THE ASSOCIATION OF EARLY NEONATAL FEEDING ON CLINICAL OUTCOMES AND CYTOTOXIC T LYMPHOCYTE (CTL) RESPONSES IN HIV EXPOSED LOW BIRTH WEIGHT INFANTS

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LIST OF ABBREVIATIONS:

AFASS	Acceptable, feasible, affordable, sustainable and safe
AIDS	Acquired immune deficiency syndrome
APC	Antigen Presenting Cells
APGAR	Appearance/Pulse/Grimace/Activity/Respiration/breathing of the infant at birth
ART	Antiretroviral therapy
ARV	Antiretroviral
AZT	Zidovudine
BCIP	Bromo-Chloro-Indoylphosphate
CD4	Cluster of differentiation 4
CMV	Cytomegalovirus
CPAP	Continuous positive airway pressure
CTL	Cytotoxic T Lymphocyte
DBS	Dried blood spot
DNA	Deoxyribonucleic acid
DOH	Department of Health
DPBS	Commercial PBS
EBM	Expressed breastmilk
EDTA	Ethylenediamine-tetraacetic acid
ELISpot	Enzyme-linked immunosorbent spot
FACS	Fluorescence activated cell sorter
FAS	Apoptosis Stimulating Fragment (programmed cell death)
FBC	Full bood count

FCS	Fecal Calf Serum
FDC	Follicular dendritic cell
FH	Flash Heat
HIV	Human immunodeficiency virus
HTEBM	Heat treated expressed breastmilk
HTST	High temperature short time
ICS	Intracellular Cytokine Staining
IFNγ	Interferon gamma
IVF	Intravenous fluid
LCPUFA's	Long chain polyunsaturated fatty acids
MHC	Major Histocompatibility complex
MO	Month
NBT	Nitroblue Tetrozolium Chloride
NEC	Necrotizing enterocolitis
NVP	Nevirapine
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PHA	Phytohaemaglutinin A
PMTCT	Prevention of mother-to-child transmission
RANTES	Regulated on Activation, Normal T Expressed and Secreted
RNA	Ribonucleic acid
RT	Room temperature
SdNVP	Single dose Nevirapine

SIV	Simian immunodeficiency virus
SLPI	Secretory Leukocyte Protease Inhibitor
TB	Tuberculosis
TLR	Toll like Receptor
TNF	Tumor necrosis factor
UNAIDS	Joint United Nations Programme on HIV/AIDS
UNICEF	United Nations Children's Fund
URTI	Upper respiratory tract infection
WHO	World Health Organisation

ABSTRACT

BACKGROUND

Sub-saharan Africa remains to date at the forefront of the HIV/AIDS epidemic. Despite breastfeeding being a significant mode of postnatal HIV transmission it remains the main nutritional source and pillar of child survival for the majority of infants born in Africa. It is therefore, not surprising that considerable research has centred on making breastfeeding safer in terms of HIV transmission. The flash heat treatment method (HTEBM) provides a unique opportunity to safely breastfeed infants but prevent mother-to-child transmission of HIV. Cytotoxic T lymphocyte (CTL) responses have been well documented in HIV-infected adults and children. However, there is a lack of literature on CTL responses in HIV exposed low birth weight infants. This pilot study attempted to examine the association of early neonatal feeding on the clinical outcomes and CTL responses in HIV exposed low birth weight infants.

METHODS

Seventy-seven patients that fulfilled inclusion and exclusion criteria were enrolled. The clinical outcomes of these patients were evaluated over a 9 month period. Fifty-five of these patients were also investigated for cytotoxic T lymphocyte (CTL) responses by means of the IFNγ ELISpot (megamatrix and confirmation) assays at the 6 weeks, 3, 6,and 9 months follow-up.

RESULTS

Two HIV-1 infected infants generated a CTL response at a single time point using the ELISPOT matrix screening assay. These responses could not be confirmed and were undetectable at any of the consecutive visits. At the time of detection of responses the infants were fed unheated breastmilk. HIV-1 exposed uninfected infants were unable to elicit a HIV-1-specific CTL response irrespective of feed. With regards to clinical

outcomes, infants born o HIV infected mothers with a CD4 count < 500cells/µl were 2x more likely to acquire other infections at birth compared to those infants born to HIV infected mothers with a CD4 count >500cells/µl. Also, infants born to HIV infected mothers with advanced disease (CD4 count 0-200 cells/µl) had a lower birth weight compared to infants born to HIV-1 infected mothers with a CD4 count > 350 cells/µl. We also investigated the feasibility of the flash heat treatment method at birth. While in-hospital, 38 HIV-1 infected women fed their infants HTEBM after receiving counseling and support from the nursing staff at the King Edward VIII hospital. The numbers decreased rapidly post hospital discharge, mainly due to mixed feeding.

DISCUSSION

In conclusion we have shown that it is feasible for HIV infected mothers to heat treat their expressed breastmilk during hospital admission. Furthermore, we were able to demonstrate in this small cohort of patients that the clinical outcomes and growth parameters of infants fed HTEBM were similar to that of infants fed either formula or unheated breastmilk. We were unable to demonstrate HIV-specific responses in the infected infants or the uninfected infants who had been exposed to heat inactivated virus in HTEBM. Our findings indicate that this pilot study was limited in its ability to detect CTL responses in HIV exposed low birth weight infants and further studies are warranted.

CHAPTER 1

1. EPIDEMIOLOGY OF HIV/AIDS

According to the Joint United Nations Programme on HIV/AIDS (UNAIDS), at the end of 2008; sub-Saharan Africa remained at the epicentre of the HIV/AIDS epidemic. This region accounted for approximately 22.4 million HIV infected people and 1.9 million new HIV infections. Sub-Saharan Africa also had 72% of AIDS deaths worldwide. Young women of child bearing age are particularly prone to infection and comprise of 60% of all infections in this region. In Kenya women are three times more likely to become HIV infected than men. This vulnerability has resulted in sub-Saharan Africa being accountable for 91% of all new HIV infections in children. Of note only 38% of these children requiring antiretroviral therapy had access to it (UNAIDS, 2009).

The world's largest population of HIV infected individuals reside in South Africa – 5.8 million in 2008 (figure 1). A key element driving this epidemic is the age-differential partnering pattern, in which younger women partner with older men (Abdool Karim et al., 2009). In 2008, the prevalence of HIV at the public antenatal clinic was estimated to be 29% .This projection has been stable over the last three years, indicating that the HIV epidemic curve is reaching a plateau. The burden of disease is distributed unevenly across the country. Currently, KwaZulu-Natal has the highest HIV prevalence of 38.7% among women attending public antenatal clinic. This translates into more than a one third of the population living with HIV in this province (DOH, 2009). Karim ***et al,*** in 2009 reported that several factors have contributed to the spread of HIV/AIDS in South Africa including overcrowded squatter settlements, migrant labour and poor health services.

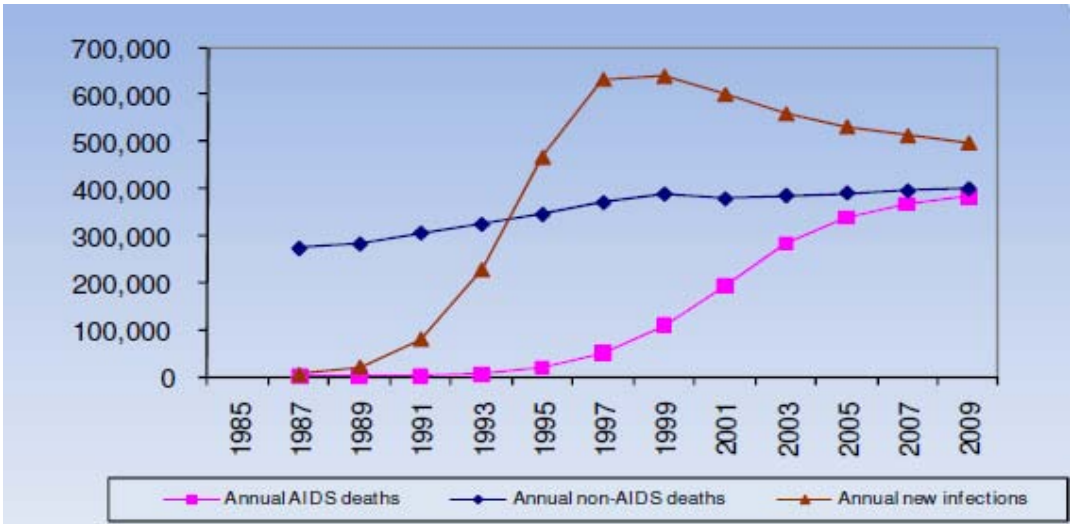

Figure 1. AIDS deaths, non-AIDS deaths and annual new infections, South Africa, 1985-2009, Source: ASSA model, 2003

Adapted from: Department of Health (2010). Republic of South Africa country progress report on the declaration of commitment on HIV/AIDS. Reporting period: January 2008 - December 2009

[www.unaidsrstesa.org/…/southafrica_2010_country_progress_report_en.pdf (Accessed on 30th June 2010)]

2. CLASSIFICATION AND ORIGIN OF HIV

HIV belongs to the genus *Lentivirus* of the family *Retroviridae.* Simian immunodeficiency virus (SIV) also belongs to the family *Retroviridae.* Accumulating evidence indicates that the HIV epidemic arose when simian immunodeficiency viruses were transmitted from non-human primates to humans in Africa perhaps through butchering and then diversified. HIV is divided into two types: HIV-1 and HIV-2. HIV-2 is phylogenetically very closely related to the SIV that infects the sooty mangabeys of West Africa and HIV-1 is phylogentically very closely related to the SIV that infects the chimpanzee. HIV-1 is the predominant type, and is responsible for the global pandemic and HIV-2 is less pathogenic

than HIV-1 and is confined to West Africa with limited spread to other countries. HIV-1 is a highly variable virus and is distinguished into three groups: group M (major) viruses include most of the HIV-1 isolates and group O (outliers) viruses are largely restricted to the central African region and group N (non-M; non- O) viruses are rare and have only been identified in a few individuals in Cameroon. Group M isolates have further been subdivided into subtypes or clades, referred to alphabetically (A, B, C, D, E, F, G, H, J and K). The subtypes are unevenly distributed globally with subtypes A, C, D and G being most common in Africa and subtype B occurring in Europe and America. In regions where multiple subtypes are circulating, recombinant viruses have been identified. A recombinant virus is a virus with mosaic genomes made up of different subtypes e.g. CRF02_AG , which is a mixture of subtypes A and G (Peeters et al., 2003; Tebit et al., 2007).

3. THE STRUCTURE OF HIV

The mature virion is spherical with a diameter of approximately 110nm. The virion has an outer lipid bilayer that is host in origin and embedded within this lipid bilayer is the surface glycoprotein (gp120) and transmembrane protein (gp41). The surface glycoprotein (gp120) is attached to the transmembrane protein (gp41). The core of the virus is conical in shape and made up of p24 capsid proteins. Between the core and the outer lipid bilayer is the matrix protein (p7). Within the viral core are two identical copies of the single-stranded RNA genome. Packaged within the virion are several proteins required for infecting the host and the initiating viral replication including reverse transcriptase, protease and integrase (Richman, 2003).

4. GENE ORGANIZATION

The human immunodeficiency virus (HIV) consists of three large open reading frames namely, ***gag, pol*** and ***env*** which are unique to all retroviruses. The ***gag*** gene encodes the Gag precursor protein (p53). Gag comprises of several functional domains, which when cleaved by the viral protease produces the following structural proteins: matrix, capsid and nucleocapsid. The ***gag*** and ***pol*** genes overlap by 241 nucleotides. The ***pol*** gene utilizes a different reading frame to the ***gag*** gene and is not transcribed alone. In a few cases, ribosomal frame shifting allows translation to continue through to the ***pol*** gene, to produce Pol proteins. The Gag-Pol precursor protein is produced during this extended translation. The Pol protein is then cleaved by viral protease to synthesize the following viral enzymes: protease, reverse transcriptase, ribonuclease H and integrase. The ***env*** gene is transcribed to produce the Env precursor polyprotein (gp160). The host protease cleaves gp120 to produce the envelope surface and transmembrane glycoproteins (gp120 and gp41, respectively). ***Tat*** and ***rev*** are two essential regulatory genes that control gene expression. These genes partially overlap each other in different reading frames and are multiply spliced. The virus also comprises of several accessory genes namely, ***vpu, vif*** and ***nef*** which function in pathogenesis and replication (Richman, 2003).

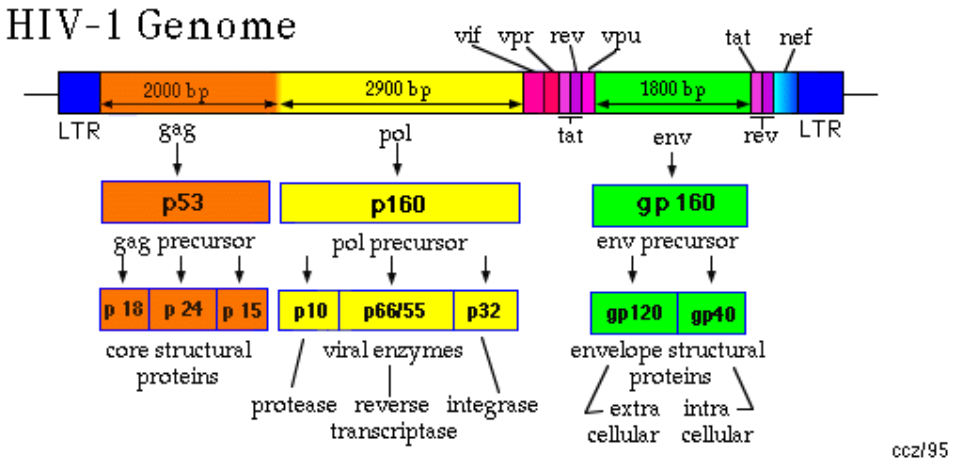

Figure 2: Genomic organization of Human Immunodeficiency Virus

Adapted from: www.yale.edu/bio243/HIV/genome.html

(Accessed 23rd February 2010)

5. THE HIV LIFE CYCLE

5.1 Cell types infected by HIV

HIV entry into cells is facilitated by the interactions between viral envelope glycoprotein and host cellular receptors and co-receptors. The receptors determine which cells the virus will infect and are important in HIV transmission and disease progression. The virus utilizes two receptors to enter cells, including CD4 and a second co-receptor belonging to the chemokine receptor family, CCR5 and CXCR4. Cells that express these receptors on their surfaces include a subset of T lymphocytes, monocytes, dendritic cells and microglial cells in the brain. Viruses that use the CCR5 co-receptor are known as CCR5 tropic (R5) and are non-syncitium inducing (NSI) and macrophage tropic in tissue culture. Viruses that use the CXCR4 co-receptor (CXCR4 tropic) are known as X4 and are syncitium inducing (SI) and T-cell line tropic in tissue culture. Some viruses can utilize both co-receptors and are referred to as dual tropic while other viruses can utilize co-receptors other than CCR5 and CXCR4 (Wilson et al., 2008).

5.2 Binding and virus entry

The interaction between viral gp120 and the CD4 molecule is essential for HIV to bind to host cells. Binding triggers a conformational change in gp120, facilitating interaction with the viral co-receptor CCR5 or CXCR4 (Turner and Summers, 1999). Co-receptor binding triggers the insertion of gp41 into the cell membrane and fusion of the virus with the cell (Turner and Summers, 1999). The viral core containing the viral RNA is released into the host cell cytoplasm.

5.3 Reverse Transcription

HIV reverse transcriptase occurs as a dimer (p51, p66) with both reverse transcriptase and RNAase H nuclease activity. This enzyme is error prone in nature, due to the absence of a proof reading function and on average one error is introduced per genome per replication cycle.

5.4 Integration

The pre-integration complex contains viral DNA, reverse transcriptase, integrase, Vpr and matrix proteins and is transported to the nucleus. The viral enzyme integrase catalyses integration of viral DNA into the host chromosomal DNA. The life cycle of the virus depends on this integration step and in its absence the transmission and the spread of the virus is curtailed. The integrated virus is referred to as a provirus.

5.5 Transcription and translation

Proviral DNA is transcribed by host enzymes utilizing host cellular machinery. Structural proteins are formed by singly spliced or non-spliced viral RNA. Regulatory and accessory

proteins are formed by multiple splicing of viral RNA. Full length unspliced RNA are shuttled to the membrane surface for inclusion into new virus particles.

5.6 Assembly, budding and maturation of new virions

Gag, Gag-Pol and Env precursor polyproteins are synthesized and accumulate in the plasma membrane, where they begin to assemble. The viral protease enzyme cleaves the Gag-pol protein to form an infectious virus. The infectious virus buds through the membrane, taking with it the cellular lipid bilayer, to form mature virus particles. Cellular enzymes cleave gp160 which is embedded in the membrane of an infected cell to synthesise functional gp120 and gp41 (Wilson et al., 2008).

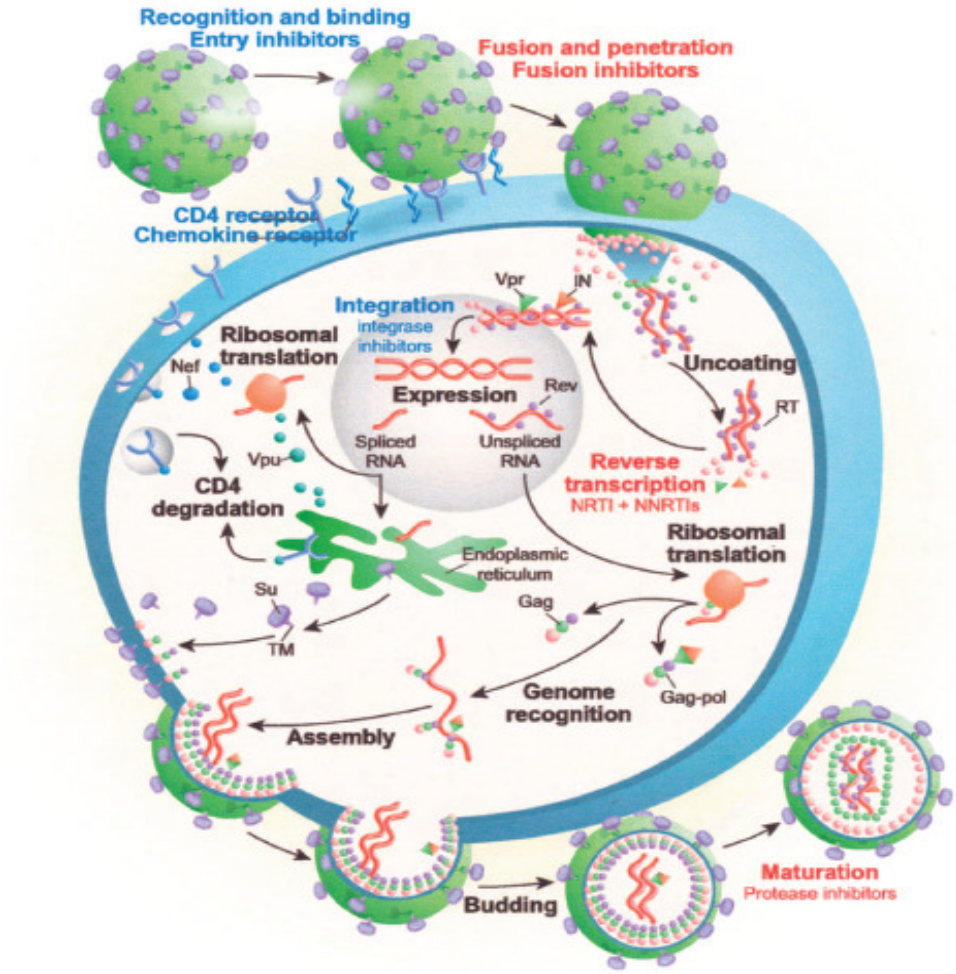

Figure 3. Viral life cycle of HIV

Adapted from Pomerantz RJ and Horn DL (2003). Twenty years of therapy for HIV infection. Nature Medicine 9:867-73. Nature Medicine adapted the figure from Turner BG and Summers MF (1999). Structural Biology of HIV. The Journal of Molecular Biology, 2851:1-32. (Accessed 15th May 2010)

6. THE HUMAN IMMUNE SYSTEM

The primary role of the immune system is to protect the host against exposure and infection by pathogenic organisms. This protection is facilitated in a two step process of innate and adaptive immunity. The innate immune response is the first line of defence against many common microorganisms and infections and comprises of macrophages and neutrophils. However, the innate system cannot always prevent or clear the infection and it does not provide immunological memory. The adaptive immune response is the second line of defence and involves T- and B-cells, which have evolved to provide a more versatile means of defence, and provides protection against re-infection with the same pathogen. There is close a relationship between the innate and adaptive immune system which is ensured via the interaction of such components as toll-like receptors (TLR) and dendritic cells (Janeway et al., 2005).

6.1. Adaptive immunity

The B and T lymphocytes originate in the bone marrow, but only the B lymphocytes mature in this central lymphoid organ. The T lymphocytes migrate to the thymus to undergo their maturation. The B- and T-lymphocytes are therefore named after the organs they are derived from. Mature lymphocytes possess antigen-specific receptors on their surface and continually circulate in the blood stream and peripheral/secondary lymphoid organs. When a mature lymphocyte recognizes its specific antigen on the surface of an activated dendritic cell, an adaptive immune response is triggered.

Lymphocytes are capable of detecting extracellular and intracellular pathogens via two distinct recognition systems. B lymphocytes provide protection against extracellular pathogens by bearing antigen-specific immunoglobulin receptor molecules on their surface

and once activated, secrete immunoglobulin as soluble antibody. T lymphocytes are specialized to recognise foreign antigen as peptide fragments of intracellular pathogens transported to the cell surface by the glycoprotein of the major histocompatibility complex (MHC). The MHC is a group of genes on human chromosome 6 and it translates into a set of membrane glycoprotein called the MHC molecules. There are two main types of T lymphocytes viz. CD4+ T helper cells and CD8+ cytotoxic T cells. CD4 cells recognise peptide fragments presented by the MHC class II molecule and the CD8 cell recognise peptide fragments presented by the MHC class I molecule (Zinkernagel and Doherty, 1974; Townsend and McMichael, 1985; Morrison et al., 1986). The CD8 T cell functions in killing infected target cells and the CD4 T cell amplifies the immune response (Bennett et al., 1997; Pardoll and Topalian, 1998; Okada et al., 1989). Therefore, the T lymphocytes are vitally important for both the humoral and cell mediated responses of adaptive immunity (Janeway et al., 2005).

6.2. Cytotoxic T lymphocytes

Effector cytotoxic CD8 T lymphocytes are essential in host defence against pathogens that reside in the cytosol, especially viruses. The CD8 T lymphocyte can recognise cells infected with foreign pathogens by recognizing foreign peptides that are presented by the MHC class I molecule on the cell surface (Doherty et al., 1992; Jamieson et al., 1987). CD8 T lymphocytes are able to kill infected cells effectively by releasing two types of preformed cytotoxic protein: the granzymes and perforin (Okada et al., 1989; Akashi et al., 1994). The granzymes are able to induce apoptosis (programmed cell death) in any type of target cell and perforin makes holes in the target cell membrane allowing granzymes to enter. This ability allows CD8 T cells to destroy any cell infected with a cytosolic pathogen. Fas (Apoptosis Stimulating Fragment) ligand is a member of the tumour necrosis

factor (TNF) receptor family and occurs on the membrane surface of CD8 and most CD4 T cells. Binding of the Fas ligand to Fas expressed by the target cell induces apoptosis. Interferon gamma (IFNγ) is a cytokine secreted by CD8 T cells and functions in inhibiting viral replication and activating macrophages (Janeway et al., 2005).

6.3. Cross-Presentation

A cytotoxic T lymphocyte response can be induced by either direct presentation or cross-presentation. Direct presentation is a process by which the cytotoxic T lymphocyte (CTL) recognizes the target cell e.g. infected cell or tumour cell itself. In contrast, cross-presentation/cross-priming is a process in which antigen presenting cells (APCs) e.g. dendritic cells take up antigen and present it on their cell surface, the CTL then recognizes the antigen bound to the APC and this leads to activation of the CTL. Several viral infections release copious amounts of viral particles from the infected cells and this may result in a relatively high number of cell deaths. A large amount of antigens are taken up by antigen presenting dendritic cells which then elicit a CTL response via cross presentation (Wordarz and Jansen, 2003).

7. IMMUNOLOGY OF PAEDIATRIC HIV DISEASE

As previously described, HIV-1 infects the host immune cells and impairs the immune responses. The immune system mounts both a cell mediated and humoral response against HIV; however, the response is unable to contain the infectious virions (Zeichner and Read, 2006).

7.1. Primary Infection

The majority of HIV-1 infections occur across the mucosal surface during sexual and perinatal transmission. Within 48 hours of exposure the mucosal dendritic cells transport the virus to the regional lymph nodes where CD4 T cells are the primary target of infection. Between four to eleven days post infection infected CD4 T cells can be found throughout the body. In adults, during the first few weeks of infection, the level of HIV in the blood increases exponentially and then declines dramatically, reaching a stable set point in approximately 6 months. The level of HIV-1 specific cytotoxic T lymphocytes is associated with the decline in HIV viral load. Cell mediated HIV-1 specific CTLs develop before antibodies and play an important role in suppression of initial viremia. Innate immune response is also responsible for clearance of plasma HIV-1 levels. Cytotoxic T lymphocytes suppress HIV-1 replication by secreting soluble factors including β-chemokines Regulated on Activation, Normal T Expressed and Secreted (RANTES), macrophage inflammatory protein-1 and α-defensins 1, 2 and 3. These soluble factors bind to the co-receptors CCR5/CXCR4 and block entry into the target cell.

In children rapid progression of HIV disease is seen because their immune system only reaches maturity between two and six years of age hence impacting on viral load reduction. Perinatally infected infants reach peak viremia at 1-2 months of life but unlike adults have a minimal decrease in plasma viral load over the next few months. Infants with rapid HIV disease progression have no decrease in viral load over the first year of life. Whilst in children with slow progression of disease show only a 0.5-1 $\log_{10}$ decline in the plasma viral load. HIV infected infants mount a poor CTL response against the infection in the first year of life. This has been hypothesized as an explanation for the high levels of plasma viral load that persist in infants during initial infection. Another possible reason

may be that the vertically infected infant has acquired a strain of virus that has mutated to escape the maternal immune response. The cytotoxic T cell response only matures to levels similar to that found in adults during the second year of life (Zeichner and Read, 2006).

7.2. Chronic HIV infection

The progression of HIV disease destroys the architecture of the lymphoid tissue. The germinal centres of the lymphoid tissue regress and there is an inability to mount a new immune response. Lymph nodes harbour actively replicating HIV in follicular dendritic cells (FDCs) and the FDC network is destroyed during chronic infection, resulting in the spill over of HIV into the circulatory system. The virus is continually replicating causing immune activation. Persistent immune system activation results in an increase in programmed cell death, T-cell turnover and inability of the thymus to produce new T-cells. This ultimately results in the exhaustion of the immune system (Appay and Sauce, 2008).

During advanced disease both the HIV infected adult and child can maintain detectable HIV-1-specific cytotoxic T lymphocyte and antibody responses. But there is a decrease or even absence of HIV-1-specific lymphoproliferative responses. HIV-1 preferentially infects CD4+ T helper cells and may directly result in its destruction. CD4+ T helper cells provide initial help in activating CD8+ T cells therefore the destruction of CD4+ T helper cells has been attributed to a loss in CD8+ T cell function (Jansen, 2006). There are a minority of adults and children who have no evidence of HIV disease for 10 years or longer and posses low plasma viral loads. These individuals have been termed long term non-progressors (LTNP) and both genetic and viral factors have been implicated (Rowland-Jones et al., 2001).

7.3. Cross Presentation during HIV infection

During HIV infection there is an impairment of virus specific CD4+ T helper cells. The main function of the CD+ 4 T helper cell is to activate antigen presenting cells which is necessary for cross-presentation and CTL induction. Therefore, CD4+ T helper cell impairment results in reduced cross-presentation relative to direct presentation due to the failure of dendritic cells to become activated. The virus thus shifts the dynamics away from efficient immunity towards tolerance (Wordarz and Jansen, 2003).

7.4. CTL responses in HIV infected infants and children

There have been reports that even the foetus is capable of generating an HIV-specific CD8+ T cell response after in-utero exposure to HIV (Rowland-Jones et al., 1993; Luzuriaga et al., 1995; Wasik et al., 1999). Several studies have shown that HIV seropositive infants have variable and inconsistent CD8+ T cell responses (magnitude and breadth) against HIV antigen (Buseyne et al., 1993; Luzuriaga et al., 1995; Scott et al., 2001, Sandberg et al., 2003, Lohman et al., 2005, Thobakgale et al, 2007). These studies suggested that the infrequent detection of HV-1 specific CTL responses in early infancy may be due to a delayed capability of vertically infected infants to generate specific cytokines. The difference in the immune response may also be attributed to the age-related differences in the dynamics of antigen specific CD8+ T cell activation and expansion (Scott et al., 2001). Infants infected in-utero had weaker responses to HIV-1 peptides than infants infected peripartum. However, during the first year of life both groups had a similar increase in the magnitude of HIV-1 specific IFN- responses (Lohman et al., 2005). The timing of infection and the progression of disease in children has also been shown to play a critical role in immune activation (Huang et al., 2008). Slow progressors have detectable ex-vivo and in-vitro cytolytic activity against HIV. Infants that are rapid progressors have

transient and weak anti-HIV cytolytic activity and these responses are absent in infants that progress to AIDS within the first year of life (Buseyne et al., 1998; Lohman-Payne et al., 2009).

During acute infection the infant elicits an Env-specific CD8+ T cell response and during chronic infection the principle target regions are Gag, Pol and Nef (Pikora et al., 1997; Thobakgale et al., 2007). Env and Nef are characterized by a high degree of sequence variability which are primary targets for a rapidly evolving virus, whereas, the Gag protein is relatively conserved. Infants that generated a Gag specific T cell response had a decrease in the HIV RNA level compared to those infants that did not mount such a response. The frequency of Gag specific T cell responses correlated inversely with viral load (Huang et al., 2008). Feeney ***et al***, (2003) showed that older children between 6-17 years of age mount a broader and more vigorous HIV-specific CTL response, which is similar to that seen in adult infection.

Mansoor and colleagues (2009) compared the CD4+ and CD8+ T cell response in untreated HIV infected infants, HIV exposed but uninfected infants and HIV unexposed infants during the first year of life. The HIV infected infants had a skewing of predominantly CD8+ T cell population compared to the other groups. At 3 months of age the frequency of naïve CD8+ T cells were lowest in the HIV infected group. This may be associated with thymic dysfunction and rapid progression of disease in perinatally infected infants. The effector memory cells that re-express CD45RA were also higher in the HIV infected infants than the HIV exposed uninfected infants.

7.5. CTL responses in HIV exposed uninfected infants and children

The majority of infants born to HIV-1 infected mothers remain uninfected despite recurrent exposure in-utero, peripartum and postpartum via breastfeeding. One of the first studies to describe the presence of HIV-specific CTL responses in exposed uninfected infants was Cheynier ***et al.***, (1992). Three children who reverted to HIV Seronegative after clearance of maternal antibodies had detectable HIV-specific cytotoxic activity. Two of these children mounted an intense CTL response against Env, Gag and Nef viral peptides. This response is indicative of continuous viral replication and persisted until 35 months in at least one child. At 5 months of age an HIV-gag-specific CTL activity was detectable in an infant born to an HIV infected mother. The level of CD45RO marker expression was also elevated at 5 months, however, by 13 months the CTL response had disappeared and the CD45RO marker expression was normal for age (Rowland-Jones et al., 1993). The infant tested HIV negative up to 18 months of age using various assays (EIA, p24 antigen, HIV DNA PCR and HIV RNA PCR). Several other studies have been able to detect HIV-specific CTL activity in a minority of HIV exposed uninfected infants (De Maria et al., 1994; McFarland et al., 1994; Wasik et al., 1999). The various studies describing HIV-1 specific CTL responses in exposed uninfected infants and children is summarised in Table 1.

There are various theories that have been proposed to explain this phenomenon. The first theory is that in-utero exposure to human immunodeficiency virus may induce a cell mediated immune response, and this may be true even in the presence of low viral stimulus and at low level of immune activation. The foetus could have been exposed to either high quantities of non-infectious HIV-1 particles in-utero or infectious maternal lymphocytes or antigen presenting cells that may micro transfuse the placenta, activating the foetal

immune system. The second theory is that the foetus was transiently infected with HIV and was able to effectively clear the virus prior to delivery. The last theory is that prolonged low level exposure to antigen during gestation may trigger differentiation of memory cells with a transition through the full effector phase (Legrand et al., 2006). Most recently, John-Stewart (2009), showed that infant exposure to human immunodeficiency virus type 1 (HIV-1) via breastfeeding may elicit HIV-1-specific immunity against infection. Forty-seven percent of the infants (HIV exposed but uninfected) enrolled in the study had at least one positive ELISPOT assay during follow-up. The data are summarised in table 1.

Table. 1 HIV-1 specific CTL responses in uninfected infants and children with HIV-1 exposure

Adapted from: Farqhar C. and John-Stewart G. (2003). The role of infant immune responses and genetic factors in preventing HIV-1 acquisition and disease progression. Clini Exp Immunology, 134:367-377

AUTHOR, YEAR	AGE RANGE	PREVALENCE OF RESPONSE
Cheynier, 1992	*2D – 35 months	3/3 (100%)
Buseyne, 1992	11D – 36months	0/4 (0%)
Rowland-Jones, 1993	Cord blood – 13 months	1/1 (100%)
Aldhous, 1994	6D – 18 months	2/11 (18%)
De Maria, 1994	12D – 50 months	7/23 (30%)
McFarland, 1994	6D – 23 months	2/8 (25%)
Luzuriaga, 1995	1D – 18 months	0/10 (0%)
Wasik, 1999	Cord blood – 14 months	2/9 (22%)
Legrand, 2006	1D - 9 months	9/9 (100%)
Thobakgale, unpublished data	1D – 144 months	0/4 (0%)
John-Stewart, 2009	1D – 12 months	141/217 (47%)

*D = day/s

8. BREASTFEEDING AND HIV INFECTION

Mother-to-child transmission accounts for the majority of (> 95%) HIV infection in children and occur either in uteri, perinatally or through breastfeeding. Vertical transmission of HIV through breastfeeding was first described in 1985 in women newly infected via blood transfusion or heterosexual exposure after delivery. In the developed world, the use of antiretroviral therapy, prevention of mother-to-child transmission (PMTCT) prophylaxis, caesarean section and the avoidance of breastfeeding has virtually eliminated vertical HIV transmission (2% to 4%) (Townsend et al., 2008).

In the developing world, perinatal transmission rate in the absence of such interventions varies between 20% and 30%. Breastfeeding especially when practiced as mixed feeding contributes an additional 10 to 15%. Hence, HIV positive mothers in the developing countries are faced with the difficult dilemma of whether to breastfeed or formula feed their infants. Breastfeeding will expose their infants to HIV infection, while formula feeding will increase their risk of developing gastrointestinal and respiratory related morbidity and mortality during the first year of life. Because of the high prevalence of HIV among antenatal clinic attendees in South Africa, and inadequate access to effective prevention of mother-to-child transmission (PMTCT) programmes, large numbers of HIV infected children will still be born.

The mechanism of postnatal HIV transmission by breastfeeding is unclear and several hypotheses have been proposed. A previous study has shown that the tonsillar region is laden with target cells and that this may serve as a primary portal of HIV entry (Campo et al., 2006). During mucosal damage, inflammation may result in an increase in susceptible target cells that may enhance HIV replication (Devito et al., 2000a). Several other

mechanisms of entry have been proposed with an intact mucosal surface including, transcytosis through epithelial cells, M cells in the Peyer's patches or enterocytes-expressing galactosyl ceramide or Fc receptors and dendritic cells (Devito et al., 2000a; Devito et al., 2000b).

During infection, breast milk contains both cell free and cell associated human immunodeficiency virus. Mature milk has detectable levels of cell free HIV and if the strain of virus is infectious then infants are more susceptible to acquiring HIV infection (Lewis et al., 1998). Semrau and colleagues (2008) showed that consistent shedding of human immunodeficiency virus in breast milk and high breast milk viral loads are strong predictors of HIV mother to child transmission. Breast milk has also been shown to be a reservoir of latently infected resting T cells that have a greater capacity to enter viral replication than latently infected T cells in peripheral blood (Becquart et al., 2006). There are maternal, infant and viral factors which contribute to an increase in postnatal acquisition of HIV-1 through breastfeeding. These factors include the duration of breastfeeding, non-exclusive breastfeeding in the first 6 months of life, maternal CD4+ count of less than 400 cells/µl, maternal nipple lesions, maternal acquisition of HIV infection during breastfeeding, infant oral thrush, prematurity, viral load in breast milk and plasma and clade C virus (Embree et al., 2000; John et al., 2001; Willumsen et al., 2003; Coovadia, 2009; Lunney et al.,2010).

In a study conducted in Durban, HIV infected mothers self selected to either breastfeed or formula feed there infants (Coutsoudis et al., 2001). Those infants that were exclusively breastfed for at least 3 months had no excess risk of acquiring HIV infection compared to those infants that were formula fed i.e. 19.4%. However, the authors showed that those

infants that were fed a mixture of breast milk and other liquids and solids (i.e. mixed fed) were at increased risk (26.1%) of mother to child transmission of HIV (Coutsoudis et al., 2001; Coutsoudis et al., 1999; Lunney et al, 2010). At 15 months the rate of transmission was lower among those infants that were exclusively breastfed for at least 3 months compared to those infants that were mixed fed, i.e. 24.75% vs. 35.9%. The authors hypothesized that contaminated fluids and food given to infants during non-exclusive breastfeeding damaged the infants gut and mediated the entry of HIV into the tissues.

In a similar study, results revealed that the risk of acquiring HIV infection in infants that were mixed breastfed before three months of age was fourfold higher than those infants that were exclusively breastfed (Iliff et al., 2005). In the presence of interventions including the provision of short term antiretroviral prophylaxis and free infant formula in Cote d'Ivoire, breastfed infants had no increased risk of mother to child transmission of HIV when compared to the formula fed infants. However, the formula fed infants had increased risk of morbidity and mortality from diarrhoeal and respiratory illnesses (Becquet et al., 2007).

9. BENEFITS OF BREASTFEEDING

The immune system of the neonate is immature at birth and this is the primary cause of the increase in neonatal morbidity and mortality. Breast milk contains a large number of specific immunological and non-immunological factors that enhance the infant's immune response against infectious organisms, by providing both passive and active immunity (Lawrence and Lawrence, 2005).

Breast milk contains large quantities of long chain polyunsaturated fatty acids (LCPUFA's) which is an essential component in neural and vascular membranes (Koletzko et al., 2001). Children that are breastfed tend to have a lower fasting plasma glucose level and a reduced diastolic blood pressure when compared to children that are formula fed (Baur, 1998; Wilson et al., 1998; Singhal et al., 2001). A cohort of children that are born prematurely and breastfed at birth were shown to have a higher intelligence quotient at 7.5 – 8 years of age than the formula fed premature children (Lucas et al., 1992).

In 1990 Lucas *et al,* conducted a prospective multicentre study to investigate the relationship between early feeding choices and the development of necrotizing enterocolitis (NEC). Results revealed that those infants that were exclusively formula fed had a 6 fold increased risk of developing NEC compared to those infants that were breastfed. Furthermore, the results also showed that those infants that were exclusively formula fed had a 3.5 fold increased risk of developing NEC compared to those infants that were fed a mixture of breast milk and formula.

In the first year of life infants are susceptible to the development of recurrent and non-recurrent otitis media. Breastfeeding was found to play a protective role against this common infant infection (Duncan et al., 1993). This study showed that increasing the duration of exclusive breastfeeding in the first year of life decreases the total number of acute otitis media episodes. This holds true for recurrent and non-recurrent otitis media.

Breast milk is a rich source of cholesterol and animal studies have suggested that postnatal ingestion of large quantities of cholesterol protects against high cholesterol challenge later

in life (Mott et al., 1990) . A study by Wong (1993), demonstrated that adolescents that are breastfed have a reduced low density lipoprotein to high density lipoprotein ratio.

High leptin concentrations relative to fat mass have been associated with obesity (Considine and Caro, 1996; Considine et al., 1996). Adolescents that were breastfed had lower leptin concentrations relative to fat mass compared to those adolescents that were formula fed (Wilson et al., 1998; Singhal et al., 2002).

Exclusive breastfeeding has been associated with reduced morbidity and mortality due to respiratory and diarrhoeal illnesses (Cesar et al., 1999; Oddy et al., 1999; Bahl et al., 2005). Several mechanisms have been proposed that suggest that exclusive breastfeeding offers protection against these illnesses. These include reduced exposure to environmental pathogens and dietary antigens, an increase in the number of beneficial intestinal micro flora, the antimicrobial, anti-inflammatory and immunomodulating factors present in breast milk, and maintaining the integrity of mammary gland epithelial tight junctions (Lawrence and Lawrence, 2005; Kraehenbuhl and Neutra, 2000). In view of the many benefits of breastfeeding it is not surprising that breastfeeding promotion was suggested to be able to reduce 13% of the 11 million needless deaths among children < 5 years of age (Jones, 2003).

10. HEAT TREATMENT OF EXPRESSED BREAST MILK

During the time of the study the 2006 WHO infant feeding guidelines were implemented. The WHO recommended that an HIV-infected woman exclusively breastfeed for the first six months unless replacement feeding is acceptable, feasible, affordable, sustainable and safe (AFASS criteria), followed by weaning only if a nutritionally adequate and safe diet can be maintained (WHO, 2006). When replacement feeding fulfils the AFASS criteria

then the avoidance of all breastfeeding by the HIV-infected woman is recommended. In 2010 these guidelines were revised. Mothers known to be HIV-infected and whose infants are HIV uninfected or of unknown HIV status should exclusively breastfeed for the first 6 months of life, introducing appropriate complementary foods thereafter, and continue breastfeeding for the first 12 months of life. The guidelines further emphasized that breastfeeding should only stop once a nutritionally adequate and safe diet without breastmilk can be provided (WHO, 2010). And finally, when an HIV-infected mother decides to stop breastfeeding they should do so gradually within one month. This recommendation is based on the finding that risk of HIV transmission is lower in infants exclusively breastfed compared to those that are mixed breastfed (Coutsoudis et al., 2001).

The WHO also recommend modifying breastfeeding to reduce HIV transmission while still retaining the immunological benefits of breast milk to protect the infant from common childhood illnesses. The alternative recommendation is the heat treatment of manually expressed breast milk.

A few heat treatment methods have been proposed which include:

a) **Direct boiling**: which causes significant nutritional damage,

b) **Pasteurization**, which can be done in 3 ways

1. **Holder Pasteurization** (62.5ºC for 30 mins): commonly used in breast milk banks and has been shown to inactivate HIV while retaining most of the breast milk protective components. However, it requires the use of commercially manufactured pasteurisers, temperature gauges and timing devices that are not available in resource limited settings.

2. **Pretoria Pasteurization** (56 - 62.5ºC for 15 minutes): A simple method but requires timing. The method is effective in inactivating HIV, destroying common pathogens (***E.coli*** and ***S.aureus***) and retaining breast milk immune components (Jeffery and Mercer, 2000; Jeffery et al., 2003; Jeffery et al., 2001).
3. **Flash Pasteurisation** (72ºC for 15 seconds): high temperature, short time pasteurization method (Terpstra et al., 2007).

The Flash heat treatment method is a simple, cost effective method that can be used in the domestic third world setting which imitates flash pasteurisation of breast milk. The method is capable of inactivating cell free and cell associated human immunodeficiency virus in naturally infected breast milk samples. Flash heating of expressed breast milk reaches a temperature of 72ºC and higher for a few minutes and the authors hypothesize that cell associated HIV provirus is inactivated and undergoes cell death under these conditions. The surface protein on the cell free virus is disrupted during heating and the virus is rendered non-infectious. The heat sensitive reverse transcriptase enzyme is exposed to the high temperature and is destroyed (Israel-Ballard et al., 2007). There is an increase in the level of vitamin A, B12, folate, riboflavin and thiamine post heat treatment. This effect may be due to the release of vitamins from binding proteins during heating (Israel-Ballard et al., 2005b; Israel-Ballard et al., 2008).The heat treatment reduces some of the lactoferrin and the vitamin C and E content of breast milk. During the flash heat treatment method the antigen binding capacity of most of the immunoglobulin's present in breast milk were retained (Chantry et al., 2009).

Israel-Ballard and colleagues also concurrently evaluated the Pretoria pasteurization method and the Flash heat treatment method and showed that the Flash heat method is the

preferred method (Israel-Ballard et al., 2005b). The antimicrobial properties of breast milk are also protected during flash heating. Heat treated samples that were spiked with *S. aureus* and *E. coli* had the least growth of bacteria compared with unheated spiked samples. Flash heating was also effective in eliminating bacteria and preventing substantial bacterial growth for up to 8 hours when heat treated breast milk samples were stored at room temperature (Israel-Ballard et al., 2006a). The flash heat treatment method is capable of inactivating HIV while retaining the nutritional content of breast milk while being a simple technology to implement in a resource limited settings burdened by the HIV epidemic. Recent qualitative studies in Zimbabwe have shown that the flash heat treatment method may be acceptable and feasible to implement in this setting but warrants further investigations (Israel-Ballard et al., 2006b; Mbuya et al., 2010).

11. STUDY RATIONALE

Infants born to HIV-1 infected women are potentially exposed to virus in-utero, at delivery and again via breastfeeding. This may be analogous to recipients of a prime boost vaccine. In this study a portion of the infants were given heat treated breast milk which contains heat inactivated virus or dead virus. We hypothesised that the mucosal lining of the gut would induce CTLs in response to the heat inactivated virus from the heat treated breast milk. Furthermore, although considerable work has been done on the CTL responses in full term HIV infected infants very little is known about the CTL responses in infants born prematurely. It is important to be able to elucidate the CTL responses shown in preterm infants who receive formula feed from birth and those that receive heat treated expressed breast milk from birth.

12. AIM OF STUDY

A pilot study to describe clinical outcomes and CTL responses in HIV exposed low birth weight infants.

13. OBJECTIVES

1. To describe cumulative 9 month clinical outcomes (growth and morbidity) in HIV exposed low birth weight infants fed on heat treated expressed breastmilk (HTEBM) vs. a group never exposed to breastmilk (formula fed infants).
2. To describe immune responses to HIV at 6 weeks of age, in HIV exposed low birth weight infants fed on heat treated expressed breastmilk (HTEBM) vs. a group never exposed to breastmilk (formula fed infants).
3. To describe immune responses to HIV at 3, 6, and 9 months in infants exposed to breastmilk and infected at 6 weeks vs. those who are not infected at 6 weeks.
4. To assess multifunctionality of HIV-1 specific T cells by multicolour flow cytometry.

CHAPTER TWO

METHODOLOGY

2.1 Study design

This was a pilot study that was conducted in a prospective longitudinal design in order to describe the clinical outcomes and cytotoxic T lymphocyte (CTL) responses in preterm infants with different feeding methods. CTL response testing was only commenced after the first 22 infants were enrolled. The sample size for the pilot sub-study (CTL response testing) was set at 25 infants. Although we only planned to study the CTL responses in 25 infants because we had to make allowances for an expected large drop out rate due to demise of the infant or other clinical complications, an extra 30 infants were recruited to ensure success in obtaining complete longitudinal data on the 25 infants in the study.

A total of 77 infants were enrolled into this cohort. Of these 38 infants were enrolled into the heat treated expressed breastmilk (HTEBM) arm, 10 infants were enrolled into the expressed breastmilk arm (EBM) and 29 infants were enrolled into the formula fed arm (Figure 4). Of the 77 infants, 55 were enrolled into the pilot sub-study. A schematic representation of the design of the sub-study is illustrated in figure 5. Due to the overwhelming success with the implementation of the Flash heat treatment method at the King Edward VIIIth Hospital Nursery, the majority of HIV positive mothers decided to heat treat their expressed breastmilk instead of feeding their infants unheated (raw) breastmilk. As a result, a decision was made to combine the expressed breastmilk feeding group (EBM) and the heat treated expressed breastmilk feeding group (HTEBM), into one group.

All infants enrolled into the study received a single dose of NVP at birth and either 7 or 28 days of AZT depending on whether the mother had received antiretroviral prophylaxis during pregnancy. Each infant was followed longitudinally and provided four testing times each allowing a longitudinal investigation over a 9 month period.

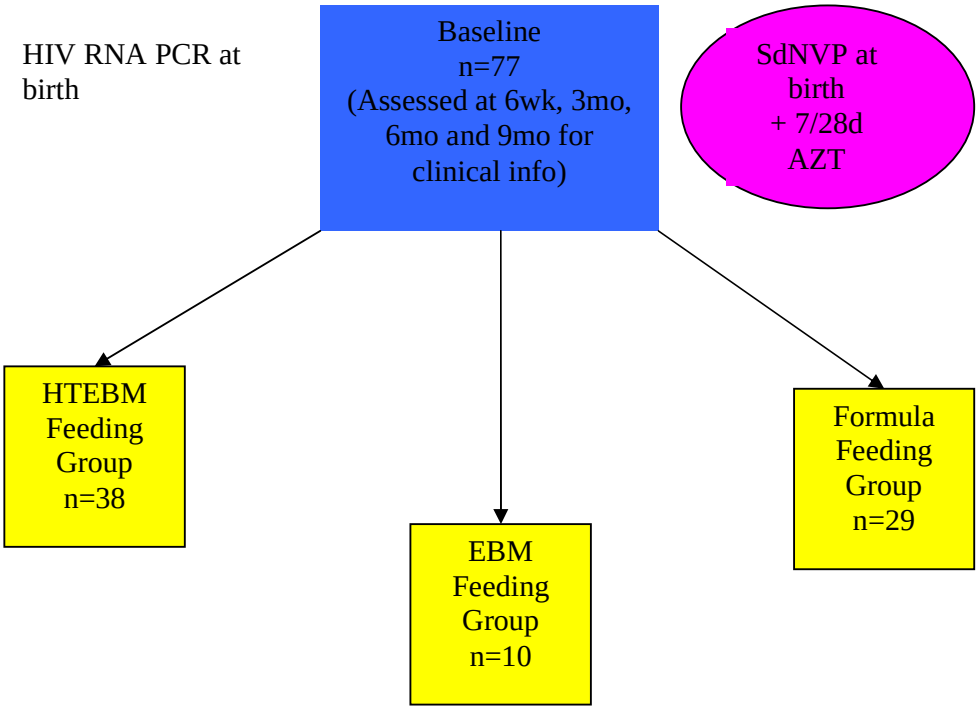

Figure 4. Schematic representation of the design of the main study

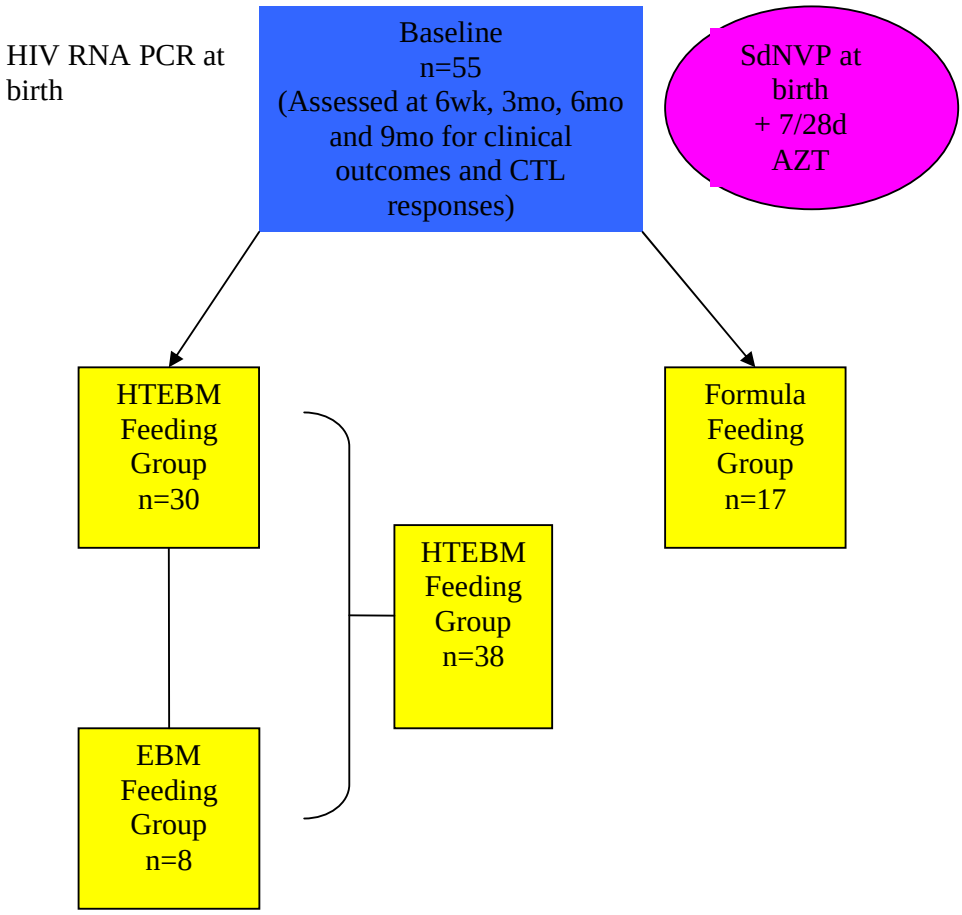

Figure 5. Schematic representation of the design of the sub-study

2.1.1 Study site and study population

Study site

Infants were be enrolled from the neonatal nursery at King Edward VIIIth Hospital, Durban, South Africa from February 2008 – September 2009.

Study Population

All pregnant women who tested positive and delivered a preterm, low birth weight infant between 1200-1800g were eligible for enrolment. All HIV positive mothers received

counselling on infant feeding choices according to WHO/UNICEF/UNAIDS guidelines and chose to either formula feed or breastfeed. Mothers who opted to breastfeed were informed about the possibility of heat treating their expressed breastmilk. The method was demonstrated to them and support was given to enable them to heat treat their milk in the nursery. Enrolment took place once the feeding and counselling had been given. Mothers were asked for written consent to have their infants participate in the study (Appendix 1).

2.1.2 Inclusion and exclusion criteria

The inclusion criteria were as follows:

- infants who were born to HIV infected mothers ≥ 18 years of age,
- infants with a birth weight of 1200-1700g,
- infants with a gestational age of < 34 weeks,
- infants whose mothers were not on HAART or eligible for HAART and
- Infants whose mothers had given written informed consent.

The exclusion criteria comprised the following:

- infants born to HIV infected mothers <18 years of age,
- infants with a birth weight of <1200g and >1800g,
- infants with a gestational age of >34 weeks,
- women who had advanced disease (CD4 < 200 or stage 3 and 4 disease) were not eligible for participation and were referred to the national ARV treatment programme,
- and finally, women who did not intend to stay within a 30 km radius of the clinic and were not able to attend the follow up visits were not eligible to participate in the study,

- any baby born with any congenital abnormality or with any complications during birth requiring specialised management was not eligible to participate and
- Finally infants who switched their feeding method while in hospital (i.e. infants who receive formula milk and switched to breastmilk during there hospital stay and vice versa).

2.1.3 HIV diagnosis and clinical & growth monitoring

Follow up: Infants were followed up at 6 wk, 3mo, 6mo, and 9mo from birth. The 6 week follow-up was conducted in the nursery if the patient was not yet discharged. Once discharged, infant follow-up visits took place in the neonatal clinic for low birth weight infants. Blood was taken at each visit. All infants had a 6 week HIV DNA PCR test as per government guidelines. Additionally, at birth a blood spot on filter paper was taken and stored for later testing by RNA-PCR if the 6wk sample was positive. At each follow up visit, a blood spot was taken and stored for all infants for later checking of HIV status. Final status was determined at 9 months of age by the Rapid test in infants who ceased breastfeeding at least 6 weeks before and were still HIV negative. Infants who were still being breastfed at 9 months of age had a DNA PCR test done to determine their status at 9 months, which was the end point of the study.

All babies were seen at 6wk, 3mo, 6mo, and 9mo for clinical, growth, developmental and nutritional assessment and to identify clinical signs of disease progression and opportunistic infections. At each follow up visit, a detailed feeding history was collected in order to categorise infants into exclusive breastfeeding with non- heat treatment, exclusive breastfeeding with heat treatment or formula feeding and the duration of each feeding mode. The HIV infected infants had WHO disease staging at each visit. All

infants testing positive were assessed for eligibility for antiretroviral therapy (ART) as per national guidelines and if requiring treatment were referred to an onsite ARV rollout clinic.

2.1.4 Patient management

During the time of the study all infant feeding counselling was conducted in accordance with the current WHO/South African guidelines for HIV infected mothers concerning infant feeding (WHO, 2006). The standard of care is that women are counselled around infant feeding options at the antenatal clinic visit and encouraged to make a choice to either exclusively breastfeed or formula feed their infants. This choice is then recorded on their maternal chart and made available to the nurses when the mother delivers. However, some mothers may deliver prematurely before they have made an infant feeding choice as a result the study had 2 groups of mothers; those who had been counselled and those who did not receive counselling. For mothers who had already been counselled we spent a short time with them ensuring that they understood the choice they had made and provided reinforcement of the counselling they received. Mothers who did not receive antenatal infant feeding counselling were provided with counselling. Basically the guidelines recommend that women should exclusively breastfeed for 6 months unless formula feeding is acceptable, feasible, affordable, sustainable and safe (AFASS criteria).

The counsellor discussed with the mother whether she has AFASS criteria in place for safe formula feeding and based on this assisted the mother to make her choice. The study had one dedicated counsellor who received training on the WHO breastfeeding counselling course. Women who experienced infant feeding problems were encouraged to return to the clinic for evaluation and care.

Mothers received additional counselling support at delivery (breastfeeding was encouraged within the first hour of birth), and postnatal counselling. The counselling on infant feeding was continued at the scheduled follow up visits. Counsellor training was based on infant feeding training materials from the World Health Organization materials and Department of Health (DOH) (WHO, 2006). The mother's baseline and 6 monthly CD4 counts were done as per national guidelines. All mothers requiring treatment as per national guidelines were referred to the onsite ARV rollout clinic.

2.1.5 Infant feeding practice

A structured questionnaire of the infant dietary consumption was given at each clinic visit to determine infant feeding patterns including exclusive breastfeeding. Mothers were asked about infant feeding practices including current breastfeeding status, total number of breast feeds over the last 24 hours, lactational amenorrhea, and mother's working status, and proximity to infant while at work. In addition the reasons for introduction of liquids or foods were solicited.

2.1.6 The Flash heat treatment method

The Flash heat treatment method demonstrated to the mothers was adapted from Israel-Ballard *et al*, (2005a). In brief, 50-100ml of breastmilk was manually expressed into a glass jar; the uncovered jar of milk was placed into a 1L aluminium pan. The water level was 2 finger widths above the level of milk, and the water and milk were heated on an electric hot plate until the water reached a rolling boil. The breastmilk was removed from heat source and allowed to cool to a lukewarm temperature tolerable to infant. The jar may be held under cool running water for up to a minute, or by letting the milk to stand.

The breastmilk was fed to the child by nasogastric tube, syringe, cup or spoon based on the child's maturational level.

2.1.7 Data collection methods and tools:

Quantitative and qualitative data was collected from the nursery and POPD clinic at the King Edward VIII hospital, Durban, South Africa. Data was collected by the counsellor and PI by means of study specific data forms (see Appendix 1-5) and included:

- The enrolment questionnaires which comprised of baseline data on mother and infant at enrolment and psychosocial data (Appendix 3),
- The in-hospital sheet which contained both morbidity and feeding data (Appendix 4),
- The infant follow-up form which consisted of morbidity and feeding data (Appendix 5).

2.1.8 Ethical approval

Ethical approval for the study was obtained in 2008 from the Biomedical Research Committee of the University of KwaZulu-Natal. Ethics Ref: BE 137/07.

2.2 LABORATORY METHODOLOGY

2.2.1 Blood sample collection

Approximately 3.5mls of whole blood sample **w**ere collected in a 4ml EDTA anticoagulant tube at each clinical visit for the following tests: isolation of peripheral blood mononuclear cells (PBMCs) for use on ELISpot assay (megamatrix and confirmation) and Intracellular Cytokine Staining (ICS), CD4 cell counts and isolation of plasma for RNA-PCR (in HIV positive infants). When insufficient amounts of PBMCs were recovered, the ELISpot (confirmation) and ICS tests were performed at the next visit. Figure 5 is an illustration of the various tests that were performed per individual on each visit. Blood samples were collected at the following time points 6 weeks, 3 months, 6 months and 9 months. A dried blood spot (DBS) was collected at birth for future HIV RNA PCR monitoring once the infant tests positive at 6 weeks. A dried blood spot (DBS) was collected at each time point for HIV DNA PCR to determine the timing of seroconversion and the end of the study.

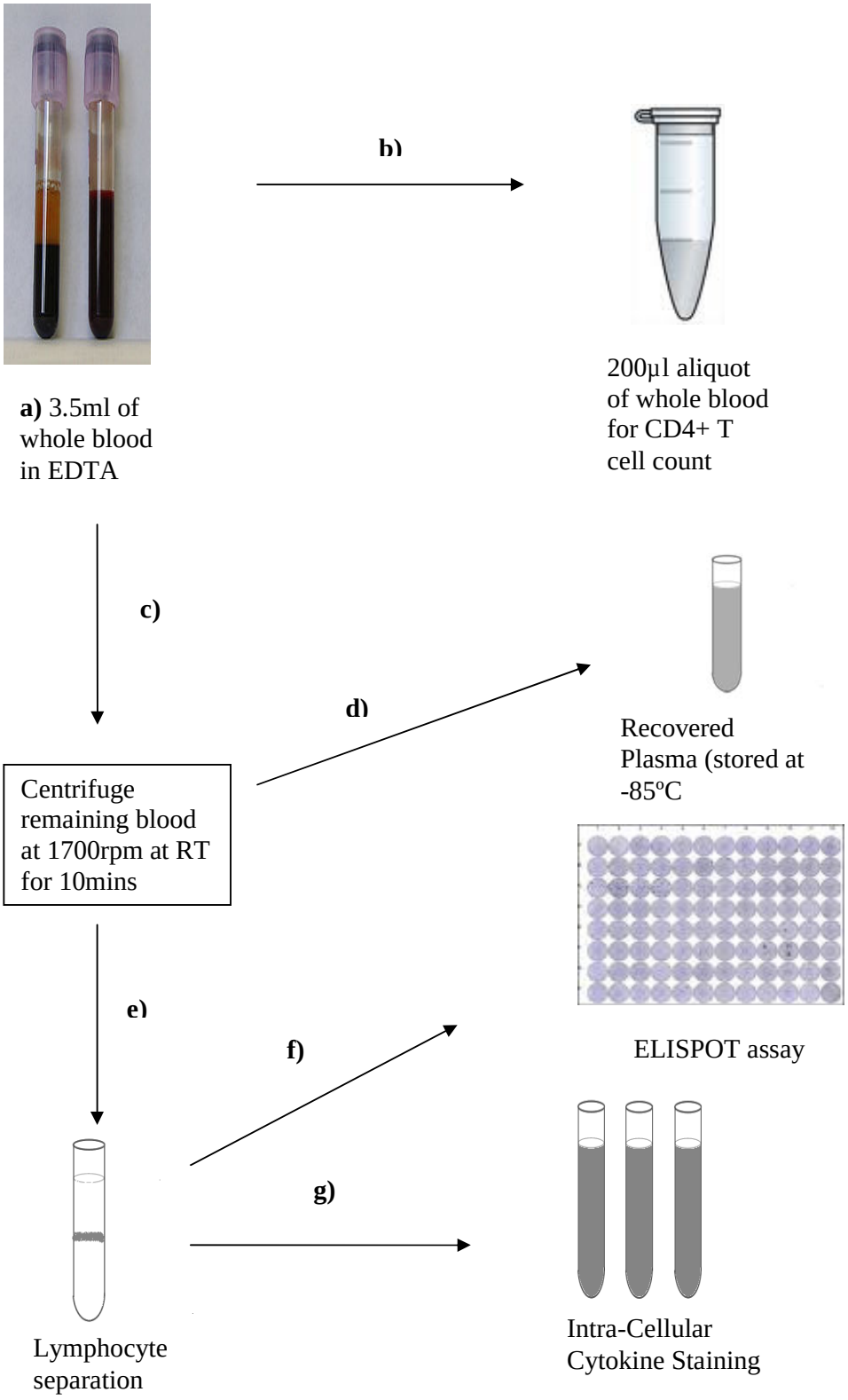

Figure 6. Schematic representation of the tests performed on the patient's blood samples upon each visit

2.2.2 CD4+ T cell count monitoring

CD4 counts were determined from fresh whole blood by Tru-Count technology using a four-color FacsCalibur flow cytometer (Becton Dickinson) as previously described (Thobakgale et al., 2007).

2.2.3 HIV RNA-PCR quantification (Viral Load)

COBAS® TaqMan® HIV-1 test (Roche Diagnostics)

The HIV RNA levels were determined from plasma using the COBAS® TaqMan® HIV-1 Test. This assay combines the automated sample processing and automated sample detection and amplification which has been previously described (Schumacher et al., 2007).

2.2.4 Peripheral Blood Mononuclear cells (PBMCs)

Separation of PBMCs

Preparation

Everything in the hood was sprayed with 70% ethanol before placing any other material in the hood. For infant samples 15ml Falcon tubes were used and for adults 30ml Falcon tubes were used. Tubes were pre-labelled with patient ID and time point. The exterior of the EDTA tube was not sterile and to prevent contamination especially around the rim, it was wiped down with 70% ethanol before transferring to the hood. The maximum amount of blood that can be processed in a 15ml tube is 13.5ml.The Histopaque-1077, PBS (Appendix 8), PBS with Antibiotics (Appendix 9) and R10 (Appendix 10) media were removed from the fridge and left at room temperature to warm for approximately one hour.

The separation was based on a ratio of 1:1:1 of the Histopaque-1077: PBS + Antibiotics: Blood Sample.

Methods

An equal volume of Histopaque-1077 was added to a sterile 15ml Falcon Tube. The blood sample was diluted with an equal volume of PBS + Antibiotics (Appendix 9). This was mixed well and carefully layered onto the Histopaque-1077. The tubes were then centrifuged at room temperature for 30 minutes at 1600rpm with slow start and the brakes off. The mononuclear cell layer (between the PBS and Ficoll) was transferred into a new sterile 15ml Falcon tube. PBS was added to the mononuclear layer to a volume of 13.5ml. If red blood cells were present, several drops of sterile water were added to lyses them, the mix was allowed to stand for a few minutes and PBS was added immediately thereafter. The tubes were centrifuged at 1600rpm for 10 minutes at room temperature ensuring that when decanting the supernatant the cell pellet was not disturbed. PBS was added for the second wash of the PBMCs. The supernatant was decanted and the tubes were taped gently to break up the cell pellet and then resuspended with 10ml of R10 medium (Appendix 10). The PBMCs were counted prior to subsequent investigations.

2.2.5 Guava ViaCount – cell counting

Method

A bead sample was prepared once the Guava Check beads and diluents were at room temperature. A 1:20 dilution of Guava Check beads: diluents (25µl beads + 475µl diluents) was freshly prepared and mixed well. The Guava check program was selected and the beads were run three times. Each reading of the beads must flag in green. If this was not the case then the clean cycle was selected, the machine was shutdown and the Guava

Check was re-run. If the Guava Check failed the Guava beads were re-prepared and read three times. A 1:10 dilution of the PBMCs was prepared (20µl of cells + 180µl of counting solution). The diluted sample was vortexed on high speed and incubated at room temperature for 8 minutes.

2.2.6 Haemocytometer – cell counting

Method

The isolated PBMCs were resuspended in 10ml of R10 medium (Appendix 10). The cells were thoroughly mixed with a pipette aid and a 10 ml pipette. The tube was then vortexed and 10µl of the cell suspension was aliquoted into a sterile 1.5ml eppendorf. 10µl of commercial PBS (DPBS) and 80µl of Tryphan blue was then added to the eppendorf and the solution was vortexed well. 10µl of the diluted suspension was transferred into the well of the haemocytometer and was then placed under the microscope and the cells were counted.

2.2.7 Freezing of PBMCs

Method

The 15ml falcon tubes containing the cells were centrifuged at 4ºC for 10 minutes at 1700rpm. After the spin the tube was aspirated down to a volume of 200µl and placed on ice. The pellet of cells was then resuspended in this volume. The cells were frozen at a concentration of 10 million cells per vial in a 1ml volume using 10% DMSO added to 100% filtered fecal calf serum. To reduce shock to the cells, half the quantity of freezing solution was added as FCS on its own and the cells were thoroughly suspended in this formula. The FCS with DMSO was thereafter added in a drop wise manner whilst gently shaking the cyropreserve vial.

The cryropreserve vials were immediately transferred to a Mr Frosty stratacooler box (which contained 100% isopropanol, since DMSO is toxic to cells) and this was placed in the -80ºC freezer overnight. The following day the cyropreserve vials were transferred to the liquid nitrogen freezer.

2.2.8 ELISPOT assay

Synthetic HIV-1 peptides

A panel of 410 overlapping peptides (18mers with 10-12 amino acid overlap) spanning the entire HIV-1 clade C consensus sequence was synthesized and used in a matrix screening assay as previously described (Kiepiela et al, 2004). Previously defined confirmation peptides were similarly synthesized.

Method

A sample of 5µl of anti-human IFN-γ (Mabtech) was diluted with 10ml commercial PBS. The solution was vortexed well and 100µl was coated into each well of a sterile 96-well polyvinylidene diflouride plate (Multiscreen, Millipore, Bedford, USA) with a multichannel pipettor. The plate was then incubated overnight at 4ºC.

The plate is removed from the 4ºC fridge and washed six times with blocking buffer (Appendix 12). Approximately 100µl of blocking buffer is added to each well to remove the unbound antibody. 50µl of R10 medium (Appendix 10) were added to each well using a multichannel pipette. For every patient's sample tested, two wells contained 10µl of Phytohaemaglutinin A (PHA) at a concentration of (33.3µg/ml) as positive controls and four wells without any peptide as negative controls. 10µl of each individual peptide is added (33.3µg/ml) giving a final concentration of 2µg/ml was added to each well.

100µl of freshly isolated PBMCs suspended in R10 medium at a final concentration of 0.5 X 10^6 was added to each well. The plate was incubated overnight at 37ºC in the 5% CO_2 incubator.

The following day cells were discarded in a disinfectant container which contained 2% Virkon solution (Appendix 11). The plate was washed six times with PBS using the ELISPOT washer, 200µl/well. 5µl of biotinylated IFN-γ antibody was diluted with 10ml of PBS and vortexed well. 100µl of the diluted biotinylated antibody was added to each well, covered with aluminium to protect he plate from sunlight and incubated for 90 minutes at room temperature. The plate was washed six times with PBS using the ELISPOT washer, 200µl/well. 5µl Streptavidin-alkaline phosphate conjugate was diluted with 10ml PBS and vortexed well. 100µl diluted Streptavidin-alkaline phosphate was added to each well, covered with aluminium foil and incubated for 45 minutes at room temperature. The plate is then washed with PBS six times using the ELISPOT washer. 100µl of Bromo-Chloro-Indoylphosphate (BCIP) (Appendix 13) and 100µl of Nitroblue Tetrozolium Chloride (NBT) (Appendix 14) was diluted in 10ml 1M TRIS Buffer (pH 0.3) (Appendix 15) and vortexed well. 100ul of the colour solution is added to each well and incubated at room temperature until blue spots develop. This usually took 15-20 minutes and the reaction was stopped before the background came up on the negative wells. The development process was stopped by washing the plate six times under running water. The plate was left to air-dry on absorbent tissue paper. The ELISPOT plate was read using the automated reader (Autoimmune Diagnostics – Germany).

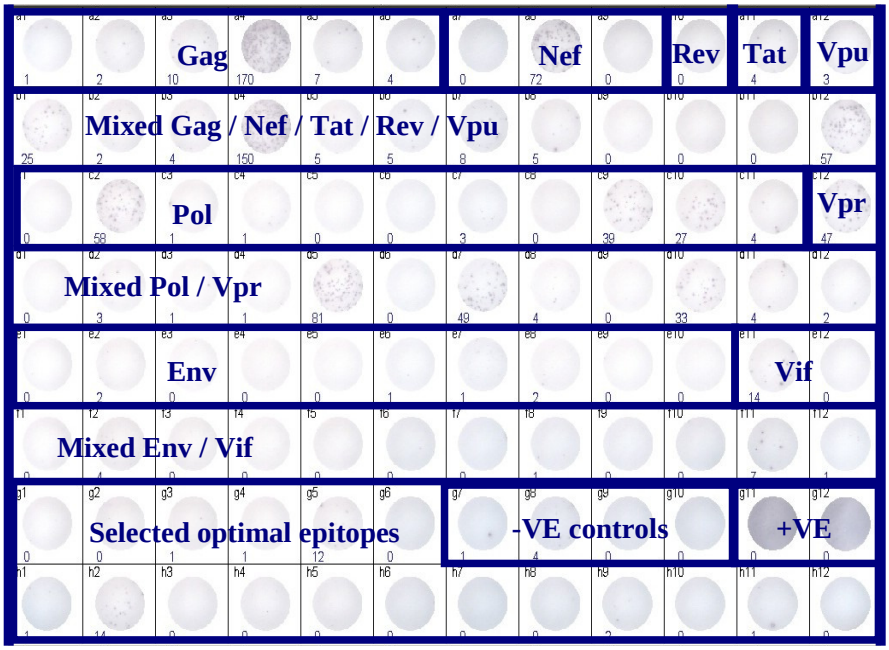

Figure 7. Pooled peptides in a matrix format on an ELISPOT plate that spanned the HIV genome

2.2.9 Intracellular Cytokine Staining (ICS)

Method

The isolated PBMCs were counted and resuspended in a final concentration of 1 X 10^6 cells/ml. A positive control (1µl of PMA and 0.5µl of Ionomycin was added) and a negative control (10µl of R10 was added) were prepared for each patient sample being tested. A master mix solution was prepared (per tube) with the following:

- 1µl of anti-CD28 antibody (Beckon Dickenson Immunocytometry Systems, San Jose, CA)
- 1µl of anti-CD49 antibody (Beckon Dickenson Immunocytometry Systems, San Jose, CA)

The master mix was vortexed and 2µl was aliquoted into each tube. 10µl of pooled peptide at a concentration of 200 µg/ml was added in the respective experimental tube. 60µl of individual peptide at a concentration of 33µg/ml was added to the respective experimental tube. 1ml of PBMCs resuspended in R10 was added to each of the experimental tubes. The tubes were incubated for 15 minutes at 37ºC in a 5% CO_2 incubator. The lids of the FACS tubes were loosened to allow the aeration of cells. After the 15 minute incubation period the FACS tubes were taken out of the incubator and 6µl of Brefeldin A (Sigma Chemical Co., St Loius, USA) and 3µl of Golgi Stop was added to each tube. The tubes were then incubated at 37ºC in a 5% CO_2 incubator for 6 hours. The tubes were then refrigerated at 4ºC overnight and processed the following day. 2ml of PBS was added to each tube to resuspend the cells and the tubes were centrifuged at 1500rpm for 10 minutes at room temperature. The PBS is then removed by inverting each tube over the waste container (making sure the pellet is not disrupted). A master mix is prepared which contains the following: 3ml of commercial PBS and 3µl of vivid dye. 200µl of the master mix is added to each tube and incubated 20 minutes on ice wrapped in foil. The cells were then washed with 2ml of PBS and centrifuged at 1500rpm for 10 minutes at room temperature. The supernatant is removed by inverting the tubes over the waste bucket and then added tittered amounts of each surface stain. The tubes are wrapped in foil and incubated at 4ºC for 20 minutes. 2ml 2% FCS PBS was added to each tube and centrifuged at room temperature for 10 minutes at 1500rpm. Cells were fixed with 100µl of Caltag Medium A (Caltag Laboratories, Burlingame, CA) for 15 minutes at room temperature. 2ml of 2% FCS PBS was added to each tube and centrifuged at room temperature for 7 minutes at1700rpm. Cells were permeabilized by adding 100µl of Caltag Medium B (Caltag Laboratories, Burlingame, CA). Tittered amounts of intracellular cytokine was added to each tube and incubated for 30 minutes at room temperature wrapped n foil.2ml of 2% FCS PBS was

added to each tube and centrifuged at room temperature for 7 minutes at1700rpm. The cells were then resuspended in 300 µl of PBS. The samples were acquired and analysed on the FACS Calibur Flow Cytometer.

Table 2. Antibody Titrations

Antibody	**Fluorophore**	**Amount**
CD3	PE-CY5.5	2 µl
CD4	APC	4 µl
CD8	APC-CY7	5 µl
CD107a	PE-CY5	15 µl
IFN-γ	PE-CY7	5 µl
TNF-α	Alexa 700	5 µl
IL-2	FITC	10 µl
MIP-1β	PE	4 µl

CHAPTER THREE

RESULTS

3.1 Descriptive analysis

3.1.1 Recruitment and follow-up for clinical outcome investigations

From February 2008 through September 2009, 77 women who fulfilled inclusion and exclusion criteria received counselling on infant feeding choices according to the WHO/UNICEF/UNAIDS guidelines (WHO. 2006) at the King Edward VIII hospital nursery and were enrolled into the study. Table 3 and figures 8 to 10 refer to background information on the hospital population from which the cohort originated.

Table 3. Enrolment Statistics at the King Edward VIII hospital nursery (February 2008 – September 2009)

Month	Number Admitted	Number of HIV Exposed Admissions	Percentage of HIV Exposed Admissions	Number Enrolled into study
Feb-08	252	81	32%	10
Mar-08	274	100	37%	8
Apr-08	300	117	39%	8
May-08	256	109	43%	9
Jun-08	253	86	34%	2
Jul-08	292	100	34%	2
Aug-08	304	106	35%	3
Sep-08	284	93	33%	5
Oct-08	246	92	37%	1
Nov-08	238	81	34%	12
Dec-08	229	70	31%	5
Jan-09	290	100	35%	4
Feb-09	260	99	38%	1
Mar-09	284	101	36%	6
Apr-09	248	98	40%	3
May-09	304	116	38%	5
Jun-09	247	114	46%	1
Jul-09	260	111	42%	1
Aug-09	249	91	36%	1
Sep-09	276	101	36%	2

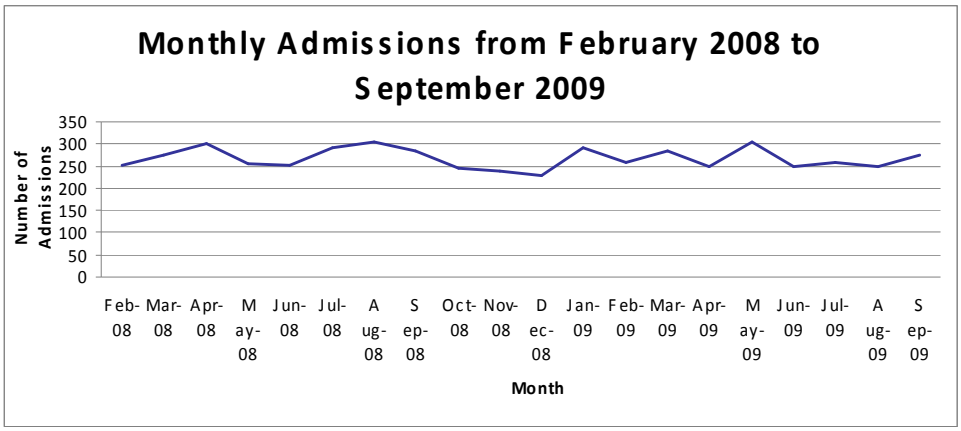

Figure 8. Monthly admissions at the King Edward VIII hospital nursery (February 2008-September 2009)

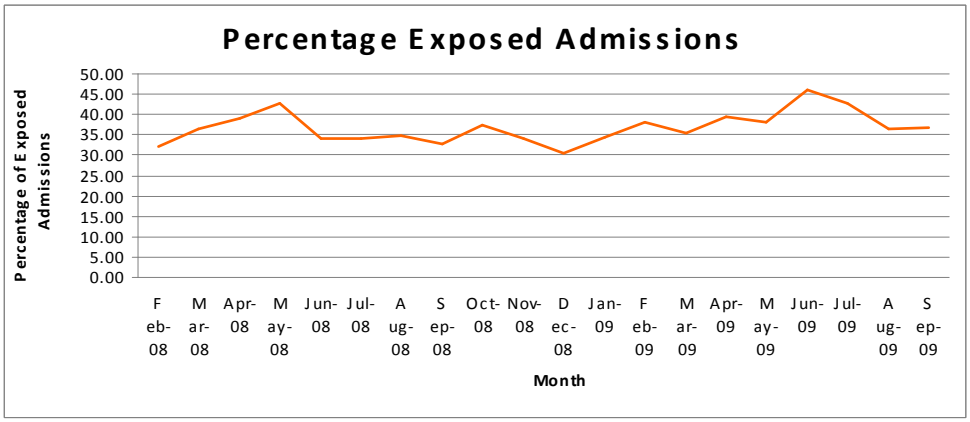

Figure 9. The percentage of exposed admissions at the King Edward VIII hospital nursery (February 2008-September 2009)

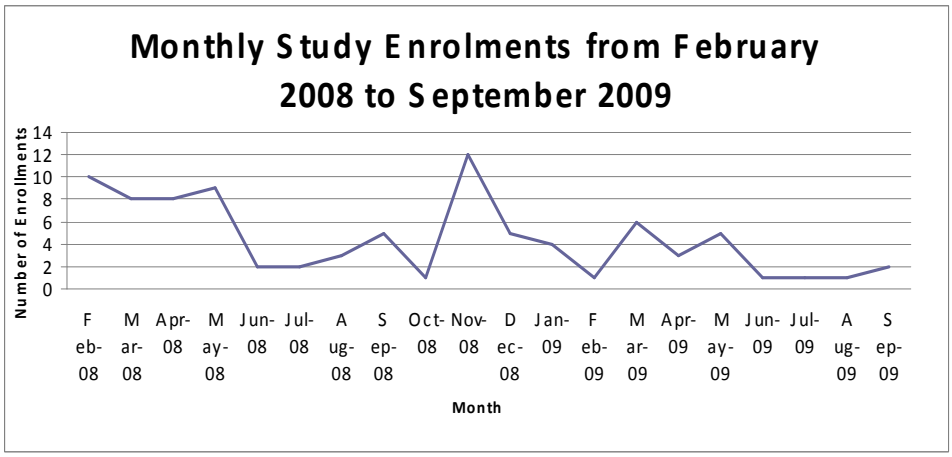

Figure 10. Monthly study enrolments of mother-child pairs at the King Edward VIII hospital nursery (February 2008-September 2009)

Delivery information was available for all 77 mother-child pairs. The HIV-1 status of the infants were determined by HIV RNA PCR at birth (day 1) or HIV DNA PCR at 6 weeks of age. By the time of discharge from hospital 19 infants were withdrawn from the study: Three infants were transferred to Inkosi Albert Luthuli Chief (IALCH) hospital for specialised care, 5 infants failed to meet inclusion criteria (mixed fed in-hospital), 9 infants were lost to follow-up and 2 infants had demised. We therefore, had 58 eligible infants with known HIV-1 status and clinical information.

Of the 58 infants, 52 infants were uninfected and 6 infants were HIV-1 infected at the 6 week follow-up visit. After the 6 week follow-up visit, 1 HIV-1 infected infant had demised, 2 uninfected infants had demised and 2 uninfected infants were withdrawn from the study due to loss to follow-up. An additional uninfected infant tested HIV-1 positive at the 3 month follow-up visit. At this time point, 6 infants were HIV-1 infected and 47 infants were uninfected. After the 3 month follow-up visit, 1 uninfected infant demised and 2 uninfected infants were lost to follow-up. During the 6 month follow-up visit, 6 infants became infected and 44 infants were uninfected. One infected infant demised and 13 uninfected infants were withdrawn from the study due to loss to follow-up after the 6 month visit. At the end of the study (9 months), 5 infants were HIV-1 infected and 31 infants were uninfected (Figure11).

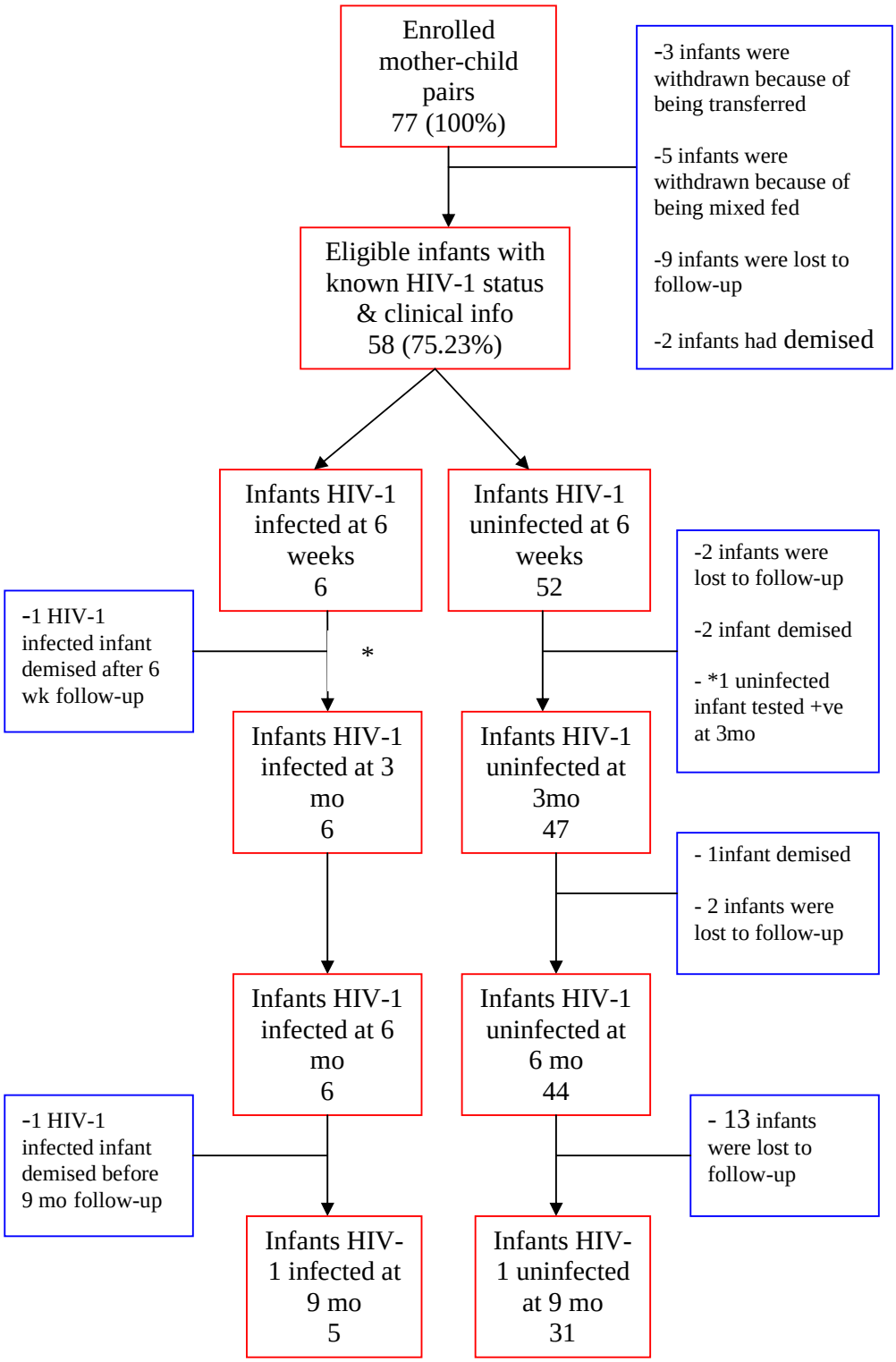

Figure 11. Participant flow from enrolment to 9 month follow-up (clinical assessment)

3.2 Maternal and infant demographics

Tables 4a and 4b tabulate the baseline characteristics of the 77 mother-child pairs. The mean age of HIV infected mothers was 27 years with a standard deviation of 5.6. The mean parity was 2 with a standard deviation of 1.1 and the mean maternal CD4 count was 420 cells/µl with a standard deviation of 228 cells/µl. The mean and standard deviation of each of the following: birth weight, birth length, birth occipito-frontal circumference (OFC), gestational age, APGAR 1 min and APGAR 5 min is described in table 4a.

At the time of the study, government guidelines changed with regards to Prevention of mother-to-child transmission (PMTCT). Previously, HIV exposed infants only received single dose nevirapine (NVP) at birth. From September 2008 exposed infants received a single dose of NVP at birth and 7 or 28 days of zidovudine (AZT) depending on the maternal prophylactic history. In this cohort, 12(15.8%) infants did not receive NVP at birth and 64(84.2%) infants received NVP at birth. Ten (13.2%) infants did not receive any course of AZT, 18(23.7%) infants received a 7 day course of AZT and 48(63.2%) infants received a 28 day course of AZT.

According to the PMTCT guidelines, HIV-1 infected women with a WHO clinical stage 4, or women with a WHO clinical stage 3 with a CD4 <350cells/µl count or a woman in WHO clinical stage 1 and 2 with a CD4 count <200cells/µl need to commence HAART during pregnancy. HIV-1 infected women who do not need HAART should receive the most effective prophylactic antiretroviral regimen. Twenty-three (29.9%) HIV-1 infected women enrolled in the study did not receive any PMTCT prophylaxis; 23(29.9%) infected women received NVP during onset of labor; 12(15.6%) infected women received AZT only; and 19(24.7%) infected women received both NVP and AZT.

Table 4a. Descriptive characteristics of mother-child pairs

	Mean ± SD	95% CI
Maternal age	27.2 ± 5.6	25.9 , 28.5
Maternal parity	2.1 ± 1.1	1.9 , 2.4
Maternal CD4	420.1 ± 228.4	362.1 , 478.1
Birth weight (kg)	1.44 ± 0.12	1.4 , 1.5
Birth length (cm)	40.3 ± 3.5	39.4, 41.1
OFC (cm)	29.6 ± 1.8	29.2, 30.05
Gestation	31 ± 2.00	31.0, 31.9
APGAR 5 min	9 ± 1.34	8.2, 8.9
APGAR 1 min	7 ± 1.82	6.7 , 7.6

Table 4b. Continuation of the descriptive characteristics of mother-child pairs

Infant Characteristics		**N (%)**
Gender	Male	46(60.5%)
	Female	30(39.5%)
Infant Received NVP	No	12(15.0%)
	Yes	64(84.2%)
Infant Received AZT	No	10(13.2%)
	Yes, for one week course	18(23.7%)
	Yes, for 28 day course	48(63.16%)
Maternal Characteristics		
Mode of Delivery	NVD	36(46.8%)
	Caesarean	40(51.0%)
	Unknown	1(1.3%)
Reason for preterm delivery	Spontaneous	41(53.0%)
	Fetal distress	11(14.5%)
	Maternal illness	9(11.9%)
	Premature rupture of membranes	2(2.7%)
	Abruption	2(2.67%)
	Fetal distress + abruption	1(1.3%)
	Maternal illness + Abruption	1(1.3%)
	Spontaneous preterm labor + maternal illness(pre-eclampsia)	2(2.6%)
	Fetal distress + maternal illness	2(2.6%)
	Unknown	5(6.6%)
Maternal prophylaxis	None	23(29.9%)
	Nevirapine	23(29.9%)
	AZT	12(15.6%)
	Nevirapine + AZT	19(24.7%)
Maternal history of TB	None	72(93.51%)
	On treatment	2(2.6%)
	Completed treatment	3(3.9%)
Maternal history of Syphilis	Not confirmed	56(72.7%)
	Confirmed	21(27.3%)
Maternal history of Pneumonia	None	75(72.8%)
	Yes	2(27.3%)
Other chronic conditions	None	74(96.1%)
	clinical	3(3.9%)
Employment of mother	No	47(61.0%)
	Yes	30(39.0)
Electricity/Fuel	No	1(1.3%)
	Yes	76(98.7%)
Piped running water	No	3(3.9%)
	Yes	74(96.1%)
Education	Did not complete primary school (grade 7)	3(3.9%)
	Did not complete secondary school (grade 12)	33(42.7%)
	Completed secondary education	36(46.8%)
	Some tertiary education	1(1.3%)
	Completed tertiary education	4(5.2%)

3.3 Maternal health and early infant infections

We examined the association between maternal CD4 counts and early infant infection during the first two weeks of life (Table 5). Episodes of infection were categorized as mild, moderate or severe. Mild or superficial infection included the skin, eye, umbilical, upper respiratory tract infection (URTI) and gastroenteritis (not requiring rehydration). Moderate infections were classified as infections that required supportive therapies without continuous positive airway pressure (CPAP) or ventilation and included respiratory tract infection/pneumonia, diarrheal disease (with intravenous fluid - IVF) and suspected sepsis with full blood count (FBC) changes. Severe infections included infections that required intensive therapies including CPAP and ventilation and include severe pneumonia, necrotizing enterocolitis (NEC), meningitis, proven tuberculosis (TB), cytomegalovirus (CMV) and zoster.

Due to the small sample size the infections were classified as absent or present. Early infant infection was classified as any infection from birth to hospital discharge. The mean number of day's in-hospital was 14 days. Due to loss to follow-up CD4 counts were available in only 61 HIV-1 infected women. HIV infected mothers were classified according to their CD4 count, 6 weeks post delivery. The following observation was made: for maternal CD4 count <500cells/µl vs. maternal CD4 count greater than >500cells/µl the odds ratio for early infant infection was 1.69. This means that the risk of an infant acquiring an infection at birth was approximately 2x higher if the maternal CD4 count was less than 500cells/µl as compared to those infants born to an HIV infected women with a maternal CD4 count > 500cells/µl.

Table 5: Relationship between maternal CD4 count 6 weeks post delivery and early infant infection

Maternal CD4 count (cells/µl)	Odds Ratio	(95% CI)
<200 vs. >200	1.47	0.45,4.72
<350 vs. > 350	1.35	0.45,4.01
<500 vs. >500	1.69	0.55,5.17

3.4 Maternal health and infant growth at birth

The Kruskal-Wallis test, which is a nonparametric version of ANOVA, was used to test whether there was a significant difference in mean birth weight, mean birth length, mean birth OFC and maternal CD4 categories (tables 6-8). There was no significant difference between mean birth weight and maternal CD4 count (p-value = 0.1394). However, the following trend was observed: infants born to HIV-1 infected women with advanced disease (CD4 count 0-200 cells/µl) were lighter in weight compared to infants born to HIV-1 infected women with a CD4 count > 350 cells/µl. The mean length did not differ significantly between categories of maternal CD4 count (p-value = 0.6533). The mean OFC also did not differ significantly between categories of maternal CD4 count (p-value=0.3770).

Table 6. Maternal CD4 count and infant birth weight (n=61)

Maternal CD4	N	Mean Birth Weight (kg)
0-200	12	1.37
201-350	16	1.41
351-500	7	1.51
>500	26	1.50

Table 7. Maternal CD4 count and infant birth length (n=57)

Maternal CD4	N	Mean Birth Length (cm)
0-200	12	29.33
201-350	14	29.45
351-500	7	30.86
>500	24	29.69

Table 8. Maternal CD4 count and infant birth OFC (n=55)

Maternal CD4	N	Mean Birth OFC (cm)
0-200	12	41.00
201-350	13	39.46
351-500	7	41.86
>500	23	39.96

3.5 Maternal education and postnatal feeding choice

Of the 73 HIV-1 infected women that had data available on their feeding choice, 48 (65.8%) women decided to feed their infants breastmilk at birth. Of the 48 HIV-1 infected women that decided to breastfeed their infants, 38(79.2%) women chose to heat-treat their expressed breastmilk(HTEBM) and 10(20.8%) women chose to feed their infants expressed breastmilk(EBM). The remaining 25(34.2%) HIV-1 infected women fed their infants exclusive formula. Of the 38 HIV-1 infected women that decided to feed their infants HTEBM, 2(5.3%) women did not complete primary school, 14(36.8%) women did not complete secondary school and 22(57.9%) women completed secondary school. Ten HIV-1 infected women fed their infants EBM birth, 6(60.0%) women did not complete secondary school, 3(30.0%) women completed secondary school and 1(10.0%) mother completed tertiary education. Twenty-five women chose to feed their infants exclusive formula, 1(4.0%) mother did not complete primary school, 11(44.0%) women did not complete secondary school, 10(40.0%) completed secondary school and 3(12.0%) women

completed tertiary education. There was no significant relationship between postnatal feeding choice and maternal education, using the fishers exact test (p-value=0.1753) (Table 9).

Table 9. Relationship between maternal education and postnatal feeding choice

FEED	EDUCATION					TOTAL FREQUENCY & PERCENTAGE
	Did not complete primary School (grade7)	Did not complete Secondary School (grade12)	Completed Secondary Education	Some Tertiary Education	Completed Tertiary Education	
Exclusive Breastmilk (EBM)	0	6	3	0	1	10
	0	8.2	4.1	0	1.4	13.7%
Exclusive heat-treated expressed breastmilk (HTEBM)	2	14	22	0	0	38
	2.7	19.2	30.1	0	0	52.1%
Exclusive formula	1	11	10	0	3	25
	1.4	15.1	13.7	0	4.1	34.2%
Total Frequency & Percentage	3	31	35	0	4	73
	4.1	42.5	47.9	0	5.5	100%

Data pertaining to maternal employment was available for 73 patients. In this cohort of mother-child pairs, 46(63.0%) women were unemployed and 27(36.9%) were employed at the time of delivery. Twenty-eight (38.4%) HIV-1 infected women that decided to feed their infants HTEBM were unemployed, 4(5.5%) HIV-1 infected women that fed their infants EBM were unemployed and 14(19.2%) women that fed their infants exclusive formula were unemployed. There no significant relationship between maternal employment and postnatal feeding choice (p-value 0.0979) (Table 10).

Table 10. Relationship between Maternal Employment and Postnatal Feeding Choice

FEED	EMPLOYMENT		TOTAL FREQUENCY & PERCENTAGE
	No	Yes	
Exclusive Breastmilk (EBM)	4	6	10
	5.5	8.2	13.7
Exclusive heat-treated expressed breastmilk (HTEBM)	28	10	38
	38.4	13.7	52.1
Exclusive formula	14	11	25
	19.2	15.1	34.2
Total Frequency & Percentage	46	27	73
	63.0	3	100

3.6 Infant infections and feeding modality

Tables 11-19 demonstrate the relationship between infant infection and feeding modality at each follow-up. Infection has been described previously (3.3), as mild, moderate or severe. Due to the small sample size infection at each follow-up infection was reported as absent or present. The Fisher exact test was used. This test analyses the independence between two categorical variables. Early feeding mode was defined as the preferred feeding choice during the first two weeks of life. There was no significant relationship between early infant feeding mode and infection at 6 weeks, 3 mo, 6 mo and 9 mo follow-up visit (p-value =0.4058, 0.1179, 0.1689 and 0.7855 respectively). We further attempted to determine whether there was relationship between 6 week infant feeding mode and infection at 3 mo, 6 mo and 9 mo follow-up visit(p-value=0.8826, 0.1403 and 0.4729 respectively). There was also no significant relationship between 3 month infant feeding

choice and infection at 6 mo and 9 mo follow-up visit (p-value=0.1403 and 0.4729 respectively).

Table 11. Relationship between early feeding mode and infection at the 6 week follow-up visit (n=56)

	Infection absent	Infection present
Breastfeeding	5(8.92%)	1(1.79%)
Exclusive Heat Treated breastmilk	19(33.93%)	7(12.5%)
Exclusive formula	13(23.21%)	8(14.29%)
Unknown	1(1.79%)	2(3.57%)

Table 12. Relationship between early feeding mode and infection at the 3 month follow-up visit (n=53)

	Infection absent	Infection present
Breastfeeding	1(1.89%)	6(11.32%)
Exclusive Heat Treated breastmilk	9(16.98%)	14(26.42%)
Exclusive formula	8(15.09%)	12(22.64%)
Unknown	3(5.66%)	0(0%)

Table 13. Relationship between early feeding mode and infection at the 6 month follow-up visit (n=51)

	Infection absent	Infection present
Breastfeeding	2(3.92%)	4(7.84%)
Exclusive Heat Treated breastmilk	8(15.69%)	14(27.45%)
Exclusive formula	11(21.57%)	9(17.65%)
Unknown	3(5.88%)	0(0%)

Table 14. Relationship between early feeding mode and infection at the 9 month follow-up visit (n=35)

	Infection absent	Infection present
Breastfeeding	3(8.57%)	2(5.71%)
Exclusive Heat Treated breastmilk	9(25.71%)	7(20.0%)
Exclusive formula	6(17.14%)	6(17.14%)
Unknown	2(5.71%)	0(0%)

Table 15. Relationship between 6 week feeding choice and infection at the 3 month follow-up visit (n=54)

	Infection absent	Infection present
Breastfeeding	6(11.11%)	12(22.22%)
Exclusive formula	10(18.52%)	13(24.07%)
Mixed feed	5(9.26%)	8(14.81%)

Table 16. Relationship between 6 week feeding choice and infection at the 6 month follow-up visit (n=52)

	Infection absent	Infection present
Breastfeeding	5(9.16)	11(21.15%)
Exclusive formula	11(21.15%)	12(23.08%)
Mixed Feed	9(17.31%)	4(7.69%)

Table 17. Relationship between 6 week feeding choice and infection at the 9 month follow-up visit (n=36)

	Infection absent	Infection present
Breastfeeding	5(13.89%)	6(16.67%)
Exclusive formula	9(25%)	8(22.22%)
Mixed Feed	6(16.67%)	2(5.56%)

Table 18. Relationship between 3 month feeding choice and infection at the 6 month follow-up visit (n=52)

	Infection absent	Infection present
Breastfeeding	5(9.62%)	11(21.15%)
Exclusive formula	11(21.15%)	12(23.08%)
Mixed Feed	9(17.31)	4(7.69%)

Table 19. Relationship between 3 month feeding choice and infection at the 9 month follow-up visit (n=36)

	Infection absent	Infection present
Breastfeeding	5(13.89%)	6(16.67%)
Exclusive formula	9(25%)	8(22.22%)
Mixed Feed	6(16.67%)	2(5.56%)

3.7 Maternal CD4 Count at 6 weeks post delivery and infant growth over time

Table 20 illustrates the differences between maternal CD4 count and infant weight over time. Infants born to HIV-1 infected women with a CD4 count of greater than 350cells/μl and infants born to HIV-1 infected women with a CD4 count of greater than 500cells/μl had a similar weight gain at 36 weeks of age (8.26 vs. 8.15).

Figure 12 and Table 21, further explore these differences. Maternal CD4 count and infant weight over time had a positive co-efficient and this was statistically significant (p value = 0.0361). Infants born to HIV-1 infected women with a CD4 count of greater than 350cells/μl had a higher mean weight during the first weeks of life compared to infants born to HIV-1 infected women with advanced disease (CD4 count < 200cells/μl). However, this effect diminishes over time.

Tables 22-23 and Figure 13 demonstrate the differences between maternal CD4 count and infant length over time. Maternal CD4 count and infant length over time had a negative co-efficient and a p value of 0.1304. Therefore, there was no relationship between maternal CD4 count and infant length over time. Tables 24-25 and Figure 14 explore the differences between maternal CD4 count and infant OFC over time. For every 1 unit increase in maternal CD4 count, the infants OFC increased by 0.0001 over time.

Table 20. The relationship between maternal CD4 count 6 weeks post delivery and infant weight at each follow-up

Maternal CD4 Count	Week	Mean Weight
0-200	0	1.30
0-200	6	2.40
0-200	12	4.18
0-200	24	6.26
0-200	36	6.86
201-350	0	1.39
201-350	6	2.33
201-350	12	3.98
201-350	24	5.91
201-350	36	7.05
351-500	0	1.46
351-500	6	2.28
351-500	12	3.72
351-500	24	6.69
351-500	36	8.26
>500	0	1.52
>500	6	2.69
>500	12	4.52
>500	24	6.97
>500	36	8.15

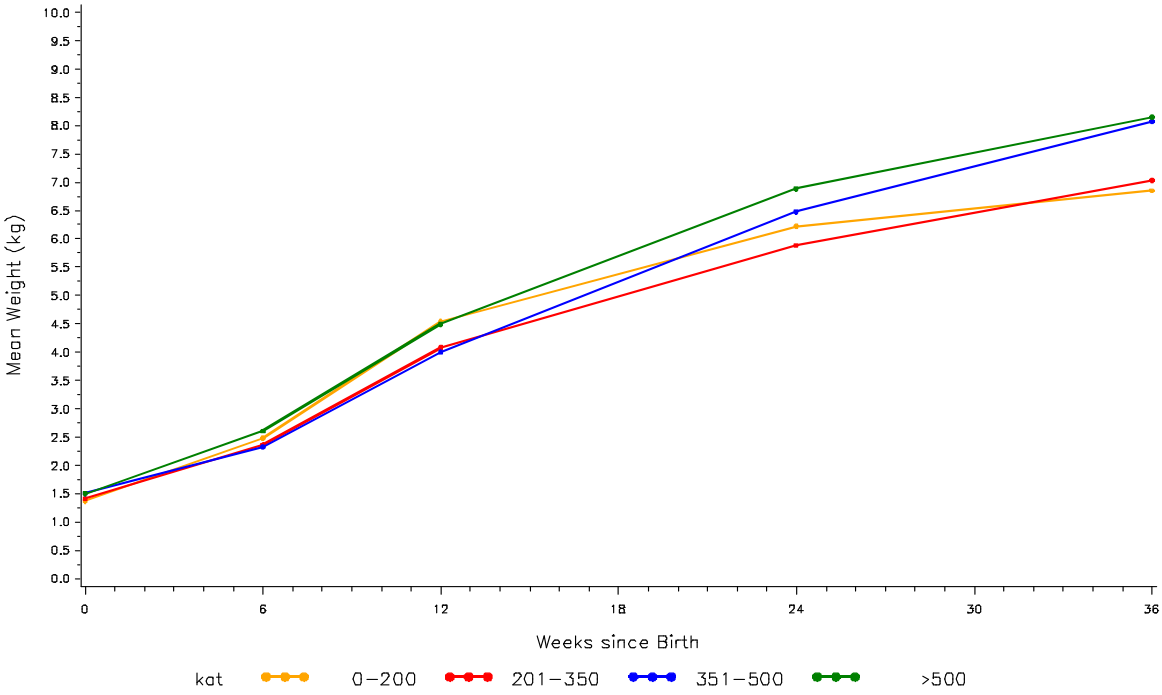

Figure 12. The participants mean weight over time

Table 21. The association between maternal CD4 count and infant weight

	Co-efficient	F-Value	P-value
Maternal CD4 Count	0.000034	0.01	0.9352
Time	0.1656	134.32	<0.001
Maternal CD4*Time	0.00006	4.61	0.0361

Table 22. Relationship between maternal CD4 count 6 weeks post delivery and infant length at each follow-up

Maternal CD4 Count	Week	Mean Length (cm)
0-200	0	40.80
0-200	6	46.00
0-200	12	52.00
0-200	24	59.17
0-200	36	67.25
201-350	0	38.90
201-350	6	44.39
201-350	12	51.10
201-350	24	57.73
201-350	36	63.00
351-500	0	41.60
351-500	6	45.80
351-500	12	51.00
351-500	24	60.75
351-500	36	65.00
>500	0	40.53
>500	6	44.83
>500	12	52.35
>500	24	60.75
>500	36	66.12

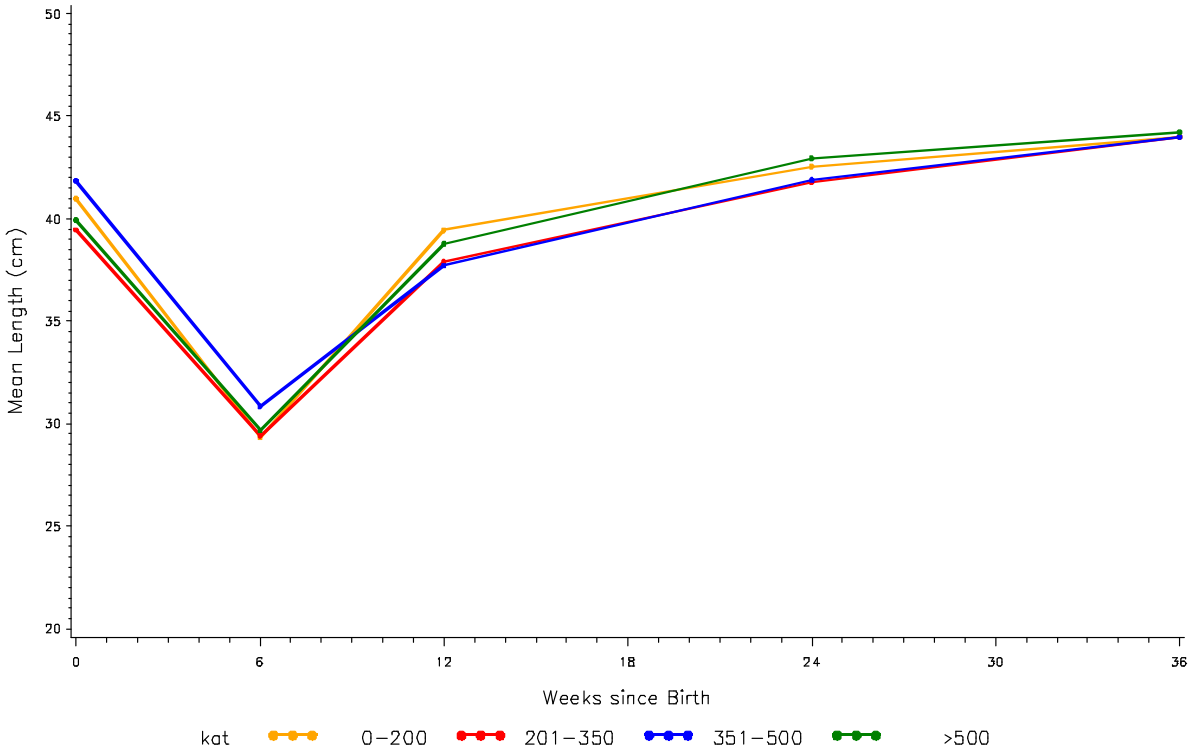

Figure 13. The participants mean length over time

Table 23. The association between maternal CD4 count and infant length

	Co-efficient	F-Value	P-value
Maternal CD4 Count	0.00061	0.09	0.7703
Time	0.6583	168.02	<0.0001
Maternal CD4*Time	0.000151	2.36	0.1304

Table 24. The relationship between maternal CD4 count 6 weeks post delivery and OFC at each follow up

Maternal CD4 Count	Week	Mean OFC (cm)
0-200	0	28.60
0-200	6	35.00
0-200	12	39.08
0-200	24	42.50
0-200	36	44.00
201-350	0	29.18
201-350	6	33.54
201-350	12	37.40
201-350	24	41.50
201-350	36	44.00
351-500	0	30.20
351-500	6	32.80
351-500	12	37.00
351-500	24	41.88
351-500	36	43.88
>500	0	29.88
>500	6	34.45
>500	12	38.87
>500	24	42.90
>500	36	44.24

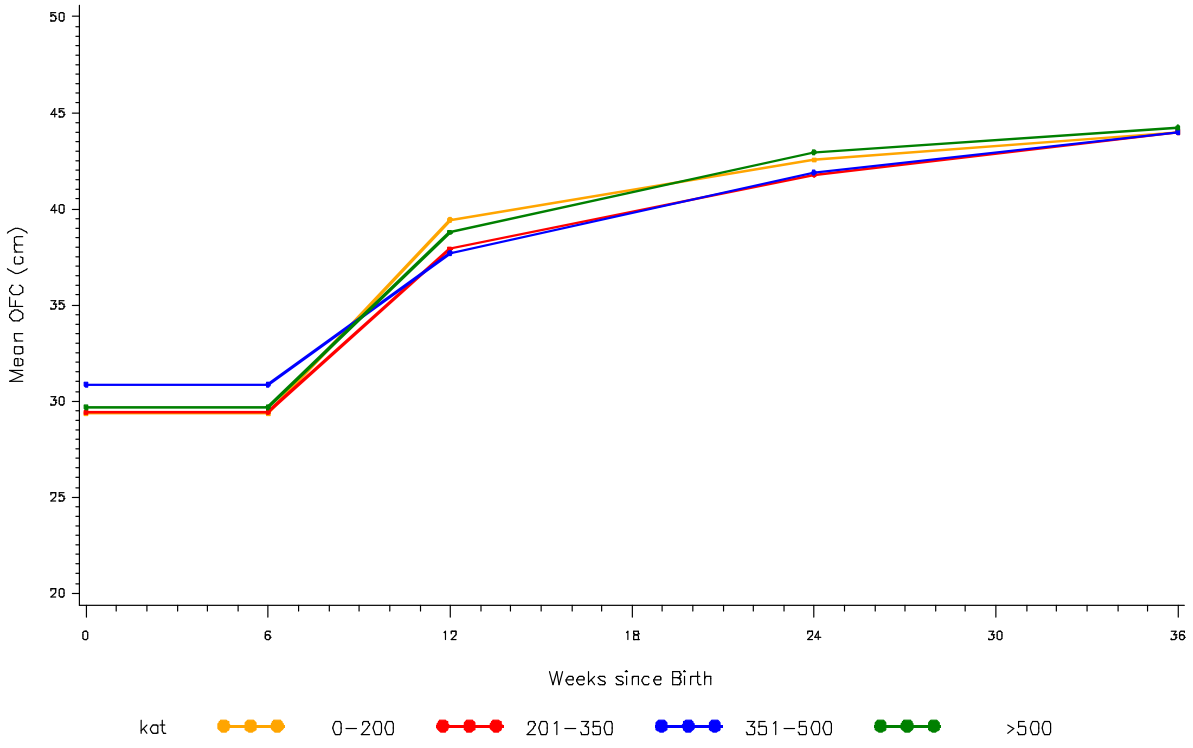

Figure 14. The participants mean OFC over time

Table 25. The association between maternal CD4 count and OFC

	Co-efficient	F-Value	P-value
Maternal CD4 Count	0.000758	0.35	0.5568
Time	0.5218	355.82	<0.0001
Maternal CD4*Time	0.000040	0.54	0.4649

3.8 The association between feeding mode and infant growth

We investigated the association between feeding mode on growth using a linear mixed model. Three models were proposed:

1.) Linear mixed model for weight as the dependent variable with feed, time and the interaction between feed and time as the explanatory variables.

2.) Linear mixed model for length as the dependent variable with feed, time and the interaction between feed and time as the explanatory variables.

3.) Linear mixed model for OFC as the dependent variable with feed, time and the interaction between feed and time as the explanatory variables.

The linear mixed model for weight indicated that there was a significant association between weight and time (p-value < 0.0001). But feed (p-value=0.4076) and time*feed (p-value=0.4930) were not significant (Table 26). The linear mixed model for length showed that there was a significant association between length and time (p-value < 0.0001). However, feed (p-value=0.6709) and time*feed (p-value=0.0648) were not significant (Table 27). There was a significant association between OFC and time (p-value < 0.0001) using the linear model for OFC. The associations between OFC and feed (p-value=0.0003) and between OFC and time*feed (p-value < 0.0001) were both significant (Table 28).

Table 26. The association of feed with weight

Effect	Numerator degrees of freedom	Denominator degrees of freedom	F Value	PR>F
Time	1	252	496.37	<.0001
Feed	4	252	1.00	0.4076
Time*Feed	3	252	0.80	0.4930

Table 27. The association of feed with length

The effect of feed on length				
Effect	Numerator degrees of freedom	Denominator degrees of freedom	F Value	PR>F
Time	1	242	354.70	<.0001
Feed	4	242	0.59	0.6709
Time*Feed	3	243	2.44	0.0648

Table 28. The association of feed with OFC

Effect	Numerator degrees of freedom	Denominator degrees of freedom	F Value	PR>F
Time	1	245	422.62	<0.0001
Feed	4	245	5.51	0.0003
Time*Feed	3	245	1.32	<.0001

3.9 The feasibility of each feeding mode

A total of 51 HIV-1 infected women decided to breastfeed their infants at birth. Of which 38 women decided to heat treat their expressed breastmilk (HTEBM) and 13 women opted to breastfeed their infants directly from the breast (EBM). The majority of HIV-1 infected women (19 or 50.0 %) chose to exclusively heat-treat their expressed breastmilk based on the health care worker recommendation. Fourteen (36.8%) women chose to feed their infants exclusive HTEBM because of its anti-infective and nutritional benefits and 3(7.9%) women chose it because it was safe and easy to do. Of the 13 HIV-1 infected women that decided to exclusively breastfeed their infants(EBM not HTEBM), 1(7.7%) mother's decision was based on the anti-infective properties of breastmilk, 2(15.4%) mothers experienced insufficient breastmilk, 3(23.1%) mothers thought that the HTEBM method

was too tedious they would rather breastfeed and 2(15.4%) mothers were uncertain of the use of HTEBM. Twenty-six HIV-1 infected women decided to formula feed their infants after intensive WHO infant feeding counselling. Ten (38.5%) HIV-1 infected women that decided to exclusively formula feed their infants at birth were either employed or attending school. Five (19.2%) women were ill after delivery, 4(15.4%) women based their choice on the health care worker recommendation, 2(7.7%) women chose to formula feed because of its anti-infective properties, 2(7.7%) women thought the method was easier to do compared to either HTEBM or breastfeeding, 1(1.37%) mother reported insufficient breastmilk and 1(3.8%) mother re-called previously infecting a child through breastmilk (Table 29).

While in-hospital, 38 HIV-1 infected women fed their infants HTEBM after receiving counseling and support from the nursing staff at the King Edward VIII hospital. At the 6 week follow-up only 9 HIV-1 infected women were able to continue feeding their infants HTEBM. As the months passed, the numbers fell to 4 infants on HTEBM at 3 months and 2 infants on HTEBM at 6 months. The decrease in number of infants on HTEBM was mainly due to mixed feeding.

Table 29. Reason for choosing each feeding modality, n = 77. (Results presented as frequency and percentage)

Feed	Anti-infective	Easy to do & Safe	Mother at school/employed	Health care worker recommendation	Maternal illness	Insufficient breastmilk	Method tedious	Previous child infected through breastmilk	Method unknown, uncertain of use	Afraid of disclosure	No Reason
Expressed Breastmilk (EBM) n = 13	1 7.7%	0 0%	0 0%	2 15.4%	0 0%	2 15.4%	3 23.1%	0 0%	2 15.4%	1 7.7%	2 15.38%
Heat-treated expressed breastmilk (HTEBM) n = 38	14 36.8%	3 7.9%	0 0%	19 50%	0 0%	0 0%	0 0%	0 0%	0 0%	1 2.6%	1 2.6%
Exclusive Formula n = 26	2 7.7%	2 7.7%	10 38.5%	4 15.4%	5 19.2%	1 3.8%	0 0%	1 3.8%	0 0%	0 0%	1 3.8%

3.10 Recruitment and follow-up for CTL investigations

Of the 77 infants enrolled in the study 55 infants were assessed for both CTL responses and clinical outcomes. Delivery information was available for all 55 mother-child pairs. The HIV-1 status of the infants was determined by HIV RNA PCR at day 1 or HIV DNA PCR at 6 weeks of age. At the time of discharge from hospital 19 infants were withdrawn from the study: Three infants were transferred to Inkosi Albert Luthuli Chief (IALCH) hospital for specialised care, 5 infants failed to meet inclusion criteria (mixed fed in-hospital), 9 infants were lost to follow-up and 2 infants had demised.

At the 6 week follow-up visit, 34 infants were uninfected and 2 infants were HIV-1 infected. After the 6 week follow-up visit, 1 of the 2 HIV-1 infected infants had demised, 2 uninfected infants had demised and 2 uninfected infants were withdrawn from the study due to loss to follow-up. An additional uninfected infant tested HIV-1 positive at the 3 month follow-up visit. At this time point, 2 infants were HIV-1 infected and 29 infants were uninfected. After the 3 month follow-up visit, 1 uninfected infant demised and 2 uninfected infants were lost to follow-up. During the 6 month follow-up visit, 2 infants were infected and 26 infants were uninfected .One infected infant demised and 2 uninfected infants were withdrawn from the study due to loss to follow-up after the 6 month visit. At the end of the study (9 months), 1 infant was HIV-1 infected and 24 infants were uninfected (Figure 14). The baseline characteristics of the 55 mother-child pairs are included in tables 30a and 30b. These descriptive characteristics were similar to that reported in tables 4a and 4b of the full cohort of 77 mother-infant pairs.

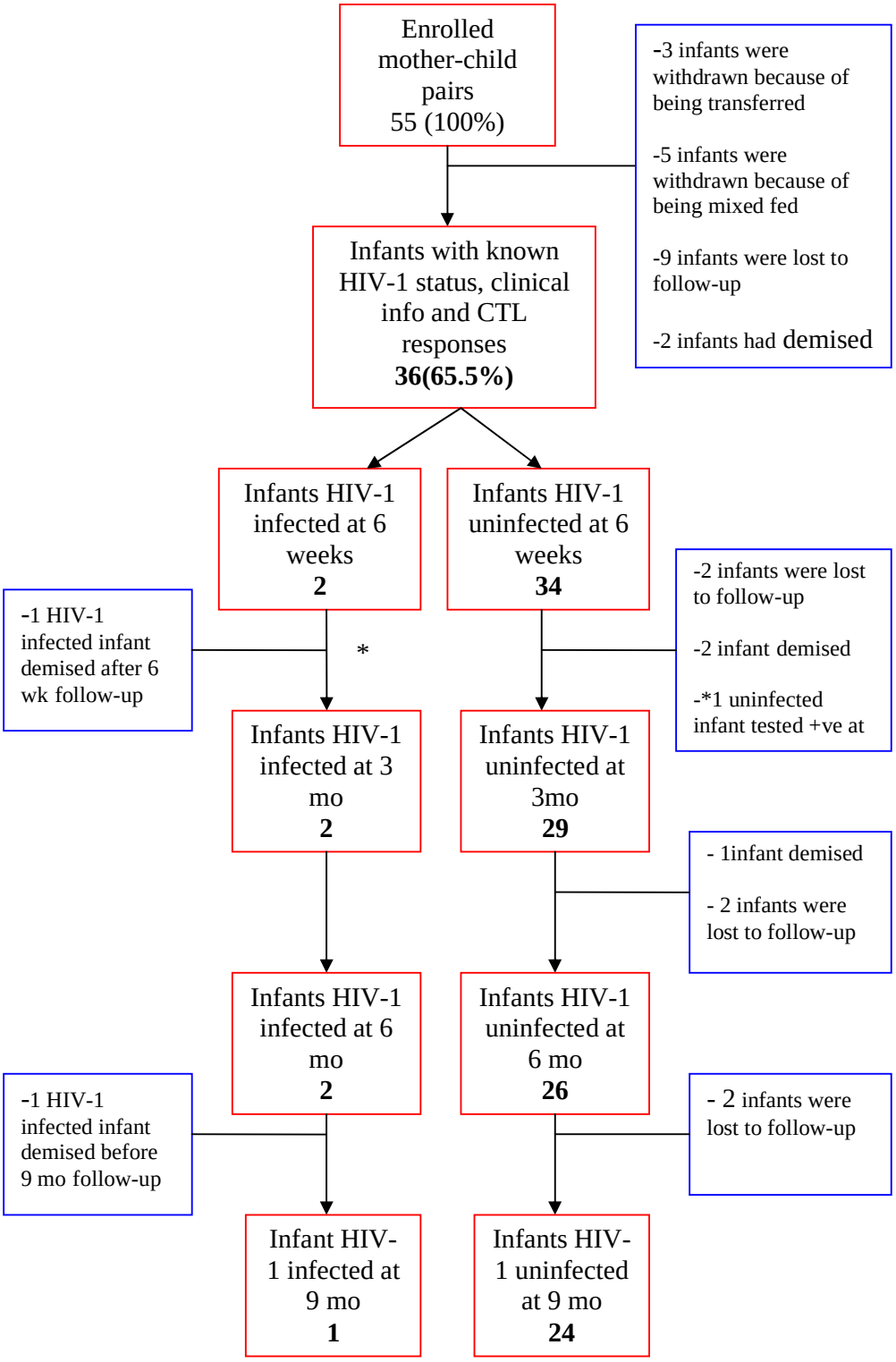

Figure 15. Participant flow from enrolment to 9 month follow-up (CTL responses and clinical assessment)

Table 30a. Descriptive characteristics of 55 mother-child pairs (sub-study)

	Mean	95% CI
Maternal Age	26.80	(25.27, 28.33)
Maternal Parity	2.09	(1.81, 2.38)
Maternal CD4	838.14	(9.68, 1666.59)
Birth Weight (kg)	1.48	(1.43, 1.53)
Birth Length(cm)	40.34	(39.31, 41.37)
OFC(cm)	29.96	(29.96, 30.44)
Apgar 1 min	7.13	(6.57, 7.68)
Apgar 5 Min	8.64	(8.28, 8.99)
Gestation (weeks)	31.47	(31.02, 31.92)

Table 30b. Continuation of the descriptive characteristics of 55 mother-child pairs (sub-study)

Infant Characteristics		N (%)
Gender	Male	31 (56.4%)
Infant Received NVP	No	8 (14.6%)
	Yes	46 (83.6%)
Infant Received AZT	No	1 (1.8%)
	Yes, for one week course	16 (29.1%)
	Yes, for 28 day course	37 (67.3%)
Maternal Characteristics		
Mode of Delivery	NVD	26 (47.3%)
	Caesarean	28 (50.9%)
Reason for preterm Delivery	Unknown	3 (%)
	Spontaneous	33 (5.5%)
	Fetal distress	7 (60.00%)
	Maternal illness	3 (12.8%)
	Abruption	1 (1.8%)
	Fetal Distress and abruption	2 (3.6%)
	Spontaneous preterm labor + maternal illness (pre-eclampsia)	2 (3.6%)
	Fetal Distress + Maternal illness	1 (1.8%)
Maternal Prophylaxis	None	12 (21.8%)
	Nevirapine	13 (23.6%)
	AZT	12 (21.8%)
	Nevirapine & AZT	18 (32.7%)
Maternal history of TB	None	52 (94.6%)
	Completed Treatment	0
	Being Worked up, Not on Treatment	3 (5.45%)
Maternal history of Syphilis	Not Confirmed	36 (65.5%)
	Confirmed	19 (34.6%)
Maternal history of Pneumonia	None	54 (98.2%)
	Yes, as per mother	1 (1.8%)
Other Chronic Conditions	None	52 (94.6%)
	Clinical	3 (5.5%)
Employment of Mother	Unemployed	37 (67.3%)
	Employed	18 (32.7%)
Electricity/Fuel	No	1 (1.82%)
	Yes	54 (98.2%)
Piped Running Water	No	3 (5.5%)
	Yes	52 (9.6%)
Education	Did not complete primary school (grade 7)	3 (5.5%)
	Did not complete secondary school (8-Matric)	21 (38.2%)
	Completed secondary education	27 (49.1%)
	Completed tertiary education	4 (7.3%)

3.11 Immune responses in HIV exposed low birth weight infants

A blood sample was taken from each patient at follow-up visits (6 weeks, 3mo, 6mo and 9mo) and immune response assays were performed on them. The assays used included ELISPOT megamatrix (day 1), ELISPOT confirmation (day 2) and Intracellular Cytokine Staining (ICS) (day 2). Immune response assay results were available for 25 infants at each time point. Both immunological and clinical data were available for these infants throughout the study. Three of 25 infants were diagnosed HIV-1 infected by HIV DNA PCR by 3 months of age. The 3 HIV-1 infected infants had a negative HIV RNA PCR at birth (day1).

At the 6 week follow-up visit only 1 HIV-1 infected infant (FH 001 H) elicited a HIV-1-specific CTL immune response towards a pool of peptides in the ELISPOT megamatrix. We were unable to confirm these responses on day 2 with the ELISPOT confirmation assay. This infant (FH 001 H) was unable to maintain these responses and therefore we were unable to detect these responses at any of the consecutive time points. The main feeding modality for this infant was exclusive HTEBM up to 6 weeks of age followed by exclusive breastmilk and complementary feeds. At the time of detection of HIV-1 specific CTL responses this infant was fed on exclusive HTEBM. The second infant diagnosed HIV-1 infected (FH 006 H) at 6 weeks of age demised before the 3 month follow-up visit and we were unable to detect HIV-1-specific CTL responses at the 6 week follow-up visit. This infant was fed a combination of HTEBM and EBM. The third infant that was diagnosed HIV-1 infected (FH 005 H) at 3 months of age and was able to elicit a HIV-1-specific CTL response against pools of peptide in the ELISPOT megamatrix. We were unable to confirm these responses on day 2 with neither the ELISPOT confirmation nor ICS

assay. This infant was also unable to sustain these immune responses up to 6 months of age and the infant demised prior to the 9 month follow-up. The infant was fed HTEBM exclusively, until 6 weeks of age followed by breastmilk and complementary feeds. HIV- 1- specific cytotoxic T lymphocyte activities were detected against pools of the following viral antigen gag, pol, env and nef in 2 infected infants (FH 002 H and FH 005 H).

We were unable to detect HIV-1-specific CTL response in any of the HIV-1 exposed uninfected infants at any of the study time points. Phytohaemaglutinin A (PHA) served as the positive control in the ELISPOT megamatrix and a detectable response was observed each time the assay was performed. Cells were even stimulated in-vitro prior to the ELISPOT megamatrix and no response was detectable.

The table below is a summary of the two HIV-1 infected infants WHO staging, CDC staging, CD4%, CD4+ count and viral load. The 3 infected infants at the time of HIV diagnosis were all fed exclusive breastmilk. All three mothers had heat treated their expressed breastmilk while in-hospital but could not sustain this feeding mode post delivery.

Table 31. Summary of the HIV-1 infected infants WHO staging, CDC staging, CD4%, CD4+ count and viral load

Patient ID:	Time Point	WHO staging	CDC staging	CD4 %	CD4+ Count (cells/µl)	Viral Load (copies/ml)
FH002 H	3 mo	3	A1	29	1745	201 082
	6 mo	4	B2	21	1700	144 439
	9mo	4	C2	18	233	123 164
FH005 H	3 mo	2	B3	12	1065	5 420 918
	6 mo	3	B3	15	1064	4 659 129
	9 mo	Demised	Demised	Demised	Demised	Demised

CHAPTER 4

DISCUSSION

Despite breastfeeding being a significant mode of postnatal HIV transmission it remains the main nutritional source and pillar of child survival for the majority of infants born in Africa. It is therefore, not surprising that considerable research has centred on making breastfeeding safer in terms of HIV transmission. The flash heat treatment method (HTEBM) provides a unique opportunity to safely breastfeed infants but prevent mother-to-child transmission of HIV. This pilot study examined the feasibility of using HTEBM and its association on clinical outcomes and CTL responses in HIV exposed low birth weight infants.

In this cohort the risk of an infant acquiring an infection at birth was approximately 2x higher if the maternal CD4 count was less than 500cells/µl compared to those infants born to an HIV infected women with a maternal CD4 count > 500cells/µl. This finding was in accordance with previous reports that infants born to women with lower CD4 counts are more susceptible to morbidity and mortality (Newell et al., 2004; Marinda et al., 2007).

Studies have shown that HIV infected women are more likely to deliver preterm and low birth weight infants (Hira et al., 1989; Ryder et al., 1989; St Louis et al., 1993; Taha et al., 1995). We evaluated the association between maternal CD4 count and infant growth. In this study, infants born to HIV-1 infected women with advanced disease (CD4 count 0-200 cells/µl) had a lower birth weight compared to infants born to HIV-1 infected women with a CD4 count > 350 cells/µl. A similar weight gain was observed at 36 weeks of age (8.26 vs. 8.15) among infants born to HIV-1

infected women with a CD4 count of greater than 350cells/µl and infants born to HIV-1 infected women with a CD4 count of greater than 500cells/µl. Infants born to HIV-1 infected women with a CD4 count of greater than 350cells/µl had a higher mean weight gain during the first weeks of life compared to infants born to HIV-1 infected women with advanced disease (CD4 count < 200cells/µl). However, this effect diminishes over time.

Several studies have reported on the impact of HIV exposure and infection on infant growth with contradictory findings. Some studies have shown no observable differences in growth between HIV exposed uninfected children and HIV unexposed children. HIV infected children had the poorest growth outcome than HIV unexposed children and HIV exposed uninfected children (European collaborative study 2003; Patel et al., 2010). While a study conducted in Malawi between 2000-2003 showed early stunting among HIV infected and uninfected boys compared to girls (Taha et al., 2010).

Although exclusive breastfeeding has been well documented to be associated with reduced morbidity and mortality due to respiratory and diarrhoeal illnesses, We were not able to show this (Cesar et al., 1999; Oddy et al., 1999; Bahl et al., 2005). The lack of finding any association between infant feed and infection (defined in section 3.3) is most likely due to the small sample number of infants in the study. Recently, studies have shown that HIV infected children that are fed breastmilk had a higher mean weight and mean length compared with HIV infected children not receiving any breastmilk (Patel et al., 2010; Taha et al., 2010).

We investigated the feasibility of HTEBM before 6 months of age. We decided to initiate HTEBM in these preterm infants at birth since their mothers were already expressing breastmilk. The majority of HIV infected women enrolled in this study chose HTEBM based on the health care worker recommendation. Our study showed that HTEBM could be initiated and sustained while in-hospital given appropriate counselling and support. It therefore, appears that HTEBM is feasible to implement in the hospital setting but it is difficult to sustain post hospital discharge.

The main reason for the lack of sustainability post discharge is that the infants now require larger volumes of feed and it is difficult for the mother to keep up with the demands of expressing sufficient milk as well as the time required to flash heat each batch of expressed milk. However, reports suggest that after 6 months the heat treatment method becomes feasible once again as the infant now receives additional complementary feeds (Israel-Ballard et al., 2006b; Mbuya et al., 2010). This was the first study to our knowledge investigating the use of HTEBM in infants less than 6 months of age. We have shown that with good counselling on optimal feeding choices and adequate support HTEBM can be implemented in the hospital setting. However, because of the small sample size we had insufficient power to show that HTEBM conferred a positive benefit on infant growth, morbidity and mortality. Further, research with sufficient power would be important to elucidate this effect.

We prospectively evaluated the association between early neonatal feeding and CTL responses in HIV exposed low birth weight infants. This study was undertaken to assess the capability of the young infant to generate HIV-1 specific CTL responses with respect to early feeding modality. Cytotoxic T lymphocyte responses have

been previously described in HIV exposed term infants (see Table 1). To our knowledge, this was the first study that investigated CTL responses in HIV exposed low birth weight infants. HIV-1 specific CTL responses were detected in 2 infected infants at only a single time point. These responses however, could not be confirmed and were undetectable at any of the consecutive time points. At the time of HIV-1 specific CTL detection these infants were fed breastmilk (heated expressed breastmilk for FH 002 H and unheated breastmilk for FH 005 H). HIV exposed uninfected infants were unable to generate a HIV-1 specific CTL response irrespective of feed. During acute HIV infection, CTL responses have been implicated in the initial containment of the virus. Previously, studies have shown that some young infants and even the foetus were capable of generating HIV-specific CTL responses (Table 1). The generation of CTL responses in infancy has been reported to be variable and develop later the course of infection.

There are a few suggestions for the lack of HIV-1 specific CTL responses in our cohort. At least 5 other groups investigating CTL responses in HIV exposed term infants were unable to detect these responses even after virus specific in-vitro stimulation (Luzuriaga et al., 1991; Buseyne et al., 1993; McFarland et al., 1994; Luzuriaga et al., 1997; Thobakgale et al., 2007). Exposed uninfected infants in this study could have completely escaped viral exposure or the small sample size may have limited our ability to detect responses. The other reason for a lack of CTL responses in this study could be that low birth weight infants are in fact not able to mount an adequate immune response as shown in 2 previous non-African studies (Chatrath, 1997; Das, 1998). Finally, the introduction of enhanced PMTCT prophylaxis during the course of the study could have had influenced the ability to

mount an immune response (outlined in section 2.1). The majority of the mother-child pairs enrolled in this cohort received both AZT and NVP. Several studies have shown that exposure to antiretroviral therapy in utero and neonatally are associated with a decrease in CD4 and CD8 cell counts (McKallip et al., 1995; Francke et al., 2000; Bunders et al., 2005; Kolber et al., 2008).

LIMITATIONS

A major confounder to the study was the initiation of PMTCT ARV prophylaxis during the course of the study. The majority of the mother-child pairs enrolled into the study received prophylactic treatment and we cannot determine to what extent it has the influenced the CTL immune response. HTEBM was introduced to preterm infants as their mothers were already expressing breastmilk. However, we are not certain of the level of prematurity of the immune system and we cannot determine how this would have influenced the CTL immune response. This was also a vulnerable population and the drop out rate due to demise or ill health was significantly higher. Therefore, it was difficult to follow-up patients longitudinally over a 9 month period. It was also difficult to maintain feeding mode post discharge despite adequate infant feeding counseling at each follow-up session. Initially one of our study objectives included investigating CTL immune responses in infants that were fed EBM (live virus) vs. HTEBM (heat inactivated virus), but due to the overwhelming success of the implementation of HTEBM at the KEH nursery we had to combine the two feeding arms (HTEBM and EBM) into one group (HTEBM).

CONCLUSION

In conclusion we have shown that it is feasible for HIV infected mothers to heat treat their expressed breastmilk during hospital admission. Within the constraints of a small sample size, we were able to demonstrate in this small cohort of patients that the clinical outcomes and growth parameters of infants fed HTEBM were similar to that of infants fed either formula or unheated breastmilk. We were unable to demonstrate HIV-specific CTL responses in this cohort of infants. Our findings indicate that additional research is required. However, due to the wide scale PMTCT programmes in South Africa it will be difficult to establish such research projects with an ARV naïve cohort. Therefore, we suggest that the scope of this project would be better replicated in an animal model.

CHAPTER 5

APPENDICES

1. Information Document for the Mothers

INFORMATION DOCUMENT

Study title: Role of flash heat-treated breast-milk in improving health outcomes in HIV-exposed preterm low-birth-weight infants: an operational study.

Hello and congratulations on the birth of your baby! We know that it is a challenge to have a small baby but the nurse will do their best to support you while you are in hospital. The Department of Paediatrics is doing research on different feeding options for premature babies of HIV-positive mothers. Research means the process of using science to learn the answer to a question. In this study we want to learn how feeding babies with heated breastmilk compares to feeding babies with non-heated breastmilk or formula. We are interested in learning which technique mothers choose to do in the hospital and at home after counselling, and which technique is most beneficial for the baby.

Invitation to participate: We are inviting you to participate and request your permission to include your child in this study.

What is involved in the study: If you decide to participate in this study, you will receive a counselling session with a trained counsellor to discuss the different ways in which you can feed your baby and the advantages and disadvantages of each method. The counsellor will also tell and show you how you can “heat-treat” your breastmilk and feed it to your baby in the way that the World Health Organization recommends. You can then make a choice to either heat-treat your breastmilk before

feeding it to your baby, breastfeed without heat-treating, or formula feed. **You will be able to make all feeding choices yourself.** We will ask you some questions about your decision, and will keep a record of how many times your baby feeds, has episodes of illness in the hospital, your baby's growth, and any information we find about your baby's HIV status from blood tests.

As part of this study, you may be asked to participate in a discussion group on infant feeding. If you are asked to be a focus group participant, you have every right to refuse to participate. Refusing will not affect your status as a study participant or the care that you and your baby receive. All comments made during these groups will be recorded and used for research purposes without being linked to your name or any other personal identification.

When your baby is six weeks old, you will return to our clinic, where we will examine your baby and you will tell us about your health, that of your baby, and give us some information on how you have been feeding your baby. We will also test your baby's blood for the HIV virus at this time, which is required in South Africa for any infant with a mother that has HIV, not just those participating in this study. You will be asked to come into the clinic again when your baby is three months old and thereafter every three months up to 9 months of age. This is the standard follow up time for babies who are born prematurely as we need to monitor their health, and is not part of the study itself. You can also come into the clinic at any point in between if you think your baby needs medical attention.

The study will require that we take two extra blood tests—one within 48 hours of your baby's birth, and one at the time that you leave the hospital. We will only analyze these blood tests if your child's HIV status changes on the HIV test that is required by the government. The purpose of these blood tests is to confirm exactly when an infant becomes HIV positive. These tests will only require a drop of blood and the blood will be collected by means of a small prick on the heel.

In addition we would request permission to take a small blood sample from your infant at the clinic visits at 6 weeks, 3, 6, and 9 months. It will be a very small sample about half a teaspoon of blood. This blood will be used to allow us to do some tests to determine how the baby's immune response is working. At discharge if your blood has not already been tested for CD4 and viral load we will take a sample from you for these tests. These results are important to allow us to advise you on your disease management. If these results show that you are in need of treatment we will refer you to the appropriate clinic.

Risks of participating: There are no risks to participating in this study. While it is possible that using the flash heat treatment may have a very small effect on a few of the vitamins and protein in the milk, laboratory studies have shown that the heating eliminates the HIV virus present in milk. For this reason, some mothers choose to heat their milk before feeding it to their child.

Benefits of participating: You will receive advice and support in the hospital on how to best feed your baby. You will specifically receive information on how to correctly heat-treat milk, which is recommended by the World Health Organization

as a way to give your baby the benefits of breastmilk while reducing the chance that your baby will get HIV from breastmilk. We will be able to closely monitor the health of your baby and provide the necessary management.

You will be given information on the study while you are involved in the project. Unfortunately the results of the study may only be available when your child is six months to a year old so it will not directly benefit your child but it will be very important to help many other mothers and their babies in the future.

Participation in this study is voluntary. This means that refusal to participate will not involve any penalty or loss of benefits to which you are otherwise entitled. You may end your participation in this study at any time without any negative impact on the care that you and your child receive.

There will be no additional cost to you associated with your participation in this study. We will provide you with R50 for each clinic visit to assist with your transport costs.

Confidentiality: Efforts will be made to keep all personal information confidential. Absolute confidentiality cannot be guaranteed. Personal information may be disclosed if required by law. Organizations that may inspect and/or copy your research records for quality assurance and data analysis include groups such as the Research Ethics Committee and the Medicines Control Council (where appropriate). All of your information will be labelled with a number code and not your name, and will be kept in a locked cabinet by the project coordinators.

If you or your baby experiences any negative events or unusual symptoms during the course of the study, please immediately contact Reshmi Dayanand or Prof Coutsoudis at 031-2604489 or Prof Adhikari on 031-2604345

Contact details of BREC Administrator – for reporting of complaints/ problems:

Ms Patricia Ngwenya, Nelson R Mandela School of Medicine, Private Bag 7, Congella 4013

Telephone: +27 (0) 31 260 4769

Fax: +27 (0) 31 260 4609

Email: ngwenyap@ukzn.ac.za

2. Consent form for the Mothers (English Version)

INFORMED CONSENT DOCUMENT

You have been asked to participate in a research study *Role of flash heat-treated breast-milk in improving health outcomes in HIV-exposed preterm infants: an operational study.*

You have been informed about the study by Reshmi Dayanand/Nozipho Makanya and the nurses in the King Edward Hospital Nursery.

You may contact **Reshmi Dayanand (073-1402914), Prof Coutsoudis (031-2604489), or Prof Adhikari (031-2604345)** at any time if you have questions about the research or if your child gets ill or if you are injured as a result of the research.

You may contact the **Biomedical Research Office** at the Nelson R Mandela School of Medicine at **031-260 4769** if you have questions about your rights as a research subject.

Your participation in this research is voluntary, and you will not be penalized or lose benefits if you refuse to participate or decide to stop.

If you agree to participate, you will be given a signed copy of this document and the participant information sheet, which is a written summary of the research.

The research study, including the above information, has been described to me orally. I understand what my involvement in the study means and I voluntarily agree to participate.

_____ _____

Signature of Participant **Date**

_____ _____

Signature of Witness **Date**
(Where applicable)

_____ _____

Signature of Translator **Date**
(Where applicable)

3. Enrolment form

STUDY NUMBER: _____
MATERNAL HOSPITAL NUMBER:_____

Enrolment Forms

Complete at enrolment into care Enter all dates as dd/mm/yy

Personal Details:	
First Name:_____	**Last Name:_____**
Date of birth: __/__/__	**Age:___(years)**
Marital Status: **☐ Married ☐ Divorced ☐Widowed** **☐ Cohabiting ☐ Single**	**Maternal Hospital Number:** ☐☐☐☐☐☐☐☐☐☐☐ **Infant Hospital Number:** ☐☐☐☐☐☐☐☐☐☐☐
Maternal ID: ☐☐☐☐☐☐ ☐☐☐☐ ☐☐ ☐	
Infant ID number (to be completed when birth certificate obtained**):_____**	
Infant date of birth:__/__/__	
Maternal Address:_____	
Maternal contact number:_____	
Name of friend/family:_____	**Contact no. of friend/family:_____**
Ante-natal clinic location:_____	**Nearest local clinic for immunizations:_____**
Date originally counselled:__/__/__	**Counselled by: ☐ Reshmi ☐ Nozipho**
Maternal ward at enrolment: ☐ O1 ☐ O2 ☐ O3	**Infant ward at enrolment: ☐ NPN ☐ ICU ☐ HC**
Date enrolled: __/__/__	
Baseline Maternal Assessment: ***(Tick appropriate block)***	
Maternal parity____	
Mode of delivery: **☐ caesarean** **☐ vaginal** **☐ unknown**	**Reason for preterm delivery:** **☐ spontaneous preterm labour** **☐ fetal distress** **☐ maternal illness**
PMTCT Prophylaxis: ***(Tick appropriate block)***	
Which prophylaxis drugs did mum receive? **☐ NVP (single dose)** **☐ AZT** **☐ UNKNOWN**	
CD 4 count: _____ **Date:__/__/__**	
Clinic location:_____	
Maternal Medical History	

(Tick appropriate block)	
Tuberculosis: **☐ none** **☐ on treatment** **☐ being worked up** **☐ not on treatment** **☐ unknown**	**Syphilis:** **☐ yes** **☐ no** **☐ unknown**
Pneumonia: **☐ yes** **☐ no** **☐ unknown**	**Other chronic conditions:** **_____**

Household Questions *(Tick appropriate block)*	
Have you disclosed your HIV status? **☐ Yes -> If yes, to whom?** **☐ partner ☐ family** **☐ Household member (not related) ☐ other** **☐No -> If no, why not? (list all)_____**	**Do you have water piped (from a tap) inside your home?** **☐ yes** **☐ no**
Do you have electricity at your home? **☐ yes ☐ no**	**Do you have a fridge at your home?** **☐ yes ☐ no**
Highest level of education **☐ did not complete primary school** **☐ did not complete secondary school** **☐ Completed secondary education/matric** **☐ Some tertiary education** **☐ Completed tertiary education** **☐ Professional training**	• **Are you currently employed?** **☐ yes ☐ no** • **Main source of household income?** **☐ family ☐ grant ☐ other**
Ante-natal feeding choice (recorded on PMTCT card):_____	**Postnatal feeding choice:_____**
Has mother breastfed before?_____	
Has mother ever expressed breastmilk before?_____	

STUDY NUMBER: **INFANT HOSPITAL NUMBER**:

Baseline Infant Assessment

Complete at enrolment into care *Enter all DATES as dd/mm/yy*

Date of birth: __/__/__

Birth weight :_____ (kg)	**Birth length: _____ (cm)**	**Birth OFC: _____ (cm)**
APGAR Score: ☐ 1min ☐ 5min	**Gender: ☐M ☐F**	**Gestational age**:_____

Problems in first 24 hrs

(Tick appropriate block)

RDS ☐ **yes** ☐ **no**	**Hypoglycaemia** ☐ **yes** ☐ **no**	**Neurological problems** ☐**yes**☐**no**	**Other:_____**

Respiratory distress at any time: ☐ yes ☐ no

Ventilation: ☐ yes ☐ no **Duration:_____day(s)**	**CPAP: ☐ yes ☐ no** **Duration:_____day(s)**	**Oxygen: ☐ yes ☐ no** **Duration:_____day(s)**
Seizures: ☐ yes ☐ no	**Feed intolerance**: ☐ **yes** ☐ **no** **Duration:_____day(s)**	**TPN given: ☐ yes ☐ no** **Duration:_____day(s)**

Infections

(Tick appropriate block)

• **Suspected sepsis:** ☐ **yes** ☐ **no** *(Please tick one)* - **proven on blood culture** ☐ - **proven on swab** ☐ - **proven on CSF** ☐ - **proven on urine** ☐ - **proven on ETT/other** ☐ - **Organism isolated** ☐	• **Suspected meningitis:** ☐ **yes** ☐ **no** *(Please tick one)* - **proven on blood culture** ☐ - **proven on swab** ☐ - **proven on CSF** ☐ - **proven on urine** ☐ - **proven on ETT/other** ☐ - **Organism isolated** ☐	• **UTI:** ☐ **yes** ☐ **no** *(Please tick one)* - **proven on blood culture** ☐ - **proven on swab** ☐ - **proven on CSF** ☐ - **proven on urine** ☐ - **proven on ETT/other** ☐ - **Organism isolated** ☐	• **Pneumonia:** ☐ **yes** ☐ **no** *(Please tick one)* - **proven on blood culture** ☐ - **proven on swab** ☐ - **proven on CSF** ☐ - **proven on urine** ☐ - **proven on ETT/other** ☐ - **Organism isolated** ☐

Jaundice: ☐ **yes** ☐ **no**	**Phototherapy:** ☐ **yes** ☐ **no**	**Exchange TF:** ☐ **yes** ☐ **no**

Tuberculosis:
none ☐ work-up pending ☐ treatment ☐ prophylaxis ☐

Apnoea: ☐ yes ☐ no	**Hypoglycaemia:** ☐ **yes** ☐ **no**	**PDA: ☐ yes ☐ no**

Necrotizing enterocolitis: suspected ☐ confirmed (XR/biopsy) ☐ None ☐

PMTCT Prophylaxis

(Tick appropriate block)

Nevirapine: ☐ yes ☐ no	**AZT**: **7 days ☐ 28 days ☐ none ☐**

Surgery in neonatal period: ☐ yes ☐ no **Indication_____**
Notes:_____

4. In-Hospital Data Form

Date	Hospital day:					Infant ward:			
Amount of feed									
Time of Feed	9	12	15	18	21	24	3	6	
Type of Feed (HTEBM/EBM/Formula									
Mode of Feeding (NGT/syringe/cup/breast)									
Supplements, fluids, or fortifiers administered									
TPN (concentration & rate)									
Total number of feeds received by infant									
Number of feeds adherent to post-natal feeding									
Description of non-adherent behavior									
Infection-related events; clinical criteria satisfied									
Other notes (Relevant medications, laboratory results, ventilator settings, etc)									

Growth							
	Date	Weight (g)	Length (cm)	OFC (cm)	Cranial scan findings	PCR result	
End of week 1							
End of week 2							
End of week 3							
End of week 4							
End of week 5							
End of week 6/Follow-up							
Details on Discharge							
Date of discharge:							
Date of death, if applicable:							
Scheduled date of next follow-up							
Medications/supplements on discharge							
Feeding practice on discharge:							

5. Clinical Follow-up Form

STUDY NUMBER:

Morbidity & Feeding data follow-up		
Date:__/__/__	**Age of infant:_____**	
Weight (kg)_____	**Length(cm)_____ __**	**OFC (CM)_____**
Infant Morbidities since Hospital admission		
Diarrhoeal illness: ☐ yes ☐ no	**Respiratory illness: ☐ yes ☐ no**	
Other illness:_____ _	**Treatment:_____ __**	
Maternal Morbidities since Hospital admission		
Maternal morbidities:_____		
Maternal ARVs: ☐ yes ☐ no	**Clinic where following_____**	
Maternal CD4:_____	**Maternal viral load**	
Clinical findings on infant		
Poor weight gain: ☐ yes ☐ no **Tachycardia: ☐ yes ☐ no** **Pallor: ☐ yes ☐ no**	**Wasting: ☐ yes ☐ no** **Tachypnea: ☐ yes ☐ no** **Oral thrush: ☐ yes ☐ no**	
Eczema: ☐ yes ☐ no	**Scabies: ☐ yes ☐ no**	
Lymphadenopathy: ☐ yes ☐ no	**Skin infection: ☐ yes ☐ no**	
LRTI: ☐ yes ☐ no	**URTI: ☐ yes ☐ no**	
Hepatomegaly: ☐ yes ☐ no	**Splenomegaly: ☐ yes ☐ no**	
Other:_____ _____		
Current problems/assessment:_____		
Specimen HIV DNA PCR to be sent to virology lab ☐ yes ☐ no **Extra sample stored ☐ yes ☐ no**		
Result:	**Date of result:**	
Current feeding practice		
What are you currently feeding baby (last 24 hrs)? **☐ breastfeeding ☐ exclusive breastfeeding**		

☐ **Expressed breastmilk** ☐ **exclusive expressed breastmilk** ☐ **Heat-treated expressed breastmilk** ☐ **exclusive Heat-treated expressed breastmilk** ☐ **Formula** ☐ **exclusive Formula**
Have you ever mixed fed your baby?_____
What was your feeding practice from your last clinic visit?_____
What prevented you from your original feeding choice (if applicable)?

Preparation of Reagents

6. Working FACS Lysing Solution (1:10 Dilution)

50µl 10X FACS Lysing solution (BD Biosciences, San Jose, USA)

450µl Sterile distilled water

Method: 500 µl of the 10 X FACS Lysing solutions was diluted in 450 µl sterile distilled water. 450 µl of the working FACS Lysing solution was used for each test.

7. Inhibitor Removal Buffer

100% Ethanol

Inhibitor Removal buffer (Roche)

Method: 20ml of 100% ethanol was added to Inhibitor Removal Buffer (IRB) and mixed by inverting 5-10 times. This is sufficient reconstituted Inhibitor Removal Buffer for 48 tests.

8. Phosphate Buffered Saline (PBS)

1 Phosphate Buffered Saline (Dulbecco A) Tablet (Oxoid Limited, Hampshire, England)

100ml Sterile distilled water

Method: One Phosphate Buffered Saline (Dulbecco A) Tablet was dissolved in 100ml of sterile water. The pH of the PBS solution was adjusted to approximately 7.2. The solution was autoclaved for 10 minutes at 115ºC and stored at room temperature.

9. Phosphate Buffers Saline (PBS) + Antibiotics

1 L Phosphate Buffered Saline

1% of 100X Penstrep/Fungizone (P/S/F) (Biowhittaker, Cambrex Bio Science, Walkersville, USA)

Method: Removed and discarded 10ml of the already prepared PBS. 10ml of the 1% Penstrep/Fungizone (P/S/F) solution was added to the remaining PBS. The solution was mixed well and stored at 4ºC.

10. R10 Media

500ml RPMI (Biowhittaker, Cambrex Bio Science, Walkersville, USA)

50ml Fetal Calf Serum (FCS) (Delta Bioproducts, Johannesburg, South Africa)

5ml of 200Mm L-glutamine (Biowhittaker, Cambrex Bio Science, Walkersville, USA)

5ml of 100X Penstrep/Fungizone (P/S/F) (Biowhittaker, Cambrex Bio Science, Walkersville, USA)

5ml of 1M Hepes Buffer (Biowhittaker, Cambrex Bio Science, Walkersville, USA)

Method: Removed and discarded 65ml of RPMI from the original volume of 500ml. 50ml heat inactivated and filtered FCS, 5ml of 200Mm l-glutamine, 5ml of 100X Penstrep/Fungizone (P/S/F) and 5ml of 1M Hepes Buffer was added to the remaining RPMI. The media was mixed well and stored at 4ºC.

11. 2% Virkon Solution

200g of Virkon Powder (Antec International, Sudbury, Suffolk)

10L Water

Method: 200g of Virkon Powder was dissolved in 10L of water.

12. Blocking Buffer

1L Phosphate Buffer Saline

10ml Fetal Calf Serum (FCS) (Delta Bioproducts, Johannesburg, South Africa)

Method: 10ml of PBS from already prepared 1L bottle was removed and discarded. 10ml of heat inactivated, filtered FCS was added to the remaining PBS. The 1L solution was mixed well and stored at 4ºC.

13. BCIP (5-bromo-4-chloro-3-indoly/phosphate)

0.015g BCIP (BioRad Laboratories, Hercules, Ca)

1ml DMF (Merck, Wadeville, Gauteng, South Africa)

Method: 0.015g of BCIP powder was dissolved in 1ml DMF. The final concentration was 15mg/ml. The solution was mixed well and stored at 4ºC.

14. NBT (Nitroblue Tetrazolium Chloride)

0.03g NBT (BioRad Laboratories, Hercules, CA)

700µl DMF (Merck, Wadeville, Gauteng, South Africa)

300µl Sterile distilled water

Method: 0.03g of NBT powder in 700µl DMF and 300µl sterile distilled water was added. The solution was mixed well and stored at 4ºC.

15. 1M Tris Buffer

12.1g TRIS (BioRad Laboratories, Hercules, CA)

1L Sterile distilled water

Method: 12.1g of TRIS powder was dissolved in 1L sterile water with the use of a magnetic stirring bar. The pH was adjusted to 9.3 with concentrated Hydrogen Chloride (HCl). The solution was autoclaved for 10 minutes at 115ºC and store at room temperature.

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