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).

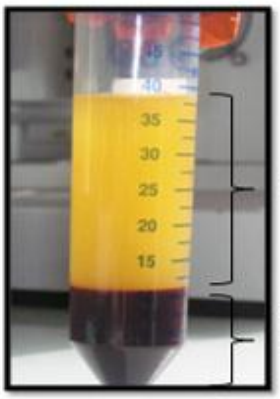

Figure 23: Plasma reduction done on each UCB unit prior to MNC isolation. The top plasma/platelet fraction was stored for subsequent HIV-1 screening with the Ultrio-Plus® assay.

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).

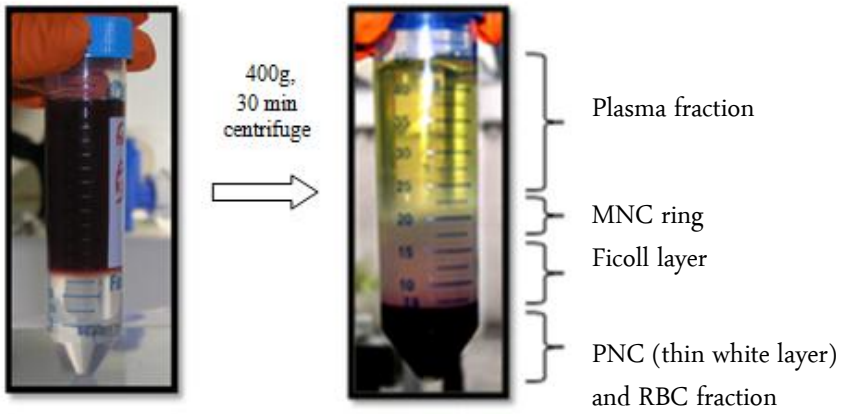

Figure 24: Ficoll (Histopaque) density gradient separation of the MNC layer. The tube on the left illustrates the blood layered on top of the ficoll before centrifugation. The falcon tube on the right illustrates the five layers obtained after centrifugation. The second layer – the MNC ring – contains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

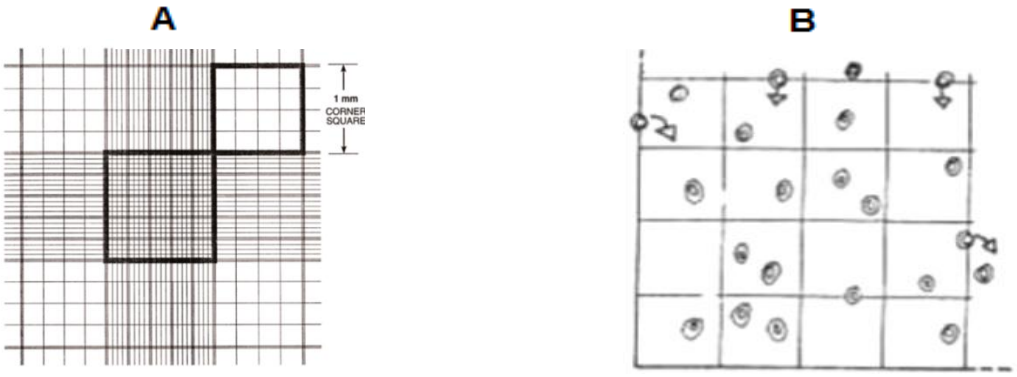

Figure 25 A: Layout of the haemocytometer. A coverslip provides a depth of 0.1mm and cells were counted in the centre square volume. Cell count per ml calculated with: NUMBER OF CELLS IN THE MIDDLE SQUARE X DILUTION FACTOR X 10^4.

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

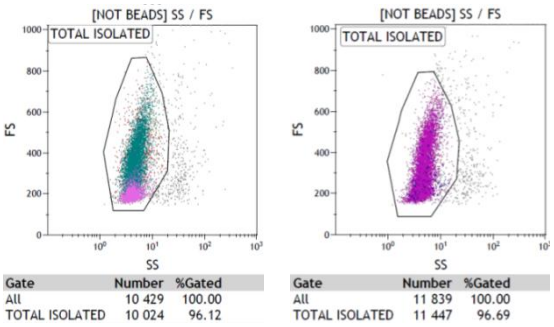

Figure 26: Comparison between flow cytometry results obtained with the Stem-Kit™, left and CD34+ Pool Kit, right, for total cells isolated.

Patient: 20120206 P1

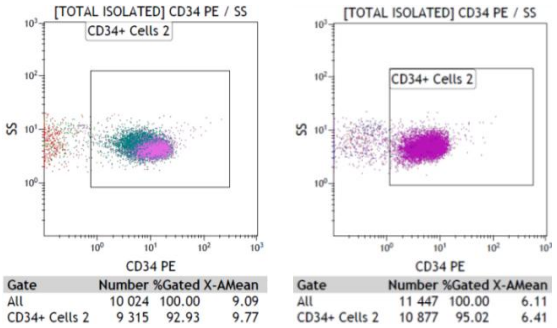

Figure 27: The percentage of CD34+ HPCs detected (Stem-Kit™, left; CD34+ Pool Kit, right). Percentages of CD34+ cells isolated are indicated under % Gated.

Patient: 20120206 P1

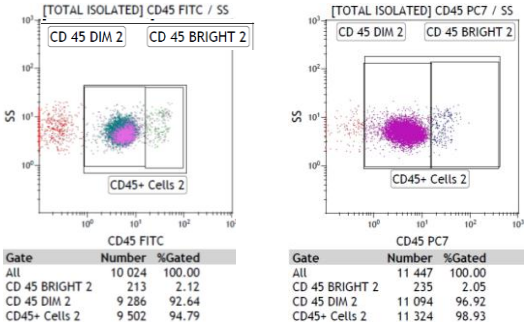

Figure 28: Percentage of isolated cells that were CD45+ (Stem-Kit™, left; CD34+ Pool Kit, right). Cells grouped on the left were CD45 dim and CD45+ HPCs on the right were CD45 bright. (Percentages are indicated at % Gated).

Patient: 20120206 P1

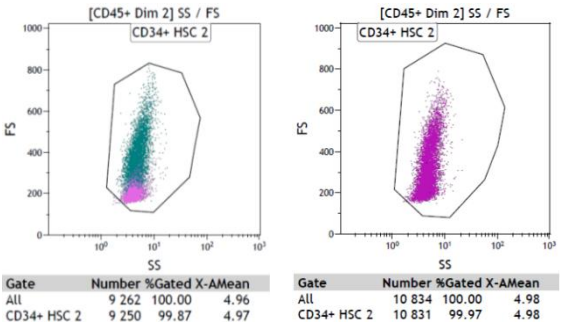

Figure 29: True HPCs, CD45 dim and CD34+ (Stem-Kit™, left; CD34+ Pool Kit, right). (Percentages are indicated at % Gated).

Patient: 20120206 P1

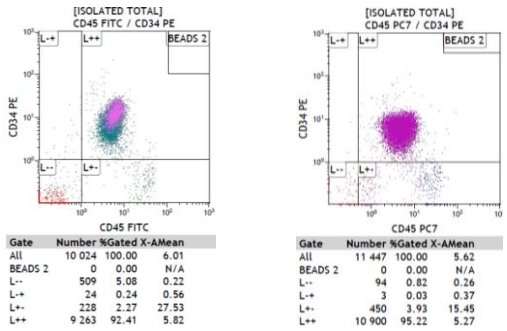

Figure 30: Characteristics of total cells isolated (Stem-Kit™, left; CD34+ Pool Kit, right) (Percentages are indicated at % Gated).

Patient: 20120206 P1

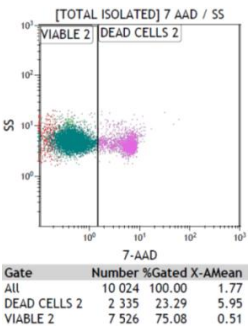

Figure 31: An example of cell viability measured with the Stem-Kit™. (Percentages are indicated at % Gated).

Patient: 20120417 P2

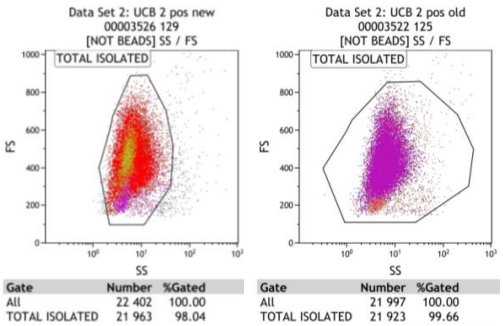

Figure 32: Comparison between flow cytometry results obtained with the Stem-Kit™, left, and CD34+ Pool Kit, right, for total cells isolated.

Patient: 20120417 P2

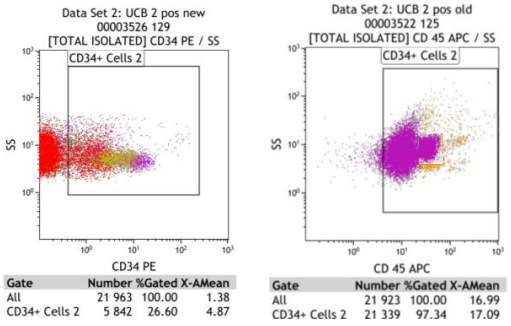

Figure 33: Comparison between the percentage of CD34+ HPCs detected with the Stem-Kit™, left, and CD34+ Pool Kit, right. Differences are clearly indicated where Stem-Kit™ detected 26.6% CD34+ HPCs and CD34+ Pool Kit detected 97.34% CD34+ HPCs for the same patient.

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.

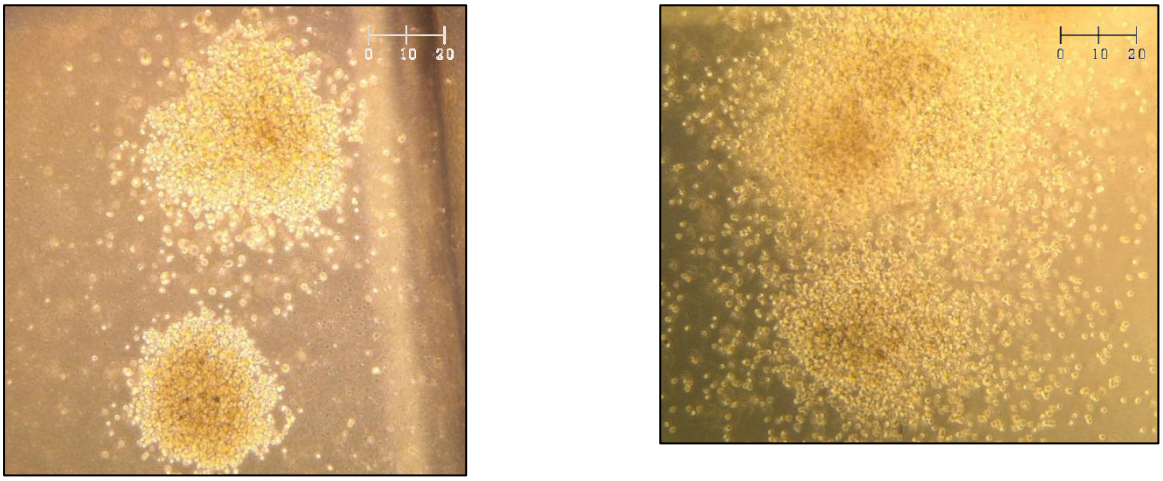

Figure 34: Normal CFU-GM colonies (14 days of culture). Pictures taken at 20X magnification. (Scale bar = 0.2 mm)

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.

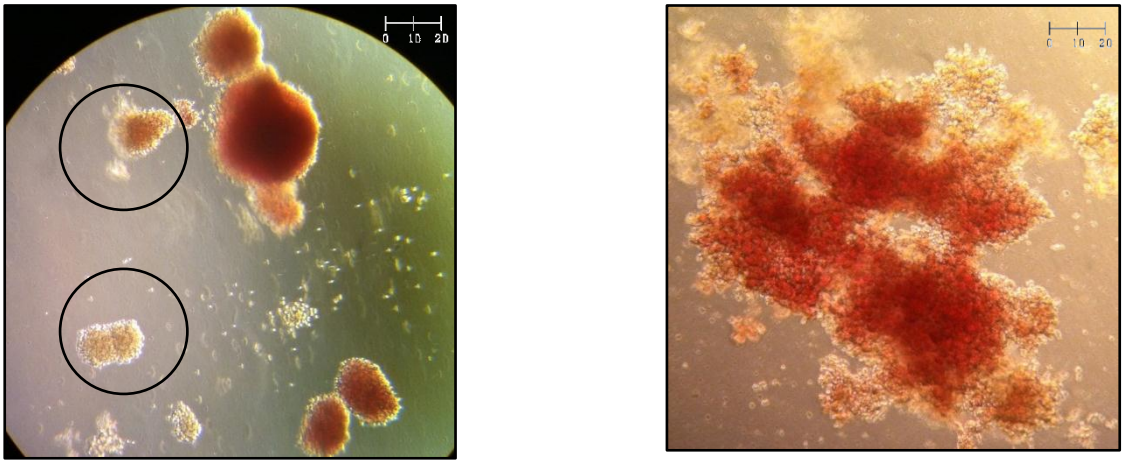

Figure 35: CFU-E (encircled left) and BFU- E (Right) colonies (14 days of culture). Pictures taken at 20X magnification. (Scale bar = 0.2 mm)

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.

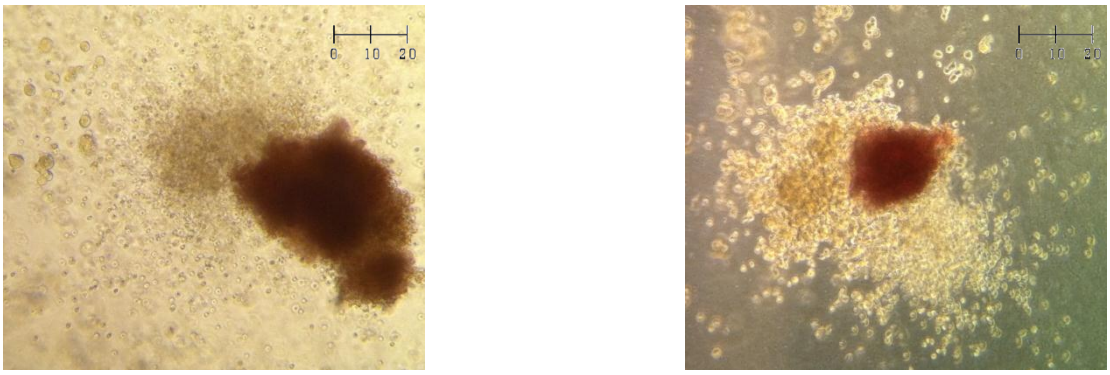

Figure 36: CFU-GEMM colonies (14 days of culture). Pictures taken at 20X magnification. (Scale bar = 0.2 mm)

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).

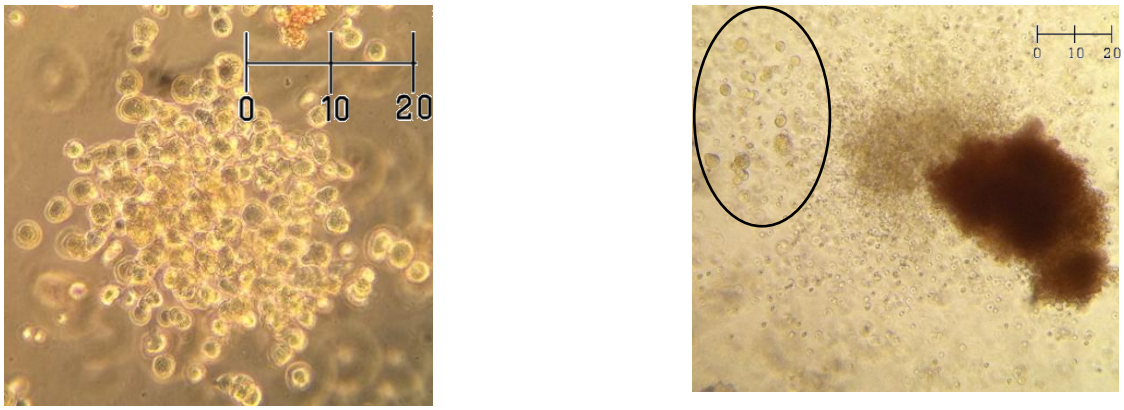

Figure 37: MK colonies (14 days of culture). Pictures taken at 40X magnification (left) and 20X magnification (right, encircled area). (Scale bar = 0.2 mm)

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).

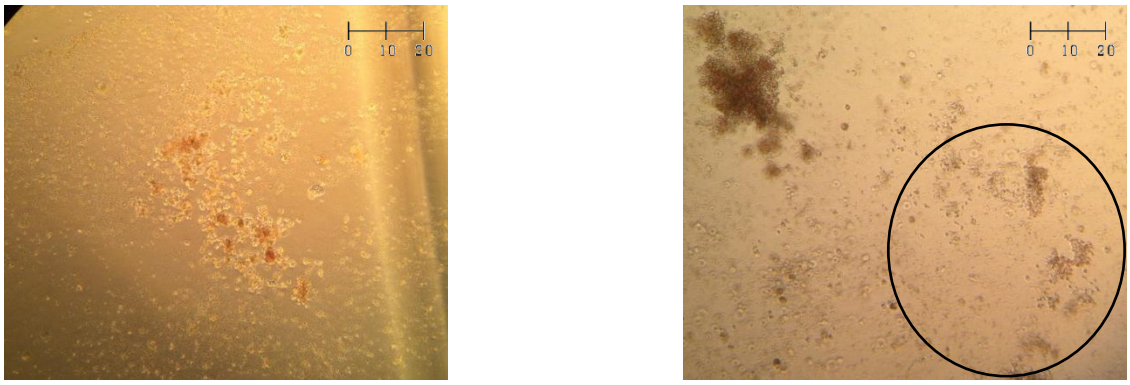

Figure 38: Poorly haemoglobinised BFU-E and CFU-E in HIV-1-spiked wells. Pictures taken after 14 days of culture at 20X magnification. (Scale bar = 0.2 mm)

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.

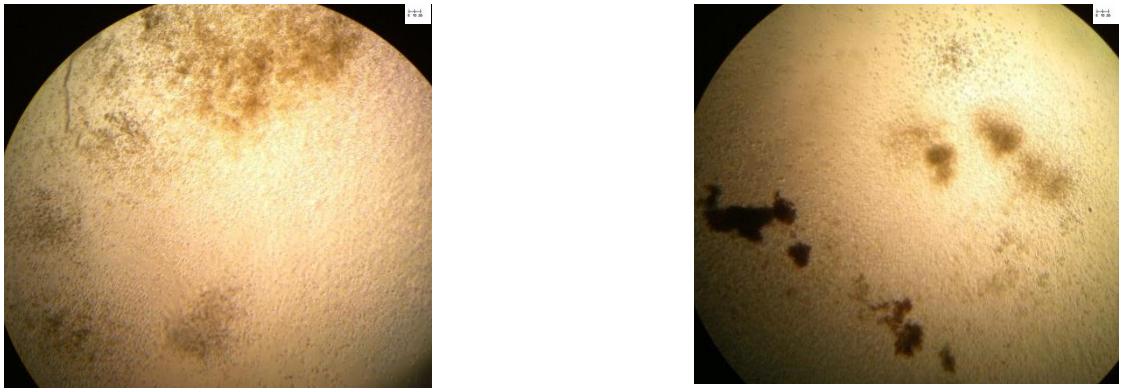

Figure 39: Within patient comparison of 14-day-old cultures. HIV-1-spiked CFUs (left) vs. Normal CFU colony growth (right) taken at 5X magnification. (Scale bar = 0.2 mm top right corner). More CFU-GM colonies seemed to grow in the HIV-1-spiked well (left) while Normal wells displayed balanced growth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

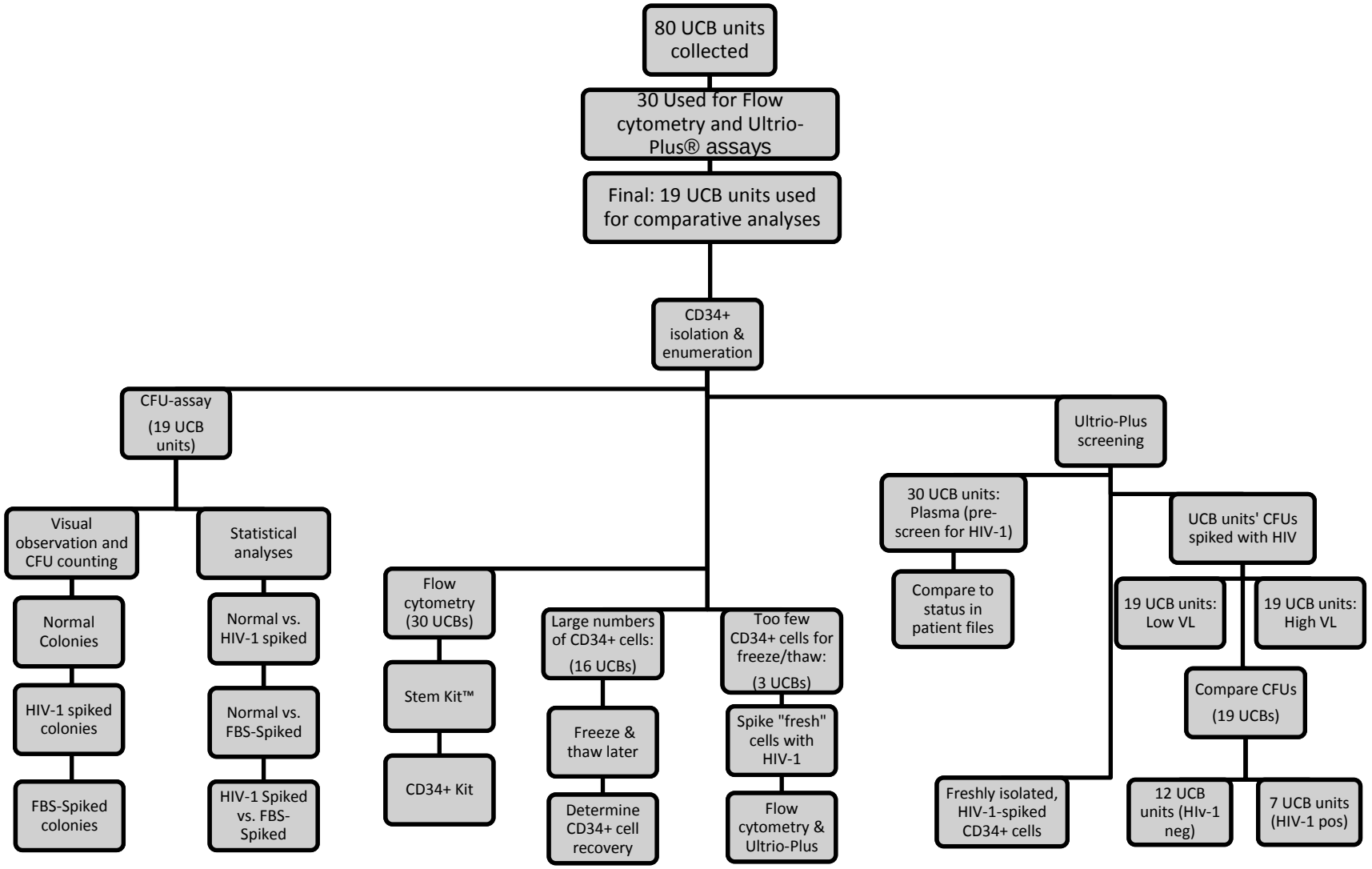

Figure 40: Outline of methods used and results obtained in Chapter 7

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.

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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.