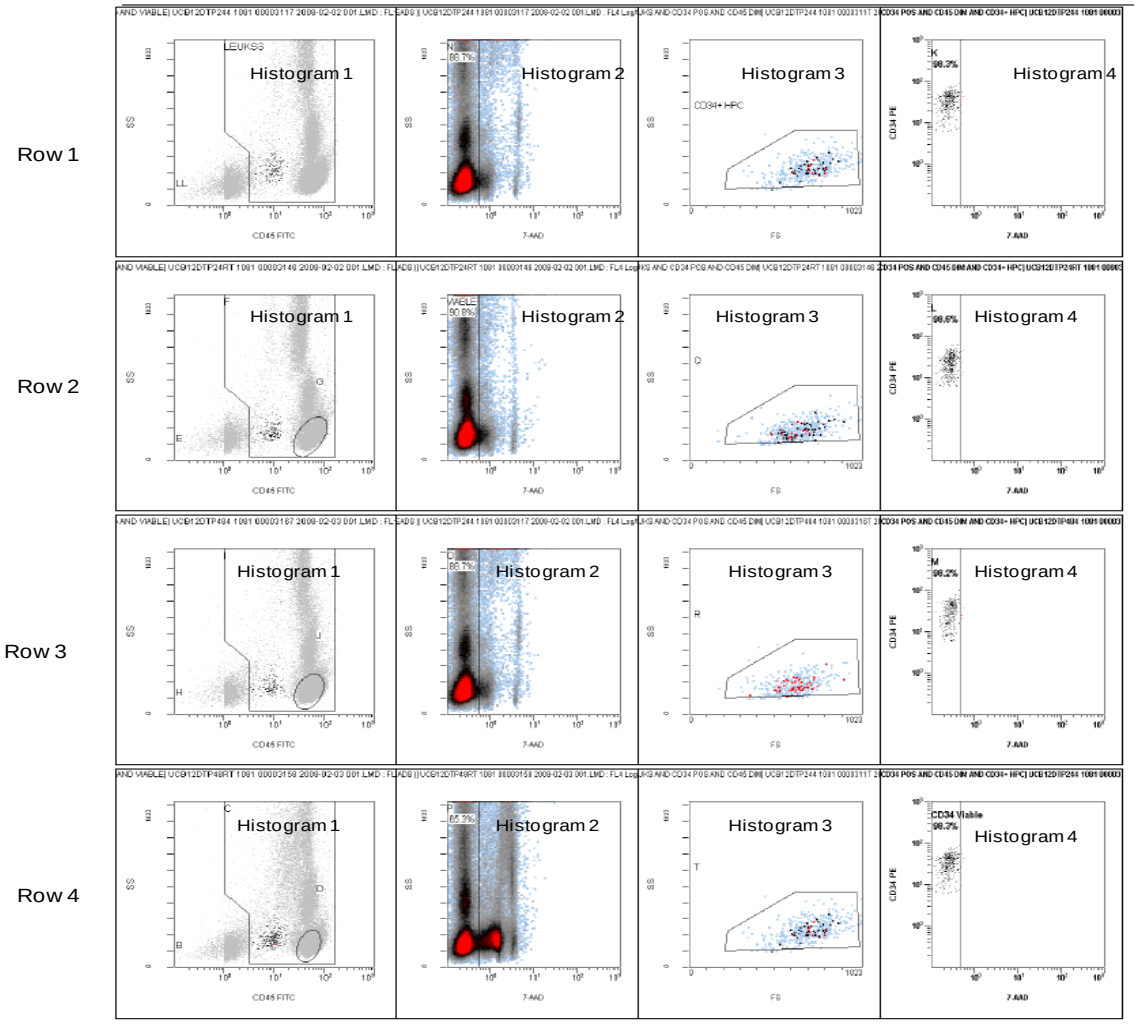

Figure 12: Leukocyte and stem cell count and viability of UCB. Row 1: UCB stored at 24h and 4ºC.Row 2: UCB stored at 24h and RT. Row 3: UCB stored at 48h and 4ºC. Row 4: UCB stored at 48h and RT. Histogram 1 (each row) = Absolute leukocyte cell count. Histogram 2 (each row) = Leukocyte viability. Histogram 3 (each row) = Absolute CD34+ HSCHPC count. Histogram 4 (each row) = CD34+ HSC viability.

4.3.3.1 The influence of variable storage temperature on CD34+ HSC cell viability

The influence of variable storage temperature on the viability of CD34+ HSC cell/ml and CD34+ HSC total cell count of processed and unprocessed UCB units were tested. Weak correlations r=0.39, r=0.17 (Table 10) were observed between unprocessed UCB and variable storage temperature. This indicates that

the effect caused by different storage temperatures is differently tolerated by the cells. The observation is statistically insignificant (p=0.62, p=0.06).

UCB Sample Type	Storage Time (h)	Storage Variable (Temperature (RT/°C))	Correlation Parameter (r)	Significance Level Propability (p)
Unprocessed UCB (CD34+ cells/ml)	24h	4°C	0.39	0.62
	24h	RT		
	48h	4°C	0.17	0.06
	48h	RT		
Processed UCB (CD34+ total cell count)	24h	4°C	0.74	0.69
	24h	RT		
	48h	4°C	0.86	0.65
	48h	RT		

Table 10: The effect of variable storage temperature (4°C and RT) on the viability of CD34+ HSC cell/ml and CD34+ HSC total cell count of processed and unprocessed UCB grafts.

The correlation parameter (r) between different storage temperatures (4°C and RT) and viability of CD34+ HSC cell/ml and CD34+ HSC total cell count is calculated. The probability that influences caused by different storage temperature on the viability of CD34+ HSC cell/ml and CD34+ HSC total cell count is tested.

Processed UCB shows good correlation with variable storage temperatures (4°C and RT) and cell viability (r=0.86 and 0.74). Whether the differences that occur are due to variable temperature storage were tested (p=0.69, p=0.65) and was found to be statistically insignificant.

4.3.3.2 The effect of variable storage period on CD34+ HSC cell viability

To determine the influence of variable time storage on stem cell viability, the correlation (Table 11) between viability of CD34+ HSC stem cells from processed and unprocessed and storage period was calculated. The associations (r=0.54, r=0.66, r=0.61) are relatively strong, except no correlation (r=0) was observed for unprocessed stem cell stored at 4°C for variable time 24h and 48h. As indicated

in Table 11 differences in cell viability as a result of variable storage periods was statistically insignificant.

UCB Sample Type	Storage Temperature (4°C/RT)	Storage Variable (Time 24h/48h)	Correlation Parameter (r)	5% Significance Level Probability (p)
Unprocessed UCB (CD34+ cells/ml)	4°C	24h	0.54	0.79
	4°C	48h		
	RT	24h	0.00	0.33
	RT	48h		
Processed UCB (CD34+ total cell count)	4°C	24h	0.66	0.09
	4°C	48h		
	RT	24h	0.61	0.24
	RT	48h		

Table 11: UCB stored at variable storage periods for 24h and 48h was tested to determine the correlation (r) between CD34+ HSC cells of processed and unprocessed UCB and time. .To determine whether observed influences caused by variable storage time was significant, probability testing was correlation parameter between different storage periods of CD34+ HSC cell/ml and CD34+ HSC total cell count is determined.

4.3.4 CD45+ cell viability

The mean percentage viabilities for leukocytes stored at RT and 4°C for 24h and 48h are listed in Table 12. There is an estimated 10% decrease in leukocyte viability when cells were stored for longer than 24h up to 48h. The mean leukocyte viability from processed and unprocessed UCB is higher (91.10>89.50)% and (91.50 > 89.90)% when cells are stored at RT compared to 4°C (81.02 > 77.40)% storage either for 24h or 48h. The only exception is CD45+ processed cells stored at 4°C for 48h (81.20 > 78.00) %, when 4°C storage has better results than RT storage.

UCB Sample Type	Storage Time (h)	Storage Temperature (RT/°C))	% Cell Viability
Unprocessed UCB (CD45+ cells/ml)	24h	4°C	89.50%
	24h	RT	91.10%
	48h	4°C	77.40%
	48h	RT	81.02%
Processed UCB (CD45+ total cell count)	24h	4°C	89.90%
	24h	RT	91.50%
	48h	4°C	81.20%
	48h	RT	78.00%

Table 12: The mean % cell viability of CD45+cells/ml and mean CD45+ total cell count for unprocessed and processed UCB stored at 4°C and RT (room temperature) for 24hour (h) and 48h.

4.3.4.1 The effect of variable storage temperature on CD45+ cell viability

Unprocessed CD45+ cells stored at 4°C and RT for 24h have a 0.70 correlation, while these same cells stored for another 24h have 0.75 correlations. The effect of variable storage temperature on CD45+ processed cells stored for 48h was 79.0% (Table 13). There is a random non linear relationship (r=0.06) between CD45+ processed cells stored at RT and 4°C for 24h. . Possible effect of variable storage temperature on leukocyte viability was proven to be statistically insignificant (Table 13). Statistically it was proven that variable storage temperature significantly influence unprocessed CD45+ stored for 48h.

UCB Sample Type	Storage Time (h)	Storage Variable (Temperature (RT/°C))	Correlation Parameter (r)	Significance Level Propability (p)
Unprocessed UCB (CD45+ cells/ml)	24h	4°C	0.70	0.21
	24h	RT		
	48h	4°C	0.75	0.02
	48h	RT		
Processed UCB (CD45+ total cell count)	24h	4°C	0.06	0.26
	24h	RT		
	48h	4°C	0.79	0.07
	48h	RT		

Table 13: CD45+cell viability of unprocessed and unprocessed UCB grafts was stored at variable temperatures (4°C and RT).The correlation parameter (r) between different storage temperatures (4°C and RT) and viability of CD45+ cell/ml and CD45+ total cell count is determined and probability testing was done to test significance of influences.

4.3.4.2 The effect of variable storage time on CD45+ cell viability

CD45+ cells of processed and unprocessed UCB were stored at variable storage periods of 24h and 48h. This revealed a correlations of r=0.60, to very poor r=0.14, r=0.14, r=0.43) comparisons between the viability of leukocytes stored for at variable period (Table 14). From this it could be concluded that 24h and 48h periods affect cell viabilities differently, resulting in a non linear comparative relationship. Probability testing (Table 14) presented p values <0.05 significance which is statistically significant. This conclusion is clearly demonstrated by figure 12 (histogram 2 of each row). When leukocytes are stored for 48h compared to 24h storage, a dramatic increase in 7AAD fluorescence towards the right of the 7AAD axis on the histogram is observed. This indicates increased cell death.

UCB Sample Type	Storage Temperatur (4°C/RT)	Storage Variable (Time 24h/48h)	Correlation Parameter (r)	5% Significance Level Probability (p)
Unprocessed UCB (CD45+ cells/ml)	4°C	24h	0.60	0.00
	4°C	48h		
	RT	24h	0.14	0.00
	RT	48h		
Processed UCB (CD45+ total cell count)	4°C	24h	0.14	0.00
	4°C	48h		
	RT	24h	0.43	0.00
	RT	48h		

Table 14: Processed and unprocessed UCB is stored at variable storage periods (24h and 48h). The correlation parameter (r) between different storage periods of processed and unprocessed is determined. The probability that influences caused by different storage time on leukocyte viability is tested at 5% significance level.

4.4 UNPROCESSED VS. PROCESSED UCB

During stem cell processing with the Sepax cell separator, cells are exposed to mechanical effects like speed, centrifugation and gravitational force. On average, 84.5% of CD34+ HSC cells are recovered during processing while 71.3% of leukocytes are recovered irrespective of storage period and temperature of storage of UCB. Nucleated cell loss in this study (29.7%), is consistent with cell loss obtained by other UCB laboratories. Studies by Laroche et al., (2005) reported 35% nucleated cell loss after processing. These cell losses could occur as a result of cell death caused by these external forces or volume loss caused by displacement of plasma from concentrated cells. Environmental factors like temperature and period of cell storage could also contribute to these cell losses and reduced cell viability.

4.4.1 CD34+ HSC cells

4.4.1.1 The effect of processing on CD34+ HSC cell count

Strong positive correlation (Table 15) for CD34+ HSC cell counts between processed and unprocessed UCB at the different storage temperatures and times was observed (R=0.92, r=0.94. r=0.94, r=0.95. The p values (p=0.12) as illustrated on Table 15 for processed and unprocessed stem cells stored at 4°C and RT for 24h are statistically insignificant. When these cells are stored for another 24h period the difference in CD34 cell count is statistically significant. This concludes that CD34+ HSC cell count are affected by processing, together with increased storage period.

Storage Variable: Unprocessed UCB vs Processed UCB	Storage Time (h)	Storage Variable (Temperature (RT/°C))	Correlation Parameter (r)	5% Significance Level Probability (p)
Unpocessed UCB (CD34+ total cell count)	24h	4°C		
Processed UCB (CD34+ total cell count)	24h	4°C	0.92	0.12
Unpocessed UCB (CD34+ total cell count)	24h	RT		
Processed UCB (CD34+ total cell count)	24h	RT	0.94	0.05
Unpocessed UCB (CD34+ total cell count)	48h	4°C		
Processed UCB (CD34+ total cell count)	48h	4°C	0.94	0.02
Unpocessed UCB (CD34+ total cell count)	48h	RT		
Processed UCB (CD34+ total cell count)	48h	RT	0.95	0.01

Table 15: Correlation between processed and unprocessed CD34+ HSC total cell counts at 24h, 48h, RT and 4°C storage. Probability testing was calculated to determine whether processing affects CD34+ HSC total cell count.

4.4.1.2 The effect of processing on CD34+ HSC cell viability

The viability of CD34+ HSC cells for processed UCB correlated perfectly (r=1) with unprocessed UCB cells stored at 4°C and RT stored for 24h (Table 16). When processed and unprocessed UCB is stored for another 24h, weak correlation is observed (r=0.32, r=0.29. The p values as indicated for CD34+ HSC cell viability are statistically significant. Processing of UCB affects the viability of CD34+ HSC stem cells.

Storage Variable: Unprocessed UCB vs Processed UCB	Storage Time (h)	Storage Variable (Temperature (RT/°C))	Correlation Parameter (r)	5% Significance Level Probability (p)
Unpocessed UCB (% CD34+Viability)	24h	4°C		
Processed UCB (% CD34+Viability)	24h	4°C	1.00	0.00
Unpocessed UCB (%CD34+Viability)	24h	RT		
Processed UCB (% CD34+ Viability)	24h	RT	1.00	0.04
Unpocessed UCB (% CD34+Viability)	48h	4°C		
Processed UCB (% CD34+Viability)	48h	4°C	0.32	0.00
Unpocessed UCB (% CD34+Viability)	48h	RT		
Processed UCB (% CD34+Viability)	48h	RT	0.29	0.01

Table 16: Correlation between processed and unprocessed CD34+ HSC cell viability stored at 24h, 48h, RT and 4°C storage. Probability testing was calculated to determine whether processing affects %CD34+ HSC cell viability.

4.4.2 CD45+ cells

4.4.2.1 The effect of processing on CD45+ cell count

The effect of processing of UCB on CD45+ cell count was tested with the correlation parameter and probability testing. From Table 17, the correlation between cell processing or no cell processing is positive. Weak correlation was observed when processed and unprocessed cells were stored at °C. With probability testing it was concluded that differences in CD45+ cell count between processed and unprocessed UCB was statistical significant.

Storage Variable: Unprocessed UCB vs Processed UCB	Storage Time (h)	Storage Variable (Temperature (RT/°C))	Correlation Parameter (r)	5% Significance Level Probability (p)
Unpocessed UCB (CD45+ total cell count)	24h	4°C		
Processed UCB (CD45+ total cell count)	24h	4°C	0.86	0.00
Unpocessed UCB (CD45+ total cell count)	24h	RT		
Processed UCB (CD45+ total cell count)	24h	RT	0.86	0.00
Unpocessed UCB (CD45+ total cell count)	48h	4°C		
Processed UCB (CD45+ total cell count)	48h	4°C	0.29	0.00
Unpocessed UCB (CD45+ total cell count)	48h	RT		
Processed UCB (CD45+ total cell count)	48h	RT	0.75	0.00

Table 17: Correlation between processed and unprocessed UCB CD45+ total cell count stored at 24h, 48h, RT and 4°C storage. Probability testing was calculated to determine whether observed differences in CD45+ total cell count is significant.

4.4.2.2 The effect of processing on CD45+ cell viability

The effect of UCB processing on the viability of CD45+ was tested. Positive correlation between CD45+ leukocyte viability and cell manipulation or absence thereof was observed as indicated in Table 18. Statistical insignificant results were obtained with probability testing (p=0.76, p=0.67, p=0.19, p=0.21).

Storage Variable: Unprocessed UCB vs Processed UCB	Storage Time (h)	Storage Variable (Temperature (RT/°C))	Correlation Parameter (r)	5% Significance Level Probability (p)
Unpocessed UCB (% CD45+Viability)	24h	4°C		
Processed UCB (% CD45+Viability)	24h	4°C	0.98	0.76
Unpocessed UCB (%CD45+Viability)	24h	RT		
Processed UCB (% CD45+ Viability)	24h	RT	0.79	0.67
Unpocessed UCB (% CD45+Viability)	48h	4°C		
Processed UCB (% CD45+Viability)	48h	4°C	0.52	0.19
Unpocessed UCB (% CD45+Viability)	48h	RT		
Processed UCB (% CD45+Viability)	48h	RT	0.70	0.21

Table 18: Correlation between processed and unprocessed CD45+ cell viability stored at 24h, 48h, RT and 4°C storage. Probability testing was calculated to determine whether %CD45+ cell viability affected by processing is statistical significant.

CHAPTER 5

CONCLUSION

The use of haematopoietic stem cells from an UCB unit for successful transplant is limited by the collection volume. Collection volumes obtained in this study compared well with collection volumes obtained in other laboratories. From this study it was concluded that higher leukocyte and stem cell counts were obtained in UCB units with higher collection volumes. It would be very useful if factors like maternal age and infant gender could be used to predict cell content prior to cell processing. However, there was no correlation between maternal age and collection volume. Also, mothers of female infants were older than mothers with male offspring. Female offspring have not significantly higher collection volume than males, concluding that maternal age and infant gender do not affect collection volume.

It could be concluded that there is no correlation between maternal age, infant gender and post processing volume. UCB collections of female infants have higher collection volumes than males, with expected higher leukocyte counts compared to males. In contrast however, lower post processing volumes and stem cell counts were obtained in females. The differences are insignificant and this concludes that maternal age and infant gender have no affect on post processing volume or CD34+ HSC stem cell/CD45+ leukocyte cell count.

According to international requirements, the mean total nucleated cell and stem cell count achieved in this study of 913 UCB units that met the minimum leukocyte requirements are sufficient for successful transplantation of a young child. The achieved mean total leukocyte and stem cell count for this study compared well to cell counts obtained in other UCB banks.

It was shown in this study that the higher leukocyte and stem cell count and cell viability found in male infants when compared to females is statistically insignificant. Also, maternal age showed no influence on CD34+ HSC/45+ cell viability.

Additionally, this study showed that the stem cell to leukocyte cell ratio is not influenced by maternal age. Statistically significant results were obtained for differences in stem cell and leukocyte ratio that do occur in female and male infants. This concludes that the stem cell to leukocyte ratio in males is higher than in females. This result is consistent with the fact that the blood cell ratio to plasma in males (46:54)% (blood cells : plasma) are higher than in females (42:58)%.

Maternal age and infant gender do not influence laboratory parameters and could therefore not be used as factors for scrutinizing UCB collection prior to processing for ultimate successful engraftment.

CD34+ HSC stem cells from processed and unprocessed UCB, stored at RT have insignificantly higher mean cell counts than 4°C storage. This is true for storage at 24h and 48h. The stem cell count respectively between the two storage temperatures and 24h and 48h periods correlates well. From the probability testing it is concluded that 4°C or RT storage temperature and 24h or 48h has no significant influence on CD34+ HSC stem cell counts. Mean percentage stem cell viability is greater than 90% irrespective of storage period and time.

Unprocessed CD34+ HSC cells stored for 24h at 4°C have insignificantly higher mean cell viability than RT storage while prolonged storage to 48h result in insignificant higher CD34+ HSC viability stored at RT than at 4ºC. Processed CD34+ HSC cells are insignificant more viable when stored at 4°C over 24h and 48h than at RT storage. Prolonged storage of cells also doesn't significant affect

stem cell viability. From these results it could be concluded that different storage temperatures and periods of storage have no influence on CD34+ HSC cell viability of processed and unprocessed stem cells.

The mean CD45+ cell count for unprocessed UCB is insignificantly higher for cells stored at 4°C than at RT over 24 and 48h but a very good correlation occurs between the different temperatures. In contrast, processed UCB showed insignificantly higher mean CD45+ cell counts at RT storage and strongly positive correlations with these cells stored at 4°C. Probability testing concluded that differences that occur as a result of storage temperature do not significantly influence leukocyte counts of processed or unprocessed UCB. However, there is a significant difference in leukocyte cell count when cells are stored for 24h followed by another 24h storage period. Increased storage periods therefore affect leukocyte cell count.

Leukocytes consist of granulocytes (basophiles, eosinophils, neutrophils) and agranulocytes (lymphocytes and monocytes) with each cell type having a different lifespan. Neutrophils have the shortest half-life among leukocytes and normally survive for less than a day in the circulation before committing to apoptosis. Neutrophils make up 55%-70% of the total white blood count in the bloodstream. This short life span and high percentage occurrence of neutrophils contribute to the dramatic loss in leukocyte cell viability. Lymhocytes and monocytes and NK cells life span varies from 2 days to a week depending on the environmental stimuli. Prolonged storage should not be incurred as this could affect other accessory cells like monocytes, lymphocytes and NK cells that are very important for successful engraftment.

Different storage temperatures do not significantly influence leukocyte cell viability but increased storage time result in statistically significant decrease in leukocyte cell viability of processed an unprocessed UCB. Processing of stem cells and leukocytes causes significant cell loss.

In summary this concludes that stem cell count and viability from either processed or unprocessed UCB are not significantly affected by different storage temperatures or periods of storage. Temperature also does not influence leukocyte cell count and viability but prolonged storage time significantly result in decrease of leukocyte cell count and viability.

From this study the following recommendations could be implemented to increase cell counts and viability to ensure successful transplant:

- As higher cell count is associated with higher collection volume. It is recommended that collection volume of more than Netcells minimum value of 60ml should be collected without causing any harm to the infant.
- The final cell concentration after addition of DMSO should be determined as cell concentration has an influence on cell viability.
- A pre screen test to determine the total leukocyte count prior to processing must be performed on UCB collections with a borderline collection volume. This procedure will prevent unnecessary processing of UCB units with borderline collection volume that will fail Netcells criteria of 2×10^8 total nucleated cell count after processing, thereby saving processing costs.

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APPENDIX 1

NETCELLS INFORMED CONSENT

MOTHER'S PARTICULARS

1. Full Name: .....
2. Identity Number: .....
3. Physical Address: .....
4. Postal Address: .....
5. Mobile Telephone Number: .....
6. Landline Telephone Number: .....
7. E-Mail Address: .....

I am the Mother of the unborn child ("my Child") whose Cord Blood is to be collected at the time of its birth.

I am entitled and duly authorized to sign this document on behalf of my Child.

I authorize Netcells to receive, Process and Test blood from the placenta and umbilical cord and to Cryopreserve and Store the Stem Cells contained therein (collectively, "Cord Blood") in accordance with the accompanying Cord Blood Storage and Related Services Agreement.

1. **What are Stem Cells?**

Stem Cells are the earliest cells of a developing embryo that differentiate to form all the cells of the human body. Researchers have found that Stem Cells remain in our bodies to maintain and repair our organs throughout life. Stem Cells are also found in the blood of the umbilical cord and placenta of newborn babies, and are normally discarded after birth. These Stem Cells contain blood forming (haematopoietic) Stem Cells as well as other Stem Cells that can differentiate into the different components of blood. If these cells are harvested, they can be transplanted into a patient for therapeutic purposes. To date, the main uses for Cord Blood and Stem Cells is in the treatment of the following groups of disease:

- **Malignancies –** Leukaemia, lymphoma, multiple myeloma, solid tumours.
- **Anemia –** Fanconi's Anemia, sickle cell disease, Thalassaemia.
- **Bone marrow failures –** Aplastic anaemia, pancytopenia.
- **Immunodeficiency** – SCID, Kotsmann syndrome, Wiskott-Aldrich syndrome, lymphoproliferative disorder, ataxic telangiectasia, Di George syndrome.
- **Inborn errors of metabolism –** Adrenoleukodystrophy, Gaucher's disease, Krabbe disease, Niemann-Pick syndrome, Tay-Sachs disease.

Researchers have also demonstrated, mainly in pre-clinical studies, that umbilical Cord Blood and Stem Cells have the potential to be used in the future for the treatment a variety of other illnesses. Various clinical trials are underway internationally to assess the potential for using Stem Cells to treat the following conditions:

- Heart disease
- Diabetes
- Joint cartilage replacement
- Spinal cord injury
- Stroke

- Auto immune diseases
- Parkinson's and Alzheimer's disease

The Stem Cells will not be used for cloning purposes.

I understand:-

1.1 that my Child's Stem Cells are stored exclusively for use for my Child or any family member that I, the mother of the Child or the Child, decides to donate the Stem Cells to;

1.2 Netcells do not do HLA typing of the Cord Blood prior to storage and will therefore not be able to suggest donation of my Child's Cord Blood to persons not known to me, as compatibility is not known.

2. **How is the Cord Blood Collected?**

2.1 A Collection Kit is provided for the collection of the Cord Blood, which is taken to the birth of my Child and handed over to the Medical Practitioner that will perform the collection.

2.2 The collection bag is packaged in a sterile manner and needs to be opened on a sterile tray.

2.3 Once my Child is born, via normal vaginal delivery or Caesarian section, the umbilical cord is clamped and cut and my Child is handed to me or the nursing staff.

2.4 The Medical Practitioner will then insert the needle of the collection bag into the umbilical vein of the umbilical cord. Through gravity the bag will fill with Cord Blood. Once the flow of blood stops, the needle will be removed from the vein.

2.5 The Cord Blood will then be packed into the temperature coollant bag and placed back into the Collection Kit for courier to the laboratory.

3. **Collection Risks and Consent**

I:-

3.1 will request that my Medical Practitioner collect the Cord Blood using the Collection Kit provided by Netcells;

3.2 understand that, under normal circumstances, collection should cause me no discomfort or pain nor interfere with the birthing process;

3.3 understand that there is a risk of contamination when collecting Cord Blood and that there is no guarantee or assurance of the success of the collection procedure;

3.4 understand that the Cord Blood collected may be insufficient and/or not viable for storage and/or transplantation and/or any other purpose;

3.5 understand that there may be complications at birth that will make it impossible or problematic to collect the Cord Blood, and for these reasons my Medical Practitioner would be entitled to refuse to collect the Cord Blood.

I record and agree that I have been fully informed about the procedure for collecting Cord Blood and hereby consent to allow my Medical Practitioner to collect the Cord Blood after the birth of my Child and to furnish it to Netcells for Testing, Processing, Cryopreservation and Storage.

4. **Risks Involved in Storage and Use**

I:-

4.1 understand that there are no assurances that any benefits of storing my child's stem cells will be obtained;

4.2 understand that the transplantation of Cord Blood and Stem Cells is a relatively new procedure that may offer possible future benefits to my Child and other beneficiaries in treating certain diseases;

4.3 understand that there are several instances in which the use of my Child's Stem Cells for her/himself would not be recommended;

4.4 acknowledge that there are alternative sources of Stem Cells such as bone marrow and peripheral (circulating) blood, that could be accessed from a suitable matching donor, should I decide not store my Child's own Cord Blood and Stem Cells;

4.5 understand that the likelihood of using my Child's Stem Cells is limited and that it is highly likely that they may never be used;

4.6 understand there are no guarantees that the Cord Blood and Stem Cells will be a match for other family members, although there may be a suitable match;

4.7 understand that the cord blood stem cell harvests are typically low in volume and could be insufficient (without expansion) for a transplant.

4.8 understand that there is a documented and unavoidable cell loss during the cryopreservation and thawing process, even though Netcells uses proven methodology and the most up-to-date technology.

5. **Maternal Blood Testing and Health Information**

I:-

5.1 agree to fill out the Maternal Health Questionnaire in the Collection Kit and return the form to Netcells with the Collection Kit;

5.2 understand that I must have a sample of my own blood taken by venipuncture (the usual method for blood tests) within 7 Days prior to delivery or 7 Days after the delivery, in order to be tested for Transmissible Diseases;

5.3 will have my blood taken by the one of following laboratories: Ampath Laboratories, Bouwer Laboratories and Pathcare laboratories, as they are ISO 17025 accredited;

5.4 understand that there is a slight risk of bruising, discomfort, inflammation, or infection at the site of the blood draw. My blood will be tested for certain infectious diseases, including:

5.4.1 Human Immunodeficiency Virus (HIV)-1 and -2 – AIDS causing virus;

5.4.2 Hepatitis B and Hepatitis C viruses – viruses that affect the liver causing hepatitis, jaundice and liver dysfunction;

5.4.3 Human T-lymphotrophic virus (HTLV)-I and -II – a rare virus that can lead to paralysis and a rare type of lymphoma;

5.4.4 Cytomegalovirus (CMV) – a common virus that is usually undetected in adults but can cause sight and hearing loss in immunocompromised individuals. If the mother is infected in early pregnancy, it can cause severe congenital defects in

the foetus;

5.4.5 Syphilis – a contagious disease that is characterized by genital ulcers, skin rashes and lesions that can affect all organs of the body.

5.5 understand that these test results are required by various regulatory authorities.

5.6 understand that abnormal results will be reported to me. I hereby consent to Netcells' disclosure of the results of my blood tests to my Medical Practitioner, to any other Cord Blood storage facility to which I have authorized transfer of the Stem Cells, to the transplantation service in the event the Stem Cells are released for use, and to any government agency to which Netcells may be required to report such results under applicable law and regulations. Should my test results be positive for any of the following: HIV, Hepatitis B or C, HTLV or Syphilis, I will have a confirmatory test done i.e. another sample of my blood will be taken and tested. If these confirmatory tests are positive, the Cord Blood and Stem Cells will not be allowed storage in the Netcells Laboratory, in line with Netcells' Policy. (Refer to Section 7)

5.7 Netcells' Medical Director will review the results of the Testing.

6. Cord Blood Testing

I:-

6.1 understand that Netcells will undertake tests on my Child's Cord Blood to ascertain the nature, number and viability of the Stem Cells to be Cryopreserved;

6.2 understand that prior to the treatment of the intended recipient of the Stem Cells, should the recipient not be the same person from whom the Stem Cells were obtained, the Cord Blood will need to be tested for an HLA match, which cost are for the Client's own account;

6.3 understand that Netcells will not release the Stem Cells until Netcells has received the HLA typing results (if required) and approval from the recipient's transplant physician;

6.4 understand that reference samples of the Cord Blood will be Stored for future Testing of HLA type, and cell count and viability upon thawing. These are small in quantity and do not significantly affect the overall number of Cord Blood and Stem Cells Stored.

7. Disclosure of Health Information

7.1 Netcells will maintain the confidentiality of my health information that I provide to them concerning myself and my Child.

7.2 I do however understand that health care providers may need such information to provide treatment to me and/or my Child and that government agencies may be entitled to obtain such information under applicable law and regulations.

7.3 I authorize Netcells to disclose such information to health care providers that are treating me and/or my Child and to government agencies as may be required under applicable law and regulations.

7.4 I authorize Netcells to disclose such information for research purposes in an anonymous manner and always maintaining Netcells' confidentiality obligations in terms hereof.

8. Cord Blood Disposal

8.1 If the Cord Blood that is collected has a low cell count or low cell viability and is not recommended for Storage according to Netcells standards, I will be informed by Netcells about the possible options available to me.

8.2 I have the choice to continue Storage and will sign a disclaimer in order so to do, or if I decide to discontinue Storage I will sign a disclaimer to either donate the Cord Blood for research purposes or to discard it.

8.3 I understand that Netcells will retain confidentiality of the sample and that the Stem Cells will not be used for any other purposes including cloning. Non-conforming Cord Blood i.e. specimens that are positive for any of the following: HIV, Hepatitis B/C, HTLV or syphilis, will not be stored by Netcells and need to be Stored at an alternative Authorized Organization, Institution or Person, if so required by the Client.

8.4 I will be responsible for the additional cost and transport of the Cord Blood and Stem Cells to the alternative bank. I will also assume the additional costs and will agree to the conditions of the alternative Cord Blood Bank.

8.5 Netcells may assist me to find an alternative Cord Blood Bank anywhere in the world that will accept the Cord Blood and Stem Cells, should it not be acceptable by Netcells for storage, but makes no representation or gives any warranty in this regard.

9. Disclosure of Information

I authorize Netcells to use (research, study and publish) information obtained from the collection, Testing, Processing, Cryopreservation and Storage of the Cord Blood and Stem Cells for marketing purposes and use the information in ethically approved research and in an anonymous manner.

10. Release from Liability

10.1 Subject to clause 12 of the Cord Blood Storage Agreement, in consideration for Netcells agreeing to Process, Test, Cryopreserve and Store the Cord Blood, I, (including respective guardians and other legal representatives, heirs and estates) irrevocably and unconditionally release and discharge Netcells and its processing laboratory(s) and their respective shareholders, directors, officers, employees, agents, representatives, and affiliates and their respective legal representatives, estates, successors and assigns, from and against any and all claims, damages, losses, causes of action or rights, known and unknown, that may arise from, or relate to, the activities and services described in this Informed Consent and Cord Blood Storage and related services agreement save for such claims, damages, losses, causes of action or rights which arise directly and solely from the gross negligence of Netcells.

10.2 Without limiting the foregoing, I further acknowledge that Netcells is not responsible in any way for the actions of others including my Medical Practitioner, the birthing hospital or medical facility, staff of the hospital or medical facility, laboratory staff, and transporters of the Cord Blood.

SIGNED :

MEDICAL DIRECTOR
BIOCELLS (PROPRIETARY) LIMITED

SIGNED:

THE CLIENT:
DATE:

CLIENT NUMBER :.....

APPENDIX 2

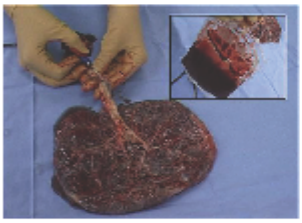
netcells
cryogenic storage for life

PROTOCOL FOR PROCEDURE OF UMBILICAL CORD BLOOD COLLECTION

The following outlines the correct procedure for collecting umbilical cord blood for the processing and storage of stem cells. This procedure is done after the baby is safely delivered to minimize the disruption of the normal management of labour. The collection must never interfere in any way with the care given firstly to the mother and secondly to the infant. This protocol should never, under any circumstance, be compromised.

It is of the utmost importance that the **procedure is done in a sterile manner**. This will ensure maximum chance of a successful collection.

1. Prepare a sterile tray on which to place the blood collection system. The contents of the 'blood collecting system' are sterile and needs to be **handled by a sterile operator at all times**. Open the bag and place the contents on the tray.
2. Following birth of the baby, either natural or Caesarian, clamp the cord with two clamps as close to the baby's umbilicus as possible (observing good obstetric practice at all times). Cut the cord between the clamps. *The longer the cord, the easier it is to collect the appropriate volume of blood*. You have a **maximum of 10 minutes** from clamping and removing the baby from the umbilical cord, to collect blood before it starts clotting in the vein.
3. **Try to collect the blood prior to delivery of the placenta**. If it is necessary to deliver the placenta for obstetric reasons, then place the placenta on a sterile tray and continue with the blood collection.
4. Swab an insertion site on the umbilical cord with Steret solution/Steret swabs provided. The insertion site should be just above the clamp distal to the placenta.



5. Insert the needle into the umbilical vein in the prepared area of the cord. *Blood must be taken from the vein as the stem cell count is much higher than in the arteries.*
6. The bag must be positioned as far as possible below the insertion of the needle, without touching the floor. This will assist drainage via gravity into the bag.
7. The anticoagulant in the blood bag totals 35ml. We need a minimum of **100 mls, excluding anticoagulant**. Therefore, an **optimum** collection should be cord blood 100mls + anti-coagulant 35mls = **135mls in total**. This volume is about half the bag full. Volumes less than this stand a lower chance of having a successful stem cell harvest. **NOTE: Total volumes of <60ml (Blood 25ml + anticoagulant 35ml) will undergo a pre-screening assay and if found unsuitable will not be accepted for processing and consequently storage.**

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8. If you are experiencing difficulty with that particular site, you may insert the needle in a different site on the cord. Swab the second insertion site prior to inserting the needle to maintain sterility. It may be necessary to utilize several sites - just work your way up the cord, maintaining sterility at all times.
9. After the blood is collected, allow the blood in the tube to run into the bag, clamp the tube, remove the needle and discard it into a sharps container. Two knots are tied in the tubing. The knots must be a hands width above the top of the bag.
10. **Turn the bag over gently several times so to mix the blood and anticoagulant.**
11. The blood bag is then given to the father/partner who places an **ID sticker on the bag** and then places the blood bag into the temperature coolant bag, inside the box provided.
12. The father/partner will contact a Netcells consultant to arrange collection.

Please Note: The collected blood must never be placed in a fridge, freezer, near a heater or direct sunlight

If you require any further information, please feel free to call:

Netcells Medical Director: Dr Yvonne Holt on (082) 569 1888

APPENDIX 3

STANDARD OPERATING PROCEDURE FOR THE OPERATION OF THE BIOSAFE SEPAX

1. **Purpose**

   To process Umbilical Cord Blood on the Biosafe Sepax with UCB-HES protocol using the CS-530 single use kit prior to the final product being cryopreserved.
   To volume reduce the Umbilical Cord Blood Collection by the depletion of plasma and red blood cells.

2. **Scope**

   This procedure will be used by Netcells staff involved in the processing and cryopreservation of stem cells.

3. **Reference**

   Sepax Cell Processing System Operator's Manual OM-114-EN - November 2006.

4. **Definitions / Abbreviations**

   HES – Hydroxyethyl starch
   RBC – Red blood cells
   BC - Buffy coat faction from Umbilical Cord Blood Collection

5. **Safety Health and Environment.**

   5.1. Staff must use personal protective equipment such as disposable latex gloves, face mask overshoes and gown while working in the clean room.
   5.2. Waste must be disposed of in accordance with SHE-SOP-001.

6. **Procedure:**

   Responsible persons: Netcells Laboratory Staff

   6.1. Switch on the Sepax device and the machine performs self check. (Ensure display indicates **UCB-HES.**
   6.2. Ensure Martel printer is on. (Provides printout of processing)

6.3. Record all client details on UCB – Processing Worksheet PRO-FRM-001. (Collection and processing dates and times, Client details, Netcells number etc.)
6.4. Inspect Collection Bag (Initial Product Bag) and seal off and remove excess tubing which may have blood clots.
6.5. Thoroughly mix contents of Collection Bag and take initial sample
6.6. Weigh Collection Bag and record weight.
6.7. Calculate the volume of the product. Record details on Worksheet.
(Collection Volume = Weight of Collection Bag - weight of empty bag)
6.8. Calculate the volume of HES to add. (40% of the collection volume). In cases where the collection volume is less than 70ml, in addition to 40% of the calculated HES volume, an additional 10ml of HES is added to the collection bag.
6.9. Working under Laminar Flow follow aseptic techniques insert a sterile sampling site coupler to the Collection Bag. Swab the sampling port with an alcohol swab.
6.10. Remove the HES from the cold store and swab sampling port with alcohol swab.
6.11. Draw up the required volume of HES into a sterile syringe and slowly add the required volume at 2 seconds/ml to the collection bag while gently mixing contents.
6.12. Prepare Sepax cell separation kit (CS-530) (**Fig. 2.**)
- Check expiry date
- Check sterility indicator and integrity of tyvek lid seal
- Working under laminar flow open kit onto working surface and spread out to inspect parts for damage or kinks - keep lid of kit to scan barcode
- Close roller clamp on inlet tubing
- Close **blue** DMSO tubing clamp and **blue** clamp on DMSO extension line
- Spike Collection Bag with inlet tubing spike

6.13. Label sample tubes, cryovials, sterility culture bottles, aluminum canister and Pall collection bag with the preprinted labels.
6.14. Install Sepax kit and Collection bag (**Fig.1.**)
- Open centrifuge pit covers and insert separation chamber, push chamber down securely and close covers
- Ensure that stopcocks are in TTT position and mount stopcock manifold on drive pins
- Insert the tubing line from separation chamber in the optic sensor and use sensor cover to insure that tubing is loaded correctly. Remove sensor cover.
- Connect filter securely to line pressure sensor
- Hang BC bag on the front pin on the left side of the Sepax and the plasma bag on the rear pin on the right side of the Sepax
- Hang the Collection Bag on the IV pole hook and place the bubble chamber in the

holder

6.15. Sepax display should read **"Start Procedure"**

- Press **ENTER**
- Display calls for scanning of initial Collection Bag
- Press **DOWN ARROW** for the next selection
- Display calls for the scanning of the final storage bag
- Press **DOWN ARROW** for the next selection
- Display calls for the scanning of the separation kit
- Press **DOWN ARROW** for the next selection
- Display calls for the scanning of the Blood bag

6.16. Follow display screen prompts Open Red roller clamp on input line and press **ENTER**

6.17. Sepax performs kit integrity check and if **"Kit OK"** message appears the procedure will start automatically.

6.18. Sepax cell separator processes blood according to protocol.

Separation principle
The unique separation chamber permits the system to separate the different cellular products into the corresponding bags as follows:

6.19. When procedure is complete:

- Follow instructions on Sepax display
- Remove bags and air filter - Press **ENTER**
- Strip Collection Bag line – Press **ENTER**
- Strip BC line – Press **ENTER**
- Remove all air from BC bag and then press **ENTER**
- Close all clamps on bags and remove kit from Sepax
- Press **ENTER**

6.20. Using a tubing sealer, seal and remove all bags from the kit and dispose of separation chamber
6.21. Transfer bags to laminar flow workstation and swab sampling ports with alcohol swabs.
6.22. Mix the contents of the BC bag by pressing the small section of the bag 20-30 times.
Take a 0.5 ml sample for Flow Cytometry using a sterile syringe.
Transfer contents of syringe to labeled EDTA test tube.
6.23. Mix the contents of the Plasma and RBC Bags by inverting the bags several times.
Take the required samples from the bags using sterile syringes and transfer the contents to appropriately labeled tubes.
- 10ml plasma into each sterile culture bottle for sterility test
- 2 - 4ml plasma into cryo-vials
- 1 - 2ml RBC into a cryo-vial

6.24. Cool the contents of the Pall bag (for addition of cryo-preservative) by either placing the bag in the Coolmix or placing the bag in the fridge. Allow at least 15 minutes for the contents to cool down.
The reference samples are to be placed in the fridge until required.
6.25. Remove printout from Martel printer of process and place into clients file.
(Place Kit lid with barcode together with unused labels into client file).
6.26. The RBC, Plasma bags and processing Kit are disposed of according to **SHE-SOP-001**
6.27. The Sepax instrument is cleaned on a weekly basis.

7. Records

7.1. The printout of the process is kept in the clients file together with lid from the Sepax Kit together with any unused labels.
7.2. The Sepax is able to store in memory a record of processing and can be recalled at a later date for printing.

8. Related Forms and Labels

PRO-FRM-001. UCB Processing Worksheet

10. Appendix

Fig.1. Biosafe Sepax cell separator
Fig.2. Sepax CS-530 Kit

11. Maintenance. Contact Haemotec service engineer at Tel: 011 6092314

APPENDIX 4

STANDARD OPERATING PROCEDURE FOR THE IDENTIFICATION AND ENUMERATION OF CD34+ HEMATOPOIETIC PROGENITOR CELLS AND LEUKOCYTES

1. Purpose

1.1. To identify CD34+ Hematopoietic Progenitor Cells (HPC) and Leukocytes.
1.2. To determine the quantity and the viability of CD34+HPC and Leukocytes.
1.3. To lyse RBC that could interfere with the analyses of HPC.

2. Scope

2.1. This procedure will be used by Netcells staff involved in the flow cytometry identification and enumeration of CD34+HPCs and Leukocytes.

3. Reference

3.1. Cytomics FC 500 with CXP Software - stemCXP System Guide (PN 627260B) September 2005
3.2. Cytomics FC 500 with CXP Software - Instructions for Use (PN 624923B) June 2004
3.3. Cytomics FC 500 with CXP Software – Operators Manuals (PN 177627C) June 2004
3.4. Stem-Kit Reagents Package Insert
3.5. GLP Good Laboratory Practice

4. Definitions / Abbreviations

4.1. GLP Good Laboratory Practice
4.2. HPC Hematopoietic Progenitor Cells
4.3. RBC Red Blood Cells
4.4. 7-AAD 7-Amino-Actinomycin D
4.5. UCB Umbilical Cord Blood
4.6. PBSC Peripheral Blood Stem Cells
4.7. MCL Multi Carousel Loader

5. Safety Health and Environment.

5.1. Staff must use personal protective equipment such as rubber gloves, protective clothes and eye protection when working with blood products and reagents.

5.2. Special care should be taken when handling and disposing of 7-AAD which is a potential carcinogen.
5.3. Stem-Count Fluorospheres contains 1% Formaldehyde and may cause irreversible effects on the eyes and skin.
5.4. Waste must be disposed of in accordance to accepted procedures (SHE-SOP-001 and SHE-SOP-009).

6. Equipment and Reagents

6.1. Cytomics FC500 Flow Cytometer
6.2. stemCXP System software version 2.0 with CXP Software version 2.2
6.3. 1000µl, 0-200µl micropipettes and tips
6.4. Vortex
6.5. Stem-Kit™ Reagent
6.6. Polypropylene test tubes
6.7. Water Bath

7. Procedure
Responsible persons: Netcells Laboratory Staff.

Important note:
Ensure that the flow Cytometer is properly aligned and standardized for fluorescence intensity before any analysis of CD34+HPC are performed.
Bring all reagents to room temperature (18-25˚C) prior to use.

7.1. Label two 12 x 75mm polypropylene test tubes with the Specimen ID and respectively as 45/34/7AAD #1 and 45/34/7AAD #2.
7.2. Label the control tube with the Specimen ID as CD45/CTRL/7AAD.
7.3. Pipette 20µl of CD45-FITC/Isoclonic Control PE into the labeled control test tube.
7.4. Pipette 20µl of CD45-FITC/CD34-PE antibody into test tubes labeled #1 and #2.
7.5 .Pipette 20µl of 7-AAD Viability Dye into each of the three test tubes prior to sample preparation. For PBSC specimens ensure that the white blood cell (WBC) concentration is no greater than 30 x 10^3 WBC/µl. If so dilute the sample with Phosphate Buffered Saline to an average value of 15 x 10^3 WBC/µl.
7.6. Pipette 100µl of the specimen blood into each of the test tubes.
7.7. Vortex the test tubes gently.
7.8. Incubate the three test tubes at room temperature for 20 minutes in the dark.
7.9. Prepare daily 1 X NH_4Cl lysing solution by adding nine volumes of deionized

water to one volume of 10X concentrated NH_4Cl lysing solution.

7.10. After incubation add 2ml of prepared 1 X NH_4Cl lysing to each of the test and control tubes and vortex each tube for 5 seconds.

7.11. Incubate the two test tubes and control tube for 10 minutes at room temperature in the dark.

7.12. After incubation gently invert the Stem-Count Fluorospheres and pipette 100µl of the Stem-Count Fluorospheres into each of the tubes.

7.13. Immediately vortex the tubes for 5 seconds and analyze within 1 hour.

7.14. The patient information must be entered into the Database information screen prior to acquisition or listmode replay. This information prints on the panel report.

7.15. From the Report Generator toolbar  select the open Database Information screen (Fig.1.) and complete the following:

- Patient ID
- Birth date
- Sex
- First name and surname
- Sample ID1 (this information needs to be the same as in the as in the Sample ID1 in the Resource Explorer i.e. Netcells Nr)
- Sample type and date of collection
- Dilution factor and harvest volume

7.16. Save all this information.

7.17. Clear the Acquisition Manager 

7.18. From the Resource Explorer window select the Cord Blood panel form the Panel menu  and drag it into the Acquisition Manager. The panel consists of the following protocols:

- stemCXP.pro
- stemCXP Contol.pro

7.19. In the Acquisition Manager window enter the:

- Carousel Number
- Sample ID1 (Netcells Number)
- Sample ID2 (Donor surname and sample type i.e. UCB or PBSC and pre/post/thaw processing)
- Cal factor  form the Absolute Count Calibration window

7.20. Place the two test tubes with CD45-FITC/CD34-PE antibody labeled #1 and #2 in positions one and two followed by placing the control tube with CD45-FITC/Isoclonic Control PE in position three of the carousel.
7.21. Place the loaded carousel in the FC500 MCL and close the cover.
7.22. Ensure that the FC500 is in the "Awaiting Sample" mode in the Status Bar and press the Acquisition icon  to start the analysis.
7.23. Select the rate of analysis i.e. low, medium or high.
7.24. In the Active Window of the CXP Software screen, nine Histograms are displayed involved in the analysis of the CD34+HPC and leukocytes and will print on a Flow page (Fig.2.)
7.25. A minimum of 75,000 CD45+ events must be analyzed with the Flow Cytometer according to the ISAGHE Guidelines for CD34+ cell determination with flow cytometry.
7.26. The flow diagrams and Panel report will automatically print. (Fig.3.)

7.27. When the analysis is completed remove the carousel and close the MCL cover. Discard of the test and control tubes as defined in SOP-ENV-031.
7.28. Following analysis review the data:
- if the Autogating function has gated the cell populations (Histograms) to satisfaction for the Cord Blood analysis - accept the results.
- if the Autogating function has not gated the cell populations (Histograms) for the Cord Blood analysis to satisfaction or when PBSC samples are analyzed - the Histograms must be regated by the Listmode playback.

7.29. Form the CXP Software Window Main Menu, click on Tools menu select Listmode Playback.
7.30. Select form the Listmode Playback window:
- Insert Panel 
- Panel files (pnl) and open Cord Blood Panel file.

7.31. On the Listmode Playback window:
- Mark the Save Boxes
- Open the LMD file icon and select the required LMD file
- Select Print Flow pages form the options menu
- Press the Start/continue acquisition

7.32. The Playback will start. Regate the cell populations (Histograms) and update it. For the second and third protocol only regate the CD45FITC/SS Histogram, as the

updated histogram gating form the first protocol will automatically carry over.
A flow page will print after each update, followed by a panel report.

7.33. Close the Listmode playback window and continue to analyze samples or cleaning of the Flow Cytometer (SOP-FC-001)

7.34. Report results as per SOP-FC-004.

8. Records

8.1. The Flow pages and Panel Report for each CD34+HPC and Leukocyte analysis must be kept in the patient/client file according to QAS-SOP-003.

8.2. LMD files created during flow Cytometry analysis of CD34+HPC and Leukocytes will be backup on a monthly basis on CD Disk and kept according to QAS-SOP-003.

8.3. There is controlled access to the Netcells Database for Netcells Employees.

8.4. Regular backups of information must be performed.

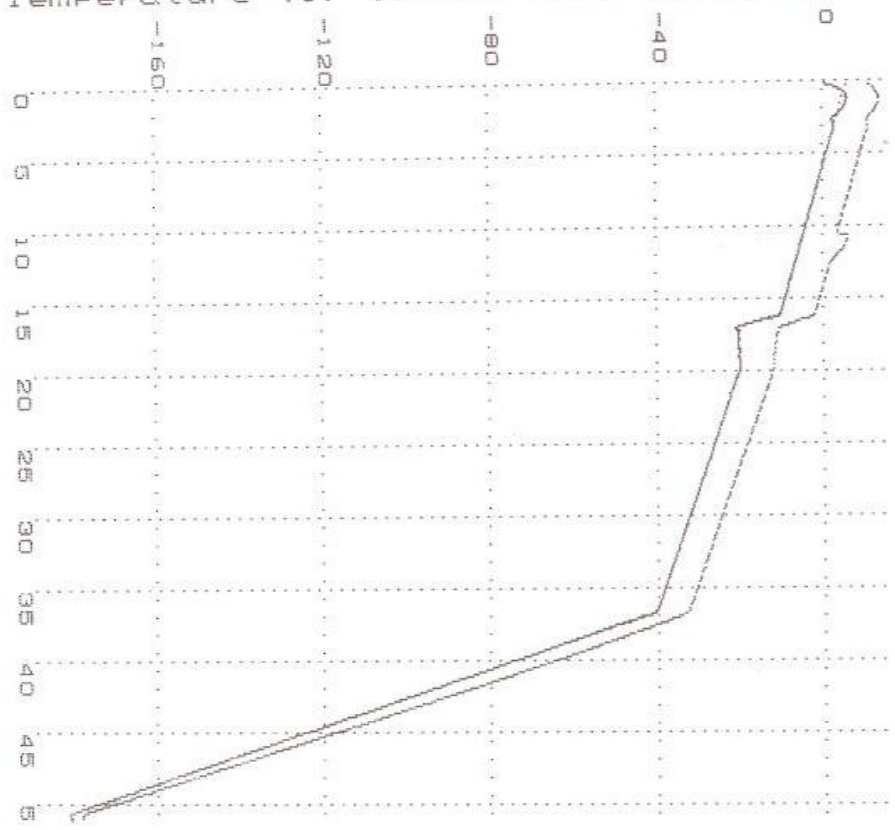
9. Related Forms and Labels

N/A

APPENDIX 5

```
www.planer.co.uk
MRV User Interface
v5.09  release
19699 19649 NETCEL
11:39 21 Jan 2010
RUN.980-_____________________
UCB
Start temp  +006.0 C
No seeding selected
#01 -01.00 C/min to -010.0 C
#02 -12.00 C/min to -020.0 C
#03 hold  03 mins 00 secs
#04 -01.20 C/min to -040.0 C
#05 -10.00 C/min to -180.0 C
```

Temperature (C) versus time (minutes)



Run completed normally.

APPENDIX 6

STANDARD OPERATING PROCEDURE FOR THE COOLING OF THE CORD BLOOD AND ADDITION OF CRYOPRESERVATIVE

1. Purpose

To ensure that Umbilical Cord Blood after red cell depletion and volume reduction on the Sepax is cooled sufficiently to add the cryopreservative.
The cryopreservative is used to protect the cells during the controlled freezing and storage process.

2. Scope

2.1. This procedure will be used by Netcells staff involved in the processing and cryopreservation of stem cells.

3. Reference

3.1. Coolmix AS-210 Operator's Manual O-0628 Version: 7 Nov. 2005.

4. Definitions / Abbreviations

N/A

5. Safety Health and Environment.

5.1. Staff must use personal protective equipment such as disposable gloves.
5.2. Staff working in clean room must wear disposable face mask, gown and overshoes.
5.2. Waste must be disposed of in accordance with **SHE-SOP-001.**

6. Procedure
Responsible persons : Netcells Laboratory Staff

The cord blood in the final freezing bag can be cooled by the following methods:
Using the Coolmix
6.1. Switch on the Coolmix device and allow 15 minutes conditioning prior to use. Fig.1.(Ensure display indicates a temperature of 4°C)
6.2. After processing on the Sepax the UCB in the Pall bag is placed directly on the cooling platform of the Coolmix for 15minutes. Start mixing.
6.3. Remove a DMSO Dextran vial (55% w/w DMSO, 5% w/v Dextran) from the

5.2. Special care should be taken when handling and disposing of 7-AAD which is a potential carcinogen.
5.3. Stem-Count Fluorospheres contains 1% Formaldehyde and may cause irreversible effects on the eyes and skin.
5.4. Waste must be disposed of in accordance to accepted procedures (SHE-SOP-001 and SHE-SOP-009).

6. Equipment and Reagents

6.1. Cytomics FC500 Flow Cytometer
6.2. stemCXP System software version 2.0 with CXP Software version 2.2
6.3. 1000µl, 0-200µl micropipettes and tips
6.4. Vortex
6.5. Stem-Kit ™ Reagent
6.6. Polypropylene test tubes
6.7. Water Bath

7. Procedure

Responsible persons: Netcells Laboratory Staff.

Important note:
Ensure that the flow Cytometer is properly aligned and standardized for fluorescence intensity before any analysis of CD34+HPC are performed.
Bring all reagents to room temperature (18-25˚C) prior to use.

7.1. Label two 12 x 75mm polypropylene test tubes with the Specimen ID and respectively as 45/34/7AAD #1 and 45/34/7AAD #2.
7.2. Label the control tube with the Specimen ID as CD45/CTRL/7AAD.
7.3. Pipette 20µl of CD45-FITC/Isoclonic Control PE into the labeled control test tube.
7.4. Pipette 20µl of CD45-FITC/CD34-PE antibody into test tubes labeled #1 and #2.
7.5 .Pipette 20µl of 7-AAD Viability Dye into each of the three test tubes prior to sample preparation. For PBSC specimens ensure that the white blood cell (WBC) concentration is no greater than 30 x 10^3 WBC/µl. If so dilute the sample with Phosphate Buffered Saline to an average value of 15 x 10^3 WBC/µl.
7.6. Pipette 100µl of the specimen blood into each of the test tubes.
7.7. Vortex the test tubes gently.
7.8. Incubate the three test tubes at room temperature for 20 minutes in the dark.
7.9. Prepare daily 1 X NH_4Cl lysing solution by adding nine volumes of deionized

7. Records

7.1. Record the Lot number, expiry date of the DMSO Dextran and the time of addition and completion of the cryoprotectant to the UCB on the UCB Processing worksheet **FRM-PRO-1.**

8. Related Forms and Labels

PRO-FRM-1. UCB Processing Worksheet

9. Flowchart

10. Appendix

Fig.1. Coolmix AS-210

Maintenance for Coolmix.

Contact Haemotec service engineer at Tel: 011 6092314

FLOW CHART