Prerequisites for establishing a public human UCB SCB; assessment of public acceptance and resistance of UCB to HIV

By

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Thesis submitted in partial fulfilment of the requirements for the degree Doctor of Philosophy in the Faculty of Health Sciences, University of Pretoria, Pretoria

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31 October 2012

Psalm 33

1 Sing joyfully to the LORD, you righteous; it is fitting for the upright to praise him.
2 Praise the LORD with the harp; make music to him on the ten-stringed lyre.
3 Sing to him a new song; play skilfully, and shout for joy.
4 For the word of the LORD is right and true;
he is faithful in all he does.
5 The LORD loves righteousness and justice;
the earth is full of his unfailing love.
6 By the word of the LORD the heavens were made,
their starry host by the breath of his mouth.
7 He gathers the waters of the sea into jars[a];
he puts the deep into storehouses.
8 Let all the earth fear the LORD;
let all the people of the world revere him.
9 For he spoke, and it came to be;
he commanded, and it stood firm.
10 The LORD foils the plans of the nations;
he thwarts the purposes of the peoples.
11 But the plans of the LORD stand firm forever,
the purposes of his heart through all generations.
12 Blessed is the nation whose God is the LORD,
the people he chose for his inheritance.
13 From heaven the LORD looks down
and sees all mankind;
14 from his dwelling place he watches
all who live on earth—
15 he who forms the hearts of all,
who considers everything they do.
16 No king is saved by the size of his army;
no warrior escapes by his great strength.
17 A horse is a vain hope for deliverance;
despite all its great strength it cannot save.
18 But the eyes of the LORD are on those who fear him,
on those whose hope is in his unfailing love,
19 to deliver them from death
and keep them alive in famine.
20 We wait in hope for the LORD;
he is our help and our shield.
21 In him our hearts rejoice,
for we trust in his holy name.
22 May your unfailing love be with us, LORD,
even as we put our hope in you.

New International Version (NIV)

Acknowledgements

A special word of thanks is owed to Dr. Hennie Lombaard and the staff at the Steve Biko Academic Hospital for providing a platform to work with mothers attending the antenatal clinic, as well as assisting with collections of UCB. Thank you to Dr. Lilian Gaggia at the South African National Blood Services for her input and support in validating the Ultrio-Plus® assay on umbilical cord blood as well as subsequent screenings of colony forming unit cells. To Prof. Piet Becker and Dr. Chrisna du Randt I owe my gratitude for assistance with statistical and flow cytometric analyses respectively.

For financial support during my studies, I would like to thank the National Research Foundation (NRF), the Medical Research Council (MRC) and the Department of Immunology at the University of Pretoria. Also, attendance at the International Vatican Adult Stem Cell conference was sponsored by the National Research Foundation (NRF) and the University of Pretoria.

I want to thank my supervisor, Prof. Michael Pepper for firstly giving me the opportunity to study in his group and secondly, for his invaluable input into my work over the course of my studies. It has been a privilege to be a part of his team in which I learnt many valuable skills and life lessons.

Thank you to all my friends and family, my husband Karl in particular, for your continuous support and motivation.

I thank my heavenly Father for the gifts that He has given me, enabling me to do every work He has called me to. All the glory and honour be to the Lord from everlasting to everlasting, Amen!

Summary

South Africa is in dire need of a public umbilical cord blood stem cell bank (UCB SCB). A severe shortage of genetically compatible samples for BM transplantation precludes the majority of South Africans from receiving the relevant medical care. UCB is a viable alternative to BM but is currently disposed of post-delivery. UCB could furthermore serve as a resource of genetically compatible haematopoietic progenitor cells (HPCs) that could be used in gene therapy approaches directed towards a cure for HIV-1. Knowing whether HIV-1 affects or infects primitive HPCs is vital to determine the course of action for transplantation of UCB-derived genetically resistant HPCs. Collecting and storing UCB in a public UCB bank could thus serve as a vital resource of genetically compatible samples for BM transplantation.

It was thought that the high incidence of HIV-1 in South African patients and the persistent stigma surrounding HIV-1 would be problematic for collecting sustainable numbers of UCB units and subjecting units to compulsory screening for infectious diseases. This was however, not the case. In the South African context, we are faced with unique and rich challenges relating to cultural and religious differences that are further augmented by linguistic constraints and educational insufficiencies. Nevertheless, the majority of patients within the interviewed patient cohort were supportive of the idea of establishing a public UCB SCB in SA and were willing to undergo additional HIV-1 screening. The Ultrio-Plus® assay was verified in this study for screening UCB units for HIV-1 and could be used in routine analyses of UCB units prior to banking.

Conflicting results in the literature exist with regard to HIV-1's ability to infect or affect haematopoietic progenitor cells. Results from this study revealed that HIV-1 was not only able to affect HPCs' ability to form colonies *in vitro*, but was also capable of infecting CD34+ HPCs in some individuals. These results substantiate the theory that some CD34+ HPCs serve as viral reservoirs which could account for residual viraemia in patients on antiretroviral therapy. Results suggest that allogeneic transplantation of HIV-1 infected individuals with UCB-derived, genetically modified HPCs, should be pursued.

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AIDS	Acquired Immunodeficiency Syndrome
ASC	Adult stem cell
BFU-E	Burst-forming-unit erythrocyte
BM	Bone marrow
CFU-GEMM	Colony-forming-unit granulocyte, erythrocyte, monocyte, megakaryocyte
CFU-GM	Colony-forming-unit granulocyte-macrophage
CFU-MK	Colony-forming-unit megakaryocyte
CI	Confidence interval
ESC	Embryonic stem cell
FDA	Food and Drug Administration
G-CSF	Granulocyte-colony-stimulating-factor
GvHD	Graft versus host disease
GvL	Graft versus Leukaemia
HBV	Hepatitis B-Virus
HCV	Hepatitis C-Virus
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HPCs	Haematopoietic progenitor cells
HSCs	Haematopoietic stem cells
iPS	Induced pluripotent stem cells
ISSCR	International Society for Stem Cell Research
MTCT	Mother to child transfer
NAT	Nucleic acid testing
PBSC	Peripheral blood stem cells
SABMR	South African Bone Marrow Registry
SANBS	South African National Blood Services
SC	Stem cells
SCB	Stem cell bank
SCT	Stem cell tourism
TMA	Transcription mediated amplification
TNC	Total nucleated count
UCB	Umbilical cord blood
WMDA	World Marrow Donors Association
FBS	Foetal Bovine Serum
DMSO	Demethylsulphoxate

CHAPTER 1

1 Introduction

Stem cells and their therapeutic applications have received much attention around the world. This is due in part to the immense therapeutic potential that these cells harbour, and also to the sea of misinformation that leads to exploitation and malpractice in the current absence of adequate legislation.

South Africa has had limited exposure to stem cells and their applications. While any exploitation is detrimental to the field of stem cells, South Africa is particularly vulnerable to such misuse. Circumstances are already precarious due to a lack of information and understanding (discussed in Chapter 2). This in turn, cultivates fears born out of existing superstitions, cultural beliefs, rituals and practices. Certain cultural or religious concerns could potentially hinder the effective application of stem cell therapies in South Africa and novel ways of addressing these concerns are necessary. This invokes a paradigm shift: Regenerative medicine is an emerging field that requires a holistic approach through novel inter-disciplinary collaborations between empirical science, social science, ethics, religion and culture (discussed in Chapter 3). Understanding how scientific progress and its implementation will affect each individual and consequently the community, will be of cardinal importance to the success of the field. A failure to understand the ethical, cultural or moral ramifications when introducing new scientific concepts could hinder the efficacy and speed of bringing discoveries from bench to bedside. Neglecting proper procedure for establishing the field, would lead to the need for a lengthy recovery of public support in South Africa.

South Africa could benefit from potential therapeutic applications that stem cells have to offer. Two particular burdens that put the healthcare system under strain are 1) a large unmet need in South Africa for bone marrow transplantation to treat malignant and inherited haematological disorders; and 2) the high rates of HIV-1 infection in the population. Both of these elements require larger studies of enquiry to fully elucidate and potentially relieve these burdens. For this reason, the work described in Chapters 4 to 8 is composed of components that form a part of both of the larger studies.

1.1 Addressing the need for BM transplantation in SA: Background to the larger study and specific components involved in the current investigation.

The large unmet need for BM transplantation in South Africa necessitates the use of alternative resources for treatment (Crookes et al., 2007). Umbilical cord blood (UCB) is an important source of stem cells for treatment of haematological and non-haematological diseases and South Africa can benefit greatly from storing UCB SCs in a public UCB SCB.

The larger study therefore has as its goal, to investigate the feasibility of establishing a public umbilical cord blood stem cell bank (UCB SCB) in South Africa. The study consists of five components: (a) public response to the establishment of a public UCB SCB; (b) testing UCB units for HIV-1 (required for compliance with international regulatory standards); (c) flow cytometric analysis for enumeration of CD34+ UCB stem cells; (d) mapping of HLA genotypes/alleles; and (e) an economic feasibility study. Combined results from each of these components will determine the final feasibility of establishing a public UCB SCB in South Africa. The objectives pertaining to the work presented in this Thesis, have to do with points (a) and (b) above.

(a) Public support for and interest in a public umbilical cord blood stem cell bank (UCB SCB) is a prerequisite to establish a sustainable public UCB SCB. Therefore, public preparedness and support for a public SCB was evaluated in this study, by addressing pregnant mothers attending the ante-natal clinic at the Steve Biko Academic hospital in Pretoria. An initial enquiry led to the initiation of a pilot study (Chapter 4), followed by a principal study into the public's response to UCB banking (Chapter 5).

In addition to the findings of the investigation into the public's response to establishing an UCB SCB, other important considerations include screening UCB units for infectious diseases, and in particular in the South African context, Human Immunodeficiency Virus (HIV-1). South Africa has a high prevalence of Human Immunodeficiency Virus / Acquired Immunodeficiency Syndrome (HIV/AIDS). There remains a risk of obtaining HIV-1 positive UCB through vertical (trans-placental) transmission of HIV-1, if a mother is in the latent phase of HIV-1 infection at the time of birth. This necessitates the implementation of additional safety and quality control measures for collection and screening of potential UCB units, prior to storage or distribution. Should a public UCB SCB be established in SA, all UCB units would need to undergo routine infectious diseases screening. Once stored, samples could potentially be made available to global UCB banks and would therefore need to comply with international regulatory and quality standards. Quality control standards include screening units for HIV-1, Hepatitis B-virus (HBV) and Hepatitis C-virus (HCV) by individual donation nucleic acid testing (ID NAT).

(b) It is thus mandatory to have a robust, sensitive and reliable assay for detection of HIV-1 in UCB units prior to banking, to prevent the banking of potentially HIV-1-positive UCB. The commercially available Procleix®Ultrio® Plus Assay (Ultrio-Plus® assay) is used by the South African National Blood Services (SANBS) for simultaneous detection of HIV-1, HBV and HCV in donated blood. This sensitive screening test is currently performed on all blood donations received at the South African National Blood Service (SANBS) and is internationally accepted and highly successful (Crookes et al., 2007).

The assay has been validated for use in human peripheral blood and organ and cadaveric tissues, but has not yet been verified for UCB. Therefore, this study (in collaboration with the SANBS) set out to verify the Ultrio-Plus® assay for routine use in an UCB SCB for detection of HIV-1 in UCB plasma (Chapter 6).

1.2 Addressing the burden of high rates of HIV-1 infection in South Africa: By rendering the immune system resistant to HIV-1 infection through genetic modification of haematopoietic progenitor cells (HPCs); Background to the larger study and specific components involved in the current investigation.

South Africa is faced with enormous challenges in the areas of HIV-1 prevention and treatment. Access to anti-retroviral clinics, compliance with drug regimens, side effects of drugs and drug-drug interactions are major problems for most South Africans living with HIV-1. Furthermore due to poor drug compliance, resistance to antiretroviral drugs is becoming a problem that cannot be solved as a limited number of antiretroviral regimens are available for salvage therapy. Multidrug resistant HIV-1 will force us to explore new therapy modalities such as vaccination; however, no vaccine is currently available for HIV-1 prevention although possible candidates are in various stages of development. Alternatives to vaccination and anti-viral treatments are therefore needed and stem cell therapy might hold the answer.

Working towards a cure for HIV-1, several studies have shown how haematopoietic progenitor cells (HPCs) could potentially serve as cellular vectors in a gene-therapy approach (Hütter et al., 2009a,b; Deeks and McCune, 2010). Rendering these cells resistant to HIV-1 infection would require genetic manipulation of HPCs (Liang et al, 2010). The long term objective of this larger project is thus to generate HIV-1-resistant haematopoietic stem cells (HPCs) for subsequent transplantation into patients with HIV/AIDS, in order to replace their endogenous HIV-1-infected HPCs. This would render the immune system of the recipient of these genetically modified HPCs – at least in part – resistant to HIV-1 infection. A proof of concept study has

previously been conducted (Hütter et al., 2009a) wherein allogeneic BM from a naturally HIV-1 resistant individual was transplanted into an HIV-1 positive individual and which subsequently generated HIV-1 resistance in the recipient.

Because the South African BM registry is not representative of South African demographics (more than 70% Caucasian), finding genetically matched BM samples for allogeneic transplantation of genetically manipulated HPCs for HIV-1 infected individuals could be problematic (Crookes et al., 2007; Ruff et al., 2008). Two possibilities exist to obtain genetically compatible HPCs for genetic manipulation: 1) Harvest autologous HPCs from HIV-1 infected individuals through apheresis (to overcome problems of rejection and graft versus host disease (GvHD)); or isolate CD34+ HPCs from allogeneic, genetically compatible UCB units (which will require immunosuppressive therapies to prevent rejection and GvHD).

The larger study aims to introduce genetic resistance to HIV-1 by isolating autologous CD34+ haematopoietic progenitor cells (HPCs) and transducing them with a lentiviral construct. This construct will target various aspects of HIV-1 infection (co-receptor binding, HIV-1 replication and transcription) in order to render the cells naturally resistant to HIV-1 (mimicking the Hütter study). These cells will then be re-administered to the patient for engraftment and reconstitution of haematopoiesis.

However, it is unclear whether early haematopoietic progenitors can be infected and/or are affected by HIV-1. Should primitive HPCs be susceptible to HIV-1 infection, autologous HPCs might already be infected or adversely affected and would therefore not be suitable candidates for gene therapy. Although some literature suggests that these cells are not susceptible to HIV-1 infection or replication, it is uncertain how the presence of HIV-1 might affect the haematopoietic capacity of these cells. This could potentially account for various cytopaenias and haematopoietic abnormalities that are observed in HIV-1^{+} patients. In the case where HIV-1 affects and/or infects primitive HPCs, allogeneic transplantation of genetically modified HPCs could be an alternative for treatment. Infants born to HIV-1 infected mothers might be able to receive their own genetically modified HPCs from their UCB units while adults could benefit either through pooling of genetically matched UCB units or expansion of UCB units to obtain adequate cell numbers for transplantation purposes. An abundant supply of UCB units stored in an UCB bank would be necessary to overcome the lack of currently available BM samples and to cover the vast genetic diversity of South African patients.

Therefore, components from the larger study that were investigated in this Thesis aimed to determine whether primitive $CD34^+$ HPCs isolated from UCB could be infected and/or are affected by HIV-1. The colony forming unit assay (CFU-assay) was used as a model to establish the haematopoietic capacity of HIV-1-exposed HPCs and the potential infectablitiy of HPCs by screening CFUs for the presence of HIV-1 with the Ultrio-Plus® assay (Chapter 7).

The Thesis is written in article format, where each chapter has its own introduction, relevant literature, materials and methods, results and discussion, conclusion and references. The last chapter, Chapter 8, discusses the final conclusions drawn from results of all the chapters.

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CHAPTER 2

2 Stem cell tourism

2.1 Introduction

Interest in the field of translational stem cell (SC) research has increased rapidly in the past decade, with exciting and promising research providing hope that cures for previously incurable diseases may well be attainable in the not too distant future. Much of the excitement originates from the ability of stem cells (SCs) to self-renew, replicate and to differentiate into any one of the more than 200 cell types in the body.

Various types of SCs with different potential therapeutic applications have been discovered and, although this may appear to be a relatively new phenomenon, these cells have in fact been used routinely for more than 50 years. The best understood are haematopoietic SCs, which have been successfully applied around the world in bone marrow (BM) transplantation for treatments of various conditions including malignant and non-malignant haematological disorders, immune deficiencies and certain genetic disorders. However, with new discoveries of different types of SCs and many potential novel applications, interest in regenerative and translational medicine has increased.

One consequence has been a dramatic rise globally in companies and clinics that sell stem-cell-related products or services. In addition to improvement in personal health and wellbeing, the increased interest in cellular and molecular medicine creates opportunities for employment, business development and entrepreneurship. South Africa has great potential for the development of regenerative and translational medicine involving SC therapies. Many thousands of people could potentially benefit from currently available therapies that SCs have to offer – e.g. bone marrow transplantation. In light of South Africa's current burden of disease and the potential for job creation, we certainly stand to gain substantially (individually and as an economy) from these and similar developments, possibly more so than many affluent countries. A single major concern for implementation and operation of such companies and clinics would be compliance with national and international regulatory standards – with the supposed precondition that appropriate national legislation and governance exist.

However, even though SCs harbour the promise of potential cures for many previously incurable disorders, this promise is easily exploited.

Although SCs appear to hold promise for future therapeutic applications (in addition to their current accepted applications), charlatans and con-men have started to prematurely promote bogus 'SC cures' for various – still incurable – diseases (Murdoch and Scott, 2010; Caplan and Levine, 2010). They often portray SCs as the 'holy grail' of cell therapies and have created much uncertainty and controversy in the field.

Unfortunately, many questionable SC practices occur in countries that lack governance and regulation. This has led to the phenomenon known as "stem cell tourism" (SCT); where patients travel abroad to undergo SC treatments not provided in their own countries (Lyndvall and Hyun, 2009).

2.1.1 Concerns of patient exploitation by medical tourism: Stem cell tourism

SCT is ethically problematic in that it offers unproven, untrusted therapies as legitimate cures for currently incurable diseases (Master and Resnik, 2011). There exists a large discrepancy between published, peer reviewed literature and claims posted on these illegitimate clinics' websites (Lindvall and Hyun, 2009; Murdoch and Scott; 2010). South Africa is vulnerable to exploitation with regard to SCT because of several gaps and inaccuracies in the current legislation (Pepper, 2012). The National Health Act[2] (NHA) was implemented on 2 May, 2005 while matters related to human tissue was classified under the Human Tissue Act[3] (HTA). However, many of the currently used scientific methods involved in scientific and medical practice had not been included in the HTA. Since then, Chapter 8 of the NHA (Sections 53,55,56,68,54 and 57 to 67) has been promulgated in order to deal with blood and blood products; assisted reproductive technology; cell-based therapy; transplantation; DNA and genetic services; tissue banks; and examination, allocation and disposal of human bodies and tissues (Pepper, 2012). Although Chapter 8 of the NHA provides some direction in the regulatory vacuum present, there is still important regulation that is either lacking scientific accuracy or absent in general, leaving room for potential malpractice.

With SC translation into the clinic (regulated or not) happening at a rapid pace across the world, South Africans are bound to be exposed to some form of SC treatment sooner or later. They need to be able to accurately distinguish between legitimate treatments and fraudulent practices and would therefore, at the minimum, need a trusted source of information to assist them in making decisions.

SCT has many facets that could lead to multiple pitfalls, each posing a unique combination of moral, legal, ethical and regulatory challenges. Hype over false therapeutic claims, ranging from the sublime to the miraculous, is endangering the entire field of SC therapies. Hopefully, these fraudulent practices will soon be curtailed by the implementation of strict regulatory frameworks – such as those established by the Food and Drug Administration (FDA) and the International Society for Stem Cell Research (ISSCR) (Strauss, 2010).

2.1.2 Mechanisms for promoting stem cell tourism

While having the world at our fingertips can be tremendously beneficial, millions of people today are bombarded with myriad options and opinions, leading perhaps to the single most daunting challenge of the information age: learning to distinguish fact from fiction.

The Internet and social networking have empowered many people to gain access to information on a virtually unlimited number of topics. Many companies use the Internet and Web pages to bring their business directly to consumers through direct-to-consumer advertising. However, limited monitoring of content on the Internet often creates difficulties in verifying the accuracy and credibility of the information presented. This is especially true when it comes to the field of SCs and their current and possible future therapeutic applications and translation into modern healthcare.

Con men mainly operate by promoting their activities on the Internet and reach a much broader audience than was possible in a pre-World-Wide-Web era. They offer false hope, promises of cures and miracle healings and often advertise unsubstantiated claims on their Web pages. They play on the needs and desires of emotionally vulnerable patients to be cured and coax members of the public into signing up for unproven SC treatments provided in often precarious SC clinics. Many unsuspecting customers have fallen prey to these illicit elements, which have the potential to discredit the legitimacy of SC research and true current and future therapeutic applications (Lindvall and Huyn, 2009).

2.2 Factors that intensify stem cell tourism

2.2.1 Lag in regulatory oversight

Certain countries have more strict regulations (e.g. the USA with its Federal Drug Agency (FDA)) than others (e.g. China, Mexico, India, Costa Rica, Thailand etc.), with fewer opportunities for such scams in the former (Brown, 2012). Unfortunately, the South African National Health Act[3]

does not provide sufficient regulatory guidelines for the latest advances and discoveries concerning SCs (as mentioned previously), a large regulatory shortcoming that could potentially be exploited by opportunistic individuals. Furthermore, many South Africans have not heard of SCs. Those who have have mostly been exposed only in passing and have not been properly informed. This absence of regulatory oversight and unavailability of easily accessible, reliable information regarding SCs and their applications renders not only the individuals but also the country vulnerable to questionable global influences.

2.2.2 Lack of proper communication between scientists and the public

A substantial gap exists between scientists and medical doctors that undertake legitimate SC research and the subsequent accurate translation of such research to patients. The media often sensationalises preliminary scientific findings, creating much hype based on half-truths – dangerous territory that is often exploited by con men (Laing, 2011). In the absence of appropriate legislation, increasing awareness of SCs and their promise in novel therapeutic applications for a wide range of disorders will undoubtedly bring with it an escalation in illegitimate SC practices.

2.3 The moral and scientific dilemma

Since the first technique for isolating ESCs was introduced by James Thomson and his team at the University of Wisconsin, SC research has increased exponentially across the globe (Kaufman et al., 1999; Barclay, 2009). Discovering SCs in easily obtainable resources such as adipose tissue and peripheral blood together with less invasive isolation techniques for obtaining these cells (such as umbilical cord blood collection) has left an open invitation to many undesirable "stem cell squatters" in the field of SC research and translational medicine.

Legitimate research is being conducted, ethically approved and undergoing various registered clinical trials. Although preliminary results for some stem cell treatments – such as using HSCs to treat myocardial infarctions or ischemia – seem positive, very few clinical trials have, to date, successfully completed phase III, which would bring new therapy or treatment for yet incurable diseases to the market (Kavanagh and Kalia, 2011; Kale et al., 2003; Orlic et al., 2001). The proceedings necessary to accredit the treatment are unfortunately tedious and painstakingly slow. The translational process from science to medicine is complex and painstakingly slow. The only proven treatments involving SCs over the past 40 years include blood disorders

treated with adult SCs through bone marrow or blood SC transplantations for rare immunological and non-immunological disorders. In addition to these, some treatments have been approved for bone- and skin grafting and certain corneal diseases or injuries, using adult SCs harvested from the particular tissue (ISSCR, 2012)

What is presented on the one hand is scientists doing legitimate research while on the other are fraudster latching onto promising research by offering unproven treatments to ill, desperate but hopeful patients. With an increase in awareness of the therapeutic potential of SCs inevitably comes a surge of illegitimate opportunists. The dilemma is that research just takes too long to go through all the right channels before it can benefit patients. Patients often don't have the luxury of time to wait for these treatments to become commercially available or for potential therapies to go through regulation and accreditation. In the meantime, the numbers of con men increase and bigger numbers of desperate patients get offered the option of unproven treatments. Furthermore, patients have a right to access medical treatment and no law forbids them to undergo treatment of any nature to which they give consent. The end result is the exodus of frustrated and impatient patients, unwilling to wait for local approval of SC therapies yet willing to risk their health and livelihoods on unsubstantiated claims (Caplan and Levine, 2010).

Despite repeated warnings from acclaimed scientists against overseas clinics that offer curative SC therapies for a variety of disorders, many doctors and their patients ignore this advice and still opt for treatment (Lau et al., 2008). According to Sean Morrison, director of the University of Michigan Centre for Stem Cell Biology and treasurer of the ISSCR, many doctors are venturing into their own SC initiatives (Barclay, 2009). Whether they are compelled to get involved by the steadily increasing patient demand for various unproven forms of SC treatments – only having their patients' best interest at heart – or not is hard to decide, since many of these doctors also stand to gain commercially from the treatments.

Some doctors, however, recognise the potential of a variety of SC treatments (HSCs, MSCs) and instead of subjecting their patients to doubtful practices abroad would rather opt to treat their patients themselves, where they are more certain of the type and quality of administered SCs and the correct application of them. This not only places them in a moral and ethical dilemma but could also have serious consequences for their licences as practitioners if they perform unlicensed and ethically unapproved procedures.

It is important to note that not all doctors who offer SC treatments are imposters. Just the same, as Timothy Caulfield at the University of Alberta's Health Law Institute, Edmonton, Canada states, people that offer treatments should publish their data in scientific, peer-reviewed journals (Barclay, 2009). The substantial risks involved in uncontrolled treatments necessitate verification of purported results in a controlled environment through appropriately structured clinical trials (Cyranoski, 2009). These should include assessments of safety, efficacy, harvesting, storing/culturing of cell isolates, dosing, administration procedures and ethically approved information leaflets and informed consent forms (Lindvall and Huyn, 2009). This transparency gives other researchers the opportunity to verify the claims, safety and efficacy of the treatment.

2.3.1 Emerging stem cell clinics and treatments

The number of clinics that offer SC treatments has increased exponentially in the past four years. In 2009 there were an estimated 200 clinics and, although it is difficult to determine an exact number because of the often clandestine nature of their activities, it is thought that the current estimate well exceeds this number (Cyranoski, 2009) and that they are found in various countries, as illustrated in Figure 1. Places such as China, Mexico, India, Costa Rica, Russia, Thailand, Germany, Hungary, Korea, the Dominican Republic, Jordan, Kazakhstan and Barbados are popular destinations, since regulations are generally less strict or non-existent (Caplan & Levine, 2010).

Figure 1: Known areas where illegitimate stem cell clinics are emerging around the World.

Some of these clinics offer treatments and cures for still incurable disorders, including amyotrophic lateral sclerosis (ALS), spinal cord injury, stroke, multiple sclerosis (MS), Parkinson's disease, all forms of blindness including optic nerve damage, systemic lupus erythematosis, brain injury, cerebral palsy, Down's syndrome, Alzheimer's disease, heart disease, diabetes and autism. Of the less serious treatments, cosmetic enhancement is at the top of the list with anti-aging creams, fibroblast/collagen injections (as an alternative to botox) or general 'health enhancements' (Lau et al., 2008).

Of particular concern are the cell sources. According to a study carried out by Lau *et al*. (2008), it was found that cells harvested from adult autologous SCs were provided in nine sites, comprising 47% of their study cohort. Bone marrow comprised 37% (in seven sites) of autologous cells used while cells were also obtained from adipose tissue and blood donations. The other 53% of cells were from sources such as foetal SCs, cord blood SCs, and embryonic SCs, peripheral blood, patient fat (adipose tissue), aborted foetuses, patient's skin, animal tissues, and human placental tissue. Treatments were provided for a wide variety of disorders ranging from neurologic and cardiovascular diseases to allergies. These were generally treated by SC infusion into cerebrospinal fluid (six sites; 32%) (lumbar puncture) while IV infusion was also common (Lau et al., 2008).

In addition to obvious health and safety risks, these clinics often charge patients exorbitant amounts averaging from $15 000 to $25 000 USD for their SC treatments. Costs are often unsubstantiated and do not include patient travel or accommodation (Cyranoski, 2009).

2.3.2 Advocates for stem cell tourism

Advocates for SC treatments do not necessarily endorse SCT, but their fervour to provide treatment often clashes with opponents of SCT.

People who advocate a patient's right to access all forms of treatment, potential or real, present the following arguments to support their case:

1. Patients often don't have the luxury of time. Their diseases usually progress fast and alternative SC treatments (proven or unproven) are their last option.
2. Clinical trials are costly and finding appropriate funding for trials is challenging (Barclay, 2009). In addition, clinical trials prolong the time until treatments become acceptable and therefore available to patients.

3. Advocates propose that they have patients' best interest at heart. They work with dying patients daily and they claim, consequently, to not have time to perform studies or write articles that are subjected to peer review.
4. They claim that SC treatments have thus far yielded little or no adverse effects (citing no graft- versus host-disease (GvHD) rejection of cells from autologous donors)
5. They disagree with the FDA with regard to classification of treatments with autologous cells. The FDA classifies all cells that are not minimally manipulated as 'drugs' that need to adhere to FDA rules and regulations pertinent to administration of a 'drug'. Conversely, advocates for SC treatments maintain that a body's own cells are not drugs and should be exempt from FDA regulatory requirements. Advocates accuse the FDA of stalling developments in SC treatment since they do not stand to gain directly from emerging SC treatments and potentially stand to lose profit because of a shift in medical treatment from pharmaceutical drugs to cell therapy. A recent example is the case of the FDA against the Broomfield, Colorado, Clinic, Regenerative Sciences. On 25 July, 2012 the US district court in Washington DC ruled in favour of the FDA's injunction (made in 2010) against Regenerative Sciences. They had been treating orthopaedic problems with their Regenexx® product, which the FDA classifies as a drug since cells were not minimally manipulated. However, Regenerative Sciences' medical director, Dr. Christopher Centeno plans to appeal the court's decision and maintains their product is not a drug (Aldhous, 2012)
6. Advocates want to capitalise on the favourable climate for new business start-ups in SC treatments. They are afraid of missing the opportunity provided in the emerging market.
7. Some advocates are furthermore driven by the potential fame and recognition of potentially curing a previously incurable disease with their SC treatments. They see themselves as pioneers and argue that technology always precedes regulation.

2.3.3 Adversaries of stem cell tourism

Adversaries – opponents to unproven SC treatments – are cautiously optimistic about the potential promises provided by SC treatments. They do not oppose the development of SC treatments, but rather propose a safe and regulated environment in which to practise and develop new treatments.

The case against SCT centres on the following points:

1. There should always be balance in new developing fields. Emphasis on scientific progress cannot override a scientist's responsibility towards the public to ensure safe and reliable treatments. Harmony should always exist between risk and benefit that should be obtained through phased and structured assessment of safety, efficacy, dosing and administration of treatments. Informed consent procedures must be assessed and approved and all innovation outside of research must be demonstrable, scientific and clinical (Cyranoski, 2009).
2. Opponents object to unrealistic, incomplete and false marketing often associated with unproven SC treatments that are made available to the public. Advertisements are generally over optimistic or positive, understating potential risks involved, and have numerous unsubstantiated claims of treatment efficacy without the necessary scientific proof to back up the claims (Master and Resnik, 2011; Caplan and Levine, 2010).
3. Adversaries object to the lack of transparency from companies providing SC treatments. At best, selective information is made available to patients and the public, creating opportunities for exploitation (Lau et al., 2008). Furthermore, little or no information is provided on experimental protocols, procedures or controls that allow for an independent analysis of the claims (Cyranoski, 2005).
4. It is imperative to subject all work to objective, peer-reviewed assessment, proper regulatory oversight and to conform to requirements from ethical councils (Lau et al., 2008). Patients are generally not followed up after treatments and there are no records kept of potential side effects. Without these measures, it is impossible to know the lasting effects of treatment, whether potential improvements are due to placebo effects or whether they are only temporary, and whether there are any side effects related to the cells administered or their route of harvesting or administration. This information is vital to the entire scientific community and could aid in developing effective, lasting treatments. With a lack of FDA-approved clinical trial data, 'evidence' is anecdotal and controversial at best.
5. Opponents of SC tourism maintain that false claims of safety and efficacy of treatments will eventually jeopardise legitimate SC research and its clinical translation. Once public confidence in these treatments is shaken, it will be difficult to convince people otherwise.
6. Another concerning factor is the lack of understanding by providers of SC treatment of the underlying biology of many of the disorders and their proposed SC treatments (Barclay,

2009). The fate of injected cells is not well understood and could lead to serious side effects or even death.

7. Erroneous therapeutic misconceptions abound and have been turned into lucrative business models (Cyranoski, 2005). Furthermore, there are no cost regulations for any of the provided treatments and patients are vulnerable to financial exploitation.

2.4 How to curb stem cell tourism?

Legitimate SC entities could curb malpractice and corruption through transparency, peer review, clinical trials, by addressing current misconceptions with regard to SC therapy and by raising public awareness of current clinical applications and exploitations of SC treatments (Lindvall and Huyn, 2009).

This calls for better governance of genuine research and open and accurate communication from scientists to the public. Creating an overarching, global, independent regulatory body could be one way in which to curb the wealth of inaccurate information and criminal activities on the Internet. This regulatory body could serve as a platform for translating legitimate research through open communication with the global public. In turn, each country should have its own regulatory body preferably linked to the global governing body. The regulatory bodies must follow up on and monitor all therapeutic claims. They should actively raise public awareness of current SC therapies and provide an accurate and clear consumer's guide to approved uses for cell therapies.

By enabling legitimate SC practices to operate under proper legislation and simultaneously increasing awareness of these legitimate practices, one could potentially reduce the appeal offered by illegitimate practices.

All doctors, scientists and suppliers of SC treatments must adhere to the minimal ethical, scientific and medical standards for treating patients with any therapy. Because many clinics fail to do so, the ISSCR has put together a task force and posted these minimal standards, together with a list of guidelines and clinic requirements, on its website (www.isscr.org) (ISSCR, 2012). This includes a list of questions about the treatments offered that patients could ask the specific clinics and that ought to be answered. Through this effort, the ISSCR aims at publishing a list of clinics that it believes adhere to the minimal standards of operation as suggested by the task force (Taylor et al., 2010). The ISSCR patient handbook could also be used both by patients

seeking treatment abroad and their physicians to make informed decisions about SC treatment and the necessary questions to ask the treatment providers prior to signing up for treatments (ISSCR, 2012) (http://www.closerlookatstemcells.org).

A similar approach has been taken by the International Cellular Medicine Society (ICMS). The ICMS has realised that there is virtually no stopping patients from going for so-called SC treatments and has therefore opted to encourage doctors and clinics to treat their patients on the basis of ICMS guidelines, which can be found on its website.

Caplan and Levine (2010) have mentioned how the neglect from mainstream medicine to act decisively on the issue of quackery has aggravated the problem, leading to whole countries purposefully gearing themselves to capitalise even more on the steady inflow of patients from abroad.

2.5 Conclusion

The vibrant field of SC research and treatment consists of dramatically different stakeholders, all of whom have specific interests and agendas. For all parties involved, the stakes are high and understanding the dangers of SCT is imperative. It should be pointed out that the use of the word "tourism" has arisen from the propensity for patients to travel long distances to receive treatments in foreign countries. However, the principles outlined above are equally applicable to activities that may exist in one's own country.

In order to reap the greatest benefit from what SCs have to offer, it is imperative to understand the current SC milieu. It is necessary to find the balance between scientific soundness in new discoveries and medical innovations and uncontrolled, experimental treatments that abuse the current regulatory vacuum. The focus should be on the creation of safe, effective, scientifically sound treatments in a controlled regulatory environment without compromising patient health care. These therapies should furthermore offer patients greater than or at least equal benefit to what conventional available therapies can provide. Unless the therapy provides benefit to the patient, it will be unethical and thus unacceptable to administer (Lindvall and Huyn, 2009).

Caplan and Levine (2010) succinctly summarise the problem:

> *Those who are drawn to SC therapies are often confused about the innovative status of these interventions, overly reliant on unsubstantiated claims about the quality of biological material being administered, or unable to readily locate balanced assessments of what medical tourism may have to offer for their particular problem. Professional societies and mainstream SC researchers have an obligation to do more.*

In light of these considerations, South Africa is especially vulnerable. With SC research still in its infancy in South Africa, we still have a clean slate to write on. As researchers we are in a position to do more for our country and its people.

The global atmosphere around SC treatments underscores the importance of distinguishing legitimate research and therapeutic application from potential fraudulent practices. As any virus spreads, so too will the infection of SCT, in this way rendering South Africa vulnerable to elements that could taint emerging SC research in SA.

One manner in which to further legitimate SC practices in South Africa is by establishing a reliable and easily accessible source of information. This information should be freely

accessible to the public, provide reliable information on SC donations and therapeutic applications and in this way establish safe and legitimate avenues for regulated SC therapies that will allow patients to distinguish legitimate SC therapies from fraudulent practices. A public umbilical cord blood stem cell bank could be a first trusted and publicly accessible entity to provide and distribute the correct information pertaining to legitimate SC therapies in South Africa.

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CHAPTER 3

Article adapted from this chapter and published in the South African Journal of Science;

Meissner-Roloff M., Pepper M.S. 2012. Stem cell research engenders interdisciplinary collaboration in science, ethics and religion. *S Afr J Sci,* 108(5/6), http://dx.DOI.org/10.4102/sajs.v108i5/6.1244

3 Stem cell research engenders interdisciplinary collaboration in science, ethics and religion:

RECONCILING SCIENTIFIC PROGRESS WITH CULTURAL PRACTICES AND RELIGIOUS BELIEFS

Science has the potential to impact significantly on society, and the South African Government's drive towards a knowledge-based economy aims to harness this potential. One example of particular interest is the field of stem cells. Although the field is likely to have a significant impact on healthcare, it is intrinsically fragile. This fragility results from the convergence of science, ethics and religion around issues such as the origin of life (in the case of embryonic stem cells). In addition, there is lack of concordance between the rapid rate of technological innovation on the one hand and the implementation of appropriate legislation on the other. Appropriately focussed inter-disciplinary collaborations considering science's impact on society, are necessary to avoid the negative consequences of this fragility. The joint conference co-hosted by the Vatican and NeoStem, an American-based international biopharmaceutical company, in Rome in November 2011, is an important step in this direction.

Stem cells are the building blocks from which all the different cells of the body are derived in response to a finely orchestrated series of chemical and mechanical signals. Stem cells have been used successfully for several decades to treat patients with a variety of diseases, and their future potential as therapeutic agents has opened up an exciting new field in medicine. In order to harness the full potential of the rapidly growing field of stem cells, it is important for the diverse disciplines implicated therein to align their goals and values. Stem cells and their application in "personalised medicine" extend far beyond the disciplines of medicine and pharmacology. There are humanitarian, social, ethical and religious concerns that will arise from developments in this field, and to ignore their impact on our communities would be to the detriment of all concerned. The establishment of interdisciplinary networks is necessary in order to retain and even accelerate the current momentum of research and to ensure that therapeutic benefits and patient safety are maximized.

The seemingly unorthodox collaboration between the Vatican and NeoStem is an example of the type of interaction between religion, science, medicine, bioethics, economics and philosophy that could pave the way for an interdisciplinary approach to stem-cell research.

Their unusual collaboration made headlines last year when the Vatican donated $1-million to NeoStem's Stem for Life foundation, the objective of which was to develop 'ethical stem cell research'. The worlds of science and religion do not necessarily oppose each other *per se*, the Vatican has not been seen as an unbiased partner with regard to support for science, since it will be recalled that the Vatican condemned Galileo Galilei as a heretic for his theories on the universe. Rev. Tomasz Trafny, a Polish-born priest, gave the LA times (20 October, 2011) two reasons for the Vatican's interest in collaborating with NeoStem:

> First, they [NeoStem] have a strong interest in … searching for the cultural impact of their own work, which is very unusual. Many companies will look at the profit and only at the profit. And the second, of course, is that they share the same moral, ethical sensitivity.... Because of that ethical position, we entered into this unique collaboration.

NeoStem has interests in adult cellular regenerative therapy, both in harvesting and storing adult cell units as well as in manufacturing adult stem cell (ASC) therapeutics. At first glance, there are no specific qualities that distinguish NeoStem from other players in the ASC therapy field. Although Neostem's interaction with the Vatican has met with some opposition, credit needs to be given for their creative approach to bridging the divide across disciplines. The reasons for NeoStem's collaboration may differ from those espoused by the Vatican. For example, there might be more to gain for NeoStem in the collaboration from a commercial perspective than the Vatican cares to admit. However, the collaboration was showcased under principles of morality and ethics, and although NeoStem will almost certainly benefit commercially, the focus was nonetheless on how to make stem cell therapies more ethical by focusing on ASCs rather than embryonic stem cells (ESCs).

The most widely publicized moral opposition to the use of stem cells comes from the use of ESCs. The Vatican's stance against the destruction of human embryos for the production of ESC therapies made them the perfect partner for NeoStem's ASC campaign. The Vatican is of the opinion that destroying an embryo does not uphold the ethics that maintain respect for life, regardless of the stage of the embryo's development (Pope Benedict XVI, 2006) and therefore gladly support NeoStem's alternative 'ethical stem cell research' which is limited to ASCs. Public awareness of ASCs is very limited to say the least, and their potential future applications are not well understood.

The Vatican-NeoStem conference strongly promoted the potential therapeutic applications of ASCs and the impact that cell-based therapy will have on society's social, cultural and religious interactions. A limited number of new insights were offered at the conference, at which various kinds of ASCs and their potential therapeutic applications were presented as better 'ethical alternatives' to ESC therapies. Current misconceptions regarding ASCs were discussed, together with patient testimonials that highlighted several successes in ASC therapies. Speakers strongly contrasted the apparent moral and ethical high ground of ASC therapies to that of ESCs. Reasons for the Catholic church's support for ASCs were given from a theological, philosophical and humanitarian point of view, with emphasis on the moral and ethical responsibility that scientists have towards society. In short, the public platform created by the conference was cleverly utilised to convey the scientific message of "adult stem cell hope" (inevitably mixed though with a little "stem cell hype" – specifically where patient testimonials were used to underscore the effectiveness of stem cell treatments).

The translation of innovation in the fields of science and medicine into therapeutic products - also known as translational medicine - has revolutionised the way scientists view modern medicine and health care in general. Despite opposition, the Vatican ventured into the stem cell arena, attempting to address preconceived misperceptions about their support for regenerative therapies. By backing NeoStem, the Vatican showed their support for regenerative therapies using ASCs as alternative to ESCs. To their credit, they have realised that in our modern society, the church cannot afford to stagnate or to be indecisive. Modern-day believers are increasingly confronted with how to marry their belief with scientific progress. This dilemma becomes more difficult when religious beliefs are contrasted with developments aimed at alleviating human suffering. The church thus needs to provide a solid foundation for dealing with contemporary issues.

Despite the obvious marketing benefits to NeoStem, it is the authors' opinion that the company used the Vatican as the 'moral microphone' through which to market their ASC therapies to the religious masses. They hoped to reach people who have been confused by ethical concerns and debates surrounding ESCs and who as a consequence have avoided the stem cell arena altogether. NeoStem appears to have wanted to raise public support for the use of ASCs by pacifying concerns related primarily to ESCs and by placing the Vatican's "religious stamp of approval" on their progress, as articulated by their chairman Dr. Robin Smith: "It's like when you have the Good Housekeeping seal of approval, this is the Vatican seal of approval."

Even though their motives were probably less altruistic than claimed, there is a lot to learn from the Vatican-NeoStem interaction. The conference and the ideas that emanated therefrom emphasize the need for a holistic approach to science and scientists' roles in the community and the world. Philosophical questions regarding human existence and suffering continue to challenge our implied and expected ethical and moral responsibility, requiring scrutiny and dissection of own motives, agendas, values and beliefs.

Can science and religion truly find common ground as partners in a mutual effort to find cures to alleviate the suffering of many? The Vatican believes they can, on condition that life is sustained and improved without compromising social and scientific integrity and clearly defined ethical and moral principles.

The unique South African mix of diverse cultures and religious practices and beliefs adds a further degree of complexity to the situation and necessitates a solution to the science vs. religion debate. Inefficiency to bring about effective scientific translation to the public could potentially have a deleterious impact on the efficacy of implementing (novel) medico-scientific discoveries in the South African context. It is therefore imperative for South Africans to find a solution to educate the public with regards to medico-scientific progress while being sensitive to cultural and religious practices.

The author believes that science and religion should complement and strengthen each other and that interdisciplinary collaborations are required to bring a holistic view to an increasingly interconnected world. However, for someone with a different world view, this might not hold true and in that regard, compromise can only extend so far… Certain core values and beliefs are irreconcilable because those values often define an entity, and compromise of those values will necessitate a change in identity, giving rise to a loss of character, which few are willing to accept.

Subsequently it was important to establish the extent of cultural and religious influences on public support for the establishment of an umbilical cord blood stem cell bank in South Africa.

CHAPTER 4

Article adapted from this chapter and published in the South African Journal of Science;

Meissner-Roloff M., Young W., Rangaka I., Lombaard H., Dhai A., Tsotsi N., & Pepper M.S. 2012. Pilot Social Feasibility Study for the Establishment of a Public Human Umbilical Cord Blood Stem Cell Bank in South Africa. *Stem Cell Reviews and Reports,* DOI: 10.1007/S12015-012-9390-7.

4 Pilot social response study for the establishment of a public human umbilical cord blood stem cell bank in a selected South African population

4.1 Introduction

There is a large unmet need in South Africa for bone marrow transplantation. Since its establishment in 1991, the South African Bone Marrow Registry (SABMR) has registered circa 70 000 potential donors. More than 70% of these individuals are Caucasian and are hence not representative of South African demographics (BMDW, 2011). Most of the samples available in the SABMR are thus not genetically compatible with the majority of South Africans and precludes them from benefiting from the SABMR.

The SABMR, as with bone marrow registries across the world, has as a further drawback, namely a donor attrition rate of about 25% (Crookes et al., 2007). This greatly hampers efforts that go into finding an adequate match with further time and cost implications. For this reason umbilical cord blood (UCB) is seen as a viable alternative source to bone marrow (BM) (BM itself or mobilized peripheral blood stem cells (PBSCs)) for bone marrow transplantation.

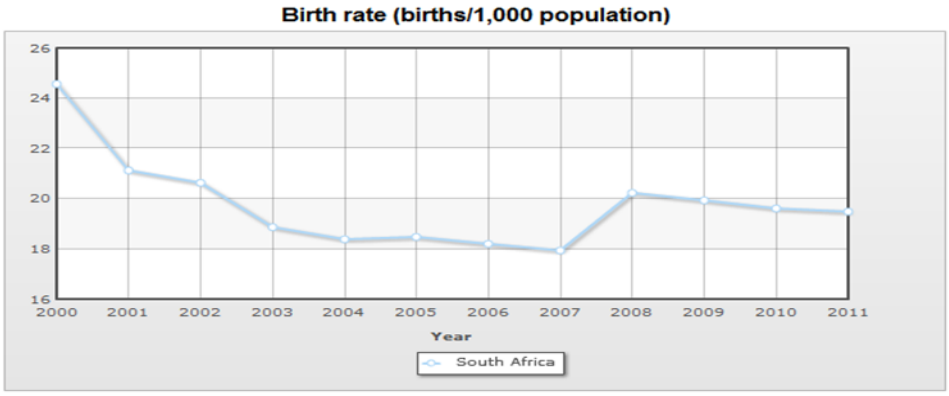
Haematopoietic stem cell transplantation is indicated for the treatment of various conditions including malignant and non-malignant haematological disorders, immune deficiencies and certain genetic disorders. UCB is an important source of haematopoietic stem cells (HSCs). Haematopoiesis, the formation of blood cells, is achieved by lineage-specific differentiation of haematopoietic stem cells (HSCs). HSCs are undifferentiated precursors of myeloid and lymphoid cells, mainly residing in adult bone marrow but can also be found in peripheral and umbilical cord blood.

HSCs are characterized by multipotency and the ability to self-renew. They are known to home to sites of injury and have the capacity to reconstitute the entire haematopoietic system post ablation (irradiation or myeloablation)) (Kavanagh and Kalia., 2011; Lewis, 2008; Shizuru et al., 2005). Furthermore, UCB HSCs display a greater tolerance toward HLA-mismatch than stem cells from bone marrow or peripheral blood which increases the chances of finding an adequate match for transplantation. With cord blood a minimum requirement of a 4/6 match HLA-A, -B, -DRB1 maternal and paternal alleles is needed while BM and PB require a 9-10/10 match of HLA-

A, -B, -C, -DRB1 and -DQB1 maternal and paternal alleles (Wagner et al., 1996; Hough et al., 2009).

According to Brunstein (2007), multiple studies have demonstrated the efficacy of cryopreserved UCB stem cells from 4-6/6 HLA-A,B antigen and DRB1 allele matched unrelated donors. These studies were done in patients with haematological malignancies and the UCB units were found to contain sufficient numbers of HSCs to engraft most paediatric patients (Brunstein et al., 2007; Gluckman and Rocha, 2006).

In South Africa, blood found in the umbilical cord and placenta is routinely discarded post-delivery. With around 20 births per 1000 individuals in South Africa (Figure 2), we theoretically have an abundant and regular supply of UCB units that are likely to be genetically compatible to the majority of South Africans (Anon, 2009). Furthermore, the umbilical cord itself contains large numbers of mesenchymal stem cells (MSCs), in what is known as the umbilical cord's 'Wharton's jelly'. Mesenchymal stem cells are known to have immune-suppressive properties and co-transplantation of these cells with HSCs could contribute to successful transplantation with decreased risks of GvHD.



Country	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011
South Africa	24.56	21.12	20.63	18.87	18.38	18.48	18.2	17.94	20.23	19.93	19.61	19.48

Figure 2: Annual birth rates from 2000 to 2011. This crude birth rate indicates the number of live births per 1,000 mid-year South African population. (Used with permission from The World Factbook (Anon, 2009)).

Collecting UCB is a non-invasive procedure and once collected, the purified stem cells can be stored indefinitely until a unit is needed. Storing these units in stem cell banks (SCBs) throughout South Africa, could thus serve as a vital, easily obtainable resource.

It is still unclear how much cell viability is affected over longer periods of cryopreservation (>25 years). However, promising new techniques in UCB expansion and innovative uses for UCB - such as the generation of induced pluripotent stem cells (iPSCs) - potentially increases the value and need for stored UCB units for the future (Broxmeyer, 2010; Gilmore et al., 2000; Kedong et al., 2010). iPSCs are differentiated somatic cells that have been de-differentiated through genetic engineering, to revert back to their embryonic-like state. These cells are thus pluoripotent, have the ability to migrate and self-renew. They could be coaxed into differentiating into several cell lineages and has enormous potential for future autologous use.

Together with a greater tolerance for HLA mismatches, the use of UCB for bone marrow transplantation results in a lower incidence of graft vs. host disease (GvHD). There are no ethical concerns for using UCB as source of stem cells as opposed to those encountered with embryonic stem cell research and no risk of donor attrition (Hough et al., 2009). This makes UCB a safe, convenient and reliable resource of stem cells (Crookes et al., 2007; Rogers and Casper, 2004).

Many South Africans are currently unable to access these treatments due to a lack of HLA-matched bone marrow (BM) or UCB units and would therefore benefit directly from UCB units stored in a public bank. Establishing an UCB bank would assist the government to give effect to the right of each person to access to health care services. The state is obliged to progressively realise this right as stated in section 27(1)(a) and 27(1)(b) of the Constitution of the Republic of South Africa, 1996.

However, South Africa has an extremely high prevalence of Human Immunodeficiency Virus / Acquired Immunodeficiency Syndrome (HIV/AIDS). In mid-2007, following the latest antenatal survey, the Department of Health, in collaboration with UNAIDS, WHO and other groups, published an updated estimate for HIV prevalence of 18.34% in people aged 15-49 years old in 2006. This equates to around 5.41 million people living with HIV in 2006, including 257,000 children. The ASSA2003 model provides a similar estimate of 5.4 million people living with HIV in mid-2006, or around 11% of the total population. It predicts that the number will exceed 6 million by 2015, by which time around 5.4 million South Africans will have died of AIDS (UNAIDS, 2010). .

Statistics from 2011 show a slight decrease, estimated at 10.6%, in overall HIV prevalence since 2006. The total number of infected individuals is estimated to be 5.38 million with 16.6% of adults aged 15-49 years being infected. The total number of new infections was estimated to be 316 900 of which 63 600 were children below the age of 14 (UNAIDS, 2010). Consequently, all UCB units considered for banking, will need to undergo rigorous testing and screening, prior to acceptance for storage.

The sensitive Ultrio-Plus® assay is currently performed on all blood donations received at the South African National Blood Service (SANBS) and is internationally accepted and highly successful (Crookes et al., 2007), but has not previously been verified for screening of UCB.

4.2 Materials and Methods

The process of informed consent is often reduced to only obtaining consent without truly informing the patient of benefits, risks and alternatives and ensuring that the patient understands the information. However, investigators have a moral and ethical duty not only to obtain consent, but also to ascertain that all components of informed consent are satisfied, i.e., disclosure, understanding and appreciation, capacity and voluntariness. The challenge of obtaining informed consent was particularly demanding due to our unique South African context.

The objective of the social response pilot study was to gauge public reaction towards and support for UCB stem cell donation and banking, as well as their perception of the processes of donation and subsequent HIV testing of UCB (necessary for compliance with international regulatory standards). A concise interview and questionnaire were constructed to convey information about the study. Balancing simplicity with accuracy and comprehensiveness was essential in order to make the information accessible to everybody and simultaneously address related questions and concerns. The anonymous questionnaire – in the structure of an informed consent document – was completed by the target population: mothers attending the ante-natal clinic in the Steve Biko Academic Hospital in Pretoria and consisted of three questions:

Question 1: Would you be willing to donate your placenta (afterbirth) to medical research?

Question 2: Would you be willing to undergo an additional HIV test seven days before or after the birth of your baby?

Question 3: If it is against your cultural or religious belief to donate your placenta for medical research, would you be willing to donate the blood from the placenta?

77 mothers were randomly selected to partake in the pilot study. Signed informed consent was obtained after they completed the closed questionaire.

Visual aids (a doll with an umbilical cord and placenta and relevant posters) were used to explain concepts relating to UCB banking during the interview. Questions that arose from the interview were annotated by the investigators and used to reconstruct the interview process and questionnaire for the principal study. The quantitative data gathered from the

questionnaires was captured and analysed using Microsoft Excel while qualitative data was gleaned from patient comments written on the questionnaire.

Approval for this study was obtained from the Main Research Ethics Committee at the University of Pretoria (131/10) and subsequent amendments that were deemed necessary were submitted and approved by the same committee. Ethical approval was valid for the duration of the study (2010 – 2012) and the final approval document is attached as Annexure 5.

4.3 Results

4.3.1 Qualitative results

4.3.1.1 Introduction

With these principles in mind, the investigators addressed the mothers attending the ante-natal clinic at the Steve Biko Academic hospital in Pretoria. Their responsibility was to introduce the mothers to stem cells in an easily accessible manner while maintaining a realistic scientific view of the field. Although conflicting views and opinions regarding current, acceptable stem cell treatments exist, it was important for the investigators to convey a balanced, realistic view of current and future possibilities with stem cell treatments. Imparting understanding to the patients equipped them to make informed decisions. The patients' contribution to the study was clarified at the onset of the interview, giving them the freedom to choose to participate or not.

The investigators encountered various obstacles which eventually shaped the structure of the principal study. These obstacles are discussed as qualitative results from the interview process and include; Ill prepared clinical settings for obtaining informed consent, cultural differences, religious practices, traditions and superstitions together with language constraints and educational disparity. Clarification regarding cell harvesting, storing and administration was compulsory together with an explanation of existing and possible future applications of stem cell treatment.

4.3.1.2 Patient interaction

It was vital to establish and maintain good inter-personal relationships with hospital staff and attending mothers to ensure a good and effective working relationship. People interaction is dynamic and required a perceptive and creative investigator to ensure active and continued

patient involvement. A relaxed and spontaneous environment facilitated a trust-relationship and patient participation. Given South Africa's history of Apartheid, dispelling fears and enkindling trust in a country wrought with racial conflict and mistrust is no small feat to accomplish.

Physical and logistical distractions provided by the environment in which the interviews were conducted complicated the interview process. The interview had to proceed concomitantly with routine administrative procedures taking place within the waiting room. Attending patients were constantly distracted by staff during the interview for administrative purposes related to patient files and by being sent away for blood tests, urine tests and patient weight). Furthermore, no time-slots were allocated to arriving patients who were treated on a first-come-first-serve basis, which contributed to the disarray. These circumstances effectively limited the interview to an interrupted 15 min. contact period.

Questionnaires were only handed out after the interview so as not to serve as an additional distraction. Asking general questions that served as leads to the next explanation in the presentation was very successful with regard to involving the patients. Upon first introduction to the study, some patients were reluctant to fill out the questionnaire and requested to discuss the information with family or friends. However, patients visit the clinic frequently and some returned with questions, while others filled out the form on a second or third exposure to the study.

This could have been due to patients becoming better acquainted with the study, the investigator and concepts which improved participation and dismissed initial prejudices. Initial concerns might have been addressed on a second exposure while peer pressure could have also coaxed patients into participation.

Given the circumstances, it was most beneficial to address the patients as a group. Patients were less intimidated by foreign concepts by not being isolated from the group; they were exposed to questions raised by others; were able to ask related questions and to interact amongst themselves in their own language in discussions which the investigator facilitated. One-on-one interviews were performed with individuals that did not understand English. One-on-one interviews were unfortunately not always possible, given time constraints, inadequate space and the lack of an interpreter.

The above-mentioned constraints necessitated a concise interview and informed consent questionnaire. The use of visual aids (in the form of posters and a doll with umbilical cord and placenta) greatly contributed to the clarity of the information presented and improved patient interaction. It held patients' attention and clarified foreign or difficult concepts concerning UCB collection and banking. The waiting room was unfortunately not equipped to use projectors or video, valuable tools to convey information – which could be especially beneficial in the absence of a translator.

4.3.1.3 The language barrier

English was used to convey the information during the informed consent presentation. South Africa has 11 official languages and many South Africans speak English only as a third or fourth language. Many patients' understanding was thus limited to that of basic conversational English. This understanding was often insufficient for them to grasp more technical concepts concerning stem cell banking and was arguably the biggest stumbling block in obtaining consent.

A succinct presentation, clear and to the point, with comprehensible English terms and short sentences, helped to make the presentation more accessible to non-English speaking patients. Concepts such as "placenta", "stem cells" and "public SCB or facility" remained foreign. These concepts were better explained through analogies or translating the words into other languages e.g. "placenta" translates to "ingubo" in Zulu and "gobo" in Tswana and means "blanket". It is commonly understood to "cover the baby in the womb".

There was limited access to a translator at the onset of the pilot study. After identifying a need for translation, a colleague joined the investigators during the presentations to assist in translation of the presentation, or parts of the presentation, into Zulu and Tswana.

Having a translator present was beneficial even to the patients that understood most of the presentation in English. It gave some patients more confidence to clarify concepts and ask questions in their own language. It particularly helped when patients with no understanding of English came to visit the clinic and the translator often conducted one-on-one interviews with these patients. Translation of the informed consent questionnaire into some of the commonly spoken languages will ease the strain of communication and clarification of concepts.

4.3.1.4 Culture and Religion

Culture and religion are often closely associated and certain cultural aspects or religious practices had to be taken into consideration during the interview. Together with cultural practices from South African citizens, the investigator encountered cultural practices in immigrants from neighbouring countries. Some of the countries that were represented included Somalia, Zimbabwe, Angola and the Congo.

4.3.1.5 Cultural practices

The pilot study's questionnaire unfortunately did not make provision for recording patient ethnicity. For the purpose of building public SCB facilities in different provinces, it would have been beneficial to segregate the data into cultural or religious groups. This segregation in turn could aid in the identification of customs potentially conflicting with the requirements for establishing a SCB. Knowing whether certain practices are more likely connected to a specific language or cultural group from a certain geographical area would have aided in choosing locations for cord blood collection. These changes were incorporated for the compilation of the principal study's informed consent questionnaire.

Given South Africa's diverse cultural and religious background, it becomes imperative for investigators to at least understand the fundamentals of cultures and religions when addressing the patients, in order to anticipate sensitive topics or misconceptions and address them appropriately.

4.3.1.6 Social rules of engagement

As mentioned previously, all mothers attending the ante-natal clinic at the Steve Biko Academic Hospital were addressed during an interview. At the onset of the study, patient interaction was limited. Some of the older women (between the ages of 30-50) seemed to be more hostile towards the investigators. Although the study did not set out to test the influence of pre-existing prejudice, this could be due to such prejudices regarding people from different races, a sad memento from our past, or could be ascribed to differences in "social conduct". Even though our country bears the scars of Apartheid, most women addressed at the clinic seemed to nevertheless have moved past the prejudices of race.

The investigators probed patients about their reluctance to participate and reasons given related to topics of "social conduct" and "social hierarchy". Certain cultures regard elderly

people as having a higher social status, which demands more respect than peers it is also regarded as disrespectful for a younger person i.e. the interviewer, to ask an older female about childbirth beliefs and practices. A young person would not be allowed to ask questions or speak out when an older person is present, regardless of the younger person's knowledge concerning the topic. It is seen as disrespectful and would be frowned upon by members of that community. Similarly, when a speaker addresses a group – such as the investigator - that speaker is seen as learned, wise or knowledgeable. Only certain elders would be allowed to address that person directly or ask him/her questions. Questions should thus be kept to oneself or should be directed at others present after the presentation, for fear of insulting the speaker or the elders present.

The investigators were unfamiliar with these concepts and fears as would be most people who are not of African origin. Patients' fear and hesitation translated into unasked and unanswered questions contributing to a lack of understanding of the concepts involved with UCB banking. Consequently patient feedback was lacking and many questionnaires were left unanswered.

By addressing these concerns, encouraging patients to ask questions (even if only amongst themselves) and leading them with questions, the investigators were able to overcome these cultural hindrances. By facilitating a discussion amongst the attending mothers, many more mothers were involved and supportive of the study.

When questions were asked, they served as valuable tools to structure the presentation and questionnaire, to eradicate all ambiguities and clarify concepts in the interview. Without this necessary feedback, it was difficult to gauge the patients' level of understanding.

4.3.1.7 Religious ritual practices

Certain ritual practices related to the placenta were also encountered. Instead of discarding the placenta in the normal way (incineration at the hospital), some religions retain the placenta after birth for various reasons. Reasons range from rituals of burying the placenta to eating it. These patients were not willing to donate their baby's placenta and umbilical cord to medical research or in some cases for the blood to be collected from the placenta after birth.

Together with some of these ritual practices, there are many superstitions and beliefs, often accompanied by fear. Some groups believe that blood constitutes a "special life power". Should anybody thus take blood from e.g. the placenta, they will take the "life power" from the

child and put this child at a disadvantage. Other patients expressed concerns in seeing the placenta, since they believe this will put a curse on the child's life with deleterious consequences for the child's future.

4.3.1.8 The influence of traditional medicine

Many Black South Africans strongly adhere to traditional medicine in various forms, some more accepted than others. Some mothers-to-be were afraid that blood samples (or the placenta) would be used for "muti" – a controversial form of traditional medicine. The stigma surrounding "muti" comes from occasional "muti-murders" where mostly very young or very old people are either murdered or mutilated in order to obtain organs or body parts for use as ingredients in "muti-medicines". When the investigator unwittingly talks in layman's terms of how stem cells can be used as a type of a "medicine" to "cure" certain diseases, he/she could immediately raise concerns about "muti" if this is not clarified and taken into consideration by the investigator during the interview.

4.3.1.9 Stigma surrounding HIV status

HIV/AIDS education has come a long way in South Africa but a lot more still needs to be done to address social prejudices and fears. This is particularly true when considering the very high HIV prevalence in our country and the continuing stigma and mysticism that surrounds HIV/AIDS.

Patients were often afraid that their HIV status would be revealed during or as a result of the interview process. Some were afraid of being victimised, of not receiving proper treatment or of being treated with contempt or disdain. Some mothers were reluctant to take HIV tests for fear of knowing their status while some were afraid of needles and the drawing of blood. One patient admitted to the investigator to being HIV infected and was reluctant to donate blood in fear of accidentally infecting another person with HIV.

Since all UCB samples need to be screened routinely for infectious diseases, including HIV, in order to be accepted nationally and internationally, a patient's refusal to do an additional HIV test at the time of delivery of the child would lead to the non-inclusion of that sample into the storage facility.

4.3.1.10 Educational disparities

As a general rule, the interview and questionnaire were structured to be easily accessible and understandable. Some patients were unable to read while others could not read in English and

were mostly reliant on understanding the information from the presentation. Some of the attending mothers had not completed their schooling, not uncommon in South Africa, and consequently lacked foundational understanding about English and often biology.

Questions that arose from the presentation can be divided into three main categories: Questions concerning female biological processes, questions related directly to the presentation and medically related questions.

4.3.1.11 Biologically related questions

Some patients lacked a general understanding of normal female health and reproduction. This led to questions relating to the origins of the placenta, menstruation, the role of the placenta during and after pregnancy. A few patients had concerns primarily centred around the placenta: how many children they could have after donating a placenta; whether a person can live without a placenta; how many placentas a mother expecting twins has; and whether the placenta could be donated after a miscarriage.

4.3.1.12 Questions directly related to the study

Patients were initially unsure of the blood collection procedure. Before introducing visual aids to support the presentation, some patients confused donating the blood from the placenta with organ donation. Some patients were afraid of, while others were excited at, the prospect that they could allegedly be sterile after donating the placenta or blood from the placenta.

Other questions included the volume of blood to be collected, where and from whom the blood would be collected, and the subsequent storage and redistribution of the collected blood. Some did not understand the necessity for additional HIV tests on patients that have already undergone HIV tests.

Additional questions regarding the technical side of UCB banking had to be addressed, complicating the interview process: Mothers had to understand the current uses of UCB, together with the prevalence of disorders that could be treated with a BMT using UCB. They often enquired about an individual's eligibility for a transplant and the subsequent procedure of obtaining an appropriate UCB unit. Some mothers were concerned about the availability of a sample and about finding an appropriate match should they require it.

4.3.1.13 Medically related questions

These included questions related to congenital disorders, alcohol abuse, the subsequent eligibility of that person to donate their UCB and situations relating to twins.

Some of the above mentioned questions could have been easily addressed by rewriting the interview and clarifying concepts during the presentation. However, as the investigators were not qualified medical physicians, these questions were referred to the doctors on duty. Learning which questions to answer or refer to medical doctors required sensitivity to each situation and added to personal development of the interviewers. Processes of Banking

The notions of stem cells and banking were foreign to most of the attending mothers and they therefore needed to be assured of the stem cell banking technology, its efficacy and applications. The processes involved in UCB banking - from harvesting the UCB immediately after delivery, to sample processing, storage and redistribution - had to be explained thoroughly. This was particularly important given the fears of organ trafficking and the use of body parts in certain traditional medicine cocktails referred to earlier ("muti"). Furthermore, it was necessary to provide a clear motivation for the study and how South Africa could benefit from having a public SCB. The benefits of having a public and/or private bank had to be addressed without partiality – a contentious topic requiring unbiased diplomacy.

Based purely on our circumstances in South Africa, there is a strong motivation for establishing a public SCB. One of the strongest motivators is the great genetic diversity in our population and the consequent lack of compatible sources of bone marrow or UCB to treat the majority of South Africans. The people's need for access to healthcare and specialised treatment outweighs most arguments against the establishment of a public SCB in South Africa, with the exception perhaps of financial considerations. This would respond to the ethical imperative of access and equity, and hence social and distributive justice in the country.

4.3.1.14 Personal obstacles

Learning how to build a patient-interviewee trust relationship was vital to ensure successful interactions with the patients. Learning what to say, how to say it and when and how to refer difficult questions, was one of the fundamental hurdles that needed to be overcome in order to successfully complete the interview.

Due to the novelty of the particular study, it lacked appropriate protocols and format for addressing the patients. Information conveyed was too broad, lacking direct relevance to the study. Instead of informing patients, details given regarding rituals and practices fed existent concerns and uncertainty amongst the patients. It gave way to a fear of unknowingly becoming involved in cultural or religious taboos. As a consequence, patients were reluctant to fill out the questionnaire and wanted to consult with their elders regarding their cultural practices before becoming involved in the study.

All irrelevant information confused the patients and detracted from information that was important. Questions from the mothers-to-be helped to point out discrepancies and uncertainties that arose as a direct consequence of the lack of experience or training of the interviewers. Feedback from patients was encouraged at all times and used as positive criticism to improve the questionnaire and interview process.

The nature of the interview/presentation and difficult questions (because of moral, ethical or legal reasons) that arose in these areas prompted a revision of the questionnaire, presentation and interview process. All changes were incorporated into a principal social response study (discussed in Chapter 5).

4.3.2 Quantitative results

4.3.2.1 Introduction

Although a few routine medical procedures exist that involve UCB transplantation, there are many more possibilities for potential treatments that could be explored. With vastly improving techniques for expanding haematopoietic stem cells (HSC) in culture, it is possible that samples stored in public banks will become a vital resource for novel forms of therapy in the future. It was therefore deemed beneficial to include in the questionnaire the possibility that samples could be used for medical research and/or public use. The term “medical research” was used to describe all downstream applications that involve current and possible future treatments with UCB. A clear distinction was made during the interview process between the current use of UCB for transplantation and research purposes.

4.3.2.2 Results

Results from the social response pilot study questionnaire are summarised in Figure 3.

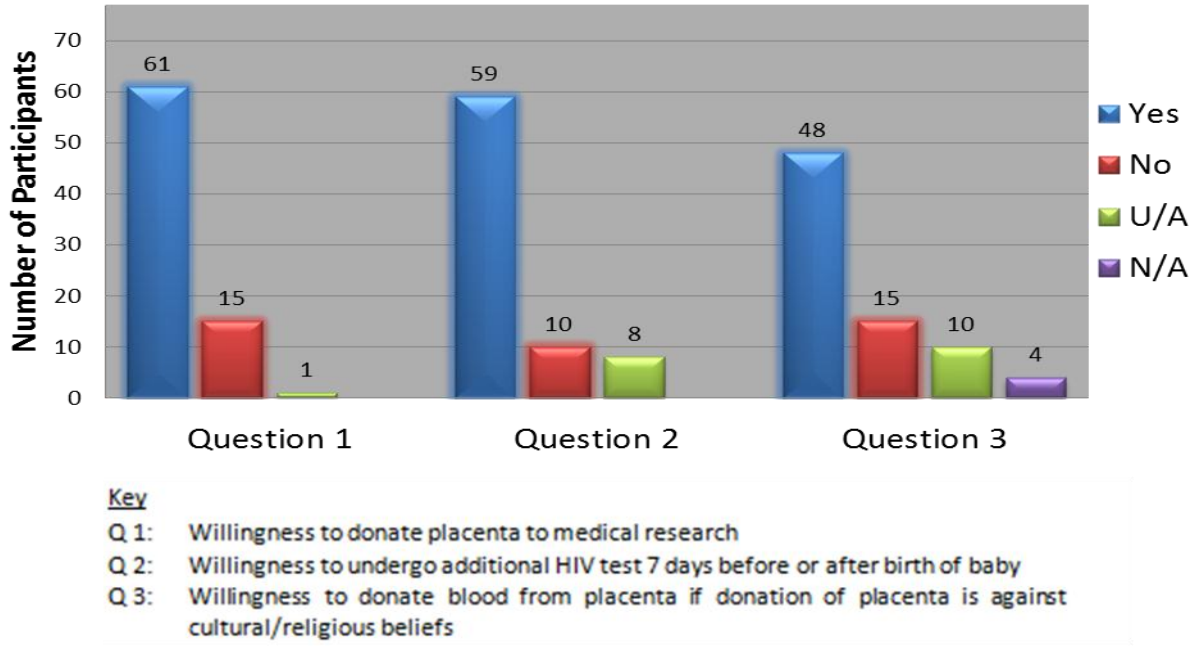


Figure 3: Summary of results from the informed consent questionnaire. Numbers above the bars in the graph indicate the number of patients that answered the particular question, out of a total of 77. Answers with either "Yes" – indicated by blue bar, "No" – red.

The patient cohort in this pilot study was intentionally small (77 patients) and was limited to patients attending the ante-natal clinic at Steve Biko Academic Hospital. Many of the mothers-to-be attending this clinic are high risk pregnancies and are often referred by their local clinics. It can be argued that these mothers receive more information regarding their pregnancies and might be more educated and have better access to information concerning their pregnancies than mothers attending other clinics. This could have facilitated the presentation of information to the mothers at this clinic, and it was understood that it might be more difficult to convey the same information in rural clinics.

As previously mentioned, initial participation in the study was poor. Many more patients filled out the questionnaire after adjusting the interview process to eliminate some of the uncertainties and fears discussed. Data gathered from the questionnaire seemed to indicate that administrative procedures, interview language and a person's education played the biggest roles in determining participation in the study. Cultural and religious concerns and HIV status only accounted for 7% of expressed reluctance towards establishing a SCB. These results need

to be confirmed however, in a larger study with different patient demographics in order to obtain statistical significance.

Despite the interviewer's efforts, some questions in the questionnaire were sometimes left unanswered (U/A) (Figure 3). This could either be an indication that the patients did not understand the question, were undecided or reluctant to answer (e.g. to undergo an additional HIV test) or were not able to complete the questionnaire because of time constraints (e.g. called by a nurse or doctor).

Of the 77 participants, only 51% (40 participants) answered "yes" to all three questions. 79% of the patients (61 patients) answered "yes" to Question 1, indicating a willingness to donate their placenta. Seventy six percent of the patients (59 patients) were willing to undergo an additional HIV test (Question 2) and 62% (48 patients) were willing to donate the blood from the placenta to medical research (Question 3) (Figure 3).

There was a discrepancy found between Question 1 and 3 in 8% of the questionnaires: 12 participants that were willing to donate the placenta itself, were unwilling to donate the blood from their placenta (Question 3).

It might be argued that 8% of the participants answered "no" to the first part of Question 3 "If it is against your religious or cultural belief…" indicating that it is not against their cultural belief and that they would donate the blood from the placenta. However, it could also indicate that they did not understand that by donating the placenta, they were giving permission to use the blood from the placenta, thus contradicting their answer in Question 1.

Only 3 patients (4%) had a problem with donating their placenta, but all 3 were willing to donate the blood from the placenta. This could be an indication that cultural or religious practices might play a much smaller role in obtaining informed consent than initially anticipated. It cannot be ruled out though that people living in the city possibly adhere less to their cultural practices than people of the same culture that live in rural communities due to different exposures and influences from other cultures. The ages of the patients completing the questionnaires were also unknown. Younger people could be more willing to fill out the questionnaire and might be more liberal concerning cultural practices and traditions.

Patients seemed less intimidated by undertaking an additional HIV test. Of all the patients that were willing to either donate their placenta (Question 1; 79%) or the blood from their placenta (Question 3; 62%), only two patients (3%) were unwilling to undergo an additional HIV test (Question 2). This could be due to the fact that all of the attending patients at the clinic had previously undergone an HIV test and their HIV status was known to them. The issue of a possible change in HIV status after testing in early pregnancy was not addressed. Mothers that attend a rural clinic for the first time might however not know their status and additional fears regarding HIV status and testing might be an obstacle to deal with in the principal study.

Not all of the cultural groups in South Africa were adequately represented and results obtained from the pilot study can thus not be extrapolated to the whole of South Africa. Similar studies will need to be conducted in different provinces and in clinics, private and public hospitals, in order to obtain a clearer picture of the influence of demographics, cultural background and socio-economic status across South Africa with regard to social acceptance and response of UCB banking.

4.4 Conclusion

Obtaining informed consent is an intricate process. In our efforts to address mothers at the Steve Biko Academic hospital, a few hurdles needed to be addressed prior to considering the question of establishing a public UCB SCB.

During the first few months various difficult situations and potential problems were encountered. Mothers were initially unreceptive and reluctant to fill in the questionnaire. Administrative, time and physical constraints impeded the efficacy of the interview process. Because there were many uncontrolled distractions, the investigators had to take heed not to add more distractions to the interview themselves with an incoherent presentation. The investigators had to address patients and situations with care and sensitivity toward opinions and perspectives. These initial difficulties were leveraged to construct a better organised and more coherent interview process as the pilot study evolved.

Certain aspects of the interview process can in future be facilitated by improving infrastructure at the clinic and by creating a specific uninterrupted time-slot to obtain consent, while others could be addressed through educating the general public on these matters.

During the interview, the subsequent inclusion of visual aids contributed to facilitating the explanation of concepts inherent to stem cell banking. It promoted the mothers' involvement by providing a platform for accessibility to the new technology and gave an opportunity to ask questions. This helped to visualise components of what was to many, a new and strange concept, thereby helping to minimise fears and superstition. In addition, translating key concepts or words - such as "placenta" - into different languages enabled a more coherent understanding of the process of UCB collection prior to banking. The questionnaire was expanded to include six questions related to UCB SC banking as well as personal information about the patient (Annexures 1 and 2). Results for the revised questionnaire are discussed in Chapter 5.

Preliminary results shed light on cultural and religious practices and linguistic and academic insufficiencies that could influence community support for a public SCB. Gathering information on the ethnicity of these patients would thus be imperative.

Some inconsistencies in answering the questionnaire indicated a need for better structured questions and improved clarity in the interview. These inconsistencies highlighted areas of confusion and shortcomings in the interview process as well as the questionnaire layout which necessitated amendments to both the interview outline and the questionnaire. A revision of our methods in conveying the purpose and utility of a public SCB helped to overcome fears and public misconceptions and contributed to educating the public as to their role in public banking.

Educating the general public with regard to the workings and benefits of public SCB is the first step to determine the viability of such an undertaking – a unique and rich challenge in our South African context. Education will have the greatest impact if children or young adults could be educated about stem cells, the future of stem cell treatments and UCB banking through integrating the work into the school curriculum.

In our South African context, we are faced with unique and rich challenges relating to cultural and religious differences that are further enriched by linguistic constraints, educational insufficiencies and logistical and administrative limitations. However, none of these provide an insurmountable challenge, and despite cultural or religious constraints, the majority of the general public that we interviewed are positive about establishing a public UCB SCB for South Africa. The results are however not definitive and need to be verified in different demographic and socioeconomic settings across South Africa. It is thus up to the team of investigators to find innovative solutions to the few remaining obstacles and to verify the initial positive results through a more extensive and definitive social response study.

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CHAPTER 5

5 Principal study: establishing a public umbilical cord blood stem cell bank (UCB SCB) for South Africa

5.1 Introduction

As mentioned in Chapter 4, South Africa faces a large unmet need for bone marrow (BM) transplantation which could be alleviated by establishing a public umbilical cord blood stem cell bank (UCB SCB). A BM registry, under representative of South African demographics, and donor attrition rates of about 25% (Crookes et al., 2007) reduces the possibility of finding an adequate match for haematopoietic stem cell transplantations for many South Africans.

Umbilical cord blood is seen as a viable source to BM (BM itself or mobilised peripheral blood stem cells (PBSCs) for BM transplantation. Establishing a public UCB SCB would therefore be a positive step towards growing South African health care, while similarly addressing the tremendous demand in public health and patient care.

UCB is an important source of haematopoietic stem cells (HSCs). Haematopoiesis, the formation of blood cells, is achieved by lineage-specific differentiation of HSCs. HSCs are undifferentiated precursors of myeloid and lymphoid cells, mainly residing in adult bone marrow, but can also be found in peripheral and umbilical cord blood.

5.1.1 The role of haematopoietic stem cells in bone marrow transplantation

Haematopoietic stem cell transplantations have been used as a successful form of treatment for haematological, non-haematological and certain genetic disorders since the identification of the HLA complex in the early 1960s (Copelan, 2006). Common examples include BM transplantations for treatment of leukaemia, myeloma and aplastic anaemia (Pepper, 2010). Treating an individual with an HLA-matched donor sample greatly reduces the occurrence of graft versus host disease (GvHD) and has an added graft versus leukaemia (GvL) effect (Copelan, 2006).

The success of BM transplantation can be attributed to the innate ability of HSCs and HPCs to self-renew and, subsequently, to differentiate in order to reconstitute the entire haematopoietic system post ablation (Caneth et al., 2010). Haematopoiesis – the formation of blood cells – is achieved by lineage-specific differentiation of HSCs. Numerous studies have been conducted to try to identify the phenotype and characteristics of primitive HSCs, but with limited success. These cells are principally characterised by multipotency (for HPCs) and

pluripotency (HSCs) and the ability to self-renew. It is estimated that approximately 1 in every 100 000 cells in circulating blood is an HSC (Bethesda, 2009). This number can, however, be increased by inducing the release of HSCs from BM into peripheral blood by means of cytokine stimulation (Ivanovic et al, 2009). Chemical signals and regulatory factors (transcription factors, cytokines etc.) involved in the process of HSC differentiation are numerous and their effects are not yet fully understood.

One property of HSCs that enables long-term haematopoietic reconstitution is the phenomenon of 'homing', which in turn, is accompanied by subsequent cellular engraftment. Homing is a controlled process in which circulatory HPCs find their way back to their stem cell 'niches', or sites of origin. These cells display various cellular markers (including CXCR4 – a chemokine co-receptor) that react to chemokine stimuli secreted in the BM stroma, that cause these cells to migrate toward the stimuli and bind to the adhesion molecules in the BM niche (Caneth et al., 2010; Lapidot and Petit, 2002).

HSCs and HPCs in the BM are responsible for replacing dead and dying blood cells and replenishing cells lost in the case of trauma and have the ability to reconstitute the haematopoietic system throughout an individual's lifetime (Wilson et al., 2008). Haematopoiesis thus consists of the self-renewal of primitive HPCs and HSCs, subsequent expansion of the generated lineage-specific progeny and these cells' eventual maturation into unipotent differentiated cells. In order to conserve the pool of pluripotent, self-renewing HSCs and multipotent HPCs, these cells enter a low proliferative state called "quiescence", required to maintain self-renewal capabilities (Tripp et al., 2005). Quiescence is believed to slow down cellular proliferation by keeping the HSCs at rest in the G0 phase and only allowing these cells to enter cellular division at infrequent intervals – generally in response to BM injury or stimulation by, for example, granulocyte-colony-stimulating-factor (G-CSF) (Wilson et al., 2008). These activated HSCs return to their dormant state after re-establishing homeostasis.

Bone marrow transplants can either be allogeneic, from a donor to a different recipient, i.e. another person's cells, or autologous, where the donor is the recipient – i.e. one's own cells (Watt et al., 2007). Autologous transplants are advantageous in posing no risk of rejection or GvHD (although graft failure could result), but could potentially contain intrinsic tumour cells. Autologous transplants also lack the graft versus Leukaemia (GvL) effect seen with allogeneic transplantations (Caneth et al., 2010). The first HLA-matched allogeneic transplant for

treatment of an immunodeficiency took place in 1968 and treatments for aplastic anaemia and leukaemia were routinely performed by the 1970s (Perry et al., 1996; Caneth et al., 2010).

In addition to other problems experienced with BM registries, BM aspirations are painful procedures with the risk of not obtaining adequate numbers of HSCs for successful transplantation (known as a "dry tap"). Currently, most BM transplants are however performed by using peripheral blood (PB) – harvested through apheresis. UCB units on the other hand are readily available and contain HSCs that have high proliferation rates and display a greater deal of immunological tolerance than BM stem cells (Broxmeyer et al., 1990; Fong et al., 2012). UCB units have therefore become a viable alternative source of HSCs for BM transplantation. Since the first successful transplant in 1988, many UCB banks have been established for allogeneic transplantation (Gluckman et al., 1989; Welte et al., 2010). The number of UCB units available for unrelated UCB transplants has increased dramatically over the past ten years, from 129 000 in 2002, to 531 000 units in 2012. Approximately 534 724 UCB units are currently registered with the Bone Marrow Donors Worldwide (BMDW) and the total number of stem cell donors are indicated to be approximately 19.8 million (BMDW; 2011).

5.2 UCB banks around the world

UCB SCs can be cryopreserved for long periods of time. This makes UCB units an attractive source of SCs for BM transplantation, specifically for unrelated donors. In order to benefit from UCB units stored worldwide, UCB banks need to adhere to strict international regulatory standards which assure the quality of the UCB units available to the national and/or international community.

The World Marrow Donor Association (WMDA) in connection with the Worldwide Network for Blood and Marrow Transplantation (WBMT) (amongst others) are working on the development of requirements for standardised practices in cellular therapy. Bodies exist that are necessary national regulatory entities: stem cell registries and stem cell banks.

Stem cell banks are repositories where actual samples are stored. They can be public, commercial, institutional etc. The samples contained in each bank need to be registered at a specific registry where all of the information pertaining to the samples is contained and made available the public (Isasi and Knoppers, 2011). Stem cell registries, on the other hand, do not store specific cell lines. They instead list all information pertaining to the specific stem cell lines

registered with them. Information consists of, for example, the cell line's origin (cell line derivation), where it is stored (which storage facility), and how to obtain that cell line. The information available about the cell lines vary according to the nature of the registry. Registries could be regulatory or more research oriented and this orientation would determine the scope of the registry (i.e. the kind and amount of information available about the samples) (Knowles and Adair, 2007).

The primary goal of the WMDA is thus to ensure the quality and safety of international UCB units by providing minimal operational guidelines to all registries (Hurley et al., 2010). It wants to create unity in practice worldwide, throughout stem cell registries, by unifying them under the umbrella of WMDA standards. For this reason the WMDA facilitates all aspects related to accreditation of bodies involved in cellular therapy using unrelated donor transplants. In addition to compliance with WMDA standards, registries are expected to comply with their own country's governmental regulations and individual transplantation community standards (Hurley et al., 2010). All haematopoietic stem cell registries that would like to become a part of the global registries network would thus be subjected to WMDA accreditation and have to adhere to WMDA standards. These WMDA standards serve as minimal guidelines for registries, but do not cover the requirements of other organisations such as the Joint Accreditation Committee-ISCT (International Society for Cellular Therapy) or the European Group for Blood and Marrow Transplantation (EBMT) (Hurley et al., 2010).

These groups are responsible for overseeing collection/harvest centres, cord blood banking and tissue typing. EuroCord, the international registry for the EBMT, founded its division for international cord blood banking – NetCord – in 1998. NetCord and the Foundation for the Accreditation of Cellular Therapy (FACT) have joined forces in compiling the international standards for UCB collection and banking. FACT was funded by the American Society for Blood and Marrow Transplantation (ASBMT) and the International Society for Cellular Therapy (ISCT) in 1996 (Anon., 2010a).

The respective functions of NetCord and FACT are to oversee the quality of UCB banking and the subsequent clinical use of these UCB units for allogeneic SC transplantation. FACT's mission is to promote quality medical and laboratory practice of cellular therapy through accredited standards, transparent peer review and accreditation.

The Standards are intended to ensure high standard medical and laboratory practices throughout the whole process of UCB banking and storage. This is particularly important in order to be able to reliably and consistently reproduce high-quality UCB products, intended for routine transplantation purposes.

The standards as described by NetCord-FACT entail: "donor management, collection, processing, testing, cryopreservation, storage, listing, search, selection, reservation, release, and distribution to clinical programs". It lists the requirements for UCB collection, sample screening, processing and cryopreservation, storage and characterisation of these units, as well as processes involved to locate specific units intended for release for subsequent administration. Handling of UCB units, including transport or shipment, is also included and detailed in the NetCord-FACT International Standards for Cord Blood Collection, Banking and Release for Administration manual, 4th Edition (Anon., 2010a).

Important additional parameters that need to be standardised are listed by Watt et al., (2007) to be: "(1) transit times and storage temperatures following harvest, (2) pre-processing prior to cryostorage, (3) the selected cryoprotectant, (4) cooling and thawing rates, temperatures, and protocols, and (5) longer-term storage temperatures" (Watt et al., 2007).

It is thus of utmost importance to ensure from the outset that all UCB banks, registries and UCB collection sites adhere to the requirements and standards as stipulated by the WMDA and other international regulatory bodies.

5.2.1 Public vs. private banking

Since UCB units can be cryopreserved for extended periods of time, they serve as vital reserves of UCB units for use in allogeneic transplantation. Many banks have thus been established worldwide, in order to generate an ever-increasing pool of potential HLA-matched UCB units that could be accessed both locally and internationally (Malgieri et al., 2010; Armson, 2005).

5.2.1.1 Public banking

Public banking consists of the anonymous donation and subsequent storage of UCB units for unrelated, allogeneic transplantation (Jordaan et al., 2009). According to the WMDA (2006), more than 1,500 allogeneic transplants occur worldwide each year and are steadily increasing (Anon, 2006). These banks operate out of altruism and mutuality for the purpose of benefiting the public (Jordaan et al., 2009).

Public banks operate on a not-for-profit basis, and any member of the public can donate their UCB blood and access to UCB units is equal for all members of the public, provided that the recipient of a unit is an adequate HLA-match. Many professional organisations and national governments support public banking and its successful application has been extensively documented (Anon, 2006).

A sub-category included under public banking is what the WMDA classifies as "Medically Indicated, Directed Family Cord Blood Storage". In this case, some public banks would provide storage of a UCB unit intended for family usage, where a family with a sickly child wishes to store the UCB of a second expected sibling for the treatment of the first, provided that the first child could potentially benefit from a UCB transplantation (Anon, 2006). There is a 1/4 (25%) chance per sibling of finding an adequate HLA-match between siblings, which thus increases the likelihood of using the specific sample.

5.2.1.2 Private banking

The WMDA defines private banking as banking for autologous or family storage. These banks differ mainly from public banks in that they sell their service of storing UCB units for exclusive use of the donor family and at the discretion of the donor. Unlike public banks where all units are anonimised and the public has equal access to any of these units, people storing their UCB privately retain the right to exclusive access to the unit (i.e. autologous use or use within the family) (Jordaan et al., 2009).

Private banks operate on a for-profit basis, often charging exorbitant fees (usually between $1,000 to $1,500 USD) excluding an annual storage fee (circa $100). They extensively market their services to the public. Their methods often create contention among cellular therapy and transplantation communities since they may hinge on false advertising and incorrect portrayal of the current state of UCB transplantation. Furthermore, patients that store privately often do not have known risk factors that would justify personal usage of the units, with very low likelihoods of these samples ever being used (Jordaan et al., 2009; Anon, 2006).

5.2.2 Controversial aspects contributing to the public-private debate

The current debate around public vs private UCB banking centres mainly around the fact that commercial UCB banking leads to many ethical dilemmas and – many believe – should thus be avoided (Thornley et al., 2009; Sullivan, 2008). The European Union Group on Ethics' stance

against private (otherwise known as "commercial") UCB banking is that it is unethical since private banks sell a service without any immediate tangible use regarding therapeutic options (Malgieri et al., 2010; Anon, 2004).

Proponents for private banking on the other hand maintain that each individual has the freedom of choice to choose where to store his own UCB units and should not be prohibited to do so (Jordaan et al., 2009).

Commercialisation of UCB banking leads to the following main ethical dilemmas: 1) Some private banks having been found to incentivise doctors to recruit patients for private storage; 2) false marketing and advertising where current benefits of UCB storage are overstated; 3) patients signing informed consents without being properly informed of the processes and options involved in UCB banking and 4) some private banks adopting for sales approaches that pressure patients into giving informed consent by playing on the parents' feelings, implying that they are not good parents if they do not store their child's UCB unit for 'biological insurance' (Anon, 2007; Petrini, 2010; Anon, 2006).

5.2.3 Other factors requiring regulatory oversight

5.2.3.1 Regulation and accreditation

Regulations for UCB banks are still being refined and adapted in order to provide for the needs of the public whilst maintaining transplantation excellence, unit safety and donor anonymity. The most recent regulations (although partially still incomplete) for South African SCBs have been published in the March 2012 Government Gazette (Motsoaledi, 2012)). These regulations stipulate the use of SCs, record keeping and reporting on obligations, duties of the health officer, inspection and control measures, traceability, data protection and confidentiality, SC quality and safety, SC quarantine, processing and storage, distribution and SC bank relationship with third parties.

All UCB banks need to adhere to strict regulatory requirements and need to comply with accreditation standards as determined by the WMDA and local government authorities in cellular transplantation (as mentioned previously). At the moment, however, regulations pertaining to cellular transplantation for South Africa are still incomplete (Pepper, 2012).

Running an UCB SCB requires a great deal of financial resources. Public UCB banks are run on a cost-recovery basis and not on a for-profit basis as is the case with private banks. The money to

run public banks often comes from government institutions such as hospitals, medical centres or non-profit organisations (who often don't have adequate funds to maintain these services to the public) (Malgieri et al., 2010; Bellomo, 2006; Anon, 2004).

Private banks, on the other hand, often have shareholders and thus operate on a for-profit basis. They encourage parents to donate their child's UCB unit mostly for autologous use, but with the option of allogeneic use amongst close relatives. This drive to bring in profit and to keep the shareholders happy often leads to questionable marketing and advertising campaigns, inappropriate informed consent procedures, information being accessible to the public, and advertisement / campaigning (Malgieri et al., 2010).

Donor identity, sample anonymity and traceability and patient safety are important factors that require strict regulation (Malgieri et al., 2010). In an effort to bring some form of regulatory oversight into the practice of UCB transplantation, the Human Tissue Authority (HTA) in the UK has put a measure in place to ensure the safety of UCB transplantation in July of 2008. It put into practice the requirement that any UCB bank (public and/or private) must be licensed by them, prior to the release of any UCB unit for transplantation purposes to hospitals in the National Health Services (www.hta.gov.uk).

5.2.3.2 Obtaining informed consent

Information given to the public should be scientifically correct and as extensive as necessary in order for each parent to make a truly informed decision regarding the banking of their child's UCB unit.

There are many aspects involved in obtaining informed consent from a potential UCB donor. Some of the elements, stated by Beauchamp and Childress (2001), include autonomy of the individual giving consent, his or her understanding of the process of UCB donation and banking – both public and private - and his or her voluntary participation in giving consent, to name but a few aspects. It is important that each individual makes an autonomous decision regarding his UCB donation, without being coerced into making a decision or being subjected to biased and false advertising (Petrini, 2010). Obtaining consent from a mother at an appropriate time is also crucial, since it is generally agreed that obtaining informed consent from a mother in labour is a questionable practice, raising many ethical concerns (Petrini and Farisco, 2011).

Aspects that need to be discussed during the informed consent process are set out in the paragraphs below.

5.2.3.3 Informing patients of allogeneic or autologous use of UCB units

Often, patients are misinformed regarding current and potential future therapeutic applications of UCB units (Petrini, 2010). According to research done by Fox et al. (2007), many patients – and especially those planning to store their UCB units privately – had an insufficient understanding of the processes and options involved in UCB banking.

Few people in the general public are educated in current and potential future applications of UCB. Often, private banks promise cures for diseases for which no clinical results have been generated, such as the use of UCB to cure Parkinsons, ALS, MS, diabetes etc. (Anon, 2006). Many are thus misled into believing that these stored UCB units are a form of 'biological insurance' to treat some of the abovementioned disorders (Anon, 2007).

5.2.3.4 Likelihood of requiring UCB units for autologous transplantation

Private banks have frequently been found to neglect to tell the clients of the minimal likelihood of using one's own UCB unit. Primarily two reasons exist why autologous use is limited: firstly, there is a very slim chance of acquiring one of the few disorders currently treatable with UCB – i.e. ever having the need to use the stored UCB; and secondly in some cases, one's own UCB unit would be insufficient for transplantation purposes (Sullivan, 2008).

A unit could be deemed insufficient for autologous use because of the following main reasons: a) autologous units do not have the immunotherapy benefits of GvL, exhibited by allogeneic units that contribute in combatting leukaemia; b) pre-leukemic cells could be present in units of children who develop childhood leukaemia, thus rendering their UCB unit insufficient for transplantation; c) certain genetic disorders are transferred in the UCB SCs and these units can therefore not be used for autologous transplantation; these haematopoietic disorders which include hemoglobinopathies, inherited immunodeficiencies etc. can, however, be treated with allogeneic transplantation (Anon, 2006).

The WMDA estimates that "Approximately 70% of patients with blood disorders such as leukaemia, severe aplastic anaemia and congenital or other acquired disorders will not have a suitable family donor" (Anon, 2006). Siblings only have a 1:4 chance of being an adequate match whereas a 1:8 chance exists between a parent and child. However, with an adequate pool of publicly stored UCB units, the likelihood of finding an appropriate HLA-matched

allogeneic unit is at least 40% and increases as the number of publicly stored units increases (Anon, 2006).

5.2.3.5 Informing the patient of a unit's cell dose requirement

Private banks often tend to neglect to inform their clients about cell dose requirements needed for successful transplantation. UCB units often only yield cell numbers adequate to treat children and not adults (Paulin, 1992). In order to obtain successful engraftment, a unit should contain about 2.5 x 10^6 CD34+ cells/kg body weight of the individual who is to receive transplantation (Rocha et al., 2000; (Yang et al., 2005). One UCB bag (80-120ml UCB) generally contains enough stem cells (10x10^6 CD34+ HPCs) to successfully engraft a child of up to 4kg (Zhang et al., 2006). Thus, if a child is not diagnosed within the first three months after birth, the chances are that their single UCB unit would not contain enough SCs for transplantation purposes. For publicly banked units, this is not a problem, since HLA-matched samples of two unrelated donors could be pooled to overcome the issue of unit potency (Fong et al., 2012).

5.2.3.6 Banking for a nation…

Although the field of UCB banking is mostly polarised between the two seemingly opposing categories for storing UCB units (public or private), alternative models have been suggested to overcome these differences. It is generally agreed that UCB banks have a role to play in furthering future therapeutic applications of UCB, and that UCB units should be made available to the public. These two principles gave rise to so-called public-private hybrid UCB banks. There are many different models through which these hybrid banks operate, ranging from catering for both public and private banking to banks in which a certain percentage of each stored sample (e.g. 80%) is available for public access while the remaining sample volume (20%) is retained for private use (Jordaan et al., 2009). The last mentioned model is known as the Virgin model and is a rather controversial model in light of cell dose requirements discussed earlier (Martin et al., 2008). Hybrid bank models do not necessarily provide a steady solution to the on-going debates and probably contribute more towards current confusion and controversies. However, with advances in cellular therapy (e.g. induced pluripotent SC technology (iPS cells), cell expansion and tissue generation) there might be merit in investigating the benefits provided by these 'hybrid banks' in serving the public through both public and private storage of cell products.

It is important to have an intimate knowledge of the benefits provided by each of the above mentioned UCB models in order to best provide for the needs of South African citizens. A public UCB bank in South Africa would give many patients access to previously unavailable treatments by providing a large pool of genetically diverse UCB samples, representative of South African demographics. Given South Africa's genetic diversity and existent financial constraints for many citizens, there is no dispute that the country would immediately benefit from a public UCB bank.

5.3 Objective

The study presented here forms part of a larger feasibility study consisting of five components. Combined results from each of these components will determine the feasibility of establishing a public UCB SCB in South Africa. The final objective of the study presented here is, therefore, to establish whether there is public support for and interest in establishing a public UCB SCB (or banks) in SA.

The investigator's objective was thus to determine preliminary public support for the establishment of a public UCB SCB by addressing mothers attending the ante-natal clinic at the Steve Biko Academic Hospital in Pretoria. In addition to assessing public support, the investigator aimed to obtain information on potential elements that could impede the establishment of a public SCB. Some of these elements can be overcome and, when appropriately addressed, could have a negligible negative impact on public support for UCB banking. The major areas foreseen to potentially impact patient support were cultural and religious practices, language constraints, academic insufficiencies and patients' willingness to undergo additional HIV screening. Some of these elements (e.g. cultural or religious beliefs and practices that specifically have bearing on the patient's beliefs regarding blood, donations, tissues, body waste etc.) might not be dealt with in a practical manner as in the case of e.g. language constraints where a translator could be approached. If these elements were to pose significant concern they could considerably hinder the establishment of a public UCB SCB.

The results from this study should assist in the design of further more in-depth studies that must be conducted in different provinces across the country in public and private hospitals for a comprehensive overview of public support for UCB banking. The results could serve as proof of concept and the methodology could contribute to a more in-depth social-science-based protocol for addressing patients in clinics, hospitals and provinces throughout South Africa.

The investigators hypothesised that more than 50% of the public would support the establishment of a public UCB SCB in South Africa. They furthermore surmised that cultural and religious practices (related to blood donations) together with language constraints and academic insufficiencies of South African citizens might have a negligible impact on the establishment of a public human UCB SCB in South Africa.

5.4 Methodology

The objective of this public acceptability study was to gauge public reaction towards and support for UCB stem cell donation and banking, as well as to indicate which foreseen parameters could potentially impede this endeavour. Important parameters considered in addition to public support, were a patient's perception of the processes involved with UCB donation and banking and subsequent HIV testing (necessary for compliance with international regulatory standards).

An initial pilot study, involving 77 expectant mothers had been conducted previously (Meissner-Roloff et al., 2012), through which the current study's design and interview processes were optimised. Using the pilot study's refined template (Annexures 1 and 2), 217 randomly chosen, expectant mothers, attending the ante-natal clinic at the Steve Biko Academic Hospital were addressed during a 15 min. interview, followed by the completion of a closed anonymous patient questionnaire. Ethics approval for the study was obtained from the Main Research Ethics Committee at the University of Pretoria (protocol number: 131/2010) (Annexure 5).

Visual aids (a doll with an umbilical cord and placenta and relevant posters) were used to explain concepts relating to UCB banking during the interview. Participants were given the opportunity to raise questions and / or comment during and after the interview and were encouraged to write comments or questions in the space provided on the questionnaire itself.

These questions and comments were documented, together with data from the questionnaire, which was analysed using Microsoft Excel (Microsoft Corp., Redmond, WA). Confidence intervals were calculated with the help of biostatistician, Prof Piet Becker, using the Statistix program (Analytical Software, Tallahassee, FL).

The questionnaire was designed in a manner that would allow the investigator to obtain limited demographic information about the patients, while simultaneously addressing the issue of public support through a series of questions.

The demographic information was chosen for the following reasons and included:

A. <u>The patient's home language</u>

- In order to infer patient ethnicity (in an attempt to steer clear of potential racial connotations).

B. <u>Patient age</u>

- To investigate whether a patient's age could potentially impact on her understanding of and support for a public UCB SCB.

C. Number of biological children

- To determine whether prior experience with childbirth influences understanding of and support for a public UCB SCB.

D. Marital and employment status

- Considered to infer the potential influence that emotional and financial support of the individual has on her understanding of and support for a public UCB SCB.

The following six questions were designed to obtain information about patient support for a public UCB SCB and to ascertain the interviewee's understanding of associated UCB SCB processes gained from the interview:

A. Question 1 (Q1):

If there is a public cord blood bank facility, would you be willing to donate your PLACENTA (afterbirth) for medical research?

If the participants answered NO to Q1, they were asked to provide one of the following reasons for not being willing to donate:

Q1 Reason:

- Against religious belief
- Against your culture
- Don't think this bank is a good idea
- Afraid of the collection process
- Don't understand what the bank is for
- Other (please specify)

B. Question 2 (Q2):

If you answered NO in question 1, would you be willing to donate the BLOOD from your placenta?

Question 3 (Q3):

If you are willing to donate your placenta OR just the blood from the placenta and umbilical cord, would you be willing to allow your doctor to do an additional HIV test?

C. Question 4 (Q4):

Have you heard of "stem cells" before today?

D. Question 5 (Q5):

Do you think stem cells can help to treat you, your child or somebody else in the future?

E. Question 6 (Q6):

Do you think that a public umbilical cord blood stem cell bank is a good idea?

5.5 Results and discussion:

5.5.1 Introduction

Although a few routine medical procedures exist that involve UCB transplantation, there are many more possibilities for potential treatments that could be explored in the future. With vastly improving techniques for expanding haematopoietic stem cells (HSC) in culture, it is possible that samples stored in public banks will become a vital resource for novel forms of therapy in the future. It was therefore deemed beneficial to include in the questionnaire the possibility that samples could be used for medical research and / or public use. The term "medical research" was used to describe all downstream applications that involve current and possible future treatments with UCB. A clear distinction was, however, made during the interview process between the current use of UCB for transplantation and research purposes.

Many of the mothers-to-be attending the antenatal clinic at the Steve Biko Academic Hospital had high-risk pregnancies and were often referred by their local clinics. It can be argued that these mothers had better access to and received more information regarding their pregnancies and might thus have been more educated about their pregnancies than mothers attending other clinics. This could have facilitated the presentation of information to the mothers at this clinic, and it was understood that it might be more difficult to convey the same information in rural clinics.

5.5.2 Results and discussion for Question 1 to Question 6

Overview

Despite the interviewer's efforts, questions were sometimes left unanswered (BL), which delivered 'non-workable data' (non-useful data). Patients that left some questions unanswered could have done so for various reasons: It could either be an indication that the patients did not understand the question, were undecided or reluctant to answer (e.g. to undergo an additional HIV test) or were not able to complete the questionnaire because of time constraints (e.g. called by a nurse or doctor).

In order to obtain about 200 questionnaires with workable data (i.e. not left unanswered) the investigator interviewed 217 patients (to replace non-workable data with workable data). Figure 4 gives an overview of all the results obtained (useful and non-useful) for questions one to six (Q1 to Q6).

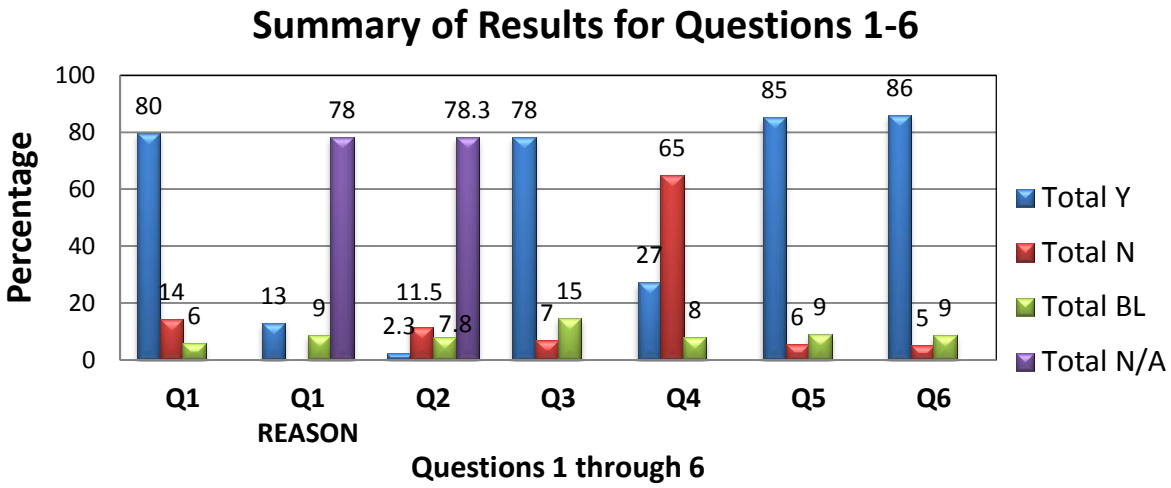

Figure 4: Summary of results obtained from questionnaire, Questions 1-6. Answers were either indicated as "Y" (yes) – indicated by the blue bars; "N" (no) – indicated by red bars; "BL" (left blank) – indicated by green bars or "N/A" (not applicable) – indicated by purple bars. *Note: All selected Q1 Reasons were considered as a positive response and therefore categorised under "Yes", to illustrate that reasons were provided as opposed to being left blank or "N/A".

Figure 5 indicates the ratio of useful data gathered for each question versus non-useful (blank or unanswered) data. Figure 5 shows that more that 85% of all the gathered data was useful and could be used for downstream analyses.

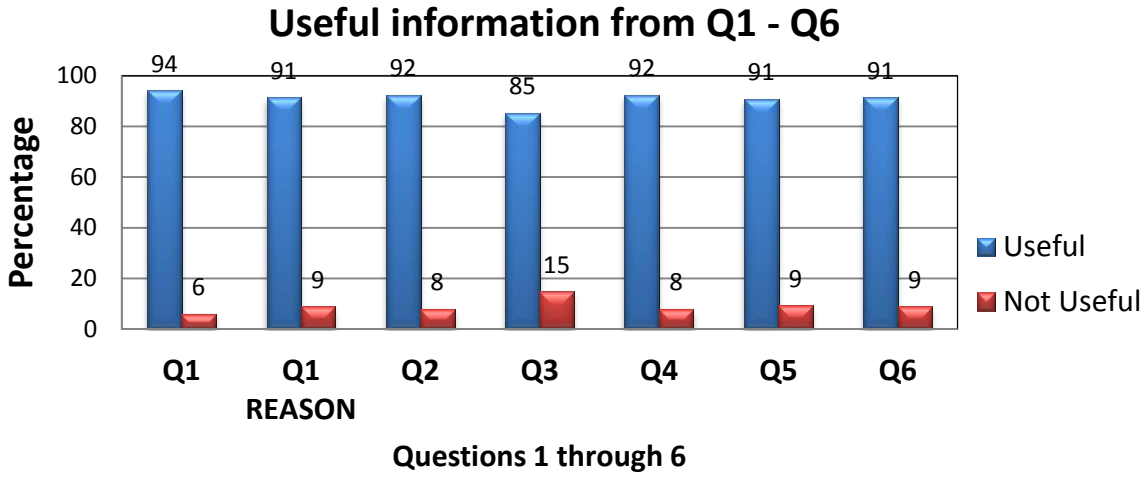

Figure 5: All answered questions were deemed 'workable data' or 'useful' and are represented above for Questions 1 through 6 (Q1 - Q6). Workable (useful) information (blue bars); blank or not useful information" (red bars).

5.5.3 Results for Q1

Q1: "If there is a public cord blood bank facility, would you be willing to donate your PLACENTA (afterbirth) for medical research?"

Support for donation of the placenta was measured in Q1 – where 80% of participants were willing to donate their placenta (Fig.7). In order to infer the reliability of this result, a 95% confidence interval (CI) was calculated. Using the Statistix software, the interval was calculated as [74.4% to 85.1%]. When results were corrected to use workable data only, the number of patients supportive of Q1 increased to a liberal 85% (Fig. 7) (95% CI of [79.9%, 89.7%]).

Patients unwilling to donate their placenta were asked to provide reasons for their reluctance in order to ascertain whether a particular concern was dominant amongst the group. Patients were provided with a list of potential concerns and were allowed to indicate more than one concern/reason for their reluctance to donate their placenta. These concerns included any of the following reasons or combinations of these reasons. Results are illustrated in Figure 6.

Q1 Reason:

1. Against religious belief
2. Against your culture
3. Do not think this bank is a good idea
4. Afraid of the collection process
5. Do not understand what the bank is for
6. Other (please specify)

It can be seen in Figure 6 that the majority of patients (78% N/A) indicated no reasons for personal concern or unwillingness to donate to a public SCB. It corresponds to the 80% of patients that were willing to donate their placentas in Q1. The discrepancy between Q2's 78% (Fig. 6) and Q1's 80% (Fig. 7) can be attributed to two patients that were willing to donate their placenta in Q1 but still gave reasons why they might not be willing to donate their placentas. It is unclear why these patients also indicated reasons for unwillingness to donate their placentas and raises the question as to what extent these two patients understood the presentation and subsequent questionnaire questions. Upon investigation of answers provided for the remainder of the questionnaire, although the first patient was willing to donate the placenta and the blood from the placenta, she indicated that donation of the placenta might be against her religion. The remainder of the questionnaire was unfortunately left unanswered and the

investigator cannot make any definitive conclusions with regard to the patient's understanding of the presentation or banking as a whole. The second patient was willing to donate the placenta but not the blood from the placenta and indicated her reason as "it is sometimes not safe". This patient left Q3 unanswered, but was otherwise supportive of establishing a public UCB SCB.

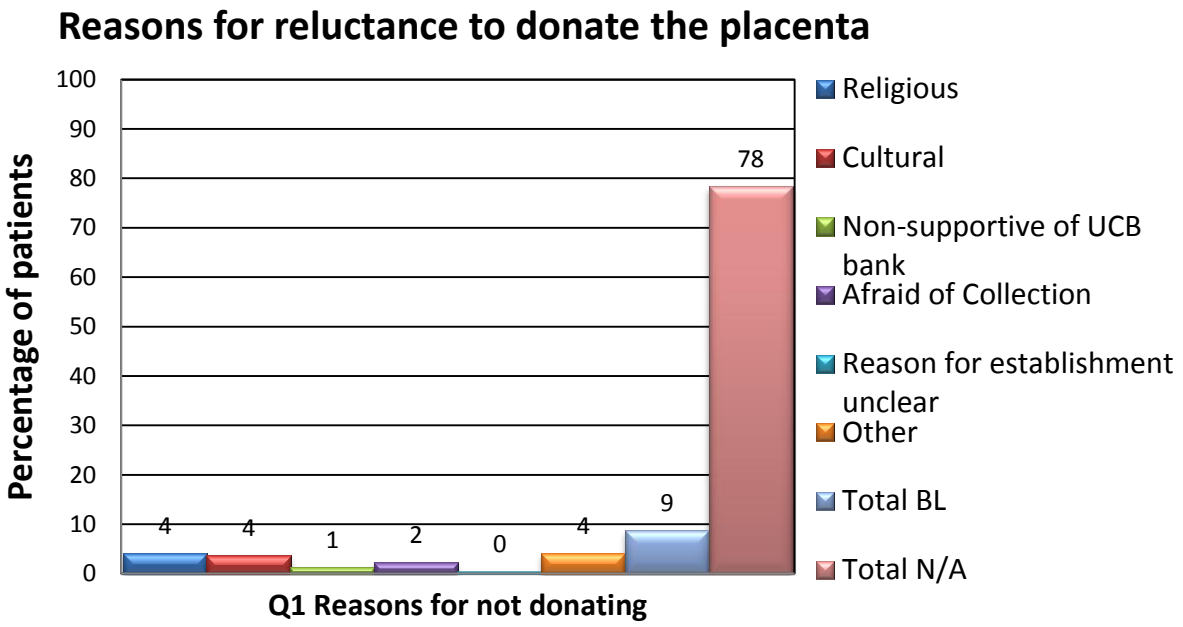

Figure 6: Question 1 Reasons: Patients' reasons why they would not support a public UCB SCB. Patients that answered "NO" to Q1 were asked to indicate why there were reluctant to donate their placentas. The investigator provided the following options: a) Against religious belief (dark blue bar); b) Against your culture (red bar); Do not think this bank is a good idea (green bar); Afraid of the collection process, (purple bar); Do not understand what the bank is for (turquoise); and Other (orange bar). Unanswered "Reasons" – (left blank) are indicated by the light blue bar. Patients that answered "YES" to Q1 were instructed to write "N/A" (not applicable) (pink bar).

No specific problematic areas were observed that related to patients' reluctance to donate their placenta. The reasons provided by the 22% of unsupportive patients were varied and not linked to a specific language group (discussed later). There was an equal amount of concern that donation of the placenta might be against people's religion or culture (4% respectively) while 4% also provided their own reasons for their reluctance to donate their placentas (Fig. 6). Only 1% of people were unsupportive of the idea of a public UCB bank while 2% were afraid of the collection process. It also seems that everybody understood the reasons provided by the investigator for establishing a public UCB SCB in SA, since nobody indicated the reason for their reluctance to be an unclear understanding of the motivation for establishing an UCB bank (Fig. 6).

Personal reasons provided by 4% of the patients were mostly concerned with fears unrelated to reasons provided on the questionnaire. These personal reasons included: 1) fears that collection procedures could be unsafe; 2) the study overwhelmed the patient and created uncertainty; 3) there was reluctance because of the person's HIV status (two individuals); 4) the person suffered from epilepsy and was afraid that the blood would not be used for intended purposes 5) – "it is part of somebody's body" 6) patient was not interested; 7) fear of donation.

These concerns could be addressed by assuring the patients of the safety and efficacy of the UCB collection, banking and redistribution procedures. Once UCB banking becomes common practice, people with these fears might feel less intimidated by the "novelty" of UCB technology while others might become more supportive once the positive effects of UCB transplantation become known in the community after successful treatments.

The investigator could not make any inference from the results that religious or cultural concerns (related to blood, blood donations etc.) were more prominently associated with a specific language group (or implied ethnic groups) for two reasons: (1) results displayed might not hold true for different demographic settings in South Africa; although the patient cohort was diverse, some ethnic groups – who might still have cultural, religious or other objections - were underrepresented; (2) the questionnaire asked the patients to indicate their first language and not their ethnicity, culture or religion; in trying to steer clear of any racial insinuations, the investigator wrongly assumed that language could be a good indicator of the person's ethnicity, culture or religion, which is not the case, as discussed later.

For these two reasons it is most likely premature to conclude that religion and culture could not significantly impede the establishment of a public UCB SCB, although the influence of religion and culture in our study cohort seems to be negligible. It is important to consider that their influence might be more pronounced in rural areas where people might adhere more to their customs, or amongst different ethnic groups not adequately represented in our patient cohort.

Unfortunately all of these reasons are conservative estimates for reasons mentioned above, since 9% of the participants left the reasons blank or unanswered, which, therefore, does not exclude them from not having cultural, religious or other more personal concerns.

Question 2:

Q2: "If you answered NO in question 1, would you be willing to donate the BLOOD from your placenta?"

Patients were requested to answer Question 2 only if (1) they answered "No" to Q1 or (2) if they were of a cultural or religious group that would, under normal circumstances, approach their physician with the request to take the placenta home after birth. This would give an estimate of patients that might support UCB banking, but because of cultural or religious practices involving the placenta, might oppose donation of their placenta. The investigator thus wanted to determine whether these patients – that were unwilling to donate the placenta – would be willing to donate the blood from the placenta and thereby still support UCB banking.

All the workable/useful data for Q2 is indicated in Fig.7. From these results it can be seen that of the initial 16% of patients who were against donation of the placenta, 3% (2.5%) were willing to donate the blood from the placenta, 13% (12.5%) were not willing and answered "No" to Q2, while 85% of the participants indicated that this question was not applicable to them (comparing well with results from Q1, Fig.7).

The 3% of patients willing to donate the blood from the placenta (thus answered "Yes" to Q2) can be divided into the following groups:

1) Patients that left Q1 blank but answered "Yes" to Q2:

   Account for 1% of the 3% who said "Yes" to Q2.

These patients were willing to donate the blood from the placenta. One of these mothers indicated that it is against her culture to donate the placenta.

2) Patients that answered "No" to Q1 but said "Yes" to Q2:

   Account for 1% of the 3% who said "Yes" to Q2.

This could indicate that these patients might want to retain the placenta, but would allow the blood to be collected from the placenta for UCB donation. One patient indicated that her reluctance to donate the placenta was due to her HIV-positive status, although this did not influence her decision to donate the blood from the placenta. A misperception that the investigator encountered a number of times during the patient interview was that some patients believed that blood "outside of the body" does not contain HIV anymore and would

thus not be able to infect people. It is probable that this perception arose from misinterpretation of safety guidelines for dealing with HIV. Although it is true that the HIV virus is fragile and does not survive well 'outside of the body', i.e. when exposed to air, heat or other chemicals, it should clearly be distinguished from collected body fluids – such as donated blood, where the HIV virus remains viable.

3) One patient (1%) answered "Yes" to Q1 and "Yes" to Q2.

This patient filled out both Q1 and Q2, although it was not necessary for her to complete Q2 (based on her answer in Q1). She was willing to donate both the placenta and the blood from the placenta.

Of the 13% that answered "No" to Q2, 12% were neither willing to donate the placenta (Q1) nor the blood from the placenta (Q2) while the remaining 1% left Q1 unanswered. Upon investigating the reasons for these patients' reluctance, it was found that 3% of these patients indicated that donation was against their religious belief while 2% indicated that it was against their culture (Table 1).

Table 1: Reasons why patients were reluctant to donate either the placenta or the blood from the placenta

Q1 Reason for not donating placenta	Number of patients indicating each reason
Against religious belief	6 (3%)
Against culture	4 (2%)
Bank is not a good idea	1
Afraid of the collection process	4 (2%)
Don't understand reason for the bank	0
Other (2%)	1 (Want to think about it) 1 (HIV status) 1 (Placenta is a part of somebody's body) 1 (Not for me)
Blank	6 (3%)

Note: Patients could supply more than one reason

Question 3:

Q3: "If you are willing to donate your placenta OR just the blood from the placenta and umbilical cord, would you be willing to allow your doctor to do an additional HIV test?"

Of all the questions, Q3 was left unanswered most often. Figure 7 indicates that 85% of data gathered for Q3 was useful, while 15% was left blank/unanswered. Figure 4 illustrates that 78% of patients were willing to undergo additional HIV testing, 7% were not and 15 % left the question unanswered.

The reasons for the patients' reluctance to answer this question are uncertain. Patients might have been uncertain about the need for a second HIV test since all attending patients at the antenatal clinic in the Steve Biko Academic Hospital had already undergone HIV testing. However, the investigator made these reasons clear during the interview. It is more likely that patients might have been fearful of undergoing additional HIV testing. Since mothers presumably know their status because of the previous HIV screening, fears could be related to either being exposed as being HIV positive or a fear that their status might have changed from negative to positive.

It is also possible that some of these patients weren't comfortable with needles and didn't want to go through the process of testing again while some of the patients could potentially not have been able to complete the entire questionnaire and therefore left this question unanswered. Although much has been done in South Africa to overcome the problems of stigmatisation because of an individual's HIV status, there are still many who hold views and fears that reveal stigma. When the results were corrected to look only at useful data, 92% of patients were willing to undergo the additional HIV test, while 8% were unwilling (Fig. 7).

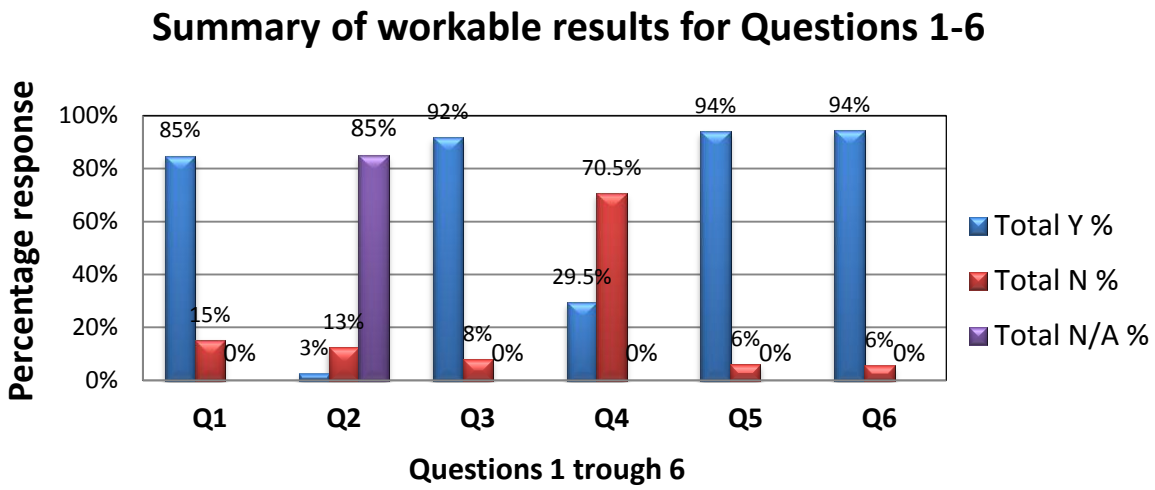

Figure 7: Summary of useful information obtained from Questions 1 through 6 (Q1 to Q6). Questions answered by "Y" (yes) are indicated by blue bars; "N" (no) indicated by red bars and "N/A" (not applicable) indicated by purple bars.

Since it is a prerequisite to test all UCB prior to banking, a person's refusal to undergo an additional HIV test would result in the donated UCB being discarded. It is therefore important to ask how many of the patients that were willing to donate their placentas (Q1) were also willing to undergo the additional HIV test (Q3). Without taking the unanswered questions into consideration, it was found that 71% of patients indicated that they were willing to donate their placenta and undergo an additional HIV test.

Question 4:

Q4: "Have you heard of stem cells before today?"

and

Question 5:

(Q5):"Do you think stem cells can help to treat you, your child or somebody else in the future?"

Question 4 was intended to serve a dual purpose: 1) to verify whether prior knowledge about stem cells and UCB SC banking could influence the patients' readiness to donate; and 2) when analysed together with Q5, to be used as a crude measure of the patients' understanding of the concepts discussed in the presentation; i.e. a person that did not know what stem cells were before the presentation (Q4) but understood that SCs could be used to treat patients with certain diseases (Q5) after the interview, presumably understood the content of the interview.

Figure 7 illustrates that almost 30% of patients had heard of stem cells before being introduced to stem cells during the interview. This number is surprisingly high and might be due to a misunderstanding of the question. Before starting with the interview, the investigator would ask the patients how many of them knew what stem cells were. The investigator's observation was that far fewer than 30% of patients knew what stem cells were, with the true number being closer to 10% to 15%. It could be argued that some patients with prior knowledge about stem cells were reluctant to raise their hands in answer to this question at the onset of the presentation, for fear of being singled out. However, during some of the one-on-one interviews, the investigator encountered a misunderstanding of the question: some patients understood the question to mean "have you heard of stem cells today" and not "BEFORE today". This could mean that patients did not always read the whole sentence or that some might have had trouble comprehending the question, which could be attributed to language constraints.

These problems could be addressed by clarifying the question or by putting it first in the questionnaire and allowing patients to answer it before the start of the presentation.

For a more direct measure of the patients' understanding, this question could in future be phrased to ask for direct feedback e.g. "what are stem cells?" or "what can stem cells do". Given the current language constraints, this might, however, complicate the questionnaire and might only be valuable if the questionnaire and interview could be translated into different languages to facilitate better understanding.

Figure 7 furthermore indicates that 94% of patients were of the opinion that SCs can be used to treat people with certain haematological diseases (Q5). This is very encouraging, since at least 70% of the patients were unaware of SCs before the presentation (Fig. 7, Q4) and did not know anything about their therapeutic application beforehand. It stands to reason then, that these patients understood the content of the presentation, which enabled them to answer in the affirmative – that SCs can be used therapeutically.

The investigator was of the opinion that having prior knowledge about SCs would be beneficial to obtaining public support for a SCB. Although this might be true in cases where patients are not thoroughly informed during the informed consent process, it does not seem to significantly impact patient support when adequate information is presented to the patients. The impact of prior knowledge (Q4) on a patient's willingness to donate their placenta (Q1), their understanding of elements presented during the interview (Q5) and support for UCB SC banking (Q6) are illustrated in Figure 8. These results highlight the importance of the "informing the patient" component when obtaining informed consent. When patients feel empowered by the knowledge presented and are not pressured into making decisions about concepts that they feel uncertain about, there are seemingly few deterrents to obtaining patient support.

Although a remote possibility in our particular context, it could also be argued that patients that had previously heard of SCs might have had their own reservations based on what they had heard. Controversies related to embryonic stem cells (ESCs) are frequently reported in the public media. If these patients had been introduced to controversies related to ESCs, they might have been more reluctant to donate their UCB because of the confusion. This might be a more plausible explanation in countries where the ESC debate has been more pronounced,

such as the USA. However, in comparison to the USA, South Africa has had limited public exposure to the ESC versus adult SC debate, rendering this possibility rather unlikely.

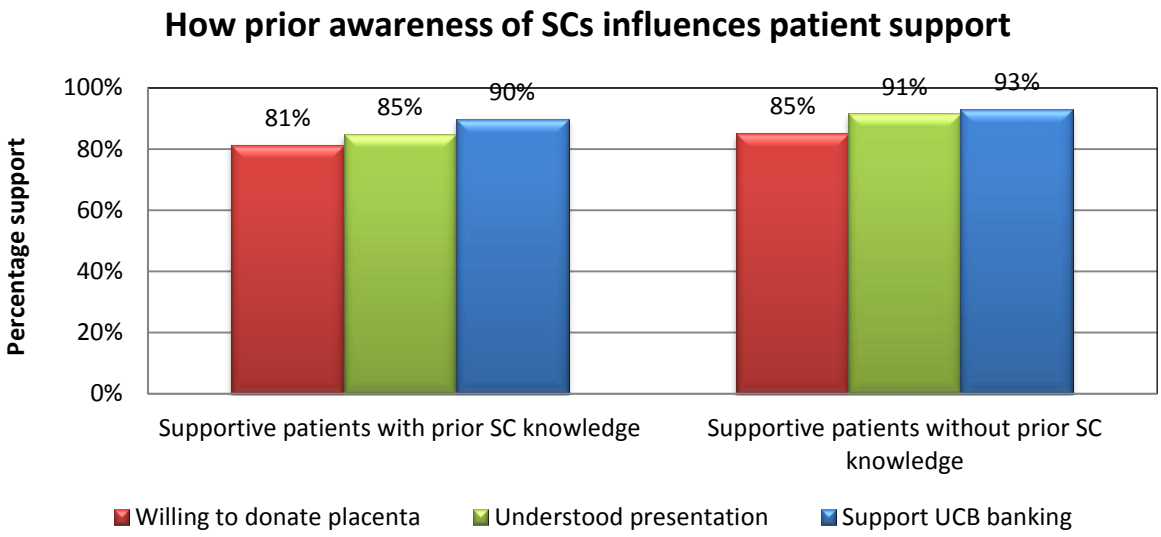

Figure 8: Illustrates how prior knowledge about stem cells influences patient willingness to donate the placenta (Q1) (red bar); their understanding of the presentation (Q5) (green bar); and their support for a public bank (Q6) (blue bar).

Question 6:

(Q6): "Do you think that a public umbilical cord blood stem cell bank is a good idea?"

This question provides direct information regarding the patients' support for the establishment of a public UCB SCB. As mentioned earlier, it could furthermore serve as a crude measure of patients' understanding about the presentation and thus processes related to UCB SC banking.

Together with Q5, these two questions received the most positive responses from the patients, with 94% (95% CI, [91.3.% to 97.6%]) of patients being supportive of establishing a public UCB SCB (Fig. 7, Q6) (a liberal estimate since it does not take blank / unanswered data into account). It should be noted that this result differs from patients that were willing to donate their placentas in Q1, where only 85% of patients were willing to donate their placentas (Fig. 7). There thus seems to be higher theoretical support for donation than actual support.

This 9% discrepancy can be attributed to the following reasons: 4% of these patients indicated that donation was either against their own cultural or religious beliefs (2% respectively) but were still supportive of the idea of banking; 2% indicated that they were afraid of the donation process involved in Q1; 2% left the reason for their reluctance to donate their UCB blank while

the remaining reasons are attributed to personal reasons. Some of these personal reasons indicate that a few patients were unwilling to donate the placenta themselves, although they were theoretically supportive of establishing a public UCB SCB. They indicated reasons such as that they would "like to think about it" or "was taken by surprise".

It might be unrealistic to think that 85% of patients would follow through with their decision to donate the placenta. The questionnaire was theoretical and very little is at stake. Once confronted with the actual informed consent documents, these patients might not all be as willing to donate their placentas to medical research. However, results presented here are very encouraging and seem to provide more than sufficient public support for establishing a public UCB SCB, provided that patients are given adequate information in order to make an informed decision.

5.5.4 Other patient information

As mentioned previously, the investigator included a section to collect information regarding patient demographics. These demographics included: patients' language, age, number of biological children, their marital and employment status. The influence of each of these demographics is explained below:

Patient language:

In order to obtain an indication of how language constraints influence the patients' understanding of the presentation as well as their support for UCB banking, patients were asked to indicate their first language.

Although the patient cohort was diverse, many of South Africa's 11 official languages were underrepresented as can be seen in Figure 9. This is mostly due to the location where the study was performed and it therefore needs to be repeated in different provinces throughout South Africa where different language distributions occur. Of interest is that some patients attending the clinic were from neighbouring countries and have either immigrated to South Africa or work in South Africa but still hold different nationalities. One patient from North Africa accounts for the French indicated in Figure 9.

Although some useful information could be gathered from the indicated languages and the corresponding answers to the questionnaire, it does not provide a complete picture of the language constraints present. Since filling out the questionnaire was voluntary, there were

some patients that were unwilling to fill out the questionnaire. Of these patients, it was evident that some were unable to understand anything during the presentation because they could not understand the language spoken (English). Their English was at best only broken English and these patients would have benefitted most from a translated questionnaire and interview (or a translator). Some were however, not interested in participating and declined to answer the questionnaire. Therefore, results obtained for the influence of patient language on the patients' understanding of the questionnaire is significantly skewed towards patients that were able to comprehend.

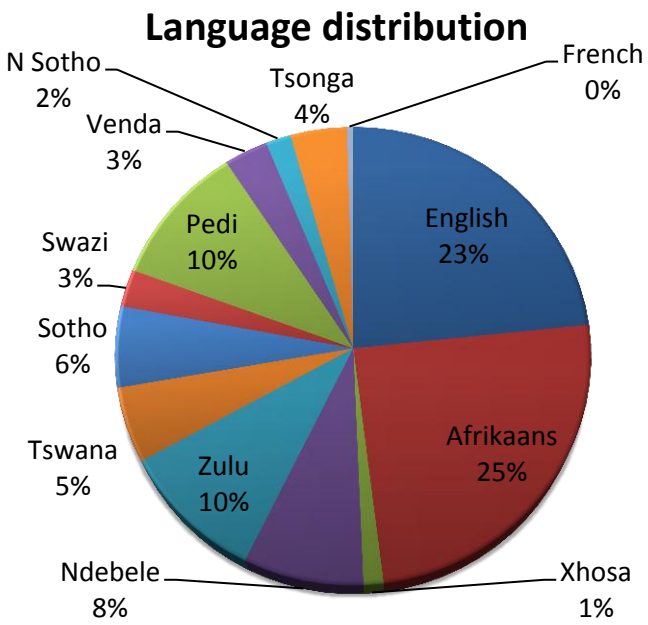

Figure 9: An illustration of the language distribution of participating patients. Although the patient cohort was diverse, all language groups were not adequately represented.

Unfortunately the investigator did not annotate the initial numbers of patients in the audience and the number of patients that were clearly unable to understand the presentation due to language constraints. These elements (audience size vs. actual participation) are extremely important parameters, which could provide useful information about public understanding and subsequent support. This information needs to be added to the final protocol if used in a national survey of patient support of an UCB SCB.

Figure 10 illustrates how language could potentially impact on a patient's understanding of the presentation. It should also be noted that it does not account for patients that could not participate in the questionnaire due to a complete lack of understanding. Therefore, these

numbers are an overestimate of the true situation. Many language groups were underrepresented; therefore, Figure 10 illustrates the proportional relationship of the most frequently encountered languages to the patients' understanding of the questionnaire (Q5), while the lesser-encountered languages were grouped together under "Other".

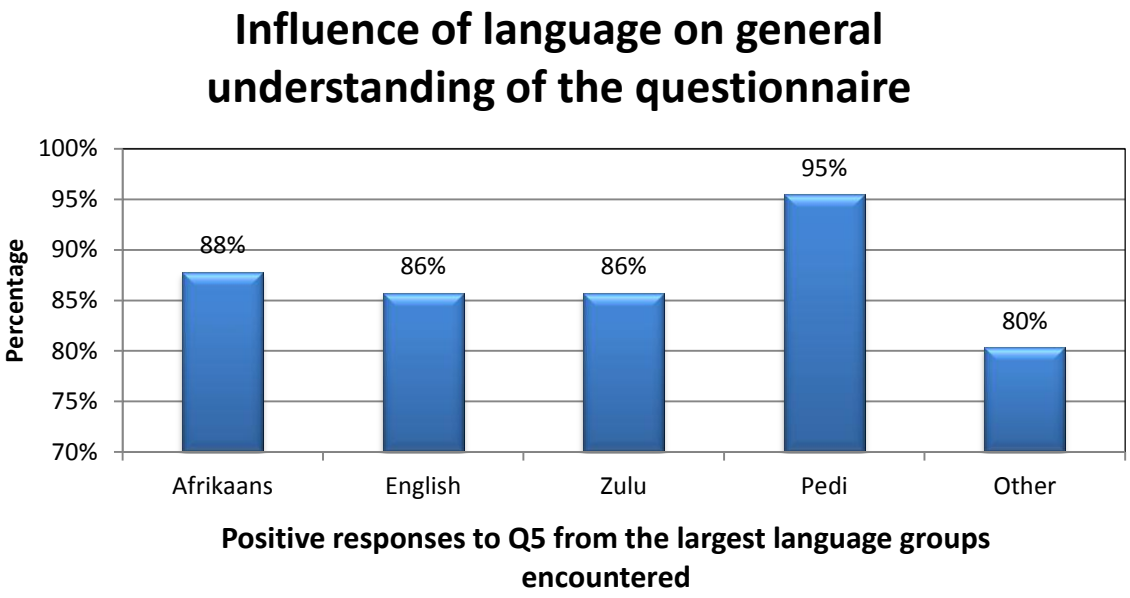

Figure 10: The influence of language on the patients' understanding of the questionnaire (Q5).

To facilitate a better understanding of the interview and questions in the questionnaire, the investigator encouraged discussions amongst attending patients. Often, a patient with sufficient English comprehension would translate some of the patients' questions to the investigator and *vice versa* in order to facilitate a better understanding among the patients. However, it must be assumed that most of the patients that completed the questionnaire had at least a fair understanding of English or Afrikaans (the two languages spoken by the investigator and used to clarify concepts and answer questions).

Results illustrated in Figure 10 seem to indicate that language does not significantly impede the establishment of a public UCB SCB. It is however an overestimation and confirms the importance of properly addressing the issues concerned in UCB banking through easily accessible language understandable to all patients. In the opinion of the investigator, this will be the single most important defining factor in influencing patient support throughout SA for the establishment of a public UCB SCB.

<u>Language is not a sufficient indicator of ethnicity, religion or culture</u>

As mentioned previously, the investigator wrongly assumed that language could simultaneously provide information regarding a patient's understanding of the questionnaire, as well as serve as a good indicator of a person's ethnicity, religion or culture. This is unfortunately not always the case; e.g. an Italian (ethnicity) born in the USA would probably speak English (language), thus this mistake renders information regarding patient ethnicity insufficient.

Although it holds true that sometimes a person's first language could be an indication of his ethnicity, it was found that many South African mothers-to-be customarily adopt the language spoken by the husband as their home language. Patients might have indicated this home language instead of their own first language and this does therefore not necessarily imply a person's ethnicity and states nothing about a culture or religion. Thus, if the husband speaks Zulu and the patient Pedi, their home language would most likely be Zulu while the patient's ethnicity could be Pedi.

Furthermore, many African patients often speak more than one African language and in some cases neither husband nor wife speaks their partner's first language. In these situations they communicate in a second language that subsequently becomes the home language.

Therefore, all inferences on relationships based on language groups (as a substitute for ethnic, religious or cultural groups) are at best a crude indicator of the influence of ethnicity on patient support and understanding of UCB banking. An example of this is found in Figure 9 where 23% of patients indicated that they spoke English while 25% of patients spoke Afrikaans. This does not indicate whether these patients were Caucasian English or Afrikaans-speaking patients, were Coloured, Indian or of African origin. While 25% of the patients were Afrikaans speaking, at most half of these were Caucasian (investigator observation) while the rest constituted Coloured and only a few African patients. In contrast, very few Caucasian English-speaking patients took part in the study, while the majority of the 25% of English-speaking patients were of Indian or African origin (investigator observation).

It will be important to establish women's cultural practices in relation to body waste (i.e. placenta) in pregnancy, as well as to tissues, blood and donation or "banking". Therefore, suffice it to say that more accurate information regarding patient ethnicity, culture or religion needs to be obtained through an in-depth and systematic study. Without this, no conclusions can be drawn about cultural or religious practices related to certain ethnic groups or more support for UCB banking from specific ethnic groups.

Influence of patient age on support for a public UCB SCB

From observations made during the pilot study (which preceded the current study), the investigator observed that younger patients seemed to be more supportive of UCB banking than older patients. In order to better quantify this observation, the questionnaire was revised to make provision for annotating patient age.

The majority of patients that attended the clinic and took part in the survey were between the ages of 26 to 30 (Figure 11). However, when patients were grouped into "younger" (ages 18 to 30) and "older" (ages 31+) groups, the numbers were almost equal, with 112 younger patients and 93 older patients (the remaining 12 patients did not indicate their age). Results, corrected for the number of patients per age group are indicated in Figure 12 and show how the patients' age affected their willingness to donate their placenta (Q1) and their support for a public UCB SCB (Q6).

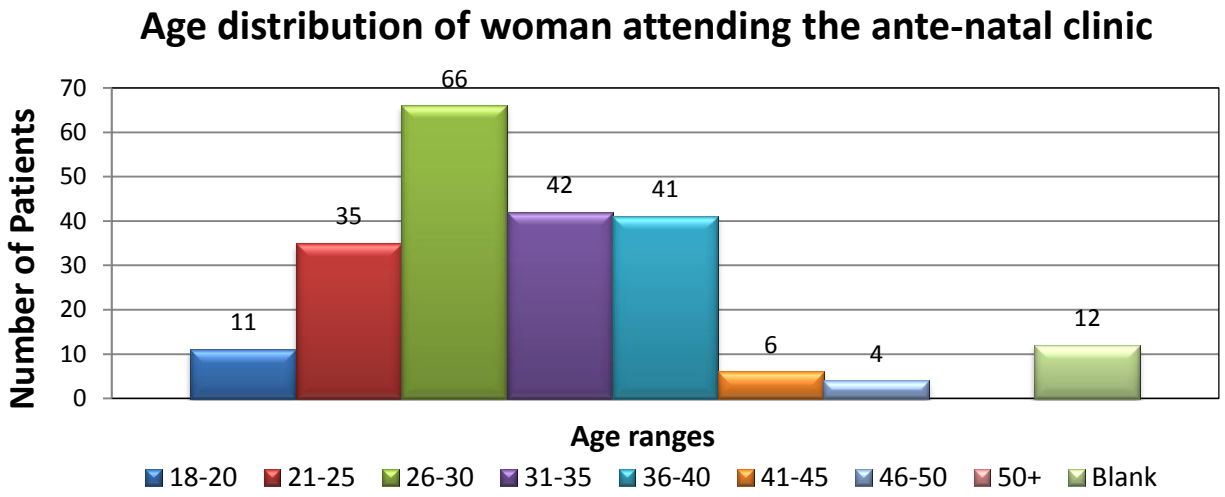

Figure 11: Age distribution of patients attending the Steve Biko Academic Hospital's antenatal clinic. Ages ranged from 18 years to above 50.

Of the younger patients, 81% indicated their support for both Q1 and Q6, while 70% support was obtained from the older patients. Assessing each question individually, younger patients were more willing to donate their placentas (84%) than older patients (77%) and younger patients were generally more supportive of the idea of establishing a public UCB SCB (92%) than older patients (82%) (Figure 12). To establish whether this observed difference in support between the age groups could be due to a difference in understanding of the presentation, the patients' ages were compared to their understanding of the questionnaire (Q5) (results not shown). It was found that 90% of the younger patients understood the information presented

and thought that SCs could be used to treat people with certain disorders. The older group's understanding (81%) corresponds well to their willingness to support the bank (82%) and could potentially imply that their weaker comprehension had a greater impact on their support than the younger group with better comprehension.

Other possible reasons that could explain greater support from younger patients include: 1) that younger patients have been more exposed to the latest technology. As a consequence, they might be more open to acceptance of new technological concepts, might be less intimidated by these concepts and more keen to explore new areas of innovation; 2) younger patients could be better equipped (schooling and thus comprehension) than their predecessors; 3) older generations could potentially be more reluctant to participate based on beliefs shaped by previous political regimes.

However, most patients that took part in the study seemed to adequately understand the presentation regardless of their age. It seems that the determining factor for obtaining support is to equip the patients with adequate and accessible information in order to make a properly informed decision. This information should be tailored to address not only people with different levels of schooling, but should also be 'age friendly'; i.e. should accommodate older people's lack of understanding of technological development and comprehension.

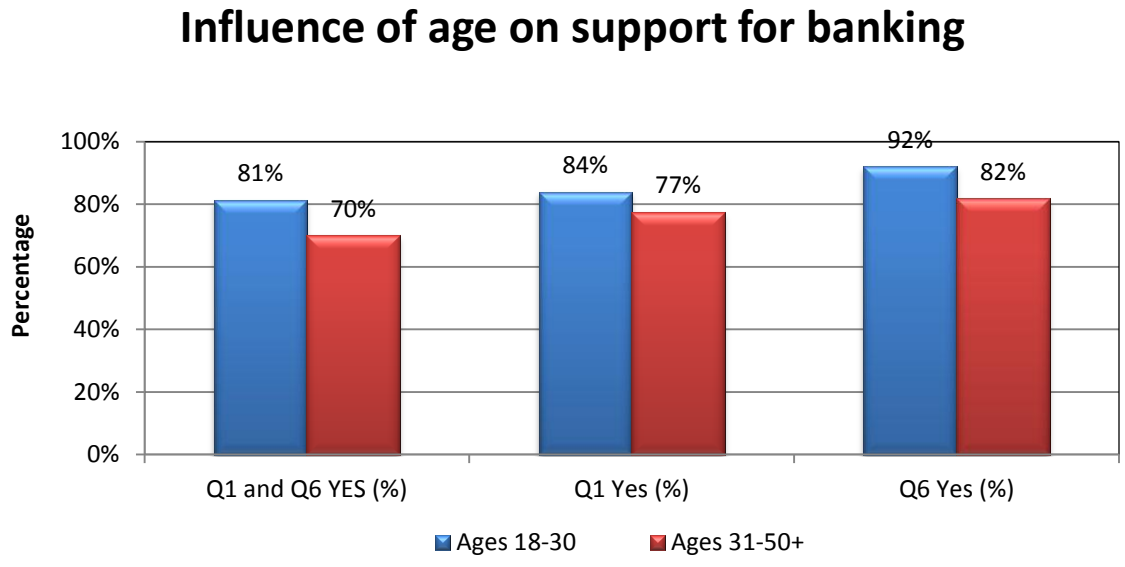

Figure 12: The influence of age on the support for establishing a public UCB SCB. Patient ages were grouped into two groups: "Younger" between the ages of 18 and 30 indicated by the blue bars, and "Older", above the age of 30, indicated by the red bars. Q1 tests a patient's willingness to donate the placenta, while Q6 tests the patient's support for establishing a public UCB SCB.

The influence of exposure to childbirth on patient support

Another factor that could influence a patient's understanding of UCB banking and related processes (discussed during the presentation) could be a patient's prior exposure to childbirth. During the pilot study (mentioned earlier), the investigator encountered patients who were unsure of what a placenta was. The word was subsequently translated in order to clarify its meaning but some patients were still unsure of the placenta's role during pregnancy and its normal disposal after pregnancy. It was therefore thought that a patient who had gone through the process of childbirth would know what to expect, understand more, be less afraid of donation and would subsequently be more supportive of a public bank. Figure 13 and Table 2 illustrate the number of children born to mothers of different age groups.

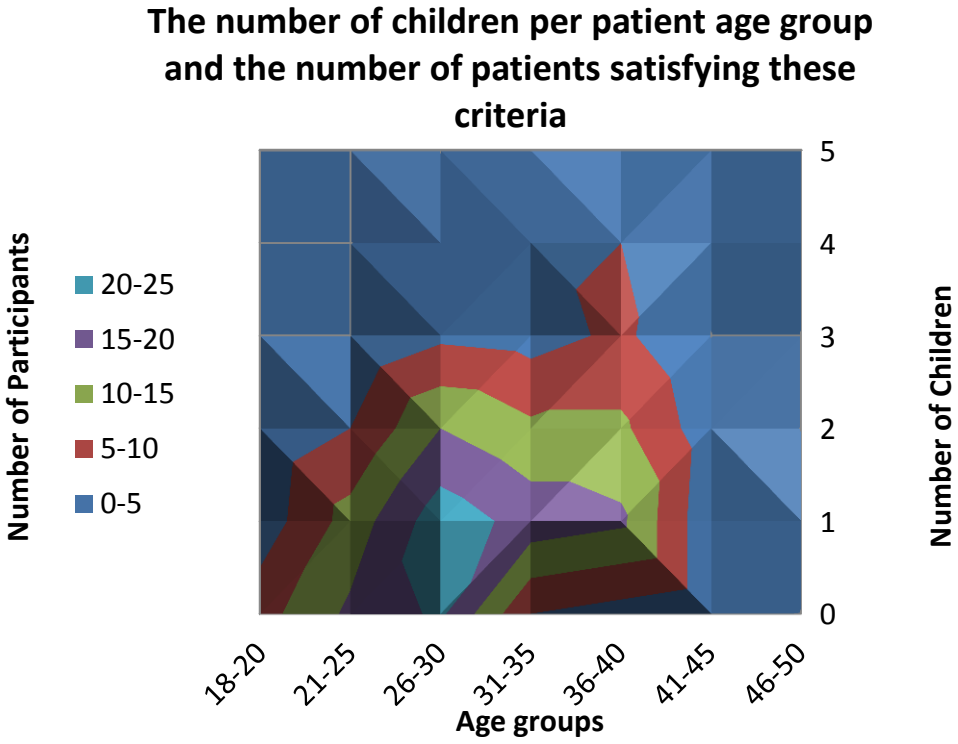

Figure 13: Indicates how many patients (indicated in colour and contour) accounted for both criteria: i.e. the a) number of children per b) patient age group. The largest group of patients (20-25 patients) is indicated by the turquoise area, followed the purple (15-20); green (10-15); red (5-10) and blue (0-5) groups.

It can be seen in Figure 13 that 20 to 25 patients (turquoise area) had one child and were between the ages of 26 and 30. Table 2 illustrates these numbers. The second highest prevalence for these categories was the 21 patients between 26 and 30 years without any children.

Table 2: Number of children for patients in different age ranges

Age	Number of Children					
	0	**1**	**2**	**3**	**4**	**5**
18-20	8	2	1	-	-	-
21-25	16	12	5	-	-	-
26-30	21	23	15	4	2	-
31-35	5	18	11	3	4	1
36-40	3	16	11	6	5	-
41-45	1	1	2	-	1	1
46-50	-	1	-	-	1	1

Patients were grouped into two categories: Patients with biological children and patients without biological children. There were 54 patients without any children and most of these (45) were below the age of 30. When comparing support from these two groups (with and without children), it seems that patients without children are slightly less supportive of establishing a public UCB SCB.

Results illustrated in Figure 14 reveal that 84% of patients without children were willing to donate their placenta (Q1), 90% of them thought that SCs could be used to treat patients (Q5) and that building a public UCB SCB is a good idea (Q6) respectively. Patients with children responded similarly but with somewhat greater support for these questions, with 85% of patients willing to donate the placenta (Q1), 95% understood the application of SCs (Q5) and 96% thought establishing a public UCB SCB is a good idea.

Collectively, all three questions related to support and understanding of a public UCB SCB (Q1, Q5 and Q6) indicated 80% support from patients without children and 85% from patients with children. In each case, it seems that having gone through the experience of childbirth (or previously being exposed to it) slightly aids the patient's understanding of the concepts involved in UCB banking – e.g. what the placenta is; what it does during pregnancy; that it is discarded after pregnancy, etc.

These patients might subsequently be less fearful of the unknowns associated with UCB collection than patients without childbirth experience. Nevertheless, the results are very similar between the two groups and the differences are not statistically significant. Although

childbirth exposure could potentially influence patients' support and understanding of UCB banking, it is unlikely to significantly impede the establishment of a public UCB SCB.

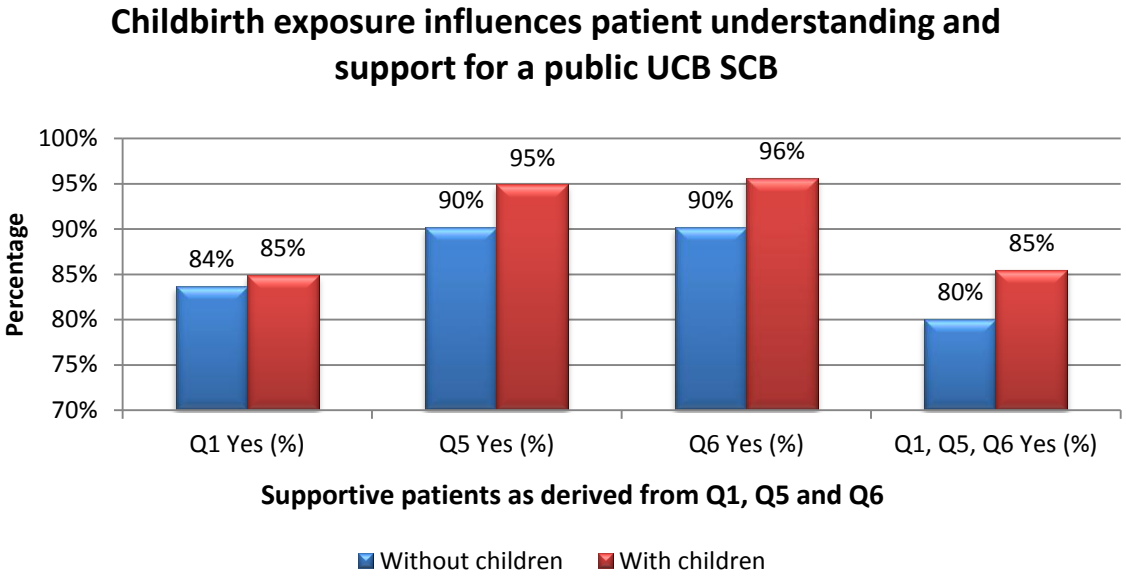

Figure 14: Exposure to childbirth influences patient of understanding and support for UCB banking. Results displayed indicate positive answers ("Yes") to Q1, Q5 and Q6 for patients with children (red bars) and without children (blue bars) respectively.

Patient demographics: Marital status and employment status

These two parameters provide personal information about patients by shedding light on their current circumstances and potential support structures. The possible relations between marital status, employment status and patient support are far too numerous to be adequately analysed with the few parameters provided by this survey. However, they could be used as initial probes into whether marital or employment status has any impact on patient support whatsoever, which could merit a more comprehensive analysis of potential contributing factors for each category.

Figure 15 indicates that the majority of patients (47%) were married (M), followed by 44% unmarried patients (U). The remaining patients were either widowed (W) (1%), divorced (D) (2%) or left the category unanswered (BL) (6%).

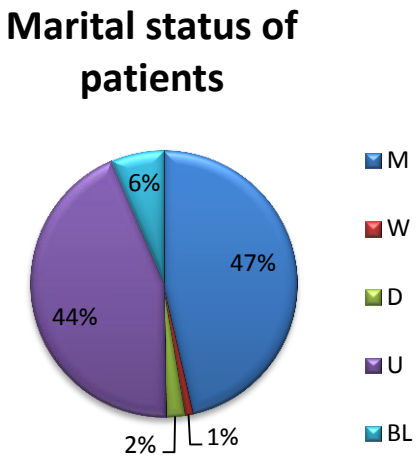

Figure 15: Marital status of patients that partook in the survey. Marital status was indicated to be: Married (M, dark blue); Widowed (W, red); Divorced (D, green); Unmarried (U, purple) or were left unanswered (BL, light blue).

Information gathered on patient employment is illustrated in Figure 16. It indicates conservative estimates of 48% unemployment (U) and 31% employment (E), since 21% of patients left the question unanswered (BL).

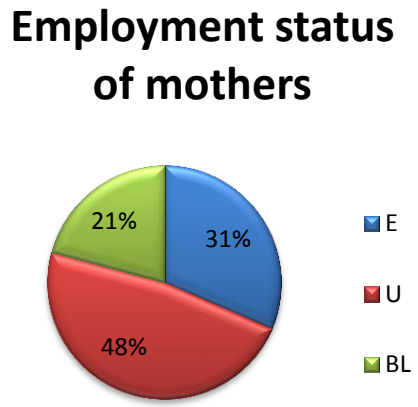

Figure 16: Marital status of patients that partook in the survey. Marital status was indicated to be: Married (M, dark blue), Widowed (W, red), Divorced (D, green), Unmarried (U, purple) or were left unanswered (BL, light blue).

Patients' answers to Q1 and their corresponding employment status and marital status are displayed in Figure 17. From this data it seems that patients were supportive of establishing a public SCB regardless of whether they were married, unmarried, employed or unemployed (**Note*: Blank data (unanswered Q1) is removed and the remaining data is corrected to display percentage support proportional to the number of patients per category; i.e. 68 patients were

employed, with 57 of them supportive (Q1), thus 84% of employed patients are supportive of public UCB SC banking).

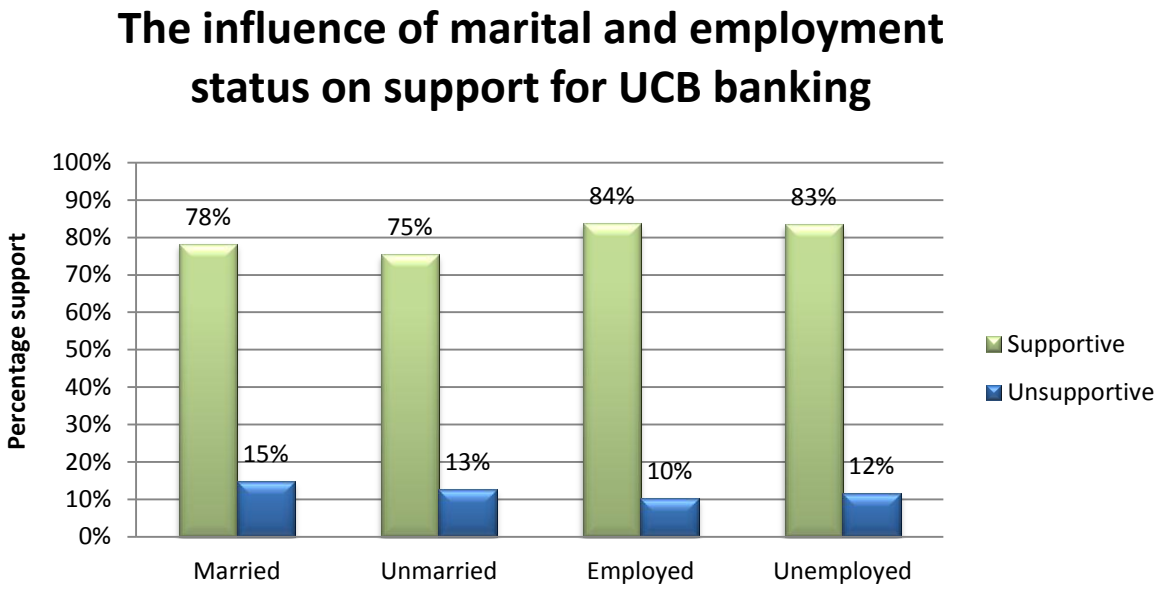

Figure 17: Influence of employment and marital status on patient support for a public UCB SCB (Q1).

From Figure 17 it is clear that marital status and employment status are not determining factors for a patient's support for UCB banking. Equal support was given from married (78%) and unmarried (75%) patients; 84% of employed people supported the bank and 83% of unemployed people supported the bank. A similar trend is observed for unsupportive patients in each of the abovementioned categories.

These results could be because very little is required of patient in order to donate their placentas to UCB collection. If they are presented with adequate information, they would not have a need to contact family or relatives for more information or support in making the decision to donate. Furthermore, eligibility for donation does not require any financial contribution nor does a patient have any additional expenses associated with donation, since the placenta and UCB is collected at the time of delivery of the child.

5.6 Conclusion

South Africa is in a favourable position to implement new avenues for access to healthcare and to increase development in the areas of cellular, molecular and regenerative medicine. Not only are many South Africans in need of these advanced medical and technological developments, but they are also enthusiastic about building a better South Africa.

Similar studies to the one presented here have been conducted globally. Results from this study are strikingly similar to results obtained from these studies abroad and are discussed below:

Fernandez *et al*. (2003) assessed the knowledge and attitudes of Canadian women with regard to testing, collection and banking of UCB SCs. Rucinski *et al*. (2010) reported on the opinions and beliefs of Hispanic and non-Hispanic woman with regards to UCB donation and banking.

Both studies encountered a large gap in information available and accessible to patients, with very few patients aware of UCB banking. As many as 70% of patients indicated poor or very poor knowledge of UCB SCs (Fernandez et al., 2003), which corresponds to data gathered for this study (Fig. 7, Q4) while Katz *et al*. (2010) reported that 79% of woman lacked basic knowledge about SCs.

In the current study the investigator concluded that educating the public with regard to UCB banking and the application thereof would be the single most important factor in generating public support for a public UCB bank. Rucinski *et al*. (2010) concluded the same reporting that their biggest barrier to patient support was a lack of basic information available to the public with regard to UCB banking, UCB harvesting and use. They furthermore suggested that patients should not only be informed on the social value of UCB banking but also be informed about the technical aspects involved in banking.

Similar to observations reported in the current study, Fernandez *et al*. (2010) and Katz *et al*. (2011) both reported a majority support for public UCB banking (as opposed to private or hybrid banking). In this study it was found that a conservative estimate for support from patients for the public bank lies between 80% and 86% (Fig. 3, Q1 and Q6). Taking only workable data into account, these numbers increase to between 85% and 94% (Fig. 7, Q1 and Q6).

Similarly, Fernandez *et al*. (2003) reported that 86% of their Canadian patients opted to store their UCB in a public bank and Katz *et al*. (2011) reported 89% of patients (from 5 European countries) would store their UCB, 76% of which would store publicly.

Additional factors assessed by previously published studies that are similar to results from this study:

Katz *et al*. (2011) found no correlation between patient income and the decision to donate UCB, which held true for all five countries surveyed. Although information gathered about patient employment in the current study is insufficient to draw definitive conclusions, preliminary data seem to correspond with results obtained for patient income from Katz *et al*. (2011).

Many studies have reported on ambiguity of words that caused confusion amongst the patients – most notably "donation" versus "banking", "cord" as explained in "cord blood" (as opposed to spinal cord) (Rucinski et al., 2010). Similarly, this study found words such as "placenta" and "bank" often confused patients. In order to clarify the concepts, words were either translated (e.g. placenta translates to "Inghubo" in Zulu) or explained in broader detail.

Rucinski *et al*. (2010) mentioned that racial and ethnic disparities were observed for donation of UCB similar to those found in organ and tissue donation. Although this does not seem to be the case in South Africa, the possibility that certain ethnic groups in South Africa might be more reluctant to donate cannot conclusively be ruled out in the current study. In order to address potential ethnic influences with regard to UCB banking, the patient cohort would need to be more representative of the population. The questionnaire should also be modified to capture patient ethnicity more accurately.

Rucinski *et al*. (2010) also reported on misconceptions with regards to the placenta, its function during pregnancy and what happens to it after pregnancy. This is similar to observations made during the pilot study (reported on earlier) accounting for the lack of patient knowledge of even basic biological concepts.

This study was conducted to estimate public preparedness and support for establishing a public UCB SCB in South Africa. It furthermore obtained information on specific parameters that could potentially impede the establishment of such a bank. Although the patient cohort was not adequately representative of the total population of South Africa, patients were nevertheless optimistic about the potential establishment of a public bank. Comments received from

patients during the study were predominantly positive, stating their enthusiasm and support for an UCB bank. Reasons from unsupportive patients were mostly reflective of unavailable and inadequate information available to the public with regard to UCB donation.

Patients were supportive of UCB banking regardless of their age, ethnicity, employment, marital status or whether they had previously experienced childbirth. Some of these factors – e.g. previous childbirth – might, however, influence the level of a patient's understanding of UCB donation.

The main determining factor in obtaining support for UCB banking in SA is thus equipping the patients with adequate and accessible information in order to make a properly informed decision. This information should be tailored to address not only people with different levels of education but should also be 'age friendly'; i.e. should accommodate older people's disadvantage with regard to technological development and comprehension. The information should include technical aspects involved in the processes of UCB donation, banking and application as well as the social value of donation.

Finally, results obtained from this study are supportive of establishing a public UCB SCB in South Africa but should be confirmed in different provinces across the country. It serves as a preliminary screening of the public acceptability response from a selected cohort of South African citizens to UCB public banking and paves the way to an in-depth social scientific enquiry. These results could potentially also allude to provinces that might be more suitable for the establishment of public UCB banks.

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CHAPTER 6

6 Verification of the Ultrio-Plus® assay on umbilical cord blood

6.1 Introduction

Umbilical cord blood (UCB) has become an acceptable alternative source of haematopoietic stem cells (HSCs) for bone marrow (BM) transplantation (Broxmeyer et al., 1990; Brunstein et al., 2007). The main function of a public UCB stem cell bank (SCB) is to collect and appropriately store voluntarily donated UCB, until such a time that any patient might need the UCB unit for transplantation. UCB units collected for a public UCB bank would thus be for allogeneic purposes.

With South Africa's particularly high rate of HIV infections, one of the biggest challenges in the establishment of a South African public UCB bank is to screen effectively for infectious diseases and in particular for HIV prior to storage of a unit. Current international screening methods involve screening of the donor (mother) for infectious diseases and potential risk factors associated with rejection of a donated UCB unit. No tests have thus far been verified to screen the UCB unit itself for infectious diseases.

6.1.1 Occurrence of HIV-1 infection in South Africa

South Africa is faced with enormous challenges in the areas of HIV prevention (including education) and treatment. With regard to the prevalence of HIV/AIDS in South Africa, the data from the South African Antenatal Sentinel HIV and Syphilis Prevalence Survey reveal the following (Anon, 2010a):

- The estimated overall HIV prevalence rate is approximately 17.9%. The total number of people living with HIV is estimated at approximately 5.57 million. For adults aged 15 to 49 years, an estimated 17% of the population is HIV positive (Anon, 2010a).
- For 2010, approximately 4.03 million people aged 15 and older and approximately 438 000 children were infected with HIV (Anon, 2010a).
- Of these individuals, 1.2 million people aged 15 and older and 102 000 children would be in need of anti-retroviral therapy (ART) (Anon, 2010a).
- The total number of new HIV infections for 2010 is estimated at 281 000 for adults, and 54 000 new infections among children 14 years and younger (Anon, 2010a).

Figure 18 below indicates the global prevalence of HIV and puts the severity of HIV prevalence in sub-Saharan Africa into perspective: where most countries have an estimated HIV prevalence below 5%, sub-Saharan Africa has an estimated HIV prevalence of more than 15% to 28%; i.e. three to six times that of most countries in the world.

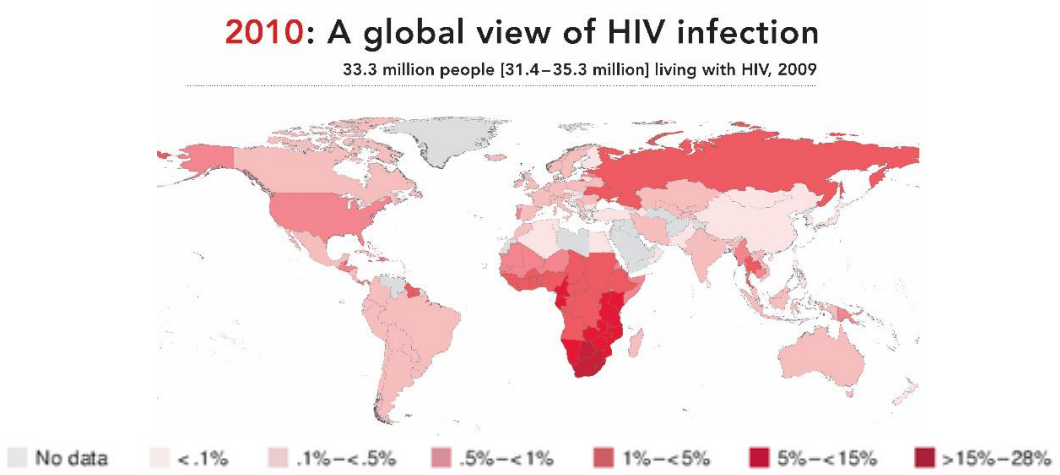

Figure 18: UNAIDS report on the global Aids epidemic, illustrating global HIV prevalence for 2010 (http://www.unaids.org/globalreport/HIV_prevalence_map.htm).

Figure 19 indicates the estimated HIV prevalence in 15 to 49 year olds for individual provinces in the country. It shows four of the nine provinces (Gauteng, Free State, Mpumalanga and KwaZulu-Natal) with HIV prevalence rates above 30% and KwaZulu-Natal almost reaching 40% prevalence.

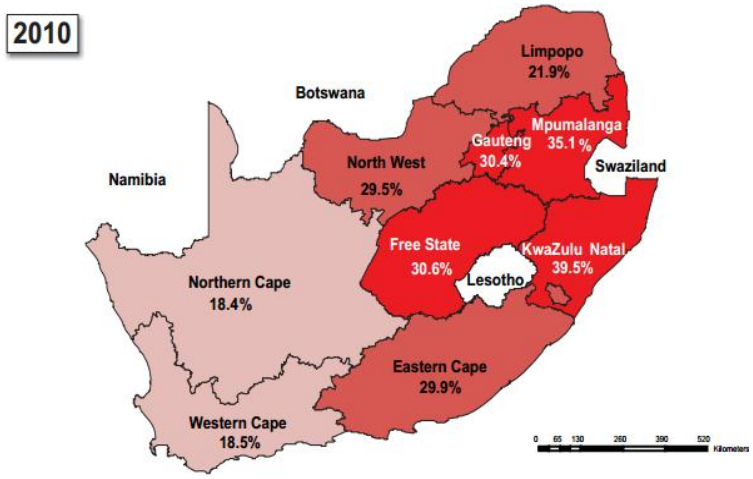

Figure 19: HIV prevalence among antenatal women, distribution by province, South Africa, 2010 (Anon, 2010a)

Of particular importance for establishing a public UCB bank is the high HIV infection rates among pregnant women, from whom UCB units would be obtained. According to this survey, the national HIV prevalence for woman attending antenatal clinics in 2010 was estimated at 30.2% (95% CI of 29.39 to 30.91). The HIV prevalence trend from 1990 to 2010 among women attending antenatal clinics is indicated in Figure 20 while Figure 21 shows the trend in individual provinces in SA from 2008 to 2010.

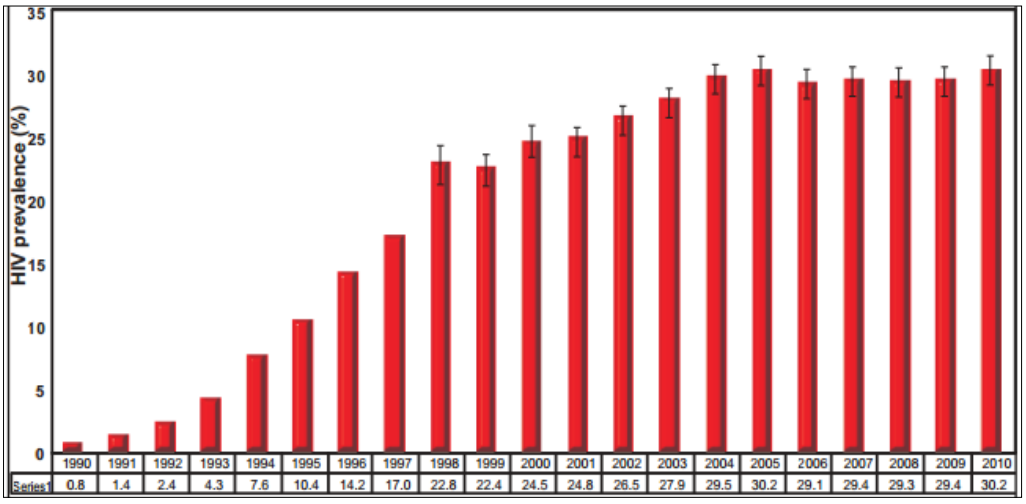

Figure 20: HIV prevalence trends among antenatal women, South Africa 1990 to 2010. The estimates from 2006 are based on a different sample from the previous years (Anon, 2010a)

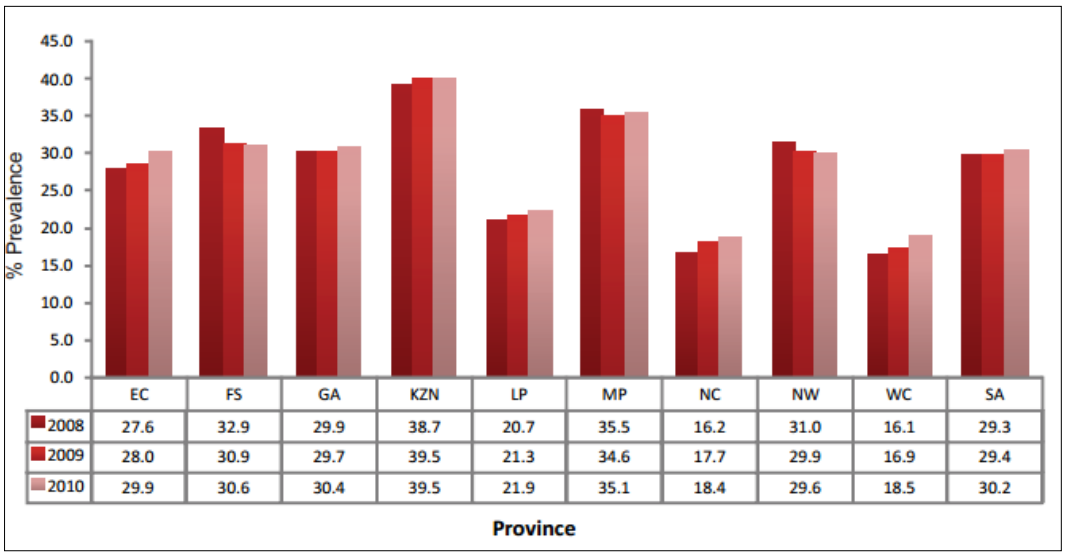

Figure 21: HIV prevalence trends among antenatal women by province, South Africa, 2008 to 2010 (Anon, 2010a)

It seems that the sharp increase in HIV prevalence from the early 1990s has levelled out since 2004 and has remained more or less stable at 29% for the past four years. These high

prevalence rates would disqualify significant numbers of potential UCB units even before collection. This underscores the importance of pre-screening questionnaires for the mothers so that only potentially viable UCB units are collected and unnecessary downstream screening expenditures are prevented.

In addition to the dramatic effects that HIV/AIDS has on individuals, families and society in general, an indication of the impact of HIV/AIDS and tuberculosis (>70% of patients with TB have HIV/AIDS) on the South African economy can be found in the Global Competitiveness Index, which is determined by World Economic Forum. In the 2012 to 2013 period, South Africa was ranked 50th overall out of 144 countries (web: http://www3.weforum.org/docs/WEF_GlobalCompetitivenessReport_2012-13.pdf).

However, when health was considered on its own, the following ranking data emerged:

Category	Rank
• Health	131
• Business impact of HIV/AIDS	135
• Tuberculosis incidence	143
• HIV prevalence	141
• Business impact of tuberculosis	132
• Life expectancy	133
• Infant mortality	107
• Business impact of malaria	100
• Malaria incidence	89

This points to the dramatic effect that infectious diseases (including HIV/AIDS) have on South Africa's global competitiveness.

6.1.2 Probability of obtaining HIV-1 positive umbilical cords: vertical transmission of HIV-1 from mother to child

HIV infection and transmission can occur in utero and is termed mother-to-child transmission (MTCT), vertical transmission or trans-placental transmission (Soilleux & Coleman, 2003). In developed countries, the prevalence of HIV-1 MTCT ranges between 13% and 32%, while it increases to between 25% and 48% in developing countries, with 30% of these HIV-positive infants being infected "in utero" (Guevara et al., 2000). A study done by Taha *et al*. (2011) furthermore suggested that the percentage of in utero infection increases with newly infected mothers. These authors found that out of a total of 73 mothers, recently infected mothers

transmitted the virus in utero at a frequency of 17.8%, as opposed to not-recently-infected mothers who had an in-utero transmission of 6.7% (Taha et al., 2011).

Studies that furthermore distinguish between true in-utero infection, intrapartum infection (occurring during the time of birth) and perinatal infection (period around birth – between five months before and one month after birth) are in agreement that around 5% to 8% of HIV MTCT occurs in utero, while 15% to 30% occurs intrapartum (Biggar et al., 1996; Mock et al., 1998).

Guevara (2000) stated that HIV RNA measurements from maternal and cord blood plasma allow for the quantitative assessment of HIV viremia in the mother and infant respectively. This statement was further supported by Biggar *et al*. (2007) who reported that they conducted polymerase chain reactions (PCR) on infants to detect the HIV genome. The infants were only considered to be infected with HIV in utero if HIV was detected by PCR done on umbilical cord blood. They furthermore concluded that the positive infants were indeed infected in utero due to HIV levels equally as high as, or higher than their mother's HIV levels.

Courgnaud *et al*. (1991) reported that they detected HIV DNA (provirus) in foetal spleen thymus and peripheral blood mononuclear cells (PBMCs). The foetuses were aborted between 16 and 24 weeks from HIV-positive mothers. Nine foetal specimens of spleen and PBMC and eight foetal specimens of thymus were tested for HIV DNA. Six out of the eight foetal thymus specimens, eight out of the nine spleen foetal specimens and five out of the nine PMBC foetal specimens tested positive for HIV DNA, demonstrating that HIV infection does occur in utero.

HIV infection of Hofbauer cells, specialised foetal macrophages, has been demonstrated by *in situ* hybridisation, *in situ* PCR and immunohistochemistry (Newell et al., 1998). HIV has also been detected in amniotic fluid (Guevara et al., 2000; Newell et al., 1998).

Townsend *et al.* (2008) found in their study on mothers receiving ARTs, that three infants (from a total of 2117 infants born) contracted HIV from their mothers despite the mothers being on ART treatment and having viral loads below 50 IU/mL. Two of these infants showed evidence of in utero transmission.

6.2 International regulatory standards for screening of UCB units

Upon receiving an UCB unit, the unit has to undergo various types of screenings in order to medically qualify it for transplantation. Each unit receives a "Cord blood unit report", which contains detailed information about the unit – e.g. total nucleated cell (TNC) count, human

leukocyte antigen (HLA) typing, specific tests performed on the CB unit and/or mother to name but a few (Welte et al., 2010).

Cord blood banks have maternal health questionnaires that serve as a pre-screening tool and aim to identify certain risk factors related to transplantation of the UCB unit prior to acceptance or storage. These risk factors vary between different cord blood banks, but the World Marrow Donor Association (WMDA) has consolidated these requirements into a comprehensive list. The list covers various blood disorders (red and white blood cells and platelets), certain genetic disorders (including monogenic disorders), cancers (Leukaemias), metabolic disorders, severe auto immune disorders and infectious diseases (Welte et al., 2010).

There are currently three potential ways of screening for infectious diseases:

1. Screening the mother within seven days of delivery
2. Retesting of maternal donors at six- month follow up
3. Testing the UCB unit

6.2.1 Maternal screening:

As per Section D 11.1.9.2 of the NetCord-Foundation for the Accreditation of Cellular Therapy (FACT) International Standards for Cord Blood Collection, Banking and Release for Administration (fourth edition) (Anon, 2010b2), the minimal evaluation of infectious agents is performed through serologic screening and nucleic acid testing (NAT) of the maternal sample as a substitute for the CB unit.

Pregnant mothers get tested for HIV at their first medical consultation. If they are found to be HIV positive, anti-retroviral regimens are administered to them in order to prevent MTCT of the virus. If a patient is negative at the first screening for HIV during pregnancy, it does not rule out the possibility that she might still contract HIV during her pregnancy. Mothers that consented to UCB donation are therefore screened again for infectious diseases – including HIV – within seven days prior to or after delivery. In order to rule out the possibility that the mother might be in the window period of infection at the time of her last screening, some cord blood banks also require an additional follow-up screening of the mother six months after delivery. In such a case, a CB sample would only be eligible for further consideration if the screening results for all the time periods are negative.

Although there are benefits to conducting a six-month follow-up screening on the mother, it places an administrative burden on the cord blood banks. It is often difficult to locate the patients after six months and many might not stay close to the hospital or clinic. The onus of re-testing the mother lies on the bank and the bank would therefore be responsible for any additional costs involved for the patients to return to the hospital or clinic for screening. The NetCord-FACT guidelines in dealing with cases where six-month follow ups of the mother are not achievable are found in Section B.2.6.6.2. If initial maternal screening results return as indeterminate or repeatedly reactive, the UCB bank cannot conclude on the interpretation of results without a follow up on the mother. Therefore, the NetCord-FACT guidelines suggest that the UCB bank inform the mother and / or physician of the test results in order to rule out potential health-related risks (Anon, 2010b2).

6.2.2 Cord blood unit screening

Another alternative would be to screen the mother at the time of delivery but to also subject the UCB unit to screening. According to Section D. 10.8 of the NetCord-FACT Cord Blood Accreditation Manual, testing of the CB units are recommended. Many test kits (for infectious diseases) have not been approved by the Food and Drug Administration (FDA) for use on UCB, but performing these tests is nevertheless recommended by the NetCord-Foundation. In the case where a screening test – which is unaccredited for UCB – is used, the UCB bank is advised to denote the outcome and annotate that the test has not yet been validated (Anon, 2010b).

Section D 10.8 of the NetCord-Foundation furthermore states: “Prior to the release to the Clinical Program, each Cord blood unit should be tested for evidence of infection by at least the following communicable disease agents using licensed donor screening tests when available according to Applicable Law”:

- Human immunodeficiency virus type 1
- Human immunodeficiency virus type 2
- Hepatitis B virus
- Hepatitis C virus
- Human T cell lymphotropic virus type 1
- Human T cell lymphotropic virus type 2
- Treponema pallidum (syphilis)

- And any additional agents required by Applicable Law at the time of the release of the CB unit" (NetCord FACT international standards) (Anon, 2010b).

The reluctance to standardise screening of UCB units stems from concerns about reducing the volume of the UCB unit for additional testing requirements. Volumes might furthermore be affected by dilutions with the anti-coagulant in the collection bags. Furthermore, if appropriate provision for testing and re-testing were not made, it might require thawing of the UCB unit, which could damage the integrity of the sample (Anon, 2010b). However, in order to overcome this last-mentioned logistical issue, small segments attached to the UCB bag are now being sealed off and frozen together with the CB unit during sample processing. These segments are representative of the UCB unit and can easily be broken off and used for additional screening or sample analyses without compromising the UCB unit's integrity or volume.

It therefore seems that the more viable option, which would also be the most stringent in screening for infectious diseases, would be to screen the UCB units in addition to screening the maternal sample within seven days of delivery.

6.2.3 Stringency in screening and acceptance criteria

The heavy burden of HIV disease in South Africa combined with the risk of MTCT highlight the important risk of obtaining and transplanting potentially infected UCB units. UCB banks make their UCB units available to patients globally: however, these risks might discourage international UCB banks from using UCB units that originate from South African UCB banks.

In order to increase stringency of detection methods for infectious diseases, tests need to be validated/verified for use on UCB units in addition to already validated tests currently performed on peripheral blood. This would increase screening comprehensiveness and improve international confidence in the quality of UCB units.

By only screening the donor (mothers) for infectious diseases, certain HIV-1-infected UCB units could go undetected. It is important to note that although the placenta serves as a barrier to entry to disease organisms, the extent of vertical transmission of diseases varies between different organisms. Conversely, it might be argued that potentially viable UCB units would be wasted if they were discarded only on the basis of the mother's history of infection. Performing screening on both the maternal sample and UCB unit would increase the safety margins and decrease margins of error when screening is performed.

6.2.4 Transplantation of UCB units for HIV-positive patients

The question has arisen whether organ donation and transplantation for HIV-positive individuals might be feasible if done between HIV-positive individuals. Because of the success of ART therapies in reducing viral load, HIV-positive patients live longer, healthier lives than before and subsequently also become subject to other diseases affecting organ function.

Most HIV patients are not eligible for transplantation purposes for numerous reasons – most notably the accompanying suppression of an already weakened immune system in order to prevent graft versus host disease post transplantation. However, studies done on kidney transplantation between HIV-positive individuals have delivered promising results (Muller et al., 2010; Frassetto et al., 2009), making the possibility of eligible HIV transplantation donors and recipients a reality.

Similarly, the question arises as to whether or not HIV-positive patients' UCB units should not be stored for potential use for another HIV-positive individual.

Currently, UCB units are not collected from patients who have received ART treatment during their pregnancies. Although there is a risk of trans-placental transmission of HIV during pregnancy, this risk decreases significantly when mothers receive ART treatment. A study done by Townsend *et al.* (2008) on perinatal transmission of HIV in 5,151 HIV-infected women in the United Kingdom and Ireland between 2000 and 2006 showed transmission rates as low as 1.2% (61/5151, 95% confidence interval: 0.9-1.5%), and 0.8% (40/4864) for women who had received ART for at least the last 14 days of pregnancy (Townsend et al., 2008).

If the viral load of a mother receiving ART is below 50 IU/mL and the subsequent screening of the collected UCB unit is negative, should this CB unit be discarded, made available to the general public (seeing that it is negative) or be stored separately for potential use in HIV-positive patients? These answers would be subject to stringency and sensitivity of tests used and the reliability of results. Many HIV-negative individuals would probably not be comfortable with receiving an UCB unit (albeit negative) from an infected mother regardless of her current health status. In these cases, it might be best to keep these samples separate from samples that were qualified as negative for both maternal and UCB unit screening.

In a country as severely affected by HIV as SA, it might, however, be necessary to create a separate storage facility that would only store UCB units collected from HIV-positive individuals. If both the mother and UCB unit are screened, then samples from HIV-positive patients could

be divided into three categories: A) Screening where both maternal and UCB unit returned positive; B) Maternal sample resulted positive, but the UCB unit came back negative; C) Mother had a history of infectious diseases and/or used ART but current viral load is undetectable and both maternal sample and UCB unit returned negative.

A critical component of clarifying these concerns will be the accuracy and sensitivity of tests used to detect the various infectious diseases.

6.3 Ultrio-Plus® assay

The Ultrio-Plus® assay is a nucleic acid test (NAT) that has been validated for the simultaneous detection of HIV type-1 (HIV-1), Hepatitis B-Virus (HBV) and Hepatitis C-Virus (HCV) in human peripheral blood (PB), bone marrow (BM) and cadaveric tissue (using plasma or serum). The test was developed, manufactured and distributed by Gen-Probe Inc. (San Diego, CA) in collaboration with Novartis Vaccines and Diagnostics, Inc (Emeryville, CA). It utilises target amplification nucleic acid probe technology and has an internal control incorporated for monitoring assay performance in each individual specimen. Although it does not discriminate initially between a positive signal for HIV-1, HBV or HCV, the technique is fast, effective and accurate in determining which samples are contaminated with these infectious diseases and should be discarded. Specimens found to be reactive in the Ultrio-Plus® assay can be run in individual HIV-1, HCV, and/or HBV discriminatory assays to determine if they are reactive for HIV-1, HCV, HBV or any combination of the three, should the need arise.

6.3.1 Procleix® ultrio® assay (Ultrio-Plus® assay)

The following section has been modified from the package insert:

The Ultrio plus assay is used internationally by blood centres (including the South African National Blood Services (SANBS)) for HIV-1, HBV and HCV screening.

It has three main steps:

1. Target capture (sample preparation)
2. Transcription-mediated amplification (TMA)
3. Detection of the amplicon (amplification products) by the hybridization protection assay (HPA)

6.3.1.1 Step 1: Target capture

The aim of the first step is to isolate the target (HIV-1 RNA, HCV RNA and HBV DNA). In the case of HIV, this involves the release of viral genomic RNA, the denaturation of proteins and the solubilisation of the viral envelope by adding a detergent to the sample in question. The next step in the target capture is to hybridise oligonucleotides (short nucleic acid polymers) that are homologous to highly conserved regions of HIV-1 to the HIV-1 RNA if it is present. Finally, in order to separate the hybridised HIV-1 RNA, it is captured by magnetic micro-particles, which are separated from the sample in a magnetic field. Subsequent wash steps remove extraneous components from the reaction tube.

6.3.1.2 Step 2: Transcription-mediated amplification

The aim of this step is to amplify the hybridised HIV-1 RNA through a process called "transcription mediated amplification". The hybridised HIV-1 RNA has to be converted into a DNA copy of the target sequence. This process is achieved by the enzyme, reverse transcriptase. In the case of the Ultrio-Plus® assay Moloney Murine Leukaemia Virus Reverse Transcriptase (MMLV reverse transcriptase) is used. The DNA copy contains a promotor sequence for the T7 RNA polymerase enzyme. This enzyme in turn produces multiple RNA copies from the DNA amplicon.

6.3.1.3 Step 3: detection of the amplicon by HPA

Detection of viruses occurs through a process known as "hybridisation protection assay" (HPA). Complementary single-stranded nucleic acid probes with chemiluminescent labels are hybridised to the specific amplicon. A selection reagent is then added. The selection reagent differentiates between hybridised and unhybridised probes and inactivates the probes on the unhybridised single-stranded nucleic acid. The hybridised probes give off a chemiluminescent signal, which is measured by a luminometer and reported as Reactive Light Units (RLU).

The Ultrio-Plus® assay is used to detect HIV-1 RNA, HCV RNA and HBV DNA simultaneously. In order to differentiate between these three viruses the Procleix HIV-1, HCV, and HBV discriminatory assays need to be done. The discriminatory assays follow the same three steps as described above except that they use HIV-1-specific-, HCV-specific-, or HBV-specific probe reagents in place of the Ultrio-Plus® assay Probe Reagent.

The Ultrio-Plus® assay has not been validated for cord blood plasma, which – for all intents and purposes – should be similar to plasma from peripheral blood. Method verification of the Ultrio-Plus® assay done on UCB plasma would be advantageous for screening any UCB

unit intended for UCB banking and subsequent transplantation. Results obtained from maternal screening could then be compared to UCB plasma Ultrio-Plus® results.

6.4 Hypothesis and objective

Should a public UCB bank be established in South Africa, all UCB units would undergo compulsory routine infectious diseases screening for compliance with international regulatory standards. It would be imperative to have a sensitive and reliable assay for detection of HIV-1 in potential UCB units prior to banking. It would also be beneficial to use the same screening test for both maternal samples and UCB units for further result comparison.

Since the Ultrio-Plus® assay has been validated for specificity and sensitivity in PB and BM samples, the investigators hypothesised that it would also be an effective, sensitive assay for successful detection of HIV-1 in UCB units. The objective is thus to verify the routinely used Ultrio-Plus® assay for sensitivity in detection of HIV-1 in UCB units.

6.5 Methodology

The Ultrio-Plus® assay has previously been validated for specificity and sensitivity in peripheral blood samples. The researchers wanted to verify that sensitivity of the assay would not be compromised when UCB plasma was used. UCB units were collected at the Steve Biko Academic Hospital from expectant mothers that had given informed consent to use their UCB for medical research. UCB was collected in UCB collection bags (Pall Medical, Midrand SA), containing citrate phosphate dextrose (CPD) anticoagulant. Units were plasma depleted during centrifugation (800 rpm) for 20 min. and the plasma stored in accordance with the Ultrio-Plus® assay protocol for human serum or plasma according to the package insert guidelines, until further sample processing could commence. Because of difficulties of obtaining HIV-positive UCB units for screening purposes from mothers that were already receiving ART, the researchers decided on spiking 16 UCB units with HIV-1 with a known viral load for validation purposes.

According to the Ultrio-Plus® protocol, whole blood, plasma, or serum may be stored at temperatures ≤ 25°C for up to 72 hours from the time of withdrawal, temperatures that exceed 30°C are acceptable for no more than 24 hours. Specimens may be stored an additional five days at 2° to 8°C following centrifugation. Plasma separated from the cells may be stored for longer periods of time at ≤ -20°C before testing. For validation purposes, collected UCB units

were stored at 2° to 4°C for less than 72 hours, after which they were plasma-depleted and plasma was stored at -20°C until the Ultrio-Plus® assay could be performed on these samples.

In order to obtain a panel of ten UCB units for validation purposes, 16 UCB plasma units were run in the Ultrio-Plus® assay to assess HIV-1 sensitivity (i.e. IUmL). The 16 UCB units were screened by the Ultrio-Plus® assay prior to spiking them with HIV-1, in order to confirm their HIV negative status. UCB samples were subsequently spiked with three dilutions (1:2, 1:4 and 1:8) of an HIV-1 positive quality control stock (diluted 1:80) with a known HIV viral load (VL) (used by the SANBS). The viral loads added to the dilutions were thus 46 IUmL (1:2 dilution); 23 IUmL (1:4 dilution); and 11,5 IUmL (1:8 dilution) respectively. All samples were run by the SANBS through the Ultrio-Plus® assay according to already existing protocols in order to verify the Ultrio-Plus® assay's sensitivity for detection of HIV in UCB plasma. Figure 22 gives a diagrammatic representation of the dilution procedure.

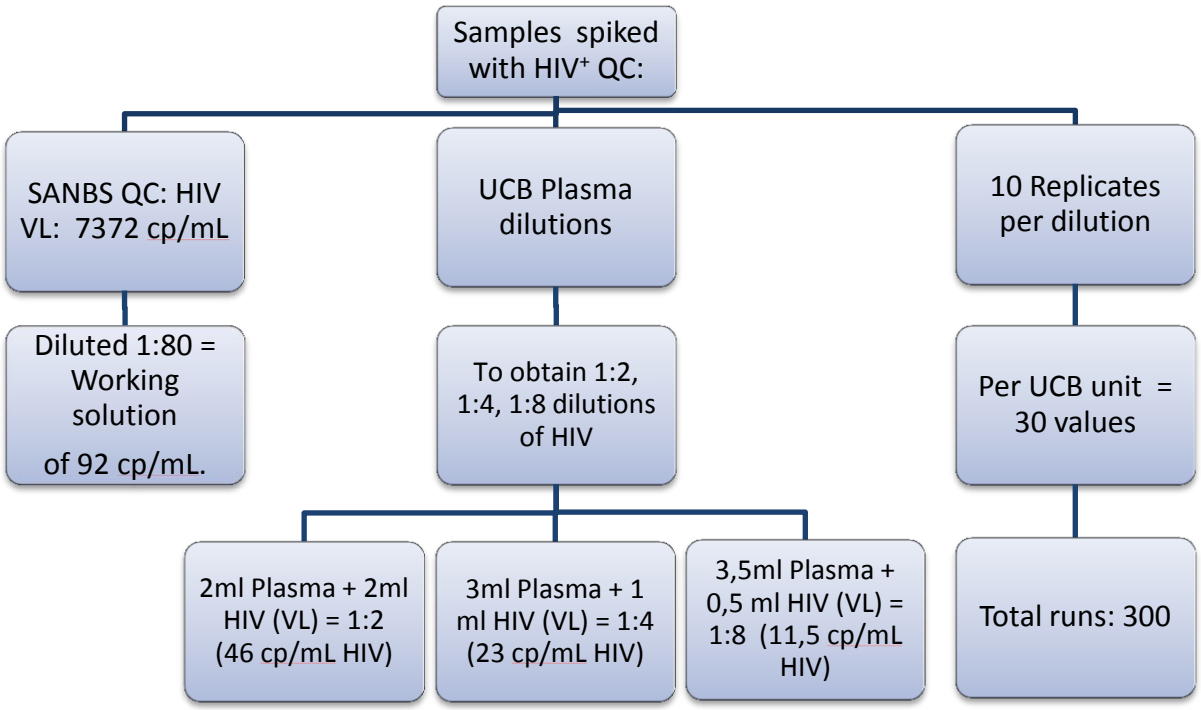

Figure 22: Procedure followed for the 10 UCB units used during validation of the Ultrio-Plus® for sensitivity. A known HIV VL QC stock solution was diluted (1:80) and used as working solution for further 1:2, 1:4 and 1:8 times dilutions. Each sample had three dilutions, which were repeated 10 times each for a total of 30 values per patient and 300 values in total.

The test was compiled so as to prove reproducibility of sensitivity of the assay on UCB units up to the lower detection limit as is currently accepted for screening of PB samples.

Samples were run through the Ultrio-Plus® assay and results documented in Microsoft Excel (Microsoft Corp., Redmond, WA). Commercially available quality control kits, as well as internally manufactured quality control (QC) samples (specific to the South African genotypes), prepared by SANBS were used. All quality control procedures, standards and acceptance criteria, as indicated on the Ultrio-Plus® assay insert, were followed for validation of UCB plasma samples.

6.6 Results and discussion

6.6.1 Introduction

Sensitivity and specificity for the Ultrio-Plus® assay have previously been determined and information can be obtained from the package insert. Specificity for the following genetic variants were previously obtained for both the Ultrio-Plus® assay, as well as its subsequent discriminatory assay: HIV-1 specimens and tissue culture isolates of group M (subtypes A, B, C, D, E, F, and G), N and O. Table 3 is taken from the package insert and indicates the specificity of the Ultrio-Assay test for HIV genetic variants.

Table 3: Procleix® System detection of HIV-1 genetic variants with the Procleix® Ultrio® and HIV-1 Discriminatory Assay

Genetic Variant	Conc. IUmL	Ultrio	dHIV-1
HIV-1 Group M Subtype A	300	7/7	7/7
	100	7/7	7/7
	30	7/7	7/7
HIV-1 Group M Subtype B	300	5/5	7/7
	100	5/5	7/7
	30	4/5	7/7
HIV-1 Group M Subtype C	300	8/8	8/8
	100	7/8	8/8
	30	5/8	8/8
`HIV-1 Group M Subtype D	300	7/7	7/7
	100	7/7	6/7
	30	7/7	7/7
HIV-1 Group M Subtype E	300	6/6	7/7
	100	6/6	7/7
	30	6/6	7/7
HIV-1 Group M Subtype F	300	4/4	6/6
	100	4/4	6/6
	30	4/4	6/6
HIV-1 Group M Subtype G	300	2/2	3/3
	100	2/2	3/3
	30	2/2	3/3
HIV-1 Group N	300	1/1	1/1
	100	1/1	1/1
	30	0/1	1/1
HIV-1 Group O	300	7/7	7/7
	100	7/7	7/7
	30	7/7	7/7
HIV-1 Variants Total	300	47/47	53/53
	100	46/47	52/53
	30	42/47	53/53

Samples were not analysed for specificity again, but only for sensitivity.

6.6.2 Sensitivity

According to the package insert, the Ultrio-Plus® sensitivity for running neat HIV-1 specimens is 99.50% with a 95% confidence interval of (CI 98.21 ; 99.94). Diluted specimens (1:8 and 1:16) are given as 98.50% (95% CI 96.76; 99.45) and 98.25% (95% CI 96.43; 99.29) respectively. Table 4 is taken from the package insert and illustrates the analytical sensitivity of the Ultrio-Plus® assay (without looking at the subsequent discriminatory assay data).

Table 4: Procleix® system - Detection of HIV-1 Type B in analytical sensitivity panels

HIV-1 B IU/mL	Number of reactive/ tested^	% Positive	95% Confidence Limits	
			Lower	Upper
300	80/80	100	95	100
100	80/80	100	95	100
30	77/79^	97	91	100
10	55/79^	70	58	79
3	24/80	30	20	41
0	0/79^	0	0	4

^Invalid reactions were not included

According to these results, the Ultrio-Plus® assay detects HIV-1B with 97% accuracy for 30 or more IU/mL. The validation test's HIV viral load dilutions were undertaken in order to go below this copy number, for detection of HIV-1 at 11.5 IU/mL.

6.6.3 Validation results

The average UCB blood volumes obtained prior to plasma depletion varied between 50 ml and 80 ml. In order to perform adequate repeats for each dilution, at least 8,5 ml of UCB plasma was needed per sample. Of the 16 collected samples, only nine UCB units delivered adequate volumes of UCB plasma required for the sensitivity analyses of the assay on UCB plasma. A tenth sample had adequate volume to perform 25 of the 30 repeats and is included in the results displayed in Table 5.

Table 5: Summary of Ultrio-Plus® screening results for 10 HIV spiked UCB units

Patient no	Number of reactive tests per dilution			
	01:02 46 IU/mL	01:04 23 IU/mL	01:08 11.5 IU/mL	Total
1	10	5^	10	25
2	10	10	10	30
3	10	10	10	30
4	10	10	10	30
5	10	10	10	30
6	10	10	10	30
7	10	9^^	10	29
8	10	10	10	30
9	10	10	10	30
10	10	10	10	30

^ *= Invalid reactions due to inadequate sample volume*
^^ *= Invalid analyses due to sample error code related to instrument mechanics*

Each of the ten samples thus had a total reactive score out of 30. For the total of 300 patient samples run, 294 were reactive. Five samples from patient 1 (for the 1:4 dilution) could not be run due to inadequate sample volume while one sample for patient 7 had a mechanical error. If these six samples are not taken into consideration, the test had 100% detection of HIV-1 up to a lower viral load limit of 11 IUmL. Although the viral loads were below those used for initial validation of the test, the results compare well with detection rates observed in Table 4.

6.7 Conclusion

All UCB units intended for storage in an UCB bank would need to undergo infectious disease screening for compliance with international regulatory requirements.

The Ultrio-Plus® assay is a nucleic acid test (NAT) that has been validated for the simultaneous detection of HIV type-1 (HIV-1), HBV and HCV in human PB, BM and cadaveric tissue (using plasma or serum). The test has not, until now, been verified on UCB units.

The current accepted detection limit for screening for HIV infection is 50 IUmL. The Ultrio-Plus® however is more sensitive, with a 95% limit of detection of 21 IU/mL. Although the possibility exists for an HIV-positive sample to go undetected (having viral loads below the currently detectable lower limit), the clinical relevance is yet undetermined.

It is important to furthermore consider the concept of a minimum HIV infective dose. The Centers for Disease Control and Prevention (CDC) reports on the effect of ARTs on the risk for HIV infection: patients that adhere to ART are less infectious than patients without ART with very low or undetectable viral loads (Anon, 2009). A report by Quinn *et al.* 2000 showed that patients who received ART, but still transmitted the virus to their partners, had higher mean viral loads. One infected partner (who received ART) with a viral load of 90,254 IUmL was able to transmit the virus to his partner. In contrast, no transmission took place between partners where the infected partner had a lower viral load of 38,029 IU/mL. It was furthermore found that no HIV transmission took place if the infected partner's viral load was below 1500 IU/mL (Quinn et al, 2000).

HIV infectability is furthermore subject to many different factors. These factors include infective titre, viral load and injection inoculum volume, area of contact (mucosa, blood, etc.) to name but a few. If the right circumstances prevail, a single virion could cause active HIV infection. The probability of HIV transmission in small blood exposures such as with needlestick injury has been investigated by Reid and Juma (2009). They concluded that HIV's 50% infective dose could range from one virion (i.e. two RNA copies) to 65 000 copies.

Until more comprehensive and sensitive methods are developed to eliminate non-detection of HIV-1 positive samples, screening of maternal and UCB units with the Ultrio-Plus® assay is recommended.

According to currently accepted standards and practices, the Ultrio-Plus® assay is as sensitive in detecting HIV-1 in UCB as it is for detecting HIV-1 in peripheral blood. The assay had 100% detection of samples up to a lower detection limit of 11,5 IU/mL and is recommended for future screening of UCB units.

6.8 References

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CHAPTER 7

7 HIV-1's effect on CD34+ HPCs: CFU ability and infectivity

7.1 Introduction

Access to antiretroviral clinics, compliance with drug regimens, side effects of drugs and drug interactions are major problems for most South Africans living with HIV. In addition, because of poor drug compliance, resistance to anti-retroviral therapy (ART) is becoming a serious problem. No vaccine is currently available for HIV prevention and there is limited likelihood of seeing an effective vaccine in the near future, although several potential candidates are in various stages of development. Alternatives to vaccination and anti-viral treatments are therefore needed.

A study conducted by Hütter *et al*. (2009a) (discussed in more detail in Section 7.2.3), illustrates the rationale behind a larger project in our group for which this chapter lays the foundation. The larger project has as its goal to genetically engineer an HIV-1-resistant immune system. Using the basic principles revealed in the Hütter study, it might in theory be possible to generate an HIV-1-resistant immune systems by collecting primitive haematopoietic stem cells (HPCs) from an (HIV-positive) individual, genetically modifying these HPCs so as to effectively introduce a CCR5 mutation, and re-infusing the patient with these HIV-resistant cells.

The proof of concept for allogeneic transplantation was provided by the Hütter *et al.* (2009b) study. However, it still remains unclear whether this approach would be successful in autologous transplantation of HIV-positive individuals; i.e. to isolate an HIV-positive individual's own CD34+ HPCs and subject them to genetic manipulation prior to re-introducing the cells into the patient. This is because it is unclear, if and how HIV-1 affects primitive HPCs. There are several theories as to how HIV could influence these cells' normal growth and differentiation by means of viral cytokines and proteins but uncertainty remains as to whether these cells could be directly infected with HIV (Alexaki and Wigdahl, 2008). It is thus important to experimentally investigate HIV's influence on primitive HPCs before considering using these cells as genetic vectors for HIV-1 gene therapy.

7.2 Literature background

7.2.1 Introduction to HPCs

HPCs were described in some detail in Chapter 4; however, little has been said about their

potency. Stem cells are characterised by the ability to self-renew (i.e. to proliferate or divide recurrently) and differentiate (give rise to more mature progeny). Stem cells differentiate through decreasing levels of stem-ness or potency.

A fertilized egg (not strictly speaking a stem cell) is referred to as "totipotent" since it is able to form all the cells of the body, including the placenta. Totipotent stem cells are found in the morula. Pluripotent cells are found in the inner cell mass of the blastocyst and are defined as cells that can differentiate into any cell found in one of the three germ layers (mesoderm, endoderm, and ectoderm) (NIH, 2001) but are not involved in the formation of the placenta. A rare population of haematopoietic stem cells (HSCs) is pluripotent and is generally kept in a low proliferative, quiescent state (Eliasson and Jonsson., 2010). Embryonic stem cells (ESCs), which are derived from the inner cell mass of the blastocyst, are also pluripotent in nature.

Multipotent stem cells are often referred to as progenitor cells, because they are more differentiated than true pluripotent stem cells. Multipotent progenitors can give rise to multiple cells, though these cells are limited to a specific lineage. An example is found in HPCs, which could give rise to all types of blood cells (haematopoietic lineage), but would not differentiate into, for example, the neural cell lineage. An HPC with multipotent capacity would therefore give rise to granulocyte-macrophage colonies (colony-forming-unit granulocyte-macrophage or CFU-GM), for example, which consist of cells from the granulocytic and macrophage haematopoietic groups (NIH, 2001).

Oligopotent progenitor cells are capable of producing only a few cell types (fewer than multipotent cells). Therefore, a macrophage progenitor cell would be able to give rise to monocytes and macrophages and not to granulocytes (which consist of neutrophils, eosinophils and basophils).

Finally, a cell that has the ability to differentiate into a single cell type (erythroid progenitor cells, for example) is referred to as "unipotent". Unipotent cells are thus tissue specific; epithelial stem cells for example have to constantly reproduce new skin to replace the dead or damaged skin tissue.

7.2.2 Culturing CD34+ HPCs in CFU-assays (potency testing)

Many experimental models have been introduced through which HPCs can be investigated. Ultimate proof that a genetically altered HSC could engraft and repopulate the haematopoietic

system lies in the long-term ability of these cells to reconstitute haematopoiesis in an animal model such as nonobese diabetic (NOD)/severe combined immunodeficient (SCID) mice (Zhang et al., 2008; Osawa et al., 1996; Schroeder, 2010). However, these models are often expensive and time consuming, creating a need for more readily accessible models. The colony-forming-unit assay (CFU-assay) is regarded as the gold standard for *in vitro* laboratory research on HPC growth and differentiation. It allows easy manipulation of culture conditions so as to mimic conditions in the body, in order to study different disease-related growth patterns of affected HPCs.

CFU-assays have been used in a number of applications. These include: 1) the evaluation of the engraftment ability (potency) of an UCB unit prior to stem cell transplants; 2) toxicity testing or drug-screening assays; 3) optimisation of gene transfer protocols following gene manipulation; 4) evaluation of haematological disorders (where CFU-assays support diagnosis and treatment); and 5) study of the effects of different cytokines, growth factors, hormones or mimetics on haematopoietic progenitors.

The CFU-assay is a clonal/clonogenic assay that gives quantitative and qualitative information about the isolated HPCs from, for example, an UCB unit reserved for transplantation purposes. As noted earlier, there is a minimum cell dose required for successful UCB unit transplantation, which can be quantitated through automated cell counting. However, since engraftment relies on the proper functioning of HPCs, it is important to know that the cells in the unit function normally.

Section D.10 of the NetCord-FACT international cord blood standards (2010) requires that CFU-assays be done to evaluate the functional capacity of the UCB unit prior to its release. It has been found in patients that have undergone UCB transplantation that the total colony-forming-cell (CFC) numbers correlate more strongly with recovery and survival of patients.

An UCB unit could contain many HPCs that are further along in their differentiation, thus lacking sufficient progenitors capable of self-renewal and haematopoietic reconstitution. In addition, ex vivo manipulations, cryopreservation and thawing could severely decrease the viability of progenitor cells and their subsequent engraftment and differentiation. The CFU-assays are thus useful in providing proof of the functionality of isolated $CD34^+$ cells and in giving an estimate of the potency of the UCB unit.

It is important that a unit reserved for transplantation purposes contains the right numbers of

all the cell types necessary for normal haematopoiesis, in order to avoid the occurrence of post-transplantation cytopenia. Total nucleated cell counts (TNCs) give an estimate of the total cell content in the UCB unit; they do not however provide an indication of primitive progenitors nor do they take nucleated red blood cells into account. CD34+ counts together with functional CFU- assays give a better estimate of potentially primitive, functional HPCs present in an UCB unit that are necessary for engraftment.

CFU-assays could furthermore reveal disease-specific diagnostic growth patterns (that would not be detected by TNCs) (Nissen-Druey, 2005). The CFU-assay is thus a good model to mimic disease specific influences on HPCs. The clonal capacity, growth and differentiation of HPCs following HIV-1 exposure, could therefore be evaluated through CFU-assays.

Early HPCs do not display a distinct morphology from which their line-commitment can be established. However, 14-day cultures of CFU-HPCs are ideal for shedding light on the intermediate phase of repopulating lympho-haematopoietic stem cells in that the cells in this widow-period show morphologically visible features of their differentiation (Nissen-Druey et al., 2005). When cultured under the right circumstances (i.e. media containing a cocktail of cytokines and growth factors), pluripotent HPCs could give rise to mixed myelo-erythroid as well as lymphoid progeny (unipotent) (Kavanagh and Kalia, 2011). In essence, CFU-assays thus provide retrospective information (after 14 days in culture) of the potency and functional diversity of HPCs in the UCB unit and whether such a unit would be adequate for transplantation or not.

In order to culture HPCs *in vitro* it is important to obtain a 'pure' cell population (Schroeder, 2010). Having a marker with which to isolate these cells is important for potential clinical application and many studies have subsequently set out to identify cellular markers specific to HPCs. No single marker exists for the identification of these cells, but CD34 is accepted as one of the important cellular markers expressed by HPCs – despite the fact that $CD34^{+}$-isolated HPCs are largely heterogeneous, containing HPCs in various stages of differentiation (Schroeder, 2010).

Since UCB HPCs are mostly heterogeneous, some of the colonies produced could potentially come from pluripotent stem cells (such as HSCs), but most come from multipotent progenitors cells (the HPCs). Early colonies (i.e. cultured in fewer than 10 days) generally arise from

oligopotent progenitors, whereas colonies grown for 12 to 14 days mostly come from multipotent progenitors (Weissman et al., 2012). Early progenitor cells have greater self-renewal and migratory capabilities thus giving rise to large, mixed colonies. More differentiated progenitors with little self-renewal capacity either form small single-cluster colonies or none at all. Thus, a larger colony (cell number) with more burst-forming units (i.e. spatial orientation of the colony) and (potential) mixed cell types is thought to have arisen from an earlier (oligopotent) progenitor (Nissen-Druey et al., 2005).

Colonies are counted as being primitive BFU-E (or burst-forming unit erythroid colonies), CFU-E (erythroid precursors), CFU-G (granulocyte precursors), CFU-M (macrophage precursors), CFU-GM (granulocyte / macrophage precursors), CFU-Mk (megakaryocyte precursors) or mixed CFU-GEMM (granulocyte / erythroid / macrophage / megakaryocytic) colonies (Eaves and Lambie, 1995).

7.2.3 Combating HIV-1 with HIV-1 resistant HPCs:

In order to manipulate HPCs or even more primitive HSCs for use as vectors in gene therapy, it is important to understand the properties that qualify these cells as the appropriate cellular vectors and whether or not these properties have been altered either through physical manipulation of the cells, or by the presence of HIV-1.

The clonal properties of HSCs, their ease of isolation and manipulation and their “stemness” (potential to self-replicate and produce identical daughter cells that differentiate along different haematopoietic lineages) make them particularly attractive cell models intended for combined cell- and gene-therapy approaches.

Hütter and colleagues conducted a ground-breaking study that indicated proof of concept of the possibility of long-term control of HIV-1 infection (Hütter et al., 2009a; Allers et al., 2011). CD4 and CCR5 are two cell surface receptors required by the HIV virus to gain cellular entry. However, a naturally occurring mutation exists in certain individuals – the CCR5-delta32 mutation – that in the homozygous state provides a high degree of natural resistance to HIV-1 infection (Liu et al., 1996; Hütter et al., 2009b). The Hütter team performed an allogeneic transplantation – using PB from a donor homozygous for the CCR5-delta32 mutation – on an HIV-positive individual with acute myeloid leukaemia (Hütter et al., 2009a). Reporting on their work 27 months post-transplantation, the previously HIV-positive individual displayed no signs of viral replication, despite the absence of ART.

This work introduced many possibilities with regard to gene therapy approaches to combat HIV-1 infection – specifically for those individuals who cannot obtain unrelated matched CCR5-delta32 negative donors. Gene-therapy approaches directed towards inhibiting viral entry (thus mimicking the results obtained by the naturally occurring mutation) might prove to be effective at some point in the future. This method might prove to be particularly useful for infants born to HIV-1-positive mothers. These infants could then benefit by receiving their own genetically modified HIV-1 resistant UCB HPCs.

For HIV-1-positive adults their own peripheral blood could be used, if it is clear that HIV-1 has not deleteriously affected primitive HPCs prior to genetic manipulation of these cells. The option of allogeneic transplantation for adults presents with a few difficulties: 1) finding an adequate match will be difficult in the South African context because of a large genetic diversity; 2) allogeneic transplantation requires administration of immunosuppressive therapy to prevent graft versus host disease (GvHD) in the patient. Suppressing the immune system of an HIV-1-positive individual is counter-productive. Even so, the Hütter *et al.* (2009a) study administered prophylaxis to their patient who subsequently developed stage 1 GvHD post-transplantation, which was reduced by adjusting the prophylaxis treatment (Hütter et al., 2009a). In the South African context, this approach would not be feasible (additional costs, finding near perfect allogeneic-matched individuals, and health risks to name but a few reasons).

Questions arise with regard to the safety and efficacy of genetically modified cells intended for cellular therapy. CCR5 is a chemokine receptor that has been implicated in various aspects of inflammatory immune responses to infection. One particularly important concern is what the consequences would be should a previously CCR5 positive individual subsequently undergo transplantation with a blood unit homozygous for the CCR5-delta32 mutation (Hütter et al., 2009a). This could potentially lead to deleterious consequences that are still unknown and all gene-therapy- and cellular-therapy approaches should be done in a cautiously optimistic manner.

7.2.4 HIV-1's effect on HPCs

The combination of genetic engineering with cellular therapy is an attractive alternative in the search for effective cures against HIV infection.

Following the proof of concept from the Hütter (2009a) study, it seems – at least in theory – to

be possible to genetically engineer an HIV-proof immune system. Stable genetic alterations could theoretically be introduced into haematopoietic reconstituting cells (HPCs or HSCs), mimicking the naturally occurring delta32 mutation, which could make these cells, at least in part, resistant to HIV. These genetically engineered cells could then be used in either autologous or allogeneic stem cell transplantations after partial myeloablation, the concept being that newly introduced HPCs would reconstitute the haematopoietic system with genetically engineered HIV-resistant HPCs, thus doing away with the need for lifelong ARV therapy. Numerous studies following these principles have been conducted in order to achieve long-term resistance against HIV (Li et al., 2005; Liang et al., 2010; Barichievy et al., 2009; Anderson & Akkina, 2007).

Isolating and genetically manipulating HPCs for autologous transplant in HIV-1-infected individuals would theoretically render these cells at least partly resistant to HIV-1 entry, replication and integration, if they are not already infected by the virus. Some recent evidence suggests that primitive progenitor cells can be infected by HIV-1 and serve as viral reservoirs (Shen and Siliciano., 2008; Alexaki et al., 2009). Furthermore, pro-viral DNA has been found in latently infected CD34+ HPCs and both active and latent infection of CD34+ HPCs (*in vivo* and *in vitro*) has been shown (Chun et al., 2005; McNamara and Collins., 2011; Mullis et al., 2012). In cases where CD34+ HPCs are infected with HIV-1, genetic manipulation of these cells would not necessarily eradicate the viral reservoirs present, but could inhibit downstream active replication of the virus. If full haematopoietic reconstitution could be established with HIV-1-resistant HPCs, then the presence of viral DNA might be negligible, since no immune-activating viral proteins would be capable of forming. However, it is unclear whether residual viraemia in primitive genetically manipulated HPCs would affect the engraftment and reconstitution ability of the HPCs. It is therefore necessary to establish whether primitive HPCs can be infected and/or are affected by HIV-1.

If HIV's indirect influence on HPCs does severely or permanently impact growth and differentiation of primitive HPCs, use of autologous engineered cells in HIV-positive individuals might not be successful.

It is unclear whether HSCs and/or HPCs could potentially be affected or infected by HIV during either their active state, in circulation, or dormant/quiescent state in the BM. Alexaki and Wigdahl (2008) indicate in their comprehensive review that HPCs might not only be directly

susceptible to HIV's influences through viral entry, but also indirectly. HPCs, for example, do display co-receptors used by HIV-1 to gain viral entry into the cells, such as CXCR4 – allowing direct access to the HPCs. For this reason, it is speculated that primitive quiescent HPCs could serve as potential latent HIV-1 reservoirs, accounting for continuous viral presence and resurgence after anti-retroviral treatments (Alexaki & Wigdahl, 2008; Carter et al., 2010).

Indirect influences of HIV-1 on HPC growth and differentiation involve effects of viral proteins present in the cellular micro-environment. These influence different components of the HSC/HPC niche environment and in turn affect HPC growth, differentiation and engraftment. The immunologic response elicited by the virus furthermore causes cytotoxicity, often resulting in apoptosis (McNamara and Collins, 2011; Mullis et al., 2012). This could potentially account for the various types of cytopenias and dysplasias found in HIV-1-positive individuals. According to Alexaki and Wigdahl, (2008), HIV-1 may influence the growth, differentiation and engraftment of the most primitive HPCs, through viral proteins and cytokines released upon HIV-1 viral entry into the host.

The full extent of HIV-1's effect on haematopoiesis is still unclear and many studies seem to have contradictory results (Alexaki & Wigdahl, 2008; Carter et al., 2010; Alexaki et al., 2009). Further studies need to be performed to elucidate this matter.

7.3 Hypothesis and Objective

The long-term objective of the larger project (as mentioned above) is to generate HIV-1-resistant haematopoietic stem cells (HPCs) for subsequent transplantation into patients with HIV/AIDS, in order to replace their endogenous HIV-1-infected HPCs. Working toward this long term objective, the proposed study's immediate objective is to develop a technique for CD34+ HPC isolation from UCB; to establish a colony-forming-unit assay and to identify individual colonies grown from UCB. This work will lead up to an understanding of the effects of HIV-1 on normal haematopoiesis and will allow us to determine the suitability of using autologous HPCs (from UCB or PB) as targets for lentiviral transduction. Based on conflicting data in the literature, we hypothesise that $CD34^+$ HPCs can be infected with HIV-1 (i.e. serve as viral reservoirs and/or undergo apoptosis) and/or be indirectly affected by HIV-1, i.e. altered growth due to viral cytokines and proteins

7.4 Methodology

7.4.1 Introduction

Donated peripheral blood is routinely processed at the South African National Blood Services (SANBS). Platelet-rich and red blood cell fractions are respectively collected from whole blood and the remaining buffy coat, which contains 70% of the donated blood's mononuclear cells (MNCs), which are routinely discarded. This source of MNCs – which is rich in $CD34^+$ haematopoietic stem cells – was collected from the SANBS and used to standardise routine ficoll-density gradient (Histopaque) separation techniques and CD34+ HPC magnetic isolation.

The techniques for isolating and culturing CD34+ HPCs in colony-forming units were acquired through a collaboration with colleagues at the Victor Segalen University in Bordeaux, France. In order to standardise the protocol in the laboratory in Pretoria, peripheral blood was used in the initial stages since it is more readily available than UCB. Once the protocol and the technique for CD34+ magnetic isolation had been standardized, CD34+ cells from UCB were isolated and used for the CFU assays. Figure 40 is a flow chart with an outline of the methods used.

CD34+ HPCs were isolated through density gradient-centrifugation and magnetic bead separation. Flow cytometric analysis of isolated $CD34^+$ HPCs was performed to verify CD34+ cell count and purity after isolation. CFU-assays were subsequently performed on the UCB units to assess the normal potency (haematopoietic capacity) of each UCB unit. A parallel CFU-assay study was conducted to investigate the effect of HIV-1 on the development and growth of primitive HPCs. The effect of HIV-1 on primitive HPCs was evaluated by comparing HIV-1-spiked HPCs in CFU-assays to CFU-assays of the same UCB unit in the absence of HIV-1.

In total, eighty UCB units were collected from mothers attending the ante-natal clinic at the Steve Biko Academic Hospital in Pretoria. Annexure 3 shows the collected UCB units and their application over the course of the study.

Umbilical cord blood was collected post-delivery, with the assistance of the Department of Obstetrics and Gynaecology at the Steve Biko Academic Hospital. After receiving each UCB unit, samples were codified in order to protect patient anonymity and were stored at 4˚C until collected.

Owing to administrative difficulties beyond of the investigator's control, all UCB units were not collected within 72 hours post-delivery. The administrative issues included doctors forgetting

to place the collected UCB in the designated refrigerator for timely collection; misplacement of UCB bags at the hospital and not being notified in time by the ante-natal staff that UCB bags were ready for collection.

Of the initial 80 UCB units, 26 UCB units were analysed by flow cytometry and the Ultrio-Plus assay (Table 8). Four of these units were excluded from the final analysis because they were not processed within 72 hours after collection. Furthermore an additional 7 units had low volumes and subsequently low CD34+ yields. The CFU-assay results for these units are also excluded and they were not included in statistical analyses. In order to have comparable samples, UCB units were collected within 72 hours post-delivery as the protocol specified, and immediately processed.

In order to conduct a preliminary investigation into HIV-1's effect on CFU-growth, colonies from the remaining 19 UCB units were grown in parallel in three conditions for each UCB unit: HIV-1-spiked; non-spiked and FBS-spiked. These 19 UCB units were cultured under comparable conditions and analysed by the same flow cytometry and Ultrio-Plus protocols and were subsequently used in statistical analyses.

7.4.2 UCB collection

Informed consent questionnaires were filled out by mothers attending the clinic. In these questionnaires, patients gave the researchers permission to collect UCB for medical research, to have access to personal patient information (that pertains to the study), and to screen the collected UCB for HIV-1. These screening tests are compulsory for future UCB banking purposes, where it will be necessary for all UCB units to undergo routine screening for infectious diseases (for compliance with international regulatory standards).

The blood was collected (with the assistance of the Department of Obstetrics and Gynaecology at the Steve Biko Academic Hospital) from the placenta after the 3rd stage of delivery via the umbilical vein, using a 16-gauge needle, into Pall medical collection bags containing Citrate Phosphate Dextrose (CPD) anticoagulant (Pall Medical, Midrand SA).

The HIV status of patients from whom UCB was collected was obtained from patient files (as determined by the Steve Biko Academic Hospital upon patient admission) for later comparison with Ultrio-Plus® screening results for the respective UCB plasma. All patients attending the ante-natal clinic were required to undergo HIV-screening. The HIV status of 7 patients was

unknown, due in some cases to the fact that the patients declined to undergo the HIV testing (Table 10). Permission to obtain patient statuses for Ultrio-Plus® screening of UCB units was received from the Main Research Ethics Committee, University of Pretoria (Protocol 89/2010; approved: 11/10/2010 and Protocol 131/2010; approved: 01/10/2010) (Annexure5).

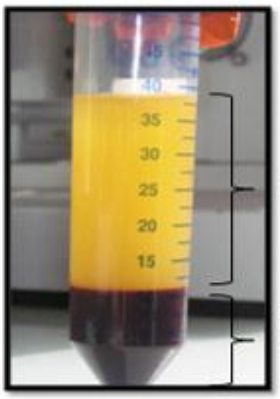

Figure 23: Plasma reduction done on each UCB unit prior to MNC isolation. The top plasma/platelet fraction was stored for subsequent HIV-1 screening with the Ultrio-Plus® assay.

Collected UCB units were plasma depleted by centrifugation at 110 x g (800 rpm) for 20 min. (Figure 23) and the plasma stored in accordance with the Ultrio-Plus® assay protocol requirements for human serum or plasma according to the package insert guidelines, until samples could be screened for HIV-1 with the Ultrio-Plus® assay .

7.4.3 MNC isolation

After plasma depletion the mono nuclear cell layer (MNC layer) of UCB units was isolated by density gradient centrifugation using Ficoll (Histopaque) 1077 (d = 1.077 g/mL, Sigma) density centrifugation (Sigma-Aldrich Co. LLC, St. Louis, MO). According to the information leaflet, Histopaque consists of polysucrose and sodium diatrizoate. The density of the solution – 1.077 g/mL – facilitates the isolation of lymphocytes at the Histopaque plasma interface. The red blood cells aggregate and the granulocytes become hypertonic and sediment to the bottom of the collection tube.

Blood was carefully layered onto the Histopaque in a sterile 50-ml Falcon tube without mixing the two phases. The blood was layered onto the Histopaque in a 2:1 volume ratio (30 ml of blood on 15 ml Histopaque) and centrifuged for 30 min at 400 x g (1500 RPM) to reduce the platelet contamination and recover the MNC layer (Figure 24).

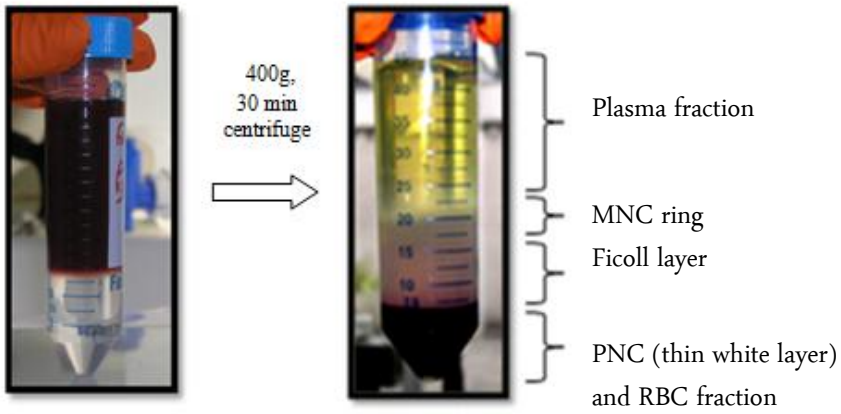

Figure 24: Ficoll (Histopaque) density gradient separation of the MNC layer. The tube on the left illustrates the blood layered on top of the ficoll before centrifugation. The falcon tube on the right illustrates the five layers obtained after centrifugation. The second layer – the MNC ring – contains the HPCs of interest. (PNC = poly nucleated cells)

The platelet fraction was aspirated and the MNC layer collected into a clean 50 ml Falcon tube. For the first wash step, the falcon tube was filled up to 50 ml with a specially prepared selection buffer (SB) and centrifuged at 260 x g (1200 RPM) for 10 min. The SB consists of 50 ml of 4% Human Albumin (Sigma Aldrich, SA) and 1.6 ml ethylenediaminetetraacetic acid (EDTA) (0.5M) made up to 400 ml with 349 ml PBS (pH 7.4).

The supernatant was removed, the pellet resuspended in SB and the wash step was repeated a second time. After the supernatant was removed, the pellet was resuspended in SB again and transferred to a 15 ml falcon tube for the final wash. The tube was filled up to 15 ml with SB and centrifuged at 260 x g (1200 RPM) for 10 min. The final pellet was resuspended in 2 ml SB for subsequent magnetic labelling and purification.

7.4.3.1 Magnetic purification

Subsequent magnetic purification of $CD34^+$ cells was done by positive selection of $CD34^+$ cells with the MACS® Technology CD34 MicroBead kit (containing CD34 MicroBeads and FcR Blocking Reagent), MS and LS columns and a MiniMACS™ Separator (Miltenyi Biotec, Germany) (http://www.miltenyibiotec.com).

Magnetic labelling

Cells were magnetically labelled by adding 150 µl of the FCR blocking reagent and CD34+ Hapten Antibody to the MNC suspension. The sample was placed in the fridge at 4°C for 15 min. and slightly vortexed every 5 min. The tube was filled up to 10 ml with SB and centrifuged at 260 x g (1200 RPM) for 10 min. The supernatant was aspirated and the pellet resuspended in 2 ml SB. This process was repeated for the addition of the Anti-Hapten MicroBeads and the final pellet resuspended in 2ml of SB.

Large column isolation

The magnetically labelled MNC layer was subsequently separated with large and small (LS and MS) columns (Miltenyi Biotec, Germany). The LS columns were inserted into the magnet, prepared by adding 3 ml SB to the column and waiting for it to pass through into a Falcon tube that will contain all the waste flow-through. Columns were not allowed to run dry. Just before the last volume of SB had passed through the column, the MNC sample was added to the column and allowed to run through the column by gravity. The column was washed three times with 3 ml of SB. Finally, the CD34+ HPCs were eluted from the column by adding 5 ml SB to the column, removing it quickly from the magnet and placing it into a clean 15 ml Falcon tube. The plunger was firmly applied to the column in order to flush out the fraction of magnetically labelled cells from the column. The sample was centrifuged at 260 x g (1200 RPM) for 10 min., the supernatant aspirated and the pellet resuspended in 500 µl of SB.

Small column isolation

The small columns were placed into the Miltenyi magnet, 500 µl SB was used for initial equilibration followed by 500 µl of sample. Three wash steps with 500 µl SB were performed and the sample eluted into a final volume of 2 ml SB. Samples were centrifuged again at 260 x g (1200 RPM) for 10 min. Pellets were resuspended in 1 ml of freshly prepared Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS).

7.4.3.2 Cell counting

A haemocytometer was used for counting CD34+ HPCs prior to culturing. 20 µl cell suspension was added to 20 µl of Trypan blue for determination of CD34+ cell viability. 10 µl of this solution was added to the haemocytometer and cells were counted with the 20X objective on a

Zeiss inverted microscope. Cells in the middle square were counted and the final count was multiplied by the dilution and again by 10^4 to obtain the final cell count (e.g. 16 cells counted: 16 x 2 (trypan blue dilution) x 10^4 = 300 cells / microliter (c/µl)). Figure 25 A and B illustrates when cells were included or excluded during counting:

The cell counts were used to dilute the cells to a final volume of 50 000 cells/ml. To plate the cells at a density of 250 cells per well, 5 µl of this 50 000 c/ml solution was used.

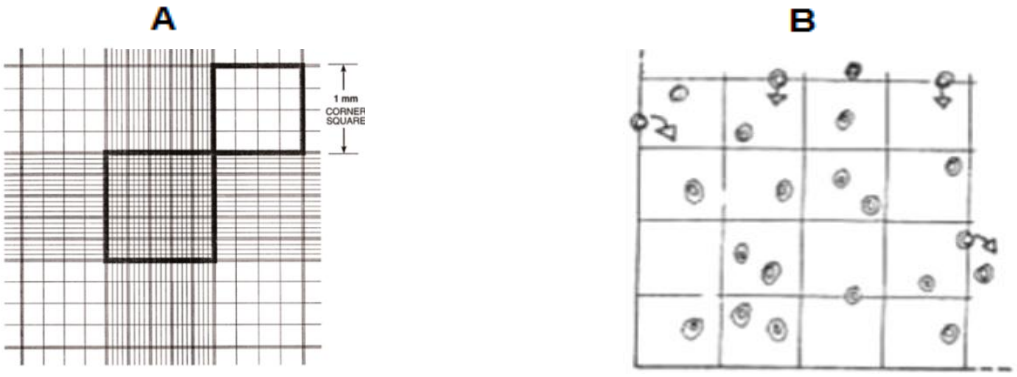

Figure 25 A: Layout of the haemocytometer. A coverslip provides a depth of 0.1mm and cells were counted in the centre square volume. Cell count per ml calculated with: NUMBER OF CELLS IN THE MIDDLE SQUARE X DILUTION FACTOR X 10^4.

Figure 25 B: Indicates inclusion or exclusion of cells during counting of the center square. Cells that lay on the left and/or top lines of the counted block were included in the cell count. Cells on the bottom or right lines of the counted block were excluded. (Stem cell Technologies, 2004)

7.4.4 Flow cytometry: Fluorescent labelling

Flow cytometry was performed on all UCB units (although flow cytometry results displayed in Annexure 4 are representative of only those units used in both flow cytometry and Ultrio-Plus® assays; i.e. 30 UCB units). It was used to evaluate CD34+ HSC cell count and sample purity and viability prior to performing the assay. Isolated $CD34^+$ HPCs from each UCB unit were assessed using two methods: 1) the Stem Cell Enumeration Kit, Stem-Kit™ from Beckman Coulter (Miami, USA); and 2) the CD34+ Pool Kit (3 pooled CD34+ PE-conjugated monoclonal antibodies (mAB) and Isotypic control IgG (1+2a)-PE) used together with a separate FITC-conjugated CD45+ (mAB) (Beckman Coulter, Miami, USA). Stem-Kit™ reagents included a two-colour fluorescent (FITC and PE) murine monoclonal antibody reagent, a two-colour murine fluorescent (FITC and PE) isoclonic control and cell viability reagent (7-AAD Viability dye). It simultaneously detected and enumerated CD45+ and dual-positive CD34+ CD45+ HPCs. The monoclonal antibodies (conjugated to their respective fluorochromes) bound to respective cell surface antigens. The

isoclonic control was used to evaluate non-specific binding. Non-viable cells were detected with the 7-AAD Viability dye. This nucleic acid dye binds to DNA base pairs of cells with disrupted cell membranes but cannot enter cells with intact cellular membranes.

The number of cells/µL was calculated with the following formula:

$$\frac{Number}{CAL} \times Cal\ Factor$$

where: Number = the total number of cells detected during flow analyses; CAL = instrument calibration; and Cal Factor = the calibration beads factor (obtained from the calibration beads package insert).

Six normal CFU-assays were also analysed by flow cytometry to confirm the presence of the different colony-forming cells that were counted during CFU-assay enumeration. Flow cytometry results were analysed with the Kaluza® Flow cytometry software program (Beckman Coulter, Miami, USA) and statistical data exported into Microsoft Excel (Microsoft Corp., Redmond, WA).

Flow cytometry data for UCB units processed within 72 hours after delivery were used in analyses.

7.4.5 CFU assay

The CFU-assay was done for 19 UCB units spiked with HIV-1 and FBS. A great deal of inter-patient sample variability existed and in order to establish whether variability seen between HIV-1-spiked CFU's and normal CFU's was truly due to the effect of HIV on CFU-assays, patient samples were split into three groups for intra-individual comparison: 1) Normal (i.e. HIV-1 negative, no additives); 2) HIV-1-spiked (HIV-1 QC added); and 3) FBS (FBS added to HIV-1 negative, normal cells). Comparisons were thus done intra-individually between the three groups, but also inter-individually between the 19 UCB units.

Because of a low probability of obtaining HIV-1-positive UCB units, it was decided to spike the UCB units with HIV-1-positive serum. Two HIV-1 viral loads were used respectively to spike the CD34+ HPCs in two sets of triplicate wells: one with a low copy number: HIV-1 QC (92 IU/ml) obtained from the SANBS (to spike the first set of triplicate wells); and one with a high copy number: 2564697 IU/ml obtained from the Department of Virology, University of Pretoria (to spike the second set of triplicate wells). All 19 UCB units were spiked with plasmas containing either the high or low viral loads (Table 16). Samples were spiked in order to investigate HIV-

1's effect on the ability of early progenitor HPCs to self-renew, migrate and differentiate into different cell types that subsequently formed colonies.

UCB units intended for use in the CFU-assays were first screened by the Ultrio-Plus® assay to detect possible HIV-1 prior to culturing. Units were therefore plasma-depleted and the plasma was sent to the SANBS for Ultrio-Plus® screening. Screening was carried out to determine whether normal cultured CFUs were infected with HIV-1 before these samples were spiked with HIV-1.

HIV-1 negative $CD34^+$ HPCs were cultured in Stem-α1D semi-solid methylcellulose medium (Stem Alpha, St. Genis L'Argentière, France, http://www.stemalpha.fr) to evaluate HPC haematopoietic capacity through CFU-assays. Cells were seeded in triplicate at a density of 250 cells per well in 24 well plates (Greiner Bio-One, Frickenhausen, Germany) containing 250 µl medium per well. Cells were incubated for 14 days at 20% O2; 5% CO2 at 37°C and ddH2O was added to adjacent medium-free wells to prevent the medium from drying out.

The assay included detection and analysis of erythroid (including burst-forming unit erythrocytes – BFU-E), granulocyte-macrophage (CFU-GM), megakaryocyte (CFU-MK) and granulocyte, erythroid, macrophage, megakaryocytic (CFU-GEMM) colonies. Cells were counted using a high-quality inverted microscope (Carl Zeiss Microscopy, Germany) equipped with low (4X) and higher power (10X; 20X and 40X) objectives and WF10X/20 ocular eyepieces.

In addition to normal CFU-assays per patient, three sets of additional wells were also cultured in triplicate: Two sets of triplicate wells were spiked with 5 µl of an HIV-1-positive serum (one with the high viral load and the other with the lower viral load). The other set of triplicate wells was spiked with 5 µl of FBS. Since HIV-positive serum was used to spike the cells, it was anticipated that the serum could have an effect on CFU growth. For this reason, FBS was used as a serum control.

CD34+ cells for all three conditions (normal, HIV-positive – high and low viral copy numbers – and FBS) were cultured in parallel and enumerated on the same day, 14 days after plating. Images of colonies were captured digitally and processed with standard image-processing software.

In addition to investigating the difference in CFU-growth and numbers, colonies were assessed for the presence of HIV-1 inside the cells with the Ultrio-Plus® assay: cell colonies for the

respective triplicate conditions of each patient were pooled and transferred to a 15 ml falcon tube (i.e. triplicate HIV-1-spiked CFUs for high viral loads were pooled together; low viral loads together; normal triplicate CFUs and FBS-spiked triplicate CFUs). Falcon tubes were filled up to 15 ml with SB and centrifuged at 260 x g (1200 RPM) for 10 min. To remove residual medium and possible HIV-1 serum, the wash step was repeated three times and the final pellet resuspended in 1 ml phosphate buffer saline (PBS). Samples were subsequently frozen at -20˚C until they could be analysed by the Ultrio-Plus® assay at the SANBS. (As positive control, the Ultrio-Plus® assay uses inactivated HIV-1-positive plasma in defibrinated normal human plasma (nonreactive for HIV-2, HCV and HBV when tested by FDA-licensed assays). Prior to running the samples on the Ultrio-Plus® assay at the SANBS, samples were "quick thawed" in a water-bath at 37˚C and sonicated for 30 sec. in an ultrasonic bath.

In order to compare all the units across all the different analyses, results were compared only for the 19 units that met the following criteria: Units were processed within 72 hours after delivery; were spiked with FBS; were spiked with high and low HIV-1 viral loads; and underwent Ultrio-Plus® screening and flow cytometry analyses (with both the Stem-Kit™ and the CD34+ Pool Kit). The raw data for each analysis performed on all 30 of the units, is however included in Annexure 4 for comprehensiveness.

7.4.6 Freezing of CD34+ HPCs

Some of the samples with a higher cell yield were cryopreserved in order to investigate the effect of the freeze-thaw process on the viability of cells. For each sample that was to be frozen 500 µl of FBS containing 20% of DMSO (DMSO HYBRI-MAX 100 ml, Sigma) and 500 µl of pure FBS was prepared. The FBS-DMSO was placed on ice while the cell sample was centrifuged at 300 x g (1500 RPM) for 5 min. The supernatant was eliminated and the cell pellet resuspended in the 500 µl of pure FBS. The 500 µl of FBS containing 20% of DMSO was drop-wise added to the cells while kept on ice. Cells were gently mixed once and transferred to an appropriately labelled cryogenic tube (AEC, Amersham, Pty, Ltd, Johannesburg). The cryogenic tubes were put into a Mr. Frosty freezing container (AEC, Amersham, Pty, Ltd, Johannesburg) and placed at -80˚C for at least 24 hours, where after the tube was transferred into a liquid nitrogen container at -196˚.

7.4.7 Thawing of CD34+ HPCs

In order to investigate the post-thaw viability of cryogenically frozen CD34+ HPCs, cells were

thawed in the following manner:

Before the onset of thawing 9 ml FBS was prepared in a clean 15 ml Falcon tube in addition to the prepared final medium (FM) for thawed cells, in a separate 15 ml Falcon tube. The FM consisted of 20% FBS and was made up to 10 ml with DMEM. The FBS and FM were placed in a water-bath at 37˚C. CD34+ HPCs were removed from the liquid nitrogen container and thawed in a water bath at 37˚C until only a small ice cube was visible. The sample was quickly removed and transferred to the 9 ml FBS. The cells were centrifuged for 5 min at 300 x g (1400 RPM) after which the supernatant was aspirated and the pellet gently resuspended in 1 ml of FM. Cells were subsequently counted and re-plated for CFU-assays as described previously.

7.4.8 HIV-1 infection of freshly isolated cells

Samples with cell concentrations too low to freeze were diluted in DMEM to 50 000 cells/ml. These fresh cells (50 000 c/ml) were subsequently spiked with 100 µL of the same HIV-1-positive plasma (with known viral load) that was used to spike the CFU-assays. Cells were incubated for two days at 20% O2; 5% CO2 at 37°C, after which cells were prepared for Ultrio-Plus® screening as discussed for CD34+ cells spiked in the CFU-assays.

7.4.9 Statistical analysis

Statistical analyses were done in collaboration with Professor Piet Becker (biostatistician, MRC) and are described below:

Statistical considerations:

One of the study's objectives was to assess the effect of HIV-1 on the colony forming unit's ability (CFU- assays) of CD34+ haematopoietic progenitor cells (HPCs). The comparison of spiked and non-spiked samples with regard to CFUs was done in respect of CFU-GM, BFU-E, CFU-MK and CFU-GEMM colonies using a pairwise Wilcoxon's matched pairs Sign ranks test.

A comparison of CD34+ HPCs with HIV- and FBS-spiked HPCs was done with respect to CFU growth. Individual UCB samples were split into three categories: normal, HIV-1-spiked and FBS-spiked samples and subsequent within-blood comparisons were done for each UCB unit – i.e. the three conditions (normal, HIV-1-spiked and FBS-spiked samples) were compared with each other within the individual. A sample size of 27 would have at least 90% power to detect a change (increase from normal CFUs to HIV-infected CFUs) of 10 CFUs in the HIV spiked samples, where in un-spiked samples, a mean value of 37 CFUs is expected with a standard deviation of

17 CFUs (inflated by $\sqrt{2}$ for paired values). A sample size of at least 17 would have power in excess of 90% (power) to detect a change (increase from normal CFUs to FBS-spiked CFUs) of 13 CFUs in the FBS-spiked samples. One sided testing at the 0.05 level of significance was assumed.

For this reason, 19 within-sample comparisons were done for HIV-1-spiked vs. FBS-spiked samples.

7.5 Results and Discussion

7.5.1 UCB collection

Of the mothers addressed at the Steve Biko Academic hospital, ante-natal clinic, 270 gave consent to donate their UCB to the study. However, only about 1 UCB unit out of every 3 patients was collected (79 collected in total) because of administrative insufficiencies at the ante-natal clinic (as mentioned previously).

Some collected UCB units were not placed in the medical store room by medical staff directly after collection. Thus, some samples were not processed within 72 hours after delivery and had to be excluded from the results. Low blood volumes and old UCB units severely affected the CD34+ isolation efficacy, quantity and quality of viable HPCs in these units and contributed to large inter-sample variability. Patient samples varied in plasma volume, viscosity (and cell clumps), cell count, viability and CFU-ability.

Assigning a dedicated doctor to in-theatre UCB collection would greatly enhance consistency in the method and volume of UCB collection. Better administration should also be implemented for better communication between hospital staff and researchers at the hospital in order to overcome delays in UCB collection and other administrative insufficiencies. Despite the protocol's recommendation for collecting and processing UCB within 72 hours after delivery, it is advised to retrieve UCB and isolate CD34+ HPCs within 24 hours after delivery in order to minimise cell losses.

7.5.2 CD34+ Isolation

The volume of UCB collected directly affects the quantity of CD34+ cells present in UCB units (Chandra et al., 2011). Various studies have been conducted to evaluate how different factors (such as mode of delivery, infant sex, infant birth weight etc.) would affect TNC, CD34+ count and total blood volume (Ballen et al., 2001, Urciuoli et al., 2010). The main factors that affected

CD34+ isolation in our study were; 1) the total blood volume collected; 2) the fraction of this total blood volume constituted by plasma; and 3) the age of the UCB unit (processed within 24 to 72 hours or after 72 hours after delivery; Table 6).

The blood volumes collected before plasma depletion ranged between 25 and 115 ml (60 ml on average) (Table 6). Also, UCB collection bags contained 10 ml CPD anticoagulant and this is not excluded from the aforementioned blood volumes. Therefore the haematocrit values are lower than would be ideal. This directly influences the physical quality of the UCB units received which had marked influences on all downstream applications of harvested CD34+ cells. This problem could be addressed by allocating a dedicated doctor to UCB collections, as mentioned in section 7.5.1. This could increase the volume of collection and the timely removal of units for subsequent processing and will be imperative for future studies that would like to make use of UCB.

Plasma volumes, obtained during plasma depletion and used to obtain each UCB unit's HIV status, ranged between 2 and 45 ml (Table 6). Larger total blood volumes did not necessarily yield high plasma volumes. Low final blood volumes directly influenced the quantity and quality (purity) of CD34+ HPCs isolated (as was indicated by flow cytometry and subsequent CFU-assays).

Table 6: Blood and plasma volumes of UCB units collected for CD34+ HSC isolation

No.	Unit ID	Total volume collected	Plasma fraction	Remaining Cellular fraction	Cells/µL isolated
1	20120229 P1	60.0	7.0	53.0	100
2	20120306 P1	55.0	15.0	40.0	120
3	20120312 P1	77.0	23.0	54.0	160
4	20120312 P2	31.0	21.0	10.0*	20
5	20120312 P3	55.0	15.0	40.0	70
6	20120402 P1	55.0	6.0	49.0**	80
7	20120417 P1	68.0	7.0	61.0	560
8	20120417 P2	115.0	21.0	94.0	460
9	20120419 P1	75.0	15.0	60.0	200
10	20120704 P1	80.0	45.0	35.0**	220
11	20120710 P1	80.0	22.0	58.0	170
12	20120724 P1	50.0	14.0	36.0	360

No.	Unit ID	Total volume collected	Plasma fraction	Remaining Cellular fraction	Cells/µL isolated
13	20120724 P2	60.0	18.0	42.0	200
14	20120725 P1	32.0	18.0	14.0*	120
15	20120725 P2	86.0	14.0	72.0	400
16	20120726 P1	39.0	19.0	20.0	160
17	20120727 P1	80.0	20.0	60.0	120
18	20120727 P3	40.0	22.0	18.0*	80
19	20120727 P4	80.0	22.0	58.0	120
20	20120727 P5	30.0	23.0	7.0*	160
21	20120803 p1	25.0	15.0	10.0*	114
22	20120803 p2	45.0	8.0	37.0	320
23	20120803 p3	35.0	7.0	28.0	100
24	20120803 p5	50.0	20.0	30.0	220
25	20120803 P4	45.0	9.0	36.0	300
26	20120803 P6	25.0	14.0	11.0*	60
27	20120806 P1	80.0	2.0	78.0	30
28	20120806 P2	37.0	14.0	23.0*	140
29	20120807 P1	60.0	17.0	43.0**	40
30	20120807 P2	100.0	35.0	65.0**	280
Average blood collection		58.33	16.93	41.40	182.80
Standard deviation		23.1	8.6	22.1	131.3

* - Low final blood volumes affected CD34+ HPC isolation purity and CFU-growth.
**- UCB processed after 72 hours could have affected CD34+ HPC isolation purity and CFU-growth.

The CD34 cell marker is not the most primitive marker for identification of HPCs. More primitive cell markers exist (c-Kit, Lin^-, CD38- etc.). However, CD34 was chosen for use in this study because of various practical factors including its utility and application for establishing a public UCB bank. The typical way for qualifying an UCB unit for storage in an UCB bank is through total nucleated cell count of the unit, cell viability, CFU- capacity and CD34+ cell enumeration. CFU-assays and CD34 counts are recommended prior to cryopreservation of the samples, as well as post-thawing in order to measure the unit's viability and potency or engraftment capacity (Section D 10.5.2 of the NetCord-FACT international cord blood standards) (Anon, 2010). Furthermore, the CD34+ marker is used for isolation of HPCs for CFU-assays and standardised kits are available for this purpose.

The number of HPCs isolated per microlitre as manually counted with the haemocytometer, ranged between 20 cells/µL and 2000 cells/µL depending on the UCB unit quality and quantity. Manual counts of individual samples compared well with results obtained by Flow cytometric analyses; however manual counting had a larger margin of error than flow cytometry.

The cell count from the haemocytometer was consistently used to prepare 250 cells/well to plate cells for the CFU-assay. However, the flow cytometry results yielded a more accurate representation of the percentage of true HPCs within the isolated population. Cell counts obtained by flow cytometry, were, however, dependent on the kits used for fluorescent labelling and identification of the CD34+ population. Three classes of mABs directed against CD34+ are currently known:

- Class I anti-CD34 mABs (recognises epitopes with sialic acid residues)
- Class II mABs (recognises carbohydrate epitopes)
- Class III mABs (recognises epitopes on the CD34 polypeptide) (Steen & Egeland, 1998)

The Stem-Kit™ contains a Class III mAB while the CD34+ Pool Kit was directed against Class I and II and was therefore able to recognise and bind to more CD34+ HPCs, thus yielding a higher cell count.

7.5.3 Flow cytometry results

CD34+ HPCs are regarded as cells in the intermediate phase of repopulation i.e. not as primitive as true stem cells, yet maintaining replication, migration- and differentiation properties that are absent in cells with morphologically distinguished features of differentiation. The CD34+ marker thus isolates a heterogeneous population of both more and less differentiated HPCs. Sample isolation is also subject to some lymphocyte contamination. Total isolated CD34+ cells was therefore constituted of two groups as indicated in Table 7: a) “True” HPCs that are a more primitive subpopulation identified through flow cytometry as being CD34+ but only dimly positive for CD45; and b) mature lymphocytes that were brightly positive for CD45 and are not a part of the “true” HPCs. The CD34+ CD45 dim population was presumably responsible for forming CFUs in the CFU-assay because of their more primitive nature (multipotency and ability to migrate and differentiate) (Deutsch et al., 2010).

The average percentage of primitive HPCs isolated (CD34+ CD45 dim) for the 26 UCB units (processed within 72 hours), obtained with the CD34+ Pool Kit and determined by flow

cytometry, was 89.52% (Standard Deviation (SD) ± 8.84). The Stem-Kit™ detected 65.0% (SD ± 30.03) of true HPCs. All the data for the flow-results are shown in Table 8. Table 7 provides a summary of the data.

Table 7: Summary of Flow cytometry results obtained with Stem-kit™ and CD34+ Pool Kit fluorescent kits

Protocol	Total CD34+ HPCs (%)	Total CD34+ HPCs consist of:		CD34+ HPCs (cells/µL)	Viability (%)	No of patients
		True HPC (CD34+ & CD45 Dim) (%)	Contaminating Leukocytes (CD34+ & CD45 Bright) (%)			
CD34+ Pool Kit	65.46	89.52	10.48	193.06	(N/A)	26
Stem-Kit™	51.30	65.00	35.00	148.84	73.32	26

The overall isolation purity averaged at 65% (as detected with the CD34+ Pool Kit) and 51% (as detected with Stem-Kit™), indicating that some CD34+ cells were not enumerated by the Stem-Kit™ reagents. This seemed to be highly dependent on the individual UCB units, since Stem-Kit™ often had similar results to the CD34+ Pool Kit and sometimes even had better detection (Figures 26 to 30; Table 8). Figures 32 and 33 illustrate results for the two kits on the same patient. This gives an indication of the large variability observed between the kits for certain patients and accounts for the results illustrated in Tables 7 and 8. Therefore, when UCB units are to be enumerated for their CD34+ content in an UCB bank setting, it will be extremely important to use CD34+ mABs that are representative of all three classes of mABs directed against CD34+.

Table 8: Flow cytometry data for the 26 UCB units that were also subjected to Ultrio-Plus® screening

Flow cytometry data for the CD34+ Pool Kit protocol						Flow cytometry data for the Stem Kit protocol				
No.	Unit ID	Average of %Gated CD34	Average of cells/µL CD34	Constitution of CD34+ cells:		Average of %Gated CD34	Average of cells/µL CD34	Constitution of CD34+ cells:		Viable
				CD45+ Dim 2	CD45+ Bright 2			CD45+ Dim 2	CD45+ Bright 2	
1	20120229 P1	98.15	27.55	79.51	20.49	65.73	17.75	91.16	8.84	79.16
2	20120306 P1	77.72	80.10	84.00	16.00	75.74	66.74	60.10	39.90	93.16
3	20120312 P1	79.82	184.84	98.04	1.96	86.55	162.15	79.43	20.57	88.64
4	20120312 P2	48.61	9.96	60.00	40.00	49.55	10.76	59.27	40.73	67.93
5	20120312 P3	97.49	118.87	93.94	6.06	84.88	10.76	94.94	5.06	83.90
6	20120417 P1	97.77	1197.79	95.84	4.16	67.16	795.78	93.05	6.95	87.98
7	20120417 P2	96.40	992.48	98.20	1.80	26.60	307.62	46.99	53.01	80.65
8	20120419 P1	87.85	265.51	98.85	1.15	91.32	278.87	93.84	6.16	95.93
9	20120710 P1	94.57	640.14	96.18	3.82	95.97	669.20	96.24	3.76	96.53
10	20120724 P1	61.40	146.23	95.31	4.69	97.33	245.29	96.95	3.05	93.80
11	20120724 P2	56.14	150.74	94.09	5.91	95.34	215.13	88.71	11.29	89.70
12	20120725 P1	61.20	55.27	95.92	4.08	2.27	2.70	44.44	55.56	42.11
13	20120725 P2	38.95	94.57	87.58	12.42	84.88	195.36	94.94	5.06	83.90
14	20120726 P1	96.86	118.15	93.21	6.79	71.17	72.02	97.06	2.94	88.48
15	20120727 P1	99.79	176.57	94.01	5.99	93.22	133.44	97.21	2.79	95.88
16	20120727 P3	99.24	90.45	88.59	11.41	11.02	6.01	60.54	39.46	79.46
17	20120727 P4	98.47	46.95	87.36	12.64	21.11	6.20	36.84	63.16	64.17
18	20120727 P5	98.44	238.73	96.22	3.78	12.75	16.63	57.53	42.47	63.80
19	20120803 p1	5.95	78.25	90.54	9.46	12.65	177.94	13.01	86.99	50.87
20	20120803 p2	5.70	51.30	86.60	13.40	23.03	66.44	3.32	96.68	52.16
21	20120803 p3	8.18	17.76	68.94	31.06	30.80	60.91	14.59	85.41	52.53
22	20120803 p5	38.13	149.74	93.04	6.96	23.40	50.24	18.11	81.89	50.62
23	20120803 P4	15.94	32.19	87.65	12.35	72.06	146.93	82.95	17.05	32.83

Flow cytometry data for the CD34+ Pool Kit protocol						Flow cytometry data for the Stem Kit protocol				
No.	Unit ID	Average of %Gated CD34	Average of cells/µL CD34	Constitution of CD34+ cells:		Average of %Gated CD34	Average of cells/µL CD34	Constitution of CD34+ cells:		Viable
				CD45+ Dim 2	CD45+ Bright 2			CD45+ Dim 2	CD45+ Bright 2	
24	20120803 P6	43.47	8.15	82.27	17.73	10.00	59.67	38.69	61.31	67.12
25	20120806 P1	52.73	23.65	89.64	10.36	7.34	93.17	82.07	17.93	63.72
26	20120806 P2	43.00	23.57	91.94	8.06	21.94	2.16	48.08	51.92	61.18
Total		**65.46**	**193.06**	**89.52**	**10.48**	**51.30**	**148.84**	**65.00**	**35.00**	**73.32**
Standard deviation		**32.31**	**290.15**	**8.84**	**8.84**	**33.87**	**191.44**	**30.03**	**30.03**	**18.25**

*** - *Note:* Data for UCB units processed after 72hours was not included in this table. Results for these 30 patients are illustrated in Annexure 4.**

Patient: 20120206 P1

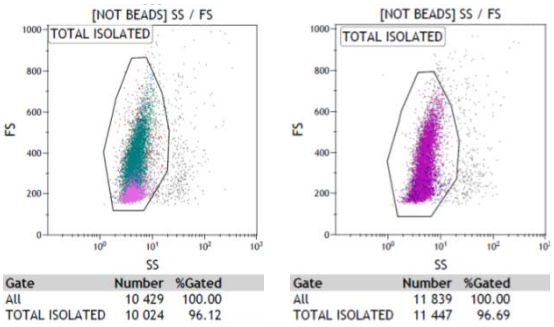

Figure 26: Comparison between flow cytometry results obtained with the Stem-Kit™, left and CD34+ Pool Kit, right, for total cells isolated.

Patient: 20120206 P1

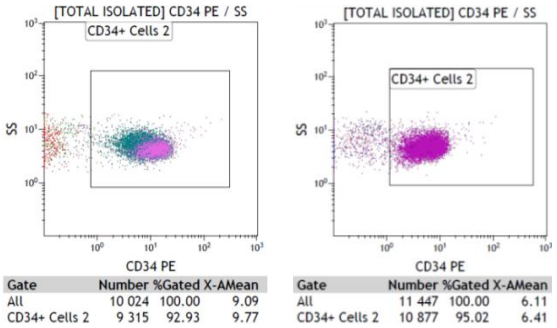

Figure 27: The percentage of CD34+ HPCs detected (Stem-Kit™, left; CD34+ Pool Kit, right). Percentages of CD34+ cells isolated are indicated under % Gated.

Patient: 20120206 P1

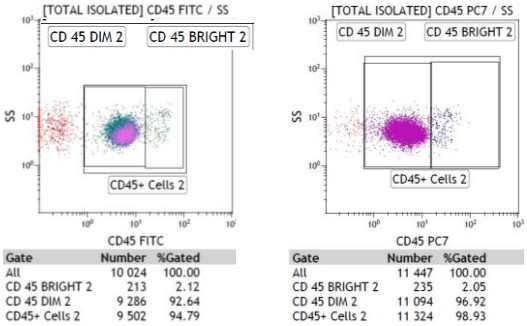

Figure 28: Percentage of isolated cells that were CD45+ (Stem-Kit™, left; CD34+ Pool Kit, right). Cells grouped on the left were CD45 dim and CD45+ HPCs on the right were CD45 bright. (Percentages are indicated at % Gated).

Patient: 20120206 P1

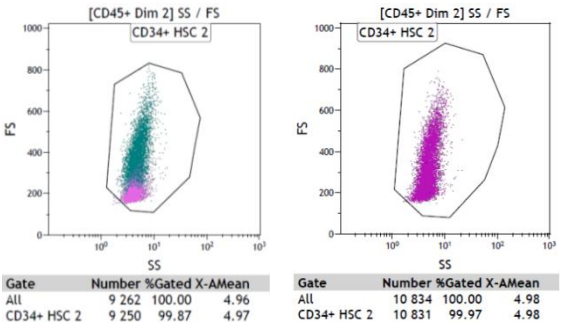

Figure 29: True HPCs, CD45 dim and CD34+ (Stem-Kit™, left; CD34+ Pool Kit, right). (Percentages are indicated at % Gated).

Patient: 20120206 P1

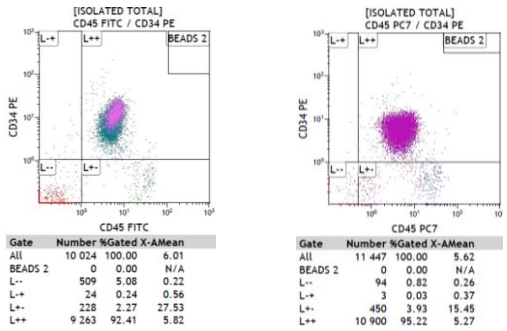

Figure 30: Characteristics of total cells isolated (Stem-Kit™, left; CD34+ Pool Kit, right) (Percentages are indicated at % Gated).

Patient: 20120206 P1

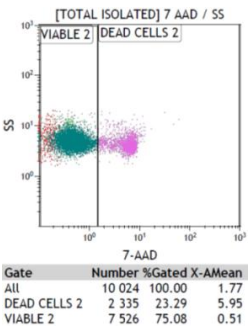

Figure 31: An example of cell viability measured with the Stem-Kit™. (Percentages are indicated at % Gated).

Patient: 20120417 P2

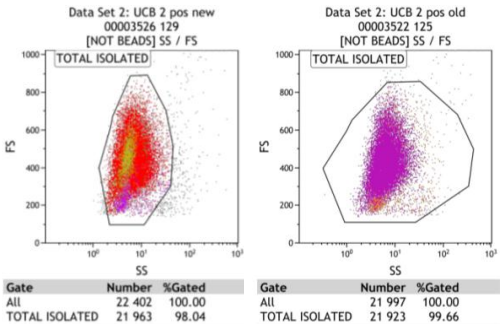

Figure 32: Comparison between flow cytometry results obtained with the Stem-Kit™, left, and CD34+ Pool Kit, right, for total cells isolated.

Patient: 20120417 P2

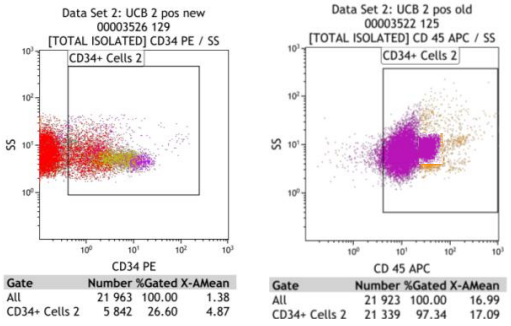

Figure 33: Comparison between the percentage of CD34+ HPCs detected with the Stem-Kit™, left, and CD34+ Pool Kit, right. Differences are clearly indicated where Stem-Kit™ detected 26.6% CD34+ HPCs and CD34+ Pool Kit detected 97.34% CD34+ HPCs for the same patient.

CD34+ cell viability (Figure 31), was not measured with the CD34+ Pool Kit. However, CD34+ viability measured with the Stem-Kit™ averaged at 73.32% (SD ± 18.25%) (Table 7). Viability is a key factor involved in enumerating CD34+ HPCs and would therefore need to be included during CD34+ enumeration to give a better indication of the UCB unit's overall engraftment ability.

The effect of cell viability and percentage of true HPCs were also reflected in the number and type of colonies grown. When UCB units were older than 72 hours, were more viscous or had very low blood volumes after plasma depletion, it was difficult to isolate a viable, pure CD34+ population. In cases where isolation purity was low (below 70%), corresponding CFU-assays had adherent cells that resembled fibroblast / mesenchymal or epithelial-like cells. Further analysis is required for identification and characterisation of these cells and was not within the scope of this study.

7.5.4 CFU-assays

7.5.4.1 Introduction

The CFU-assay is a quantitative in-vitro Clonogenic assay, used to investigate normal and abnormal haematopoietic progenitor cell growth in various types of haematopoietic progenitor cells. Each colony is formed within a finite period of time, by a single CD34+ HPC that has started to divide and differentiate into morphologically recognisable mature progeny. Cells that were more differentiated had less ability to self-replicate, migrate and differentiate in comparison with less differentiated cells that were more primitive and had greater capacity to

reconstitute a haematopoietic system (based on the number and size of colonies formed). CD34+ HPCs take 14 days to mature into morphologically distinguishable colonies. Once colonies reach maturity, they will not expand further but will eventually start to lyse. Therefore, colonies are best enumerated within 14 to 16 days after plating (Eaves & Lambie, 1995).

7.5.4.2 Principles of CFU-assays

The number of expected CFUs is highly dependent on the quality of the sample and sample isolation. Factors that had to be standardised prior to obtaining consistent CFUs included the method of isolation, cell purity and concentration, cell-culture-medium with appropriate cytokines and growth factors (bought from Stem Alpha), incubation time, CO_2, O_2, humidity and temperature to name a few. Despite standardising these variables (by using more easily obtained PB from the SANBS), the large sample variability influenced consistency in the isolation technique, which, in turn, contributed to varying numbers of colonies obtained for each sample.

Colonies scored were CFU-GM (granulocyte-macrophages), CFU-MK (megakaryocytes), BFU-E (burst-forming unit erythrocytes – which included normal CFU-erythrocytes) and CFU-GEMM (granulocyte, erythrocyte, macrophage, megakaryocyte) colonies and are illustrated in Figures 34 to 37.

Different stages of progenitors gave rise to differently sized colonies. The majority of isolated cells contain more mature (i.e. more differentiated) cells, which lyse within the first few days of CFU-incubation and subsequently do not form colonies (Eaves and Lambie, 1995). More primitive HPCs with higher proliferative and migratory capacities, on the other hand, gave rise to larger colonies (more cells present in the colony) often consisting of different clusters (due to the increased migratory capacity of primitive progenitors). The CFU-assay thus gave a retrospective estimate of the potency (proliferative and migratory capacity linked to engraftment ability) of the UCB unit.

Furthermore, CD34+ HPCs were cultured in a semi-solid medium (methylcellulose) that contained specific proteins and cytokine supplements conducive to CFU-GM, MK, GEMM and BFU-E growth. Methylcellulose is preferred to agarose or collagen medium since hemoglobinisation is superior in methylcellulose, making scoring of erythroid and burst-forming erythroid colonies easier.

The Stemα-1D medium contains FBS, human transferrin, Interleukin-3 (IL-3), IL-6, IL-11, stem cell factor (SCF), erythropoietin (EPO), granulocyte-stimulating factor (G-CSF) and granulocyte-macrophage-colony-stimulating factor (GM-CSF). Table 9 indicates the cytokines and their functions as understood in CFU-growth.

The gel-like basis of the medium allowed some colony spreading but restricted extensive cell migration. Therefore, daughter cells were localised to the original progenitor cells during the colony formation and colonies from a single progenitor could be distinguished.

By keeping the cell seeding density at 250 cells/well and the plating volume below 5 µL per well, colonies were more easily identifiable. This seeding density ensured that there were not too many colonies per well, which would have complicated identification of single colonies. Neither did the seeding volume dilute the culture medium so as to cause runny and overlapping colonies that are also difficult to score. Too high seeding densities cause excessive consumption of nutrients and cause acidic environments that are not conducive to CFU-growth. The acidity in the medium can be distinguished by discolouration of the pink medium to a brownish-yellow medium (Nissen-Druey et al, 2005).

Table 9: Cytokines and growth factors in Stemα-1D used in CFU-assays

Cytokine or Growth Factor	Function
Foetal Bovine Serum (FBS)	➢ *Rich protein variety aids in sustaining cell growth, survival and division*
Human transferrin	➢ *Transports Iron to cells for growth* ➢ *Reduces cell damage via reduction of free-radicals* ➢ *Essential for erythroid growth*
Interleukin-3 (IL-3)	➢ *Multi-lineage stimulator* ➢ *CFU-M, CFU-Mk, CFU-Eo, BFU-E (containing Epo)* ➢ *CFU-G = weakly stimulated*
Interleukin-6 & Interleukin-11 (IL-6 & IL-11)	➢ *Act synergistically for CFU-MK development* ➢ *Colony promoting activity*
SCF	➢ *Strong, non-physiological stimulating activity* ➢ *Commits precursors to erythroid line (at expense of neutrophil line)* ➢ *Anti-apoptotic* ➢ *Stimulates Eosinophils (CFU-Eo) and Basophils*
Erythropoietin (EPO)	➢ *Physiological stimulator important for hemoglobin synthesis.* ➢ *Aids in CFU-Mk differentiation*
Granulocyte-macrophage colony stimulating factor (GM-CSF)	➢ *Multi-lineage stimulator* ➢ *CFU-M, CFU-Eo, BFU-E,* ➢ *CFU-G = weakly stimulated*
Granulocyte colony stimulating factor (G-CSF)	➢ *Multi-lineage stimulator* ➢ *CFU-G, BFU-E (containing Epo)*

(Briddell et al., 1992; Ibelgaufts, H., 2008; Nissen-Druey et al., 2005)

7.5.4.3 Determining the UCB unit's HIV status:

In order to accurately determine HIV's influence on "normal CFUs" in downstream applications of CD34+ HPCs isolated from UCB units, it was necessary to establish that UCB units (from which CD34+ HPCs were isolated), were HIV-1 negative at the onset of CFU-assays. Therefore, plasma from each UCB unit (collected during plasma depletion) was screened for HIV-1 with the Ultrio-Plus® assay.

As mentioned in the previous chapter, the current standard (NetCord-FACT standards) for the first evaluation of acceptance of an UCB unit is the mother's HIV status at the time of delivery. Mothers must be screened for infectious diseases within seven days before or after giving birth. In addition, the standards advise that UCB units should be screened in addition to maternal

screening.

In this study, mothers were not screened again at the time of delivery for the following reasons:

1) All patients attending the ante-natal clinic at Steve Biko Academic Hospital undergo compulsory HIV-1 tests upon admittance to the hospital.
2) If patients were admitted to the hospital during their first trimester, they would be subjected to a second HIV-1 test in their last trimester.
3) The HIV-1-positive patients were (presumably) aware of their HIV-1 status, had access to qualified medical counsellors, and furthermore received ART.
4) Logistics involved to obtain these additional tests from the patients were difficult and need improvement going forward.

It was therefore decided to obtain the patients' HIV-1 status from their patient files (for which informed consent was also obtained).

All UCB units were negative for the presence of HIV-1 as determined by the Ultrio-Plus® assay (Table 10). In addition to the low probability of obtaining HIV-1^{+} UCB units through vertical transmission, all HIV-1-positive patients received ART, which further reduced the probability of obtaining HIV-1-positive UCB from the specific patient cohort. For the reasons mentioned previously pertaining to ART and vertical transmission, it is important to note that a negative result for the Ultrio-Plus® UCB screening does not imply that the *patient* will be HIV-1 negative. It only affirms that HIV-1 was not present in the UCB sample (Table 10).

Table 10: Patient HIV-status obtained from patient files and subsequent Ultrio-Plus® screening of UCB units

	Patient file			Ultrio screening results
No.	**Patient no.**	**HIV status**	**HIV Key**	**Normal (CFU or Plasma)**
1	GP63155959*	UNKN	1	Non-reactive
2	GP63109323*	POS	6	Non-reactive
3	GP63042607*	POS	4	Non-reactive
4	GP63142124*	POS	4	Non-reactive
5	GP63157623*	POS	4	Non-reactive
6	GP63158331*	POS	4	Non-reactive
7	20120417 P1*	NEG	N/A	Non-reactive
8	GP63155780	NEG	2	Non-reactive
9	GT63936615*	NEG	2	Insufficient volume
10	GP08692362*	POS	4	Non-reactive
11	GP63157444	NEG	1	Non-reactive
12	GT63870747	POS	5	Non-reactive
13	GP63038885	POS	7	Non-reactive
14	GP63089794	POS	5	Non-reactive
15	GT63946009	NEG	3	Non-reactive
16	GT63946062	UNKN	1	Non-reactive
17	GP63165084	POS	4	Non-reactive
18	GT63945399	POS	4	Non-reactive
19	GP63165640	DECL	2	Non-reactive
20	GT63946115	DECL	2	Non-reactive
21	GT63856998	NEG	3	Non-reactive
22	GT63946268	UNKN	1	Non-reactive
23	GP63166030	DECL	2	Non-reactive
24	GT63853442	NEG	3	Non-reactive
25	GP63049162	POS	5	Non-reactive
26	GP63159669	NEG	3	Non-reactive
27	GT63946361	POS	4	Non-reactive
28	GT42551121	NEG	3	Non-reactive
29	GT63945100*	NEG	3	Non-reactive
30	GT63946368*	DECL	2	Non-reactive

HIV Key: 1. – HIV unknown; 2. – Declined; 3. – Negative; 4. – HIV positive (not AIDS) on dual therapy (> 1 month); 5. – HIV positive (not AIDS) on dual therapy (< 1 month); 6. – HIV positive (not AIDS) no treatment; 7. – AIDS (on HAART) (Obtained from patient files, Steve Biko Academic Hospital). * - Patients not included in comparative analyses.

7.5.4.4 Identification of individual colonies:

UCB units were subjected to three plating conditions: 1) normal (30 UCB units), 2) HIV-1 spiked (30 UCB units: 30 with high VL and 19 with low VL) and 3) FBS-spiked cells (23 UCB units)

respectively. Cells were plated in triplicate for each of these conditions and the average number of colonies determined for each triplicate condition.

CFU-GM:

Colonies were counted when more than 20 to 50 cells made up the colony (Nissen-Druey et al., 2005). These colonies were composed of smaller, denser granulocytes and larger spherical macrophages and included neutrophils, eosinophils and basophils. The colony generally had a homogeneous morphology with a dense core that spread out towards the periphery of the colony (Figure 34). There is no additional diagnostic value in counting CFU-G and CFU-M separately and they were subsequently combined and counted as a single colony: CFU-GM. Normal CFU-G varies in size and neutrophils are also slightly smaller than macrophages. Therefore, various sizes of cells were visible in these colonies.

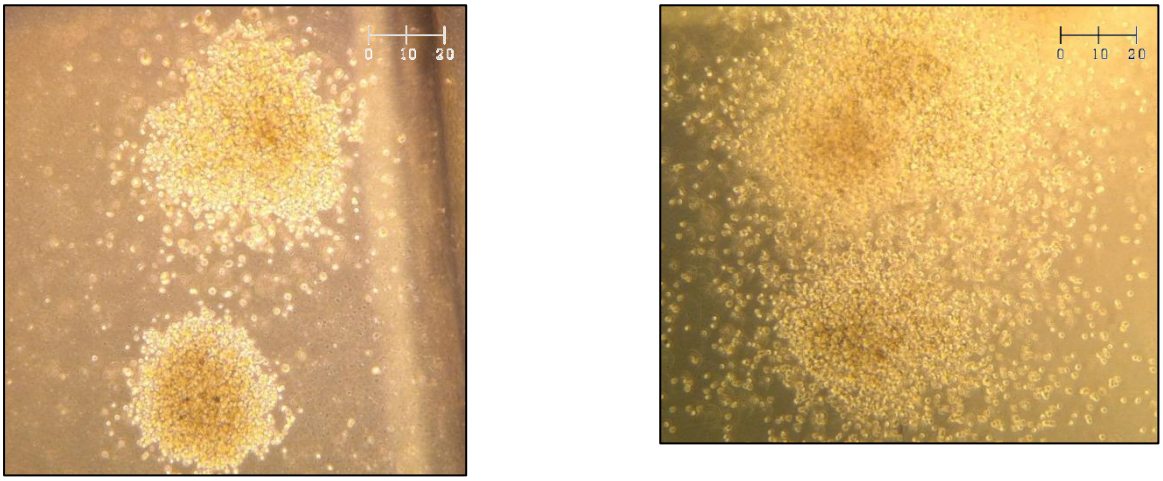

Figure 34: Normal CFU-GM colonies (14 days of culture). Pictures taken at 20X magnification. (Scale bar = 0.2 mm)

BFU-E:

BFU-E colonies are different from CFU-E colonies in that they are more primitive than CFU-E, forming much larger colonies with a burst-like pattern. These small round cells were not easily distinguishable as individual cells but were easily identified as a colony because of its red colour (due to haemoglobin synthesis inside cells during maturation) (Figure 35). Higher magnification was needed, though, (10X – 20X) in order to evaluate hemoglobinisation (in order to rule out confusion with CFU-G colonies).

Cell numbers in BFU-E colonies could range between 200 and 1 x 10^4 cells and could even contain up to 16 clusters (Nissen-Druey et al., 2005). The larger, multi-clustered BFU-E colonies were indicative of greater proliferative capacity of the initial progenitor.

CFU-E progenitors gave rise to the smallest, most rapidly maturing erythroid colonies. These colonies came from a more mature precursor (smaller colony, less differentiation and migration) and generally consisted of one or at most two clusters of between 8 and 100 to 200 erythroblasts (max) (Nissen-Druey et al., 2005).

**Note:* CFU-E and BFU-E colonies were scored independently. However, in order to simplify statistical parameters used for colony enumeration of HIV-1 spiked cells, CFU-Es and BFU-Es were grouped together as BFU-E colonies.

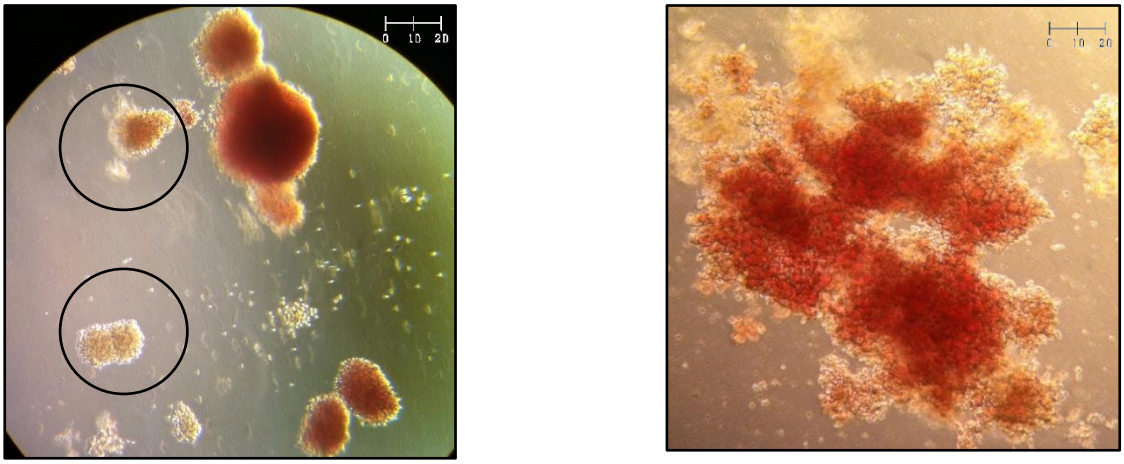

Figure 35: CFU-E (encircled left) and BFU- E (Right) colonies (14 days of culture). Pictures taken at 20X magnification. (Scale bar = 0.2 mm)

CFU-GEMM:

Truly mixed CFU-GEMM colonies (Figure 36) were indicative of the most primitive HPCs plated in the CFU assay. A single progenitor had the capacity to form this mixed colony, which contains multiple cell lineages (erythroblasts, neutrophils, granulocytes, macrophage and megakaryocytes). It had a slightly more grey/brown appearance than BFU-E colonies, because of the content of non-haemoglobinised cells. Since these cells are of the more primitive HPCs, there were fewer of these colonies present than other colonies.

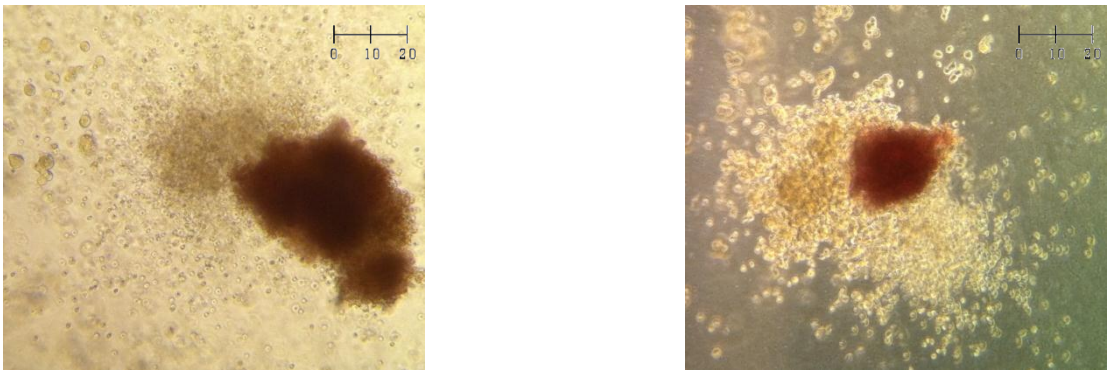

Figure 36: CFU-GEMM colonies (14 days of culture). Pictures taken at 20X magnification. (Scale bar = 0.2 mm)

CFU-MK:

These cells were often more dispersed throughout the wells with fewer, but much larger cells than other colonies. MK colonies often have fewer than 50 cells in pure colonies, which are mostly representative of MK-blast cells since they do not mature within the 14-day culture period (Eaves and Lambie, 1995). Therefore, colonies were composed of cells with varying sizes but were nevertheless easily distinguished as uniquely translucent, large blast-like cells (Figure 37).

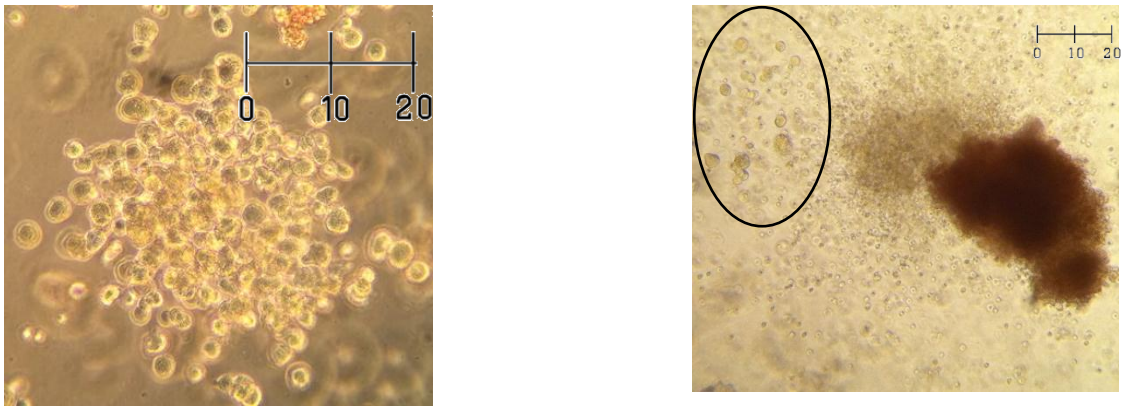

Figure 37: MK colonies (14 days of culture). Pictures taken at 40X magnification (left) and 20X magnification (right, encircled area). (Scale bar = 0.2 mm)

The average number of CFUs for each patient was calculated by counting colonies, in each of the triplicate wells, for each condition (normal, HIV-1-spiked or FBS-spiked) respectively. The overall average of colonies formed per condition (normal, HIV-1-spiked and FBS-spiked) was subsequently calculated for the 19 UCB units used in the comparative analyses (Table 12). Individual CFU wells contained between 20 and 80 colonies – indicating that around one in

every five cells plated was a viable, true HPC. However, the 19 units used for comparison yielded 21 normal CFUs, 23 HIV-1-spiked CFUs and 23 FBS-spiked CFUs respectively (Table 12). Thus, on average, only about one in ten plated cells were viable, primitive HPCs that could form colonies. Table 11 is a summary of all the data for CFU scoring of the 19 patients. Table 12 provides the summary of the final 19 patients that were compared over all analyses and displays the average colony number per CFU group (GM, MK, BFU-E and GEMM) observed for each of the three conditions under which CD34+ HPCs were cultured (normal, HIV-1-spiked and FBS-spiked).

Table 11: Average CFUs obtained for the three different conditions of culture: Normal, HIV-1-spiked and FBS-spiked for 19 UCB units used in comparative analyses

Colonies counted	Normal	HIV	FBS
CFU-GM	9	9	10
CFU-MK	4	7	7
BFU-E	5	4	5
CFU-GEMM	3	2	1
Total Average colonies	**21**	**22**	**23**
Number of UCB units	**19**	**19**	**19**

Table 13 illustrates the number of colonies grown under the three conditions (Normal, HIV-1-spiked and FBS-spiked). Colonies spiked with HIV-1 for these seven UCB units were confirmed to be HIV-1 positive by the Ultrio-Plus® assay after 14 days of incubation. Table 14 illustrates the number of colonies grown under the abovementioned conditions, however, the colonies grown from these 12 UCB units were negative for the presence of HIV-1 after 14 days of incubation.

Table 12: Colonies counted in each of the three conditions (Normal, HIV-1, FBS-spiked) for the final 19 UCB units used in comparative analyses

No.	Unit ID	Normal CFU-GM	HIV-1 CFU-GM	FBS CFU-GM	Normal CFU-MK	HIV-1 CFU-MK	FBS CFU-MK	Normal BFU-E	HIV-1 BFU-E	FBS BFU-E	Normal CFU-GEMM	HIV-1 CFU-GEMM	FBS CFU-GEMM
8	**20120417 P1**	17	18	23	5	7	4	10	13	16	2	6	4
11	**20120710 P1**	28	42	50	9	10	13	14	14	12	2	3	3
12	**20120724 P1**	5	9	7	1	3	6	0	5	6	0	0	0
13	**20120724 P2**	14	16	3	10	10	14	17	6	2	4	2	0
14	**20120725 P1**	3	3	4	3	6	5	2	0	0	0	0	0
15	**20120725 P2**	22	13	18	11	10	13	8	5	2	0	1	1
16	**20120726 P1**	20	20	31	10	11	9	4	2	2	0	0	0
17	**20120727 P1**	14	26	36	11	14	23	24	25	28	2	0	1
18	**20120727 P3**	4	5	5	4	3	3	0	0	1	0	0	0
19	**20120727 P4**	3	1	5	2	3	3	0	1	1	0	0	0
20	**20120727 P5**	4	2	5	6	7	7	0	0	1	0	0	0
21	**20120803 P1**	3	4	4	3	3	2	0	0	1	0	0	0
22	**20120803 P2**	1	2	2	2	1	2	0	0	0	0	0	0
23	**20120803 P3**	3	8	4	2	3	3	2	5	1	1	1	1
24	**20120803 P5**	8	5	9	7	11	12	2	3	0	0	0	0
25	**20120803 P4**	3	3	3	1	3	3	1	1	0	0	0	0
26	**20120803 P6**	2	2	3	4	3	3	2	1	1	0	0	0
27	**20120806 P1**	2	3	3	2	2	3	2	3	3	0	0	0
28	**20120806 P2**	20	23	18	4	6	5	10	7	11	2	0	2
Average CFUs		**9**	**9**	**10**	**4**	**7**	**7**	**5**	**4**	**5**	**3**	**2**	**1**

Table 13: HIV-1-Positive colonies for normal-, HIV-1- and FBS-spiked colonies

No.	Unit ID	Normal CFU-GM	HIV-1 CFU-GM	FBS CFU-GM	Normal CFU-MK	HIV-1 CFU-MK	FBS CFU-MK	Normal BFU-E	HIV-1 BFU-E	FBS BFU-E	Normal CFU-GEMM	HIV-1 CFU-GEMM	FBS CFU-GEMM
14	**20120725 P1**	3	3	2	0	3	6	0	0	4	5	0	0
15	**20120725 P2**	22	11	8	0	13	10	5	1	18	13	2	1
17	**20120727 P1**	14	26	36	11	23	24	25	28	0	1	1	0
19	**20120727 P4**	3	2	0	0	1	3	1	0	5	3	1	0
20	**20120727 P5**	4	6	0	0	2	8	0	0	5	7	3	0
27	**20120806 P1**	2	2	2	0	3	2	3	0	3	3	11	2
28	**20120806 P2**	20	4	10	2	23	6	7	0	18	5	3	1
Average Total		**10**	**8**	**8**	**2**	**10**	**8**	**6**	**4**	**8**	**5**	**3**	**1**

Table 14: HIV-1-Negative colonies for normal-, HIV-1- and FBS-spiked colonies

No.	Unit ID	Normal CFU-GM	HIV-1 CFU-GM	FBS CFU-GM	Normal CFU-MK	HIV-1 CFU-MK	FBS CFU-MK	Normal BFU-E	HIV-1 BFU-E	FBS BFU-E	Normal CFU-GEMM	HIV-1 CFU-GEMM	FBS CFU-GEMM
8	**20120417 P1**	17	18	23	5	7	4	10	13	16	2	6	4
11	**20120710 P1**	28	42	50	9	12	13	14	14	12	2	3	3
12	**20120724 P1**	5	1	0	0	9	3	5	0	7	6	6	0
13	**20120724 P2**	14	16	3	10	12	14	17	6	2	4	2	0
16	**20120726 P1**	20	20	31	10	11	9	4	2	2	0	0	0
18	**20120727 P3**	4	5	5	4	3	3	0	0	1	0	0	0
21	**20120803 P1**	3	4	4	3	3	2	0	0	1	0	0	0
22	**20120803 P2**	1	2	2	2	1	2	0	0	0	0	0	0
23	**20120803 P3**	3	8	4	2	3	3	2	5	1	1	1	1
24	**20120803 P5**	8	5	9	7	12	12	2	3	0	0	0	0
25	**20120803 P4**	3	3	3	1	3	3	1	1	0	0	0	0

No.	Unit ID	Normal CFU-GM	HIV-1 CFU-GM	FBS CFU-GM	Normal CFU-MK	HIV-1 CFU-MK	FBS CFU-MK	Normal BFU-E	HIV-1 BFU-E	FBS BFU-E	Normal CFU-GEMM	HIV-1 CFU-GEMM	FBS CFU-GEMM
26	**20120803 P6**	2	2	3	4	3	3	2	1	1	0	0	0
Average CFUs		**9**	**11**	**11**	**5**	**7**	**6**	**5**	**4**	**4**	**1**	**2**	**1**

7.6 Visual observation of HIV's influence on CFU growth and differentiation:

Individual patients often displayed a trend in CFU growth: Normal CFU numbers would be less than HIV-1-spiked CFU numbers, which in turn would be less than FBS-spiked CFU numbers (Normal < HIV-1 < FBS). Colonies exposed to HIV-1 looked less hemoglobinised (Figure 38) than their counterparts in Normal- or FBS-spiked colonies (Figure 35). These results correspond to results obtained by Redd *et al.* (2007b), who demonstrated a direct relation between HIV-1 subtype C infection, progenitor cell death and corresponding higher incidence of anaemia in those patients. Furthermore, colonies in HIV-1-spiked wells were smaller and seemed less viable than colonies in Normal and FBS cultures.

Colony growth was not completely inhibited by HIV-1, although morphologically, these colonies did not, in all cases, look like healthy, normal colonies. This could suggest that early progenitors were either not infected by HIV-1 or that only certain (potentially more differentiated) progenitors could be infected. These more differentiated progenitors would fail to produce colonies because they underwent apoptosis, leaving uninfected cells to produce colonies. It seemed that progenitors, capable of forming colonies were, however, affected by HIV-1, as is evident in Figure 38.

Cellular debris was visible where HIV-1 positive plasma was plated and the cultures were more acidic, with media presenting with a more yellow-brown colour than the normal pink colour. The cellular debris could be attributed to more differentiated CD34+ HPCs that became infected at the time of plating but that subsequently underwent apoptosis.

Although it is normal for more differentiated CD34+ HPCs to undergo apoptosis during CFU-assays (since only primitive CD34+ HPCs form colonies), there were no debris patterns in Normal- or FBS-spiked colonies, but only in HIV-1 colonies. This suggests that more cell death occurred in HIV-1-spiked cells. It could indicate that a certain number of (primitive) CD34+ HPCs were infected during the first days of colony growth and subsequently underwent apoptosis. However, to account for increased debris, it seems that infected cells might have also been able to affect adjacent differentiating cells (directly through viral infection or indirectly through the release of cytokines), leaving a trail of cellular debris.

Several studies have suggested that HIV-1 proteins could indirectly affect HPC proliferation; i.e. without the need of direct infection and viral replication (Maciejewski et al., 1994; Gibellini et al.,

2007; Zauli et al., 1996; Banda et al., 1997). Heat-inactivated HIV-1 has been used to show how several HIV-1 proteins (HIV-1 envelope glycoprotein (gp120); HIV-1 Gag protein, HIV-1 viral protein R (Vpr)) affect HPC colony growth. Some methods include inducing apoptosis through a Fas-dependant mechanism, suppressed myelopoietic differentiation, immune activation, and subsequent release of inflammatory cytokines (Zauli et al., 1996; Banda et al., 1997).

It has been verified, however, that CD34+ cells do express the necessary HIV-1 entry receptors (CD4 and co-receptors CXCR4 and CCR5) required for active infection (extensively reviewed by Alexaki and Wigdahl, 2008). Megakaryocyte and CFU-GM progenitors in particular have been shown to be infected by HIV-1 (Chelucci et al., 2005). Even if only a certain subset of HPCs could be infected by HIV-1 it would have the potential to affect surrounding cells and lead to cytopaenias. This could be explained by CD34+ cells' function in regulating innate and adaptive immune responses through release of inflammatory cytokines as well as influencing normal haematopoiesis through an autocrine and/or paracrine manner (Umland et al., 2004; Majka et al., 2001).

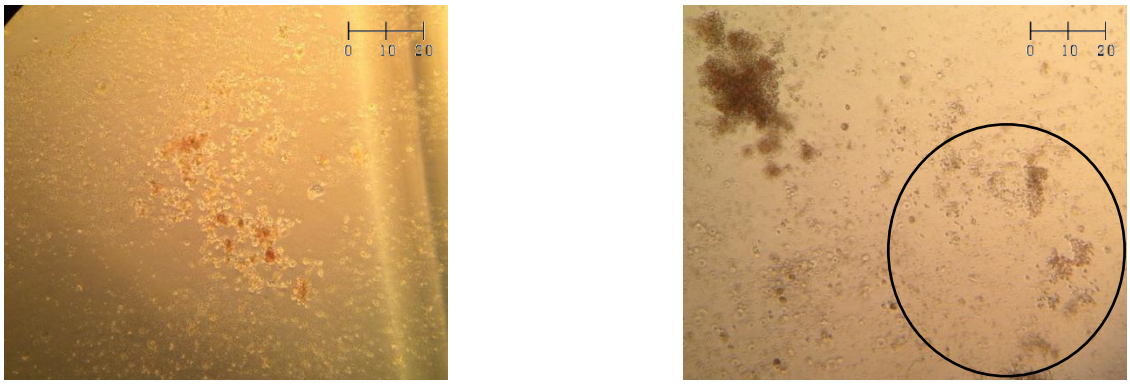

Figure 38: Poorly haemoglobinised BFU-E and CFU-E in HIV-1-spiked wells. Pictures taken after 14 days of culture at 20X magnification. (Scale bar = 0.2 mm)

Haematological cytopenias (thrombocytopenia, leukopenia, granulocytopaenia, anaemia etc.) are often associated with HIV-1 infection (Zauli et al., 1994; Marandin et al., 1996). *In vitro* colony formation of CD34+ HPCs isolated from HIV-1 individuals also yielded various cytopenias in a study done by Louache *et al.* (1992). These authors subjected individual colonies to polymerase chain reaction techniques (PCR) in order to detect HIV-1. However, they could not detect HIV-1 virus in these colonies and therefore suggested that HIV-1 indirectly affected the colony growth of HPCs (Louache et al., 1992). Furthermore, Bahner *et al.* (1997) have suggested that HIV-1 replication within the human marrow stromal microenvironment is responsible for a decrease in HPC

production during HIV-1 infection, which subsequently leads to various cytopenias (Bahner et al., 1997).

In further agreement with the results of Redd *et* al. (2007a,b), some HIV-1-spiked CFU assays in the present study seemed to produce increased growth of CFU-GM and CFU-MK in some patients, while growth of BFU-E and CFU-GEMM lagged behind (Figure 39) (Mlisana et al., 2008). This varied significantly from one patient to another and because of large inter-patient variability with CFU growth, could not be detected statistically (Table 15). Some patients had a very similar profile for HIV-1-spiked colonies vs. normal colonies and in other cases a marked difference could be observed within an individual's HIV-1-spiked and normal colonies. Such a difference is illustrated in Figure 39, where many more (and larger) CFU-GM colonies were present than, for example, BFU-E colonies in HIV-1-spiked wells. This is not due to an inherent characteristic of the patient's cells, since HPCs of the same individual grown under normal conditions yielded normal colonies representative of all colony types counted.

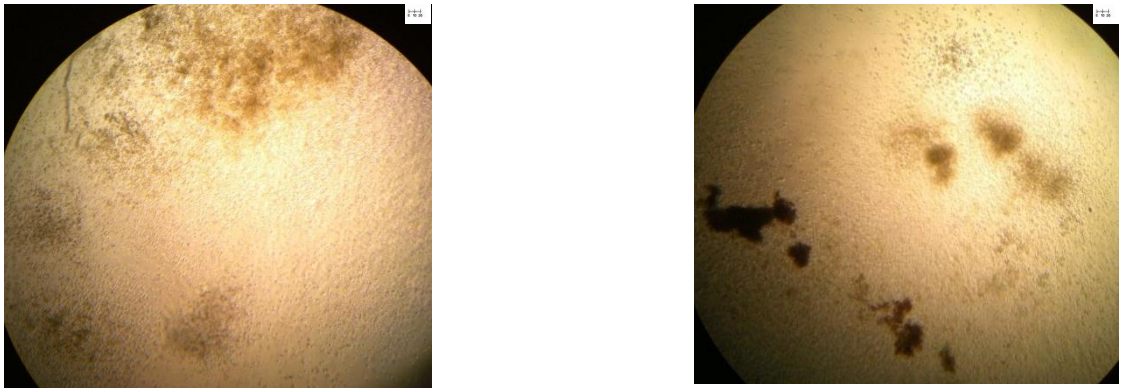

Figure 39: Within patient comparison of 14-day-old cultures. HIV-1-spiked CFUs (left) vs. Normal CFU colony growth (right) taken at 5X magnification. (Scale bar = 0.2 mm top right corner). More CFU-GM colonies seemed to grow in the HIV-1-spiked well (left) while Normal wells displayed balanced growth of all progenitors.

These results are however semi-quantitative at best and are insufficient to provide conclusive evidence of HIV-1's direct or indirect effect on HPCs' CFU-ability. Human serum would have yielded more reliable results, since HPCs grow more effectively in human serum than in FBS and results obtained for FBS-spiked CFUs are likely to be under-representative of CFU growth in human serum.

Furthermore, despite the investigator's best efforts, it is entirely possible that some colonies might have been wrongly classified/ identified. The CFU-assay and subsequent identification of the

colonies was a new technique that was acquired from colleagues at the University of Bordeaux, where PB was used to isolate CD34+ HPCs. The technique had to be adjusted to suit the circumstances in the South African laboratory, and for isolation of CD34+ HPCs from UCB. It is advisable to have a knowledgeable person available for training with CFU-assays and assistance with colony identification. Alternatively, an electronic CFU-imager and counter is available (StemVision™, Stemcell Technologies Inc.) which could aid in colony identification and reduce the counting variability between different researchers.

It would also have contributed to the validity of results if visual observations of colony size, number and haemoglobin content could have been substantiated with quantitative measurements of these units. Future studies should therefore consider the use of an automated colony identifier and cell counter which could aid in colony identification, measurement of size and estimations of cell numbers involved. Picking and staining CFU-colonies for haematological verification of colonies might also clarify possible confusion in colony identification.

7.7 Statistical analyses

The trend observed during CFU-counting (Normal CFU numbers < HIV-1-spiked numbers < FBS-spiked numbers) is confirmed in Table 15, although there were no statistically significant differences in growth between HIV-1-infected and HIV-1-negative wells.

These results differ from those obtained by Stella *et al*. (1987) for CFU growth of HPCs isolated from HIV-1-infected patients' BM, in that they reported a marked decrease in CFU growth for all colonies counted (GM, MK, BFU-E, GEMM). Similar decreases in CFU growth were noted by Marandin et al. (1996), Louache et al. (1992) and Ganser et al. (1990). Although statistical differences do not reveal severe CFU obstruction in HIV-1-spiked units in this study, certain patients did yield cytopenic colonies (with lower BFU-E, as discussed earlier) and altered CFU growth, which is in agreement with results from the aforementioned studies.

The differences in results could also be attributed to the fact that, in all cases mentioned, HPCs were isolated from BM of HIV-1-infected individuals, whereas this study used HIV-1-negative UCB HPCs that were infected *in vitro*. This suggests that HIV-1 infection in vivo predisposes CD34+ HPCs to HIV-1-infection and that the effect of HIV-1 on the haematopoietic micro-environment impairs the cells' ability to repopulate the haematopoietic system.

The average CFUs indicated in Table 12 do not take inter-patient variability and the uneven

distribution of data into account. Therefore, in order to verify the statistical significance of these observations on CFU growth (colony numbers) and constitution (colony type), a pairwise Wilcoxon's matched pairs sign ranks test was applied to the data.

7.7.1 Statistical significance of observed differences between Normal, HIV-1-spiked CFUs and FBS-spiked CFUs

The average number of CFUs for each triplicate condition (Normal, HIV spiked or FBS-spiked) was calculated per patient. The pairwise Wilcoxon's matched pairs sign ranks test was subsequently applied to the total number of CFUs for each condition (Normal, HIV spiked or FBS-spiked). The data was skewed due to inter-patient variability, therefore a logarithmic transformation was done on the data in order to normally distribute the data. The overall average for the number of colonies formed per condition (Normal, HIV-spiked or FBS-spiked) (as normalised by the geometric means) was subsequently calculated. Cells isolated from the 19 UCB units used in comparative analyses, were used to compare normal CFUs to HIV-1-spiked CFUs and HIV-1-spiked CFUs to FBS-Spiked CFUs (Table 15).

The addition of FBS to normal CFUs was done as a control for the HIV-1-positive serum. It was added as a control after it was found that higher numbers of colonies grew in HIV-1-spiked wells than in normal wells (found for the first 7 patients of the 30-patient cohort). FBS was subsequently added in order to normalise for the serum component of the HIV-1-spiked samples, which might have caused the increased CFU growth observed from normal to HIV-1 colonies. In retrospect, human serum would have been a more appropriate control since HPCs grow more effectively when spiked with human serum than FBS.

Three comparisons were done:

- Normal CFUs vs. HIV-1-spiked CFUs (high VL)
- Normal CFUs vs. FBS-spiked CFUs and
- HIV-1-spiked CFUs (high VL) vs. FBS-spiked CFUs

The data was not normally distributed, but was skewed as a result of the large inter-patient variability. For this reason, the sign rank test – a non-parametric test (applied to the log-values of the data) – was used to calculate the geometric means with a 95% confidence interval (CI). Significant differences are displayed by the p-value of the log scale's Sign rank test (significant differences are determined for $p \leq 0.05$). Results generated by Wilcoxon's test are displayed in Table 15.

Table 15: Comparison of CFU numbers for normal-, HIV-1-spiked (high VL) and FBS-spiked conditions in HIV-1-infected- and non-infected colonies respectively

Comparison of HIV-1 negative CFUs (confirmed by Ultrio-Plus)

CFU	Number of Units compared	Condition	Average	95% CI		p-value
				lower limit	upper limit	
GM	12	Normal	9.0	4.1	13.9	
		HIV-1	10.5	3.7	17.3	0.36
GM	12	HIV-1	10.5	3.7	17.3	0.44
		FBS	11.4	2.7	20.1	
MK	12	Normal	4.8	2.8	6.7	
		HIV-1	6.6	4.1	9.0	0.13
MK	12	HIV-1	6.6	4.1	9.0	0.36
		FBS	5.9	3.3	8.6	
BFU-E	12	Normal	4.8	1.5	8.0	
		HIV-1	3.8	0.9	6.6	0.33
BFU-E	12	HIV-1	3.8	0.9	6.6	0.47
		FBS	3.6	0.6	6.6	
GEMM	12	Normal	1.3	0.1	2.4	
		HIV-1	1.5	0.2	2.8	0.39
GEMM	12	HIV-1	1.5	0.2	2.8	0.15
		FBS	0.7	-0.1	1.4	

Comparison of HIV-1 positive CFUs (confirmed by Ultrio-Plus)

CFU	Number of Units compared	Condition	Average	95% CI		p-value
				lower limit	upper limit	
GM	7	Normal	8.4	3	16	
		HIV-1	7.7	2	17	0.50
GM	7	HIV-1	7.7	2	17	0.33
		FBS	8.6	2	13	
MK	7	Normal	4.1	1	14	
		HIV-1	5.4	3	14	0.44
MK	7	HIV-1	5.4	3	14	0.17
		FBS	6.0	2	8	
BFU-E	7	Normal	3.1	-1	18	
		HIV-1	3.0	-1	12	0.34
BFU-E	7	HIV-1	3.0	-1	12	0.23
		FBS	3.4	0	6	
GEMM	7	Normal	0.3	-1	5	
		HIV-1	0.1	-4	12	0.30
GEMM	7	HIV-1	0.1	-4	12	0.20
		FBS	0.4	0	1	

This sample number was also too small to accommodate for the large data variance and inter-patient variability observed between patients and subsequently yielded no statistical significant values for any of the comparisons.

7.8 HIV-1 infection of freshly isolated CD34+ HPCs

Samples with cell counts too low to allow for freezing of the cells were also spiked with HIV-1 (as described under Methods). These UCB units (three in total) were infected with HIV-1 after two to four days of incubation and led to apoptosis of many of the cells (measured cell viability with flow cytometry; results not shown). This seems to confirm that some CD34+ cells in CFU-assays could have been infected, but it does not exclude the possibility that infection of more differentiated lymphocytes within the CD34+ were responsible for viral dissemination within the fresh cells. The samples were furthermore centrifuged and sample cell pellets washed in preparation for Ultrio-Plus® analysis where samples were confirmed to be HIV-1 positive (results not shown).

CD34+ cells have been shown to contain the receptors necessary for HIV-1 entry (CD4, CXCR4 and CCR5) (Carter et al., 2010). It has furthermore been suggested that a direct relation exists between HIV-1 infection and the co-expression of HIV-1 entry receptors on HPCs (Zhao et al. 1998). Results for infection of freshly isolated HPCs seem to confirm that the heterogeneous CD34+ HPCs contained some CD34+ progenitors that were susceptible to HIV-1 infection (had co-receptors for HIV-1 entry). These cells could have become infected and could have spread the HIV-1 infection to adjacent cells, as they differentiated in the culture medium (DMEM) into mature cells before undergoing apoptosis.

In addition, the HIV subtype (in particular HIV-1 subtype C) and HIV tropism have been found to play a role in whether or not cells could be infected with HIV-1 (Mullis et al., 2012; Redd et al., 2007a). Macrophage-tropic HIV strains have been shown to be able to stably infect CD34+ cells, whereas T lymphocyte-tropic strains were unable to do so (Zhao et al. 1998; Alexaki and Wigdahl 2008).

The HIV-1 positive QC samples used to spike cells in this study were prepared from HIV-1-positive individuals (HIV-1 subtype C; prepared by the SANBS). HIV-1 subtype C is the most prevalent subtype in South Africa and has also been implicated in HIV-1 infection of primitive HPCs in other studies (Redd et al., 2007a).

However, these results do not exclude the possibility of contamination with other more mature cell types that could have preferentially been infected by HIV-1 and caused initial infection of freshly isolated HPCs.

7.8.1 Thawing of cryogenically frozen CD34+ HPCs

The method of thawing segments obtained from UCB units for post-thaw evaluation of the viability of an UCB unit will be extremely important in an UCB setting. Thawing of the UCB units prior to transfusion generally involves a rapid, warm thaw (at 37˚C) without DMSO dilution. Units are subsequently directly transfused after thawing and DMSO is diluted with the patient's own blood. This has been cause for minor concern since allergic reactions towards DMSO have been reported in a minority of cases and many studies are looking into protocols for freezing and thawing of UCB (Watt et al., 2007; (Liseth et al., 2009).

Cells in this study were quick-thawed in a water bath at 37˚C followed by washing cells to dilute the toxic effects of DMSO. Post-thaw cell viability was, however, at least 33% to 50% less than for the same individual's pre-thaw samples (as counted with the hemocytometer). These post-thaw viabilities correlate with viabilities found in other studies: 1) Lee *et al.* (2008) found a reduction in median viability down to 71% (range 31 to 89%) and found a correlation between lower CD34+ viability and lower engraftment efficacy of neutrophils and platelets; 2) Allan *et al.* (2002) had a reduction in viable CD34+ cell count from 3.6 x 10^6/kg at the time of harvest and 2.0 x 10^6/kg after thawing (55% viability post-thaw); Yang *et al*. (2005) reported a median post-thaw recovery of viable CD34+ cells of 66.4% (range between 36.1 and 93.6%). A correlation between post-thaw CD34+ recovery and engraftment of progenitors was also evident. Yang *et al.* (2005) furthermore suggested that viability of CD34+ HPCs post-thaw is a reliable tool to predict engraftment and is preferred to pre-thaw cell numbers, since it accounts for unforeseen cell loss during cryopreservation.

Similarly, our findings suggested a loss of CFU-ability in thawed HPCs, where post-thaw HPCs yielded only 58% of the CFUs compared to when they were freshly isolated (14 vs. 24 CFUs for the same 16 individuals; Table 16 and Table 17). Viabilities of UCB units must meet the currently acceptable transplantation cell dosage i.e. 2.5 × 10^6–5.0 × 10^6 CD34+ cells/kg body weight (Berz et al., 2007). Keeping this in mind, optimisation of the thawing protocol is necessary in an UCB bank setting, in order to minimise unnecessary cell losses (Meyer et al., 2006; Beaujean et al., 1998).

Table 16: Summary of CFU results obtained for the thawing of cryogenically preserved CD34+ HPCs

Colony types	CFUs from Freshly isolated UCB HPCs	CFUs from thawed UCB isolated HPCs
CFU-GM	13	6
CFU-MK	4	4
CFU-BFU-E	5	3
CFU-GEMM	2	1
Average total colonies	24	14
Sample total	16	16

Table 17: Comparative data for CFUs of freshly isolated HPCs and thawed HPCs

No.	Patient ID	CFUs from freshly isolated HPCs					CFUs from thawed HPCs				
		CFU-GM	CFU-MK	BFU-E	CFU-GEMM	Total (sum)	CFU-GM	CFU-MK	BFU-E	CFU-GEMM	Total (sum)
1	20110629 P1	18	0	8	2	28	11	5	9	6	31
2	20110629 P2	24	1	10	3	38	5	4	6	1	16
3	20110629 P3	5	1	3	1	10	5	5	7	0	17
4	20110727 P4	12	4	2	0	18	8	0	3	0	11
5	20110727 P1	13	3	14	4	34	0	0	0	0	0
6	20110727 P2	0	0	0	0	0	0	0	0	0	0
7	20111122 P1	26	7	11	5	49	3	1	1	1	6
8	20111122 P2	2	0	0	0	2	0	0	0	0	0
9	20111011 P1	4	4	2	1	11	9	7	8	0	24
10	20120209 P2	2	1	1	1	5	0	1	0	0	1
11	20111101 P1	16	6	9	1	32	11	8	1	0	20
12	20111101 P2	17	8	6	1	32	21	15	11	1	48
13	20120206 P1	11	6	7	2	26	2	1	0	0	3
14	20120214 P1	13	4	1	4	22	0	2	0	0	2
15	20120201 P3	16	6	4	2	28	18	13	8	2	41
16	20120201 P4	24	10	3	4	41	0	0	0	0	0
Total Average CFUs		**13**	**4**	**5**	**2**	**24**	**6**	**4**	**3**	**1**	**14**

Two samples were tested with a changed protocol: Instead of a rapid warm thaw of frozen cells, cells were thawed on ice while constantly rinsing them with cold FBS. Viability pre-freeze was 400 and 360 cells/µL respectively; vs. 200 and 100 cells/µL post-thaw respectively. It is difficult to comment on the results of only two samples thawed in this manner and this alternative slow thaw with constant DMSO dilution for CD34+ enumeration and viability analyses of UCB segments merits further investigation. Based on these results (although they are preliminary), it might be necessary to re-evaluate the method of thawing of UCB units for transplantation purposes.

7.9 Ultrio-Plus® CFU-screening results

Owing to the nature of CFU-assays, colonies grown give an indication of the initial progenitor content. More differentiated HPCs would not be able to form colonies whereas only primitive cells would. Therefore, infecting HPCs at the onset of the CFU-assay was thought to deliver one of these two possible results:

a) More mature, susceptible HPCs would be infected and undergo apoptosis before forming colonies *in vitro*; thus HIV-1 would not be detected in CFU screening.
b) Immature HPCs could potentially be infected and HIV-1 would subsequently be detected within colonies with the Ultrio-Plus assay (using transcription mediated amplification).

The indirect effect of HIV-1 on the HPCs' ability to form CFUs *in vitro* has been discussed earlier (Section 7.6 and 7.7). The effects seen in the aforementioned affected morphology could not directly be attributed to HIV-1 infection in previous results. However Ultrio-Plus® screening of HIV-1-spiked CFUs revealed that some progenitors were indeed infected (Table 18).

Despite being infected, these progenitors were able to produce colonies (although smaller with less hemoglobinisation in some individuals than when compared to normal CFUs). This could indicate that a small compartment of HPCs might be responsible for latent persistence of HIV-1 as is seen in individuals receiving HAART, thus serving as viral reservoirs. Many studies disagree with HPCs being infected or serving as potential viral reservoirs (as mentioned earlier). However, more recent studies are in agreement with findings from this study. These recent studies have concluded the infectability of HPCs with HIV-1 and have suggested the distinct possibility of HPCs serving as viral reservoirs (Redd et al., 2007a; Mullis et al., 2012; Carter et al., 2010; Li et al., 2012).

Of the 30 UCB units spiked with the high HIV-1 VL, six were infected during the CFU-assay, and one of the samples was infected with the HIV-1 low VL i.e. seven cultured UCB units were apparently infected (however one cannot exclude the possibility that the HIV measured was residual serum that was used to spike the samples and could therefore potentially not reflect true infection). Of these seven patients, three patients were HIV-1 positive prior to UCB collection, two were HIV-1 negative and two declined to undergo HIV-1 testing; they therefore had undetermined HIV-1 statuses. However, HIV-1 could not be detected in UCB plasma, prior to spiking of CD34+ HPCs in CFU-assays, in any of the 30 patients. Therefore, infection of CFUs must have occurred *in vitro*. It does not seem that previously infected HIV-1 positive patients were more prone to CFU infection, since HIV-1 negative individuals' CFUs were also infected (Table 18).

The viral load might also be a determining factor for infection, since six of the seven patients were infected with the higher viral load (2.5 x 10^6 IU/ml obtained from the Department of Virology, UP) while one patient was infected with a lower viral load (92 IU/ml obtained from the SANBS). A serial dilution of HIV-1 viral load could help to determine the minimum viral load necessary for infection.

Table 18: Ultrio-Plus® screening results on HIV-1-spiked and Normal CFUs

	Patient File	Ultrio-Plus® screening results		
No.	**HIV status**	**Normal (CFU or Plasma)**	**HIV spiked CFU High Viral load (Dept of Virology)**	**HIV spiked CFU Low Viral Load (SANBS QC)**
8	NEG	Non-reactive	Non-reactive	Non-reactive
11	NEG	Non-reactive	Insufficient vol	Insufficient vol
12	POS	Non-reactive	Non-reactive	Non-reactive
13	POS	Non-reactive	Non-reactive	Non-reactive
14	POS	Non-reactive	Reactive *	Non-reactive *
15	NEG	Non-reactive	Reactive **	Non-reactive**
16	UNKN	Non-reactive	Non-reactive	Non-reactive
17	POS	Non-reactive	Reactive *	Non-reactive *
18	POS	Non-reactive	Non-reactive	Non-reactive
19	DECL	Non-reactive	Reactive	Non-reactive
20	DECL	Non-reactive	Reactive	Non-reactive
21	NEG	Non-reactive	Non-reactive	Non-reactive
22	UNKN	Non-reactive	Non-reactive	Non-reactive
23	UNKN	Non-reactive	Non-reactive	Non-reactive
24	UNKN	Non-reactive	Non-reactive	Non-reactive
25	POS	Non-reactive	Non-reactive	Non-reactive
26	NEG	Non-reactive	Non-reactive	Non-reactive
27	POS	Non-reactive	Reactive *	Non-reactive *
28	NEG	Non-reactive	N/A **	Reactive **

* Patients positive according to patient files
** Patients negative according to patient files

The Welch's t-test (for unequal sample size and variance) was subsequently used to compare HIV-1-spiked vs. FBS-spiked conditions of the 12 non-reactive UCB units to the seven reactive units' conditions. Data for individual CFU-assays for the seven vs. 12 patients is shown in Table 13 and Table 14. In order to verify how poorly HIV-1-spiked colonies fared, the number of colonies formed in the HIV-1-spiked wells were calculated as a percentage of the numbers of colonies formed in FBS-spiked wells. This was done for both the HIV-1-infected CFUs (seven UCB units) and the non-infected units (12 UCB units). For example, five GM colonies in HIV-1-spiked wells vs. 10

GM colonies in FBS-spiked wells give a percentage ratio of 5/10 or 50%. The ratio indicates that in that patient, HIV-1-spiked colonies fared 50% as well as FBS-spiked colonies for the same patient. These percentages were calculated within each individual and the average percentages were determined for the total number of patients in the respective cases (average ratio for seven HIV-1-infected UCB units vs. average ratio of 12 non-infected UCB units). Table 19 illustrates the comparison between HIV-1-infected CFUs (seven UCB units) and non-infected CFUs (12 UCB units), expressing the ratios (as percentages) of HIV-1-spiked wells vs. FBS-spiked wells.

Table 19: The ratio of HIV-1-infected colonies vs. FBS-spiked colonies compared between the seven HIV-1-positive UCB units and the 12 HIV-1-negative UCB units

Comparison:	HIV-1 vs. FBS %	HIV-1 vs. FBS %	HIV-1 vs. FBS %	HIV-1 vs. FBS %	AVERAGE %
	GM	MK	BFU-E	GEMM	CFU
7 HIV POS AVERAGE	99.94	123.93	0.93	450.00	168.70
STDEV (HIV POS)	45.49	124.00	2.27	217.94	
12 HIV NEG AVERAGE	124.02	112.91	112.43	70.00	104.84
STDEV (HIV NEG)	123.33	62.14	141.40	67.08	
p-value: significance ≤ 5%	26.61	41.49	0.74	4.35	

STDEV = standard deviation (expressed as a percentage)

Table 19 indicates HIV-1-spiked wells' colony growth for BFU-E and CFU-GEMM was statistically significant between HIV-1-infected CFUs and non-infected CFUs (BFU-E = 0.74% and –CFU-GEMM = 4.35% where significance ≤ 5%). These results are in agreement with earlier results where HIV-1 spiked CFUs displayed less BFU-E (Figure 39).

Figure 40 gives an outline of all the analyses done and results obtained which were described in this chapter.

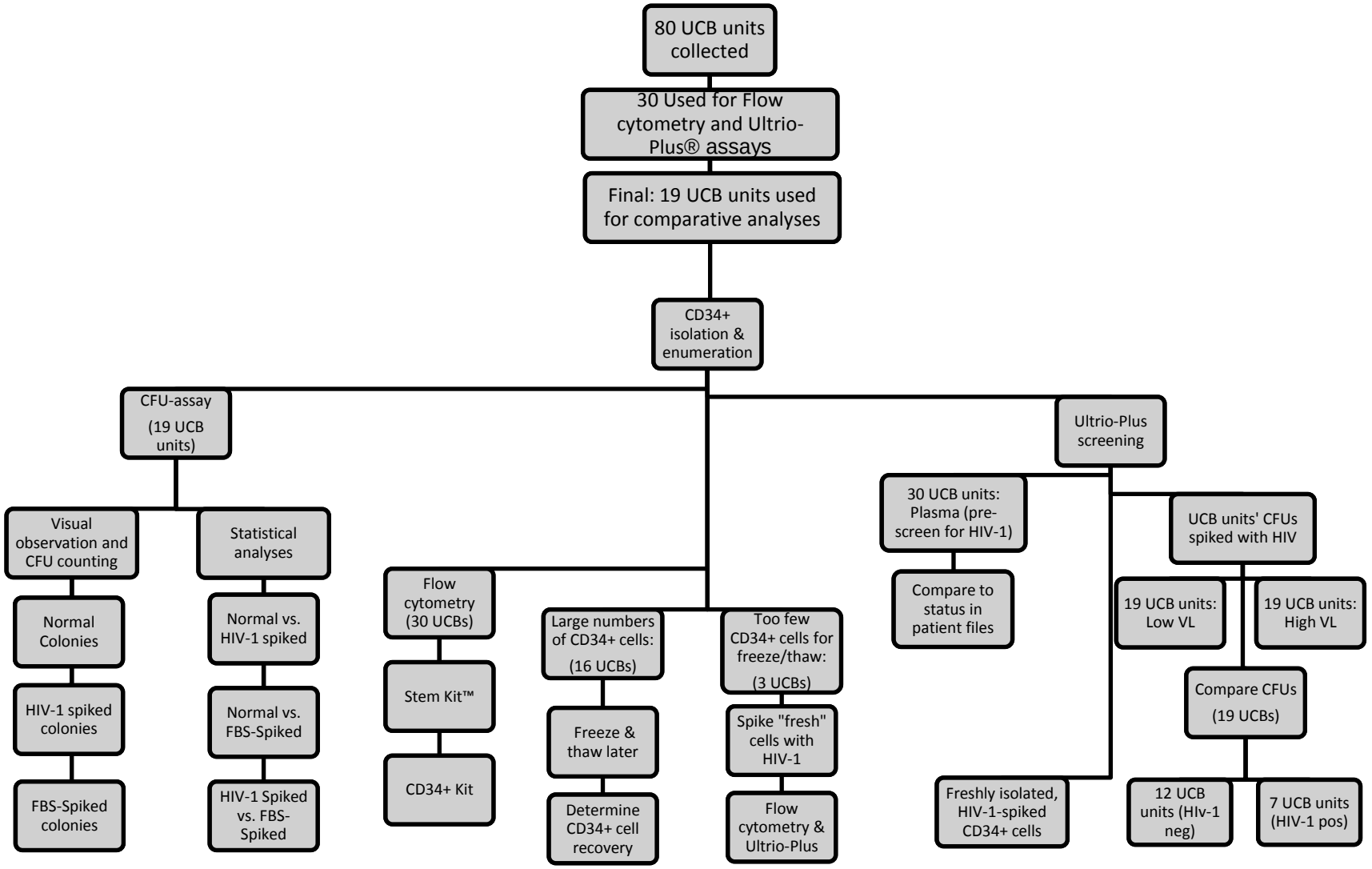

Figure 40: Outline of methods used and results obtained in Chapter 7

7.10 Conclusion

HSCs have been studied extensively through various quantitative and qualitative analyses (Schroeder, 2010). These cells have become the best understood type of adult stem cell, partly because of the relative ease with which they are manipulated (Szilvassy et al., 1990). Their potential to form CFUs was first described by Till & McCulloch (1961) who discovered them in the 1960s and defined them as cells that could produce haematopoietic nodules in the spleen of irradiated animals (Till and McCulloch, 1961; Till et al., 1964).

This study set out to develop the technique for CD34+ isolation from UCB and to establish the CFU-assays and subsequent colony identification in the laboratory in Pretoria. It furthermore set out to investigate HIV-1's ability to infect CD34+ HPCs and/or affect the colony forming ability of CD34+ HPCs. The study was a preliminary investigation into using UCB HPCs as potential genetic vectors for gene-therapy approaches directed towards a cure for HIV-1 infection. HIV-1 individuals could then potentially receive these genetically resistant HPCs via BM transplantation to combat HIV-1 infection, as was indicated in the proof of concept study done by Hütter *et al.* (2009).

HIV-1 has been implicated in affecting CFU growth through decreasing the primitive cells' clonogenic capacity through apoptosis. Cytokines and growth factors (involved in inflammation and immune activation) released upon viral entry into the host have also been implicated in decreased HSC differentiation and expansion (Gibellini et al., 2007; Zauli et al., 1996). Accordingly, HIV-1-spiked CFUs did not fare as well as FBS-spiked CFUs. Colonies were often smaller, less hemoglobinised (in the case of BFU-E or CFU-e) and surrounded by cellular debris. This is thought to be due to the effect of HIV-1 present in the well and confirms that HIV-1 had a negative impact on normal haematopoiesis. In terms of colony number, no statistically significant differences could, however, be calculated for HIV-1's influence on CFUs in this study. This might be due to the quality of UCB units collected, the small sample cohort (19 UCB units which could be compared across all the analyses), together with large inter-patient variability observed from one UCB unit to another. The fact that a marked decrease in CFU-growth was not seen could be attributed to these limiting factors, since the CFU-assay has been used for indicating decreased CFU growth of HPCs due to HIV-1infection in other studies (Redd et al., 2007a,b; Louache et al., 2012).

It is important to note that, despite the fact that the *in vitro* effect in our study seems negligible, differences could increase exponentially *in vivo,* leading to cytopaenias as observed in HIV-1-infected individuals. These differences were also varied among the patients, with some patients more severely affected than others.

Unfortunately, the study design did not allow for absolute clarification to the question of whether or not HIV-1 infects or only affects CD34+ HPCs. Although preliminary results obtained in this study seems to indicate that HIV-1 could elicit a cytotoxic effect detrimental to HPC growth and differentiation *in vitro* (i.e. affecting HPCs), these results are subjective and must be substantiated with quantitative assays e.g. immunofluorescence of GFP-labelled HIV-1 constructs and rt-PCR analysis. Preliminary results obtained for screening the CFUs with the Ultrio-Plus assay for detection of the HIV-1 genome, did however indicate that direct infection of primitive HPCs might be possible.

There is still a lot of controversy regarding HIV-1's influence on primitive HPCs however, more recent studies (Mullis et al., 2012; McNamara and Collins, 2011; Carter et al., 2010) are finding evidence for direct and indirect methods of HIV-1 infection of CD34+ HPCs. Table 20 is a short summary of some of the conflicting opinions in literature regarding HIV-1 infection of CD34+ HPCs.

Table 20: A summary of findings relevant to the enquiry of whether or not HIV-1 could infect or affect CD34+ HPCs

A summary of relevant findings from other researchers related to the enquiry of whether or not HIV-1 could infect or affect CD34+ HPCs						
Cell Source used	**Findings**	**HIV-1 infected cells**	**HIV-1 affected cells**	**Techniques used for analysis**	**Article type**	**Refe-rence**
1) CD34+ and CD133+ HPCs from UCB; 2) Purified BM-derived HPCs	HIV can infect multipotent HPCs with an immature phenotype	Active and latent HIV infection; *in vivo* and *in vitro*	Active cytotoxic infection	1) CD34+ magnetic bead isolation from HIV-infected donors; 2) CFU-assays; 3) Plasmid construct & PCR; 4) Flow cytometry	Experi-mental	Carter et al., 2010
1) CD34+ HPCs isolated from PB	In HIV-1 subtype A and D infects HPCs (but not preferentially)	*In vivo* HIV-1 infection of HPCs can affect haematopoiesis and colony-forming ability	Active HIV-1 infection might be cytotoxic to HPCs	1) Single colony infection assay; 2)CFU-assays	Experi-mental	Mullis et al., 2012
1) UCB mononuclear cells; 2) CD34+ cells from PB mononuclear cells	The HIV-1C clone could infect HPC-CFUs *in vitro* and *in vivo* at a significantly higher rate than the HIV-1B clone	HIV-1 subtype C isolates infects HPCs *in vitro*	*In vivo* HIV-1C infection associated with higher rates of anaemia	1) Single colony infection assay; 2)Quantitative proviral analysis; 3)Quantitative real-time PCR; 4) Elisa assay	Experi-mental	Redd et al., 2007

A summary of relevant findings from other researchers related to the enquiry of whether or not HIV-1 could infect or affect CD34+ HPCs						
1) CD34+ BM progenitor cells	CD34+ BM cells are infected with HIV in a subset of seropositive individuals	Subset of CD3+ BM progenitors infected *in vivo* and might serve as viral reservoirs	Depressed haematopoiesis in methylcellulose colony forming unit assays	1) Co-culture viral isolation and 2) PCR; 3) CFU-assays	Experi-mental	Stanley et al., 1994
1) CD34+ BM HPCs	HIV-1 affects CD34+ HPCs through apoptosis without direct infection	A progressive increase of apoptosis in liquid cultures of BM CD34+ cells after 2 hours exposure to HIV-I.	*In vitro* exposure of CD34+ cells to HIV-l resulted in marked impairment of their colony-forming ability, without evidence of direct HIV- l infection.	1)Isolation of CD34+ HPCs; 2) DNA labelling and Flow cytometry; 3) PCR; 4) Clonogenic assay	Experi-mental	Zauli et al., 2007
1) PB and BM mononuclear cells	Secondary colony-forming cells numbers were significantly decreased in patients with advanced disease	Secondary colony-forming cells numbers were significantly decreased in patients with advanced disease	HIV-1-associated BM failure possibly related to abnormal BM stromal function or intrinsic defects in stem or progenitor cell compartments.	1) Long term culture initiating cells; 2)CD34+ cell isolation	Experi-mental	Sloand et al., 1997
1) CD34+ BM cells from HIV-1 infected	Loss of primitive haematopoietic progenitors in	Direct infection of CD34+ HPCs not observed	Long-term CD34+ cultures from HIV-1 patients generated	1) Flow cytometry; 2) Long-term culture initiating colonies;	Experi-mental	Maran-din et al., 1996

A summary of relevant findings from other researchers related to the enquiry of whether or not HIV-1 could infect or affect CD34+ HPCs						
individuals	patients With HIV-1 Infection		much fewer colonies than controls	3)PCR		
1) CD34+ BM cells from HIV-1 infected individuals	HIV-1 infection of CD34+ HPCs lead to a reduction of in vitro colony formation in comparison to normal donors	No HIV-DNA could be detected in BFU-E- and CFU-GM-derived colonies in culture; progenitor infection could not be excluded	Hematopoietic progenitor cells of HIV-infected patients have defective in vitro growth	1) Flow cytometry; 2) Long-term culture initiating colonies; 3) PCR; 4) Indirect immunofluorescence & *in situ* hybridisation	Experi-mental	Louache et al., 1992
1) CD34+ PB-derived HPCs	*In vitro* HIV-l infection of purified HPCs in single-cell culture	A minority of primitive HPCs, but not of the multipotent type, is susceptible to in vitro HIV infection	Indicated sensitivity of HPCs to *in vitro* HIV infection	1) Enriched HPC populations challenged with purified or un-purified HIV-1 strains; 2) Cloned in uni-cellular methylcellulose culture; 3) CFU-assay; 4) PCR and reverse transcription-PCR	Experi-mental	Chelucci et al., 1995

A summary of relevant findings from other researchers related to the enquiry of whether or not HIV-1 could infect or affect CD34+ HPCs						
1) CD34+ HPCs	CD34+ HPCs carrying proviral DNA can be found in vivo in a subpopulation of HIV-1-infected patients	Evidence *for* and *against* direct infection of HPCs	References *for* and *against* indirect infection of HPCs	Review article	Review	Alexaki et al., 2009
1)Multi-potent HPCs; 2)Monocytes & tissue mast cells; 3) CD34+ BM cells	Some HPCs have the potential to generate HIV reservoirs	Evidence *for* and *against* direct infection of HPCs	References *for* and *against* indirect infection of HPCs	Review article	Review	McNamara et al., 2011
1) CD34+ BM-derived HPCs	HIV-1 Infection of BM HPCs and their role in trafficking and viral dissemination	Evidence *for* and *against* direct infection of HPCs	References *for* and *against* indirect infection of HPCs	Review article	Review	Alexaki and Wigdahl, 2008

The CFU-assay has proven to be a valuable model for detection of an UCB unit's potency/engraftment ability, but it needs to be substantiated with other quantitative analyses in order to give a better indication of HIV-1's effect on primitive HPCs' infectability. Some factors that could have influenced the outcome of the CFU-assay are listed below.

1. Purity of magnetic isolation of CD34+ cells was most notably influenced by the volumes of UCB units post-plasma-depletion and was also affected by the time it took from collection (after birth) to CD34+ isolation.

2. Apart from sample integrity, flow cytometric evaluation of purified HPCs was found to be highly dependent on the three classes of mABs directed against the CD34+ epitope that were used during analyses. The CD34+ epitope is found on essentially every HPC. However, Steen and Egeland (1998) demonstrated that Class I and Class II epitopes are down-regulated as HPCs differentiate into mature myeloid progeny. This has implications for selecting appropriate mABs directed against the CD34+ epitope, as was evident in the comparison between results obtained for the Stem-Kit™ and CD34+ Pool Kit respectively.

   The Stem-Kit™ was not always able to detect the same number of HPCs present as the CD34+ Pool kit. On average Stem-Kit™ detected about 10% fewer CD34+ HPCs than did the CD34+ Pool Kit. In order to ensure isolation of most of the primitive HPCs, both magnetic isolation beads and mABs should include at least one antibody representative of each class of mABs. This would increase the cell yield and give a better representation of the true number of HPCs in an UCB unit.

3. Other factors that could have contributed to the lack of statistical significance are: the method of UCB collection; the time until a unit was processed; the number of viable; primitive CD34+ HPCs in the units; the number of CD34+ HPCs isolated from units; humidity and percentage O_2 and CO_2 during incubation; the use of FBS instead of human serum and the variability in manual CFU identification and counting.

   Furthermore, low numbers of detected HIV-1 infection in the 19 UCB units could be explained by the reasons that Mullis *et al*. (2012) mention. These include the fact that HIV-1 has a cytotoxic effect on progenitors, causing infected progenitors to die off.

Furthermore, HIV-1 could potentially induce differentiation of primitive cells, leading to a depletion of progenitors (as was also found by Marandin et al. (1996)). Finally, a mechanism has also been suggested whereby CD34+ HPCs could exercise resistance towards HIV-1 infection through cyclin-dependent kinase-inhibitor p21 (Zhang et al., 2007).

CFU identification and scoring could be improved by using an electronic colony counter, such as the STEMVision™ instrument (http://www.stemcell.com). Increasing the number of UCB units and processing all units within 24 hours after delivery would also reduce sample variability and could potentially influence the statistical significance of differences observed.

CFU results together with the screening of HIV-1-spiked CFUs by the Ultrio-Plus® assay, indicated that HIV-1 could not only potentially affect HPCs' ability to form colonies *in vitro* (von Laer et al., 1990), but could also infect these cells.

The presence of CXCR5 and CCR4 co-receptors on some primitive HPCs has been confirmed and HPCs could thus theoretically be infected by HIV-1 (Redd et al., 2007a; Alexaki and Wigdahl., 2008). These results correspond to results obtained by Carter *et al.* (2010), who showed that a proportion of HPCs can be infected by HIV-1 *in vivo* and *in vitro*. Carter *et al*. (2010) furthermore concluded that the proportion of infected HPCs corresponded to the number of cells that expressed both co-receptors (CXCR4 and CCR5) as was also found by Zhao *et al*., (1998).

Recent studies have additionally demonstrated an interesting and much overlooked method of viral dissemination in CD4+ T cells, which involves direct cell-to-cell contact through structures known as "virological synapses", "filopodia" and "nanotubes" (Rudnicka et al., 2009; Martin et al., 2010). According to these findings, infected cells could transmit viral particles to adjacent cells and one infected cell could even infect many cells simultaneously through so-called "polysynapses" (Rudnicka et al., 2009). The possibility therefore exists that HPCs might also be susceptible to the same mechanism of viral dissemination, even in the absence of CXCR4 or CCR5, and act as viral reservoirs (Carter et al., 2010).

The fact that HPCs could not only be affected *in vitro* by HIV-1 but also infected by it has definite implications for future studies envisioned. HPCs that have already been affected and potentially infected by HIV-1 would not be ideal targets for gene therapy directed at creating an HIV-1 resistant immune system. Rather, HIV-1-negative allogeneic HPCs – which could be

obtained from UCB units – should be used for these genetic manipulations. HIV-1-negative UCB units from HIV-1-positive individuals could serve as a vital resource of these primitive HPCs for genetic engineering. These UCB units would otherwise be discarded based on the fact that the mother was HIV-1 positive and would therefore not be used for normal UCB transplantation purposes.

Future studies on the effect of HIV-1 on HPCs need to determine the effect of different HIV-1 subtypes on CD34+ HPCs' ability to form CFUs *in vitro*. Furthermore, isolation of more primitive HPCs (such as $CD34^{+}CD38^{-}CD133^{+}$ subpopulations) and HIV-1's effect on these more primitive populations could potentially shed light on the particular subpopulations of HPCs that are infected by HIV-1.

7.11 References

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CHAPTER 8

8 Conclusion

This thesis examined two interrelated matters that impact on the delivery of health care services in South Africa. The first is the lack of genetically compatible resources for transplantation purposes such as BM or UCB stem cells and; the second is the impact of HIV-1 infection on the haematopoietic capacity of primitive UCB-derived HPCs, which might - after genetic manipulation - be used in allogeneic UCB transplantation to cure HIV-1 infection.

UCB is an important source of stem cells that could be used as an alternative to BM, to treat haematological and non-haematological diseases. However, South Africa does not have a public UCB SCB and access to the two existing private UCB SCBs is limited to individuals that can afford it. The problem is compounded by a severe shortage of genetically compatible samples in the existing BM registry that are representative of South African demographics. This further aggravates the ever increasing divide between families from different socio-economic classes.

The first step towards establishing a public UCB SCB in South Africa was to investigate public support for the establishment of a public UCB SCB. Due to the novel nature of the investigation for interviewers and interviewees alike, the study was initially conducted as a pilot study. Many obstacles were met and dealt with during the pilot study, which lead to the compilation of a more comprehensive investigation in the principal study. Complicating factors encountered during the informed consent process included cultural differences, religious practices, traditions and superstitions together with language constraints and educational disparity. However, initial concerns that cultural or religious practices might hinder public support were unfounded. Determining factors that could potentially influence public support for the successful establishment of a public UCB SCB (but which can easily be overcome), had to do with educating the general public with regard to stem cells (SCs) and SC banking and overcoming language insufficiencies by translating materials into different languages. Despite these (and other) initial obstacles, preliminary results for assessing public support for establishing a public UCB SCB were favourable.

It would furthermore be helpful to investigate perceptions and opinions of people from different religious or cultural backgrounds regarding UCB donation. The patient cohort in this study was not representative of the entire South African demographics and it will be necessary to understand the attitudes and objections that all cultural or religious groups in South Africa

might have against UCB donation prior to establishing a public UCB SCB. This could potentially shed light on areas where public education with regards to UCB banking is lacking and could furthermore elucidate why the current BM registry has had great difficulty to obtain demographically representative BM donations.

The second important aspect in establishing an UCB SCB was to screen potential UCB units for infectious diseases prior to banking. Consequently, patients were assessed for their willingness to undergo additional HIV-1 screening and to allow the additional screening of their donated UCB units. Although some patients were reluctant to undergo additional HIV-1 screening, the majority of patients (78%) gave consent to do so.

Screening mothers, who wish to donate their UCB, for infectious diseases is used as the standard to determine the suitability of an UCB unit for banking. However, the NetCord-FACT standards for UCB processing and banking have suggested the additional screening of UCB units for infectious diseases. With the high rate of HIV-1 infection in South Africa there is a risk that a mother might be in the latency period of the virus at the time of delivery. The mother might thus test HIV-1 negative at the time of delivery, while in actual fact she is HIV-1 positive. In such a case, the possibility exists that trans-placental transmission of the virus into the UCB unit could have occurred. Therefore, additional safety and quality control measures for collection and screening of potential UCB units, prior to storage or distribution, are necessary.

No validated test for screening UCB units currently exist, thus this study set out to verify the routinely used Ultrio-Plus® assay for screening of UCB units. The Ultrio-Plus® assay is a useful assay, since it can simultaneously detect the presence of HIV-1, HBV and HCV in an UCB unit. The assay was found to be as sensitive and reliable in detection of HIV-1 in UCB as it is to detect the virus in peripheral blood and could therefore be used in routine screening of UCB units.

Finally, alternative treatments to combat HIV-1 infection, such as genetic modification of HPCs to render the cells naturally resistant to HIV-1, are needed. The lack of genetically compatible samples in the current South African BM registry and no alternative resources such as a public UCB SCB, leaves little room for alternative therapies. The only alternative to treat HIV-1 positive individuals with genetically engineered SCs - without the availability of HIV-1 negative allogeneic matched HPCs - be would be through autologous transplantation of their own genetically manipulated cells.

The final component of the study set out to develop the technique of CD34+ isolation from UCB and to establish the CFU-assays and subsequent colony identification in the laboratory in Pretoria. It furthermore set out to investigate HIV-1's ability to infect CD34+ HPCs and/or affect the colony forming ability of CD34+ HPCs.

Unfortunately, the study design did not allow for a conclusive answer to the question of whether or not HIV-1 directly or indirectly affects/infects HPCs. Results obtained for the influence of HIV-1's effect on HPCs correspond to those of recent studies in terms of direct infection of CD34+ HPCs with HIV-1 subtype C (as mentioned in Section 7.10 and Table 18), however, the lack of consistency between studies makes comparison of data extremely difficult (Table 18).

Some major limitations of this component of the study included:

- **The poor quality and blood volume of UCB units collected**

  Appointing a trained, designated physician or nurse to collect the UCB would greatly enhance consistency in the collection method and volume of UCB collected. Working with a single contact would furthermore ease communication between the hospital and the researcher in order to ensure appropriate handling of the sample, storage and timely collection in order to minimise losses in cell numbers and cell viability.
- **The lack of appropriate controls for investigating HIV-1's influence on CFU-assays**

  Since the HIV-spiked cultures received HIV in human serum, the most appropriate control for these experiments would have been human serum. This was not done, and FBS was used instead. In addition, FBS contains various growth factors necessary for cell maintenance and growth (Shah, G., 1999) and has been widely used as a cell-culture-media supplement, producing optimal growth and batch-to-batch consistency (Stemcell™ Technologies; http://www.stemcell/com/~/media). However, it has been shown that human serum could be more advantageous in producing larger numbers of cultured cells (Rauch et al., 2011; Ruszymah et al., 2003). Therefore, FBS might not have adequately presented the potential proliferation that could have been achieved with human-serum-spiked colonies and could potentially account for the fact that no statistical differences were observed between HIV-1-spiked- and FBS-spiked colonies.

Future studies should include the use of human serum as control sample to HIV-1 positive serum if cultures are to be spiked (as opposed to FBS used in this study).

Despite the probability that FBS did not yield potentially as many CFUs as human serum, HIV-1-spiked cells – on average – still produced fewer colonies than FBS-spiked cells (Table 11). The fact that HIV-1 colonies also contained human serum but did not always fare as well as pure FBS colonies could be attributed to HIV-1's influence on HPC's ability to form CFUs (Gibellini et al., 2007).

- **The need for an experienced and knowledgeable person locally to assist in confirmation of CFU-identification**

  Given that colony identification in CFU-assays is subjective, it is imperative to have control measures in place by which to verify the validity of the identified colonies. Although the investigator was sent to Bordeaux to learn the techniques, local expertise on CFU-assays and colony identification is limited. Therefore, the best solution to ensure consecutive, reliable results, is to automate the colony identification and scoring processes (for example through the use of the StemVision automated colony counter). This would not only have saved time in colony identification, but would also have removed inter-individual variability and subjectivity of researchers involved in colony scoring and identification. Future studies would benefit greatly from implementing such an automated colony counter and would allow for the comparison of CFU-data between individuals within the same laboratory, and also between different laboratories.

- **Absence of additional quantitative assays for measurement of colony growth in different culture conditions**

  Visual observations of colony number and size or haemoglobin content were subjective and insufficient to determine whether or not HIV-1 had a direct or indirect effect on CFU-ability of primitive HPCs. Quantitative measurements of haemoglobin content, measurements of colony size, identification with fluorescently labelled antibodies for flow sorting or flow cytometric analyses would have greatly enhanced the quality of data and could have contributed to more reliable results and should be included in future investigations of this nature.

- **Too few samples cultured under similar conditions to obtain statistically significant differences**

  Due to large inter-patient variability larger numbers of collected UCB units are required to make statistically significant claims to results obtained.

These limitations prohibit the drawing of reliable and statistically significant conclusions from the results obtained. Qualitative preliminary results obtained illustrate how HIV-1 seems to affect normal haematopoiesis (as seen in defective colony growth of CD34+ HPCs as assessed by colony forming unit assays, Section 7.6, Figures 38 and 39), but to also directly infect a subset of CD34+ HPCs in some individuals (measured by the Ultrio-Plus® assay). Thus, for future consideration, autologous use of genetically modified HPCs would need to take into account that a subset of HPCs could already have been infected by HIV-1. In order to investigate the cytotoxic effects of HIV-1 on CD34+ HPCs, future investigations could include metabolic profiles of colonies in different stages of normal development and development when exposed to HIV-1. It would also be beneficial to know whether a minimum HIV-1 viral load is necessary to infect primitive HPCs.

In order to identify the specific subset of infected HPCs that were susceptible to HIV-1 infection, future experiments could isolate more primitive stem cells (e.g. c-Kit+Lin-,CD138- cell populations) and assess these cells for the presence of HIV-1 by flow cytometric immunofluorescence. CFU-assays – together with additional quantitative assays such as cell sorting, flow cytometry or immunophenotyping – could furthermore be useful tools in future experiments to investigate the effect of lentiviral transduction of HPCs directed towards rendering them resistant to HIV-1. The proof of concept should however be illustrated in an appropriate animal model (e.g. NOC/SCID mice).

Despite the above mentioned shortcomings, the preliminary results obtained from the CFU-assays and subsequent Ultrio-Plus® screening of cultured CFUs exposed to HIV-1 serum suggest that further investigation into HIV-1's influence on primitive HPCs is merited.

Finally, concerns related to storing UCB units is the volume of UCB collected which yields only a limited number of cells for transplantation purposes. However, many laboratories abroad are working on techniques to expand HPCs while retaining their primitive state. Once these techniques have been standardised, UCB units could potentially be expanded to yield HPCs in

excess of what is required for normal use in allogeneic transplantation for haematological diseases. These extra HPCs could then routinely be made available for genetic manipulation, to provide HIV-1 resistance to patients. However, as was evident in this study, the quality of the UCB units is extremely important for all downstream applications and standardised techniques for UCB collection, storage and handling should be implemented if UCB is to be used as a regular source of CD34+ HPCs.

Another important source of potential HPCs could be HIV-1 negative UCB units, collected from confirmed HIV-1 positive mothers. These units would not be accepted in an UCB SCB for normal transplantation and would be discarded, unless they could be stored in an UCB-bank-bio-repository to be used for applications in genetic manipulation. Even if a certain percentage of primitive HPCs in these units are infected by the HIV-1 virus, all of the cells would not be negatively affected. Thus, genetic manipulation of the HPCs to express natural resistance to HIV-1, could theoretically yield normal-functioning, HIV-1 resistant HPCs from these units. Infants, born to HIV-1 infected mothers with low detectable virus that also received HAART, might in future be transplanted with their own genetically-modified HPCs in order to reduce mother-to-child-transfer of the virus through breast-feeding.

The persistent need for alternatives in the treatment of haematological abnormalities and HIV-1 infection underscores the need for a public UCB SCB in SA. This would provide more South Africans with access to previously unavailable treatment in the form of affordable, genetically compatible stem cells for bone marrow transplantation. Working towards this goal, the preliminary investigations performed in this study demonstrate that the necessary support exists to establish a public UCB SCB in South Africa. The favourable outcomes of the components investigated in this thesis contribute to the foundation for the larger projects, mentioned previously, to expand on.

9 Annexures

ANNEXURE 1: Patient interview

Good Morning Ladies,

My name is Madelein, and this is my colleague, Isabella. We are from the University of Pretoria and we are working together with staff here at the hospital in a study. Our goal is to build a public facility, called an umbilical cord blood stem cell bank, and we are here today to see what you think about this idea and if you would support a public umbilical cord blood stem cell bank.

We would like to give you some information about the stem cell bank, what it is and how it will work and then go through a questionnaire with you. This is voluntary and you do not have to fill in the questionnaire if you don't want to.

First of all, you might wonder what is an umbilical cord blood stem cell bank?

It is not like a bank where you go to save or withdraw your money. It will work almost in the same way that the South African National Blood Bank works. The blood bank collects donated blood and stores it and when someone needs blood – like when they were in an accident and has lost a lot of blood - the blood bank is able to give them the blood that they need.

In a similar way, the umbilical cord blood bank will be a facility that will collect and store umbilical cord blood that has been donated to the bank by pregnant mothers. But before we can open the new facility we would first like to know if pregnant women would like to make use of such a facility.

What is umbilical cord blood?
It is the blood that is left in the placenta (also known as the "gobo or inghubo" – the "blanket") and the umbilical cord, which can be collected after a baby is born.

So why would we like to store umbilical cord blood?

Because the blood that is left in the umbilical cord and placenta after the baby has been born, contains special cells – called stem cells. These stem cells can be used to treat people with certain types of cancers for example leukaemia (blood cancers) and certain genetic diseases. These are very rare diseases and the chance of your baby getting one of these diseases is very small. So these stem cells will not be used to treat a sick child with a cold, the flu or a stomach bug, it is for vary rare/ uncommon genetic diseases or certain cancers.

That is why we would like to ask mothers if they think that they would be willing to donate their umbilical cord blood when the baby is born and to store those cells in the bank, if such a facility were available.

This way, if a mother comes to us with a sick child with one of these rare diseases, we can find a match for the child in the bank and give the cells to a doctor that can treat the patient.

How and where will the blood be collected?
To answer this, we need to show you where the placenta comes from and when is it needed by your baby:
You fall pregnant when one of your egg cells are fertilised by a sperm from your husband. This fertilised egg then implants in the uterus and where it implants, the placenta starts to develop. The role of the placenta/ gobo is to transfer nutrients and oxygen from your body to your baby, so that your baby can grow and develop. The placenta is only important to the baby, while the baby is still in the womb, growing. After the baby is born, the placenta also comes out and the doctors usually throw it away/ send it off to be burned. The placenta has to come out, otherwise the mom can get very sick.

If you have a normal birth, your baby will be born, and the doctor will clamp the umbilical cord on two places and cut it in between the two clamps. The doctor will then give your baby either to you or to a nurse. At this stage your placenta will move away from your uterine wall, because the placenta must come out/ be delivered as well. While this is happening the doctors will draw the blood from the placenta through the umbilical vein. The doctor puts a syringe into the loose end of the umbilical cord and draws the blood. This is a quick and painless procedure and will not harm you or your baby. If the placenta does not come out, the doctor normally needs to remove it and then it gets thrown away/burnt.

The blood gets collected while the placenta is still in the mom, because there is not a lot of blood in the placenta and umbilical cord. It is usually about 60-80 ml (a quarter of a cup). If the placenta gets delivered, a lot of that blood is spilled and we cannot use it any more.

For the bank, we would like to collect the blood that has the special stem cells from the placenta, before the doctor throws the placenta away. We will then create a bank where we can store these special stem cells.

This is not a new procedure. All over the world they have public and private stem cells banks and in South Africa we already have private stem cell banks, but not a public bank. If a mother wants to store her umbilical cord blood in a private bank, she usually has to pay a large sum of money to keep the cells there, for a limited time (usually 10-15 years). If we can build a public umbilical cord blood bank, all the mothers in South Africa can donate their umbilical cord blood to be stored in this bank and they wouldn't have to pay money to store the cells, because it is a donation and anybody that needs it, would be able to get the blood.

HIV positive blood cannot be stored in the bank, because we cannot use it to treat certain diseases. This blood could however be used for medical research purposes to see how HIV affects the cells in the blood.

Before I go through the questionnaire with you, there are just a few points I would like to make you aware of

This is just a survey – meaning, we just want your opinion, if you think this bank is a good idea or not.
You don't have to answer this questionnaire - it is voluntary (nobody can make you answer these questions).
Whether or not you choose to answer the questions will have no effect on the way the doctors and nurses will treat you or your baby now or in the future.
The results of this questionnaire will be anonymous.
This survey is only to see if people will be willing to donate their placenta (afterbirth) to medical research. If you answer yes to all the questions it does not mean that you will be donating your placenta to medical research. This questionnaire is only to find out what you would be willing to do if you were given the choice.

Are there any other questions?
Can I please go through the questionnaire with you?

ANNEXURE 2: Patient Questionnaire

DEPARTMENT IMMUNOLOGY

FACULTY OF HEALTH SCIENCES

Prinshof Campus

P.O. Box 2034

Pretoria 0001

SOUTH AFRICA

Tel: 012-319-2621

Fax: 012-323-0732

Donation of Placenta (Afterbirth) survey

The University of Pretoria is doing a survey to see if pregnant mothers would be willing to donate their placenta (afterbirth) to medical research and we would like to invite you to take part in the survey by answering the following questions.

Please note:

- This is just a survey – meaning, we just want your opinion, if you think this bank is a good idea or not.
- You don't have to answer this questionnaire - it is voluntary (nobody can make you answer these questions).
- Whether or not you choose to answer the questions will have no effect on the way the doctors and nurses will treat you or your baby now or in the future.
- The results of this questionnaire will be anonymous.
- This survey is only to see if people will be willing to donate their placenta (afterbirth) to medical research. If you answer yes to all the questions it does not mean that you will be donating your placenta to medical research. This questionnaire is only to find out what you would be willing to do if you were given the choice.

Participant information:

Language group:

English	☐	Afrikaans	☐
Xhosa	☐	Ndebele	☐
Zulu	☐	Tswana	☐
Sotho	☐	Swazi	☐
Pedi	☐	Venda	☐
Northern Sotho	☐	Other	☐

Age range:

18-20	☐	21-25	☐
26-30	☐	31-35	☐
36-40	☐	41-45	☐
45-49	☐	50+	☐

Number of biological children

0	☐	6	☐
1	☐	7	☐
2	☐	8	☐
3	☐	9	☐
4	☐	10	☐
5	☐		

Marital status:

Married	☐
Widow	☐
Divorced	☐
Unmarried	☐

Employment status

Unemployed	☐
Employed	☐
*Please specify	_____

Comments and/or Questions:

_____

Question 1

If there is a public cord blood bank facility, would you be willing to donate your PLACENTA (afterbirth) for medical research?

YES	NO

If you answered NO:

Please indicate the reason:

Against religious belief ☐

Against your culture ☐

Don't think this bank is a good idea ☐

Afraid of the collection process ☐

Don't understand what the bank is for ☐

Other ☐

Please specify_____

Question 2

If you answered NO in question 1, would you be willing to donate the BLOOD from your placenta?

YES	NO

//

Question 3

If you are willing to donate your placenta OR just the blood from the placenta and umbilical cord, would you be willing to allow your doctor to do an additional HIV test?

Before you have your baby your doctor will do an HIV test to find out about your HIV status. Your test results will remain confidential. If you are HIV positive, your doctor will not ask you to join this study. If you are HIV negative, your doctor will ask you if you are willing to do another

test either seven days before or seven days after the birth of your baby to confirm that you are HIV negative.

YES	NO

Question 4:

Have you heard of stem cells before today?

YES	NO

Question 5:

Do you think stem cells can help to treat you, your child or somebody else in the future?

YES	NO

Question 6:

Do you think that a public umbilical cord blood stem cell bank is a good idea?

YES	NO

ANNEXURE 3: Different applications of UCB units collected

Table 21: An overview of the use of all UCB units collected during the course of the study

No	UCB unit ID	Method of UCB application	Comment
1	20101202 P1	Frozen UCB units	No viable CD34+ isolated
2	20110118 P1	Frozen UCB units	No viable CD34+ isolated
3	20110215 P1	Frozen UCB units	No viable CD34+ isolated
4	20110217 P1	Frozen UCB units	No viable CD34+ isolated
5	20110305 P1	Different protocol (Magnetic isolation)	Cannot compare results to final protocol
6	20110608 P1	Different protocol (Magnetic isolation)	Cannot compare results to final protocol
7	20110608 P2	Different protocol (Magnetic isolation)	Cannot compare results to final protocol
8	20110610 P1	Different protocol (Magnetic isolation)	Cannot compare results to final protocol
9	20110610 P2	Different protocol (Magnetic isolation)	Cannot compare results to final protocol
10	20110610 P3	Different protocol (Magnetic isolation)	Cannot compare results to final protocol
11	20110629 P1	Different protocol (Magnetic isolation)	Cannot compare results to final protocol
12	20110727 P1	Results incorporated	
13	20110727 P2	Results incorporated	
14	20110727 P3	No results	No colonies formed
15	20110906 P1	Results incorporated	
16	20110906 P2	Results incorporated	
17	20110922 P1	Results incorporated	
18	20111011 P1	Results incorporated	
19	20111012 P1	Results incorporated	
20	20111018 P1	Results incorporated	
21	20111018 P2	Results incorporated	
22	20111101 P1	No results	No colonies formed
23	20111122 P1	Frozen w/o CFU-assay	No CFU-assay performed
24	20111122 P2	Frozen w/o CFU-assay	No CFU-assay performed
25	20111212 P1	Frozen w/o CFU-assay	No CFU-assay performed

No	UCB unit ID	Method of UCB application	Comment
26	20111212 P2	Frozen w/o CFU-assay	No CFU-assay performed
27	20111212 P3	Frozen w/o CFU-assay	No CFU-assay performed
28	20120201 P1	Results incorporated	
29	20120201 P2	Results incorporated	
30	20120206 P1	Results incorporated	
31	20120209 P1	Results incorporated	
32	20120214 P1	Results incorporated	
33	20120219 P1	Different protocol (HIV)	Cannot compare results to final protocol
34	20120223 P1	No results	Blood volumes too low for isolation
35	20120229 P1	Different protocol (HIV)	Cannot compare results to final protocol
36	20120305 P1	Results incorporated	
37	20120312 P1	Results incorporated	
38	20120312 P2	Results incorporated	
39	20120312 P3	No results	Blood volumes too low for isolation
40	20120326 P1	No results	No colonies formed
41	20120402 P1	Results incorporated	
42	20120417 P1	Results incorporated	
43	20120417 P2	Results incorporated	
44	20120419 P1	Results incorporated	
45	20120704 P1	Results incorporated	
46	20120710 P1	Results incorporated	
47	20120710 P2	Results incorporated	
48	20120724 P1	Results incorporated	
49	20120724 P2	Results incorporated	
50	20120725 P1	Results incorporated	
51	20120725 P2	Results incorporated	
52	20120726 P1	Results incorporated	
53	20120727 P1	Results incorporated	

No	UCB unit ID	Method of UCB application	Comment
54	20120727 P2	No results	Blood volumes too low for isolation
55	20120727 P3	Results incorporated	
56	20120727 P4	Results incorporated	
57	20120727 P5	Results incorporated	
58	20120803 P1	Results incorporated	
59	20120803 P2	Results incorporated	
60	20120803 P3	Results incorporated	
61	20120803 P4	Results incorporated	
62	20120803 P5	Results incorporated	
63	20120803 P6	Results incorporated	
64	20120806 P1	Results incorporated	
65	20120806 P2	Results incorporated	
66	20120806 P3	No results	No colonies formed
67	20120806 P4	No results	No colonies formed
68	20120806 P5	No results	Blood volumes too low for isolation
69	20120807 P1	Results incorporated	
70	20120807 P2	Results incorporated	
71	20120813 P1	No results	No colonies formed
72	20120813 P2	No results	No colonies formed
73	20120813 P3	No results	No colonies formed
74	20120813 P4	No consent for Ultrio screening	
75	20120813 P5	Partial results	Cannot compare results to final protocol: HIV colonies perished
76	20120813 P6	Partial results	Cannot compare results to final protocol: HIV colonies perished
77	20120813 P7	Partial results	Cannot compare results to final protocol: HIV colonies perished
78	20120813 P8	Partial results	Cannot compare results to final protocol: HIV colonies perished
79	20120813 P9	Partial results	Cannot compare results to final protocol: HIV colonies perished
80	20120813 P10	Partial results	Cannot compare results to final protocol: HIV colonies perished

ANNEXURE 4: Complete flow cytometry data for the 30 UCB units

Table 22: Flow cytometry data for the 30 UCB units that were also subjected to Ultrio-Plus® screening

		Flow cytometry data for the CD34+ Pool Kit					Flow cytometry data for Stem Kit protocol					
No.	Unit ID	Average of %Gated CD34	Average of cells/µL CD34	Total isolated CD 45 BRIGHT	Constitution of CD34+ cells: CD45+ Dim	CD45+ Bright	Average of %Gated CD34	Average of cells/µL CD34	Total isolated CD 45 BRIGHT	Constitution of CD34+ cells: CD45+ Dim	CD45+ Bright	Viable
1	20120229 P1	90.10	24.21	13.07	85.05	14.95	65.73	17.75	10.82	91.16	8.84	79.16
2	20120306 P1	74.90	65.95	19.52	95.83	4.17	75.74	66.74	22.85	60.10	39.90	93.16
3	20120312 P1	80.90	184.04	74.87	15.36	84.64	86.55	162.15	2.96	79.43	20.57	88.64
4	20120312 P2*	29.89	81.00	2.07	95.40	4.60	49.55	10.76	4.50	59.27	40.73	67.93
5	20120312 P3	97.29	109.22	3.70	96.41	3.59	84.88	10.76	7.63	94.94	5.06	83.90
6	20120402 P1**	32.56	47.80	7.26	91.20	8.80	49.89	66.62	4.07	81.35	18.65	7.00
7	20120417 P1	98.28	1203.32	3.69	96.21	3.79	67.16	795.78	3.85	93.05	6.95	87.98
8	20120417 P2	97.34	1032.49	13.82	85.88	14.12	26.60	307.62	1.17	46.99	53.01	80.65
9	20120419 P1	89.24	1029.97	7.39	97.75	2.25	91.32	278.87	7.95	93.84	6.16	95.93
10	20120704 P1**	57.17	140.39	0.57	98.72	1.28	67.91	No beads	1.90	22.09	77.91	31.52
11	20120710 P1	93.69	649.86	1.50	98.43	1.57	95.97	669.20	4.02	96.24	3.76	96.53
12	20120724 P1	67.43	157.07	0.26	99.58	0.42	97.33	245.29	2.15	96.95	3.05	93.80
13	20120724 P2	52.70	137.77	0.55	98.95	1.05	95.34	215.13	2.44	88.71	11.29	89.70
14	20120725 P1*	70.32	89.25	1.55	97.79	2.21	2.27	2.70	4.35	44.44	55.56	42.11
15	20120725 P2	27.86	63.73	2.58	90.74	9.26	84.88	195.36	7.63	94.94	5.06	83.90
16	20120726 P1	99.66	94.60	4.41	95.58	4.42	71.17	72.02	4.23	97.06	2.94	88.48
17	20120727 P1	99.82	170.91	3.98	95.68	4.32	93.22	133.44	3.68	97.21	2.79	95.88
18	20120727 P3*	99.28	88.96	9.83	91.11	8.89	11.02	6.01	4.05	60.54	39.46	79.46
19	20120727 P4	99.17	42.96	6.26	94.22	5.78	21.11	6.20	5.69	36.84	63.16	64.17
20	20120727 P5	98.61	250.43	3.57	96.62	3.38	12.75	16.63	8.97	57.53	42.47	63.80
21	20120803 P1*	5.62	73.56	3.39	63.40	36.60	12.65	177.94	0.42	13.01	86.99	50.87

		Flow cytometry data for the CD34+ Pool Kit						Flow cytometry data for Stem Kit protocol					
No.	Unit ID	Average of %Gated CD34	Average of cells/µL CD34	Total isolated CD 45 BRIGHT	Constitution of CD34+ cells: CD45+ Dim	CD45+ Bright		Average of %Gated CD34	Average of cells/µL CD34	Total isolated CD 45 BRIGHT	Constitution of CD34+ cells: CD45+ Dim	CD45+ Bright	Viable
22	20120803 P2	6.82	221.64	7.43	50.74	49.26		23.03	66.44	0.29	3.32	96.68	52.16
23	20120803 P3	6.94	15.20	2.86	74.61	25.39		30.80	60.91	0.65	14.59	85.41	52.53
24	20120803 P5	15.89	32.07	3.95	87.65	12.35		23.40	50.24	2.81	18.11	81.89	50.62
25	20120803 P4	38.30	157.34	35.96	20.57	79.43		72.06	146.93	5.47	82.95	17.05	32.83
26	20120803 P6*	44.40	19.74	45.91	28.64	71.36		10.00	59.67	7.36	38.69	61.31	67.12
27	20120806 P1	8.34	96.86	1.16	94.73	5.27		7.34	93	0.64	82.07	17.93	63.72
28	20120806 P2*	11.59	0.83	7.93	42.11	57.89		21.94	2	9.7	48.08	51.92	61.18
29	20120807 P1**	49.09	21.69	51.92	12.45	87.55		27.19	64.48	6.75	69.57	30.43	45.44
30	20120807 P2**	41.87	22.58	34.95	17.42	82.58		52.96	30.37	2.21	59.69	40.31	17.68
Average		**60**	**210.85**	**12.53**	**76.96**	**23.04**		**51**	**139.01**	**5.04**	**64.09**	**35.91**	**66.93**
Standard deviation (SD)		34.8	321.7	18.0	29.9	29.9		32.5	186.9	4.4	29.7	29.7	24.5

*= Patients with final blood volumes below 15 ml

** = UCB units collected after 72 hours (removed from patient cohort)

ANNEXURE 5: Ethics approval forms from the Main Research Ethics Committee, University of Pretoria

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

* **FWA** 00002567, Approved dd 22 May 2002 and Expires 13 Jan 2012.
* **IRB** 0000 2235 IORG0001762 Approved dd Jan 2006 and Expires 13 Aug 2011.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee
Fakulteit Gesondheidswetenskappe Navorsingsetiekkomitee

DATE: 11/10/2010

PROTOCOL NO.	**89/2010**
PROTOCOL TITLE	Rendering the Immune System Resistant to HIV
INVESTIGATOR	**Principal Investigator:** Prof. Michael S. Pepper
SUBINVESTIGATOR	None
SUPERVISOR	None
DEPARTMENT	**Dept:** Immunology **Phone:** 012-420-5317 **Mobile:** 072-209-6324 **E-Mail:** michael.pepper@up.ac.za
STUDY DEGREE	Grant
SPONSOR	None
MEETING DATE	26/05/2010

The Protocol and Informed Consent Document were approved on 29/09/2010 by a properly constituted meeting of the Ethics Committee subject to the following conditions:

1. The approval is valid until the end of December 2012, and
2. The approval is conditional on the receipt of 6 monthly written Progress Reports, and
3. The approval is conditional on the research being conducted as stipulated by the details of the documents submitted to and approved by the Committee. In the event that a need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee
Fakulteit Gesondheidswetenskappe Navorsingsetiekkomitee

DATE: 1/10/2010

PROTOCOL NO.	**131/2010**
PROTOCOL TITLE	Feasibility study for a public cord blood stem cell bank in South Africa.
INVESTIGATOR	**Principal Investigator:** Michael S. Pepper
SUBINVESTIGATOR	Ms W M Young **E-Mail:** wendyyoung@mtnloaded.co.za Ms F Barmania **E-Mail:** barmaniaf@gmail.com
SUPERVISOR	Michael S. Pepper **E-mail:** michael.pepper@up.ac.za
DEPARTMENT	**Dept:** Immunology, University of Pretoria. **Tel:** +27(0)124203845 (Secretary) **Tel:** +27 (0)12 420 5317 (Direct) **Fax:** +27 (0)12 420 3953 **Mobile:** +27 (0)72 209 6324
STUDY DEGREE	MSc in Immunology
SPONSOR	None
MEETING DATE	**28/07/2010**

The Protocol and Informed Consent Document were approved on 29/09/2010 by a properly constituted meeting of the Ethics Committee subject to the following conditions:

1. The approval is valid until the end of December 2014, and
2. The approval is conditional on the receipt of 6 monthly written Progress Reports, and
3. The approval is conditional on the research being conducted as stipulated by the details of the documents submitted to and approved by the Committee. In the event that a need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

Ethics approval for submission of this Thesis from the Main Research Ethics Committee, University of Pretoria

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

* **FWA** 00002567, Approved dd 22 May 2002 and Expires 20 Oct 2016.
* **IRB** 0000 2235 IORG0001762 Approved dd 13/04/2011 and Expires 13/04/2014.

Denkleiers • Leading Minds • Dikgopolo tša Dihlalefi
Faculty of Health Sciences Research Ethics Committee
Fakulteit Gesondheidswetenskappe Navorsingsetiekkomitee

DATE: 30/07/2012

NUMBER	**124/2012**
OLD TITLE	~~Umbilical cord blood stem cells: a public bank social feasibility study and investigation of the effect of HIV-1 on the colony forming ability of CD34+ cells~~
NEW TITLE	The effect of HIV on the formation of colony forming units in vitro & public willingness to donate to a public cord blood bank
PRINCIPAL INVESTIGATOR	Mrs. Madelein Meissner-Roloff **Dept:** Immunology, Faculty of Health Sciences, University of Pretoria. **Cell:** 082 553 6005 **E-Mail:** mmroloff@gmail.com
SUB INVESTIGATOR	Not Applicable
STUDY COORDINATOR	Not Applicable
SUPERVISOR	Prof Michael S. Pepper **E-Mail:** michael.pepper@up.ac.za
STUDY DEGREE	PhD
SPONSOR COMPANY	Not Applicable
MEETING DATE	25/07/2012

The Protocol and Informed Consent Document were approved on 25/07/2012 by a properly constituted meeting of the Ethics Committee subject to the following conditions:

1. The approval is valid for 3 years period [till the end of December 2015], and
2. The approval is conditional on the receipt of 6 monthly written Progress Reports, and
3. The approval is conditional on the research being conducted as stipulated by the details of the documents submitted to and approved by the Committee. In the event that a need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

Members of the Research Ethics Committee:

Prof M J Bester	(female)BSc (Chemistry and Biochemistry); BSc (Hons)(Biochemistry); MSc(Biochemistry); PhD (Medical Biochemistry)
Prof R Delport	(female)BA et Scien, B Curationis (Hons) (Intensive care Nursing), M Sc (Physiology), PhD (Medicine), M Ed Computer Assisted Education
Dr NK Likibi	MBB HM – Representing Gauteng Department of Health) MPH
Dr MP Mathebula	(female)Deputy CEO: Steve Biko Academic Hospital; MBCHB, PDM, HM
Prof A Nienaber	(female) BA(Hons)(Wits); LLB; LLM; LLD(UP); PhD; Dipl.Datametrics(UNISA) – Legal advisor
Mrs MC Nzeku	(female) BSc(NUL); MSc(Biochem)(UCL, UK) – Community representative
Prof L M Ntlhe	MbChB (Natal) FCS (SA)
Snr Sr J Phatoli	(female) BCur(Eet.A); BTec(Oncology Nursing Science) – Nursing representative
Dr R Reynders	MBChB (Prét), FCPaed (CMSA) MRCPCH (Lon) Cert Med. Onc (CMSA)
Dr T Rossouw	(female) MBChB (cum laude); M.Phil (Applied Ethics) (cum laude), MPH (Biostatistics and Epidemiology (cum laude), D.Phil

2012/08/03MS: dd 2012/08/03: C:\Documents and Settings\user\My Documents\Protokolle\Grade briewe\Letters 2012\124.2012.doc