THE INTERACTION BETWEEN HUMAN LEUCOCYTE ANTIGEN–G AND NATURAL KILLER CELLS AT THE PLACENTAL INTERFACE IN HIV-1 INFECTED PREGNANT WOMEN AND THE SIGNIFICANCE, IF ANY, TO *IN UTERO* TRANSMISSION

By

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ABSTRACT

This study was undertaken to investigate the relationship between Natural Killer cells and HLA-G at the placental barrier in HIV-1 infected pregnant women and to establish the significance, if any, to *in utero* infection.

Fifty-five HIV-1 infected pregnant women were recruited into the study after consent was obtained. Blood samples were collected from both mothers and babies for viral loads and CD4+ cell counts. Placental samples were obtained from pregnancies at delivery and examined by immunoperoxidase immunohistochemistry methods using monoclonal antibodies to p24 antigens and Natural Killer (CD56+) cells. HLA-G expression was quantified using real-time polymerase chain reaction.

Analysis of viral loads and CD4+ cell counts were undertaken in categories. No significant association was observed between the viral load of mothers and their CD4+ cell counts. Eighteen percent of the women in this study population had < 200 CD4+ cell counts and were classified as having AIDS (CDC, 1993). Ninety percent of the women with AIDS had viral loads greater than 4 logs thereby, increasing the risk for transmission to the baby. Sixty percent of the women classified as having AIDS, had low haemoglobin values with a corresponding low CD4+ count. We also report here a statistically significant association between maternal viraemia and maternal haemoglobin ($p = 0.05$).

The relationship between the babies' viral load and birth weight was investigated. There was no significant difference in the median birth weight between infected and uninfected babies

(p = 0.457). The infected babies in our study had between 3 to >5 log viral loads with a transmission rate of 0.27(95% CI, 0.15 – 0. 39). Maternal viraemia was significantly associated with transmission of infection to babies (p = 0.047). The odds ratio indicated that for every 1 log increase in maternal viral load the babies were 3.1 times more likely to acquire the infection (Exp (B) = 3.137 (95%CI, 1.015-9.696). Furthermore, the study found that a higher number of female babies were infected than males. Although not statistically significant the odds ratio indicated that female babies were 3.1 times more likely to become infected than males (Exp (B) = 3.110 (95%CI, 0.819-11.808).

We report here the results of immunohistochemistry for p24 antigens and NK (CD56+) cells and compare them to the immunological responses of both mothers and babies at birth. HIV-I antigens were detected in 94.5% of all placentas by immunohistochemistry. Infiltration of CD56+ was found in 98% of placental tissue. The analysis revealed that the presence of p24 antigens in placental tissue was not influenced by maternal viral load or CD4+ cell counts. Lower median NK cell values were observed in placentas of mothers with infected babies as compared with the uninfected cluster. Although not statistically significant, the risk of vertical transmission was increased 3.4 times more in placentas which had lower NK cell values. According to the odds ratio, babies CD4+ counts were affected by every 1 log increase in mother's viral load. Overall, maternal viral load emerged as a strong predictor for risk of infection from infected mothers to their infants. Our analysis indicated that female babies were 3.7 times more likely to acquire the infection than males.

Using data obtained from real-time PCR we investigated the relationship between maternal viral load and the quantity of HLA-G expression (p = 0.045; 95%CI 1.029- 11.499).

Logistic regression models revealed that mother's viral load was the strongest risk factor for vertical transmission. No statistically significant correlation was noted with HLA-G and viral transmission. However, the odds ratio indicated that the risk of infection increased by 1.3 with every 1 fold increase in HLA-G expression. An analysis of mother-to-child transmission rates by gender revealed that the odds ratio for transmission was 3.4 times more in female babies than in males.

We then investigated the relationship between maternal viraemia and HLA-G expression. A positive correlation between maternal viral load and placental HLA-G was observed ($p = 0.038$). When gender susceptibility to HLA-G expression was explored a statistically significant association was observed in placental tissue of mothers with infected and uninfected male babies and HLA-G expression ($p = 0.013$). To conclude, the analysis found that HLA-G was up regulated 3.95 times more in placental tissue of mothers with infected babies than in mothers with uninfected babies.

DECLARATION

This study represents the author's original work and has not been submitted previously to this or any other university.

The research described in this dissertation was carried out under the supervision of Professor R Bobat (UKZN) and Professor S Carpenter (Washington State University, USA).

Shamala Moodley

DEDICATION

To

My daughters, Dharshni and Mohini, the two Positives in my life.

My Mum and Dad for teaching me the value of perseverance

PRESENTATIONS

Presentations arising from this study:

- Iowa State University, USA - Maternal Viraemia and Mother to Child Transmission of HIV/AIDS.
- Iowa State Medical School – Viral RNA copies and CD4+ cell counts in babies of HIV 1- infected women.
- The 19th SMLTSA Congress Bloemfontein. 2007. Natural killer cells at the foetal-maternal interface of hiv-1 infected pregnant women and its implication in *in utero* transmission.

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ABC	Avidin-Biotin Complex
AEC	3-Amino-9-Ethylcarbazole
AIDS	Acquired Immunodeficiency Syndrome
ANC	ante natal clinic
Approx	approximately
ART	antiviral therapy
ARV	antiretroviral
B cells	B lymphocytes
bDNA	branched deoxyribose nucleic acid
BSA	bovine serum albumin
CSFs	colony stimulating factors
CD	cell differentiation
CDC	Centre for Disease Control
CDCP	Centre for Disease Control and Prevention
cDNA	complementary deoxyribose nucleic acid
CI	Confidence Interval
CP	crossing point
CTL	cytotoxic T-lymphocytes
°C	degrees centigrade
DPX	Distrene 80-Plastizer Xylene
DNA	deoxyribose nucleic acid

DEPC	diethyl pyrocarbonate
E	efficiencies
env	envelope
GAPDH	glyceraldehydes-3-phosphodehydrogenase
GM	granulocyte-macrophage
gp	gamma protein
HCL	hydrochloric acid
HE	Haematoxylin and Eosin
HIV	Human Immunodeficiency Virus
hpf	high power field
HRP	horse radish peroxidase
HSC	haematopoetic stem cell
HLA-G	human leukocyte antigen- G
IgG	immunoglobulin G
IFN-γ	gamma interferon
IL	interleukin
IQR	interquartile range
KIR	killer inhibitory receptors
MHC	major histocompatibility complex
millitre	mls
min	minute
M	molar solution
MTCT	mother to child transmission

M-trophic	macrophage tropic
NaOH	sodium hydroxide
NASBA	nucleic acid sequence- based amplification
ng	nanogram
NIH	National Institutes of Health
nm	nanometres
NCAM	neural cell adhesion molecule
NK	natural killer
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMTCT	prevention of mother-to-child-transmission
R	relative expression
RNA	ribose nucleic acid
R sq	R square
RT	reverse transcriptase
RT PCR	Real Time Polymerase Chain Reaction
SD	standard deviation
Sec	seconds
SI	syncytium inducing
ssDNA	single–stranded DNA
STI	sexually transmitted infection
TBE	tris-borate/EDTA
TBS	Tris-buffered saline

T cells	T lymphocytes
TCR	T-cell receptor
TMB	tetramethylbenzidine
Th	T helper cells
TNF-α	tumour necrotic factor alpha
μl	microlitre
uNK	uterine Natural Killer
Δ	delta

CHAPTER ONE

CHAPTER ONE

LITERATURE REVIEW

1.1 Introduction: The Global Estimation

Human Immunodeficiency Virus I (HIV-I) is a member of the *Retroviridae* family and belongs to the sub-family of the *Lentivirida.* These viruses are non-oncogenic pathogens, and they can persist in the infected host for years causing slow progression to disease. HIV is the virus responsible for Acquired Immuno Deficiency Syndrome (AIDS) and was first described in 1981 (NAIDS, 2001). In 1983 the virus was isolated and designated first as HTLV III/LAV and later as HIV 1(Gallo and Montagnier, 2003).

The global statistics for HIV, by the end of 2005 was estimated at nearly 40 million (UNAIDS, 2006). Seventy one percent of the estimated global total of people with HIV/AIDS lives in sub Saharan Africa (Grimwood *et al.*, 2000). HIV has infected 4.4 million children worldwide leading to 3.2 million child deaths (Frye, 2004).

The HIV seroprevalence rate amongst pregnant women varies in developing and developed countries. In South America and the Caribbean the prevalence rate amongst pregnant women is $\geq$2% whilst in sub- Saharan Africa it is 35-45% (UNAIDS, 2006). The Asian magnitude of the epidemic has reached approximately 2% and the vertical

transmission rate is 24% without breastfeeding. Indian mothers infected with the virus routinely breast-feed and have transmission rates as high as 48% (Frye, 2004).
In developed countries such as the western areas of France, Spain and Italy seroprevalence rates of up to 1% has been recorded (Frye, 2004).

Vertical transmission of HIV is lower in developed countries than in developing countries, independent of treatment. The transmission rates in pregnant women left untreated is as low as 13% in Europe and as high as 40% in Africa (Frye, 2004). Initiation of antiretroviral (ARV) therapy during pregnancy has been shown to reduce the mother's viral load, thereby decreasing the risk of transmission (Cooper *et al.* 2002). The Women and Infants Transmission Study reported that ARV therapy lowered mother-to-child transmission (MTCT) rates and that transmission rates were dependant on the potency of the ARV combinations administered to pregnant women. The following transmission rates were observed: 20% (95%CI: 16.1-23.9) with no ARV intervention during pregnancy, 10.4% (95%CI: 8.2 -12.6) with zidovudine monotherapy and 3.8% (95%CI: 1.1-6.5%) with dual ARV therapy (Cooper *et al.* 2002). Several different regimens of ARV prophylaxis are administered to HIV-1 infected women in labour, without previous antiretroviral therapy ((UNAIDS, 2003). A randomized, open label trial in South Africa compared two regimens:

- nevirapine 200mg during labour and one dose to the mother and infant 24-48 hours after delivery
- Multiple doses of the combination of zidovudine and lamivudine during labour and for 1week to the mother and infant after delivery.

Transmission rates were 12.3% in the nevirapine study and 9.3% in the combination of zidovudine /lamivudine study (p = 0.11) (Moodley *et al.* 2003.)

According to UNAIDS the global epicenter of the pandemic remains in Africa (Global Report, 2006). Based on UNAIDS survey systems, the mortality data is estimated at 5.5 million living with HIV in 2005 (UNAIDS 2006). South Africa's AIDS epidemic is listed as the worst in the world with no evidence of decline. Based on the South African Department of Health National antenatal survey it is estimated that 30.2% of pregnant women were HIV positive in 2005 (The South African Dept of Health Survey, 2006).

1.2 Mother to Child Transmission of Human Immunodeficiency Virus

The HIV/AIDS epidemic is having a significant impact on child health. According to Shung-King *et. al.* (2000), in Africa, in 1999, an estimated 500 000 children were infected with HIV. By 2004 the estimate rose to 550 000 with South Africa reporting at 230 000 (WHO report, 2004). Globally, by 2006, it has been reported that there were 530 000 newly infected children under the age of 15 years (AIDS Epidermic Update, 2006).

Mother-to-child-transmission (MTCT) of HIV-1 can occur before, during or after birth. The contributions of each of the routes differ in various studies (Newell *et al.*, 1998). Several possible modes of transmission of HIV from mother to infant have been

suggested (Table I). These include *in-utero* infection of the foetus via placental transmission, contact of foetus with infected maternal blood or cervical secretions during gestation and delivery, maternal disease stage, maternal viral load and postnatal infection due to breast feeding of infants (Sprecher *et al.*, 1986, Newell *et al.*, 1998; Coutsoudis *et al.*, 1999).

Before the introduction of prophylactic treatment the MTCT prevalence was 15-25% in industrialised countries (European Collaborative Study, 1992; The Working Group on MTCT, 1995) providing that the mothers did not breast feed. In the African settings where mothers breastfed their children, the statistics on transmission rates were as high as 40-50% (Ryder *et al.,* 1989; Adjorlolo-Johnson *et al.*, 1994). Studies in non-breast fed infants have revealed that approximately 60-70% of infected infants acquired the virus during delivery whilst 30-40% was presumed to be infected *in utero* (Dunn *et al.*, 1995; Kuhn *et al*, 1999). Infection of the foetus occurs at various trimesters. The presence of virus was demonstrated in aborted foetal tissue as early as 15-20 weeks of gestation (Sprecher *et al.,* 1986). Maury *et al.* (1989) isolated HIV-1 from CD4 cells in foetal derived placental tissue. The study findings showed that the majority of the foetuses were infected during the final trimester of pregnancy. Transmission during the first trimester appears to be rare. Infection rate in the second trimester was reported at 2-5% (Brossard *et al*, 1995; Phuapradit *et al.*, 1999).

Replication of HIV-1 within the mammary epithelial cells is increased by hormonal stimulation in pregnancy (Toniolo *et al.,* 1995). Several studies have demonstrated HIV-1 transmission through breast feeding (John *et al*, 2001; Fawzi *et al*, 2002) which

carries a risk of approximately 15% in mothers with established maternal HIV-1 infection and 29% in acute maternal HIV-1 infection (Dunn *et al.*, 1992). Combination of breast feeding and bottle feeding increases the risk of the child becoming infected (Leroy *et al.*, 1998; Newell *et al.* 1998; Coutsoudis *et al.*, 1999, Miotti *et al*, 1999).

The possibility of MTCT via breast milk has led to recommendations to avoid breastfeeding by HIV-1 infected women in developing countries, in particular circumstances. Coutsoudis *et al.* (1999) argues that exclusive breastfeeding still carries a significantly lower risk of transmission than mixed feeding practices. Miotti *et al.* (1999) reported that there was a higher risk of transmission from breastfeeding in the one to five months period of the infant's life than after 5 months. The authors in the study speculated that transmission rate could be substantially higher in the first month. This was attributed to the high cell content of colostrums in early milk and/or the infant's immature immune system.

Table I: Risk Factors for Vertical Transmission*

Pregnancy Period	Risk Factors	Risk Variables
Prenatal	Quantity of virus	High maternal viral load (>10 000 copies/ml) in: • Peripheral blood • Cervicovaginal fluid
Perinatal	Duration of Exposure	Mode of delivery: • Sexually transmitted infections and genital warts. • Preterm labour • Emergency caesarean sections. • Vaginal deliveries. • Chorioamnionitis.
Postnatal	Breastfeeding	• Mixed breastfeeding. • Duration of breast feeding • Vitamin A deficiency

****This table was developed by the author from information derived from various references (Sprecher et. al., 1986, Dunn et. al., 1992; Newell et.al, 1998; Coutsoudis et.al., 1999; Miotti et.al, 1999).***

1.3 Human Immunodeficiency Virus and Target Cells

Retroviruses are unable to replicate without the aid of a living cell (NIAID, 2001). The pathogenesis of HIV infection is a function of a combination of factors including the virus life cycle, host cellular environment, and quantity of viruses in the infected individual. Although, HIV can infect various cells in the body, its main target is the CD4+ T lymphocyte which has the appropriate CD4+ receptor molecules (Levy, 1993).

After entering the body, the viral particle attaches by fusion to a susceptible cell membrane or by endocytosis and then enters the cell. The probability of infection is a function of both the number of infective HIV virions in the body fluid which contacts the host as well as the number of cells available at the site of contact that have appropriate CD4 receptors (Ferguson *et al.*, 2002). Cells with CD4+ receptors susceptible to HIV infection may include cells of the mononuclear phagocyte system, principally blood monocytes and tissue macrophages, T lymphocytes, B lymphocytes, natural killer (NK) lymphocytes, dendritic cells (Langerhans cells of epithelia and follicular dendritic cells in lymph nodes), hematopoietic stem cells, endothelial cells, microglial cells in brain, and gastrointestinal epithelial cells.

In addition to the CD4+ receptor, a coreceptor known as a chemokine is required for HIV to infect cells. Chemokines are cell surface membrane-bound fusion-mediating molecules found on many cells. Macrophages, monocytes and subpopulations of lymphocytes, as well as the cells of the central nervous system can express the

chemokine CCR5 receptor. According to Rottman *et al.* (1997) areas of inflammation contain increased numbers of mononuclear cells with CCR5, and this may facilitate transmission of HIV at those sites. Without enough T cells the body is unable to defend itself against many infections. Understanding how HIV works and reproduces within the human cell can help scientists to design therapies, which can assist in suppressing viral replication.

1.4 Basic Structure of the Human Immunodeficiency Virus

Human Immunodeficiency Virus is a retrovirus, which contains RNA rather than the DNA of human cells. The virions (individual particles) of HIV are spherical in shape with a one ten-thousandth of a millimetre in diameter. The viral capsid (CA or p24) protein encloses two copies of the RNA that comprises the viral genome and two copies of viral reverse transcriptase (RT or p66/p51). Within the capsid, copies of the viral nucleocapsid, (NC or p7) and p9 proteins are complexed with the genomic RNA (Zeichner SL, 2005). This consists of three major genes, which code for the major structural and functional components of HIV. These genes are:

1. Env- for the viral envelope glycoproteins, including the outer envelope glycoprotein gp120 and transmembrane glycoprotein gp41 derived from glycoprotein precursor gp160
2. Gag- for the RNA-containing core proteins (p55, p40, p24) , outer region of viral core includes the HIV matrix (MA or p17), and nucleocapsid (p7)

3. Pol- for the viral enzyme protease, reverse transcriptase (enzyme proteins p66 and p51) and protease (p11) integrase (p32).

The envelope, derived from the external layer of the host cells, consists of a bilayer of fat molecules (lipids) and contains proteins with carbohydrate side chains (viral envelope glycoproteins, gp). The envelope protein gp41 (a transmembrane glycoprotein) is located in the viral membrane. The other is the gp120 which projects out from the membrane like a cap (Fig.1). The viral core that includes the outer protein structure p18 and the inner protein p24 is surrounded by the envelope (http://www.Roche, 2004; Zeichner SL, 2005)

Other genes code for small proteins that are involved in the regulation of gene expression. Most of the major HIV viral proteins, which include p24 (core antigen) and gp41 (envelope antigen), are highly immunogenic. However, the antibody responses vary according to the virus load and the immune competence of the host.

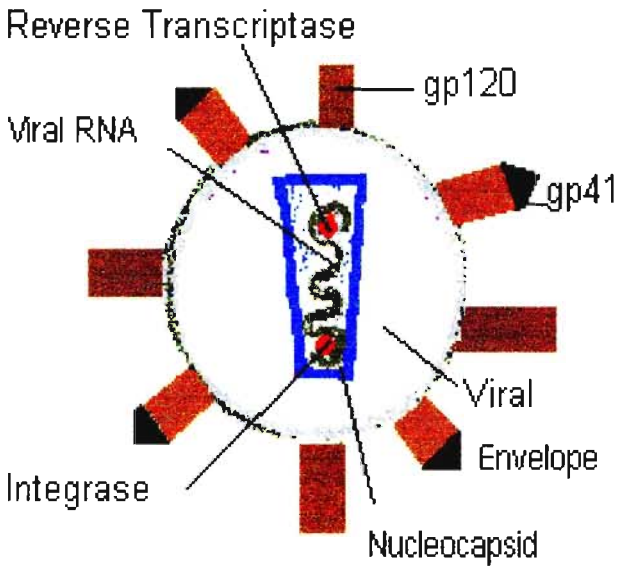

Figure 1: A schematic diagram of the HIV virion.

by HIV to latch onto the cell via envelop viral proteins gp120 and gp41. Fusion with cell occurs and viral genetic material is injected into the host cell.

- **Viral Penetration and Fusion**

After attachment is completed, viral penetration occurs. The genetic core (nucleocapsid) of the virus is injected directly into the host cell's cytoplasm. The three glycoproteins contained in gp120 spread apart once it attaches itself to the CD4 cell. This allows exposure of the normally hidden gp41 protein which, then binds to the chemokine receptor. Once this occurs both viral envelope and host cell membrane are in direct contact with each other.

- **Uncoating**

The virus is ready to release its genetic information (RNA) into the cell. The viral RNA is protected in the nucleocapsid, which needs to be partially dissolved, so that viral RNA can be converted to DNA. This process is achieved by the enzyme reverse transcriptase.

- **Reverse Transcription**

A complementary double stranded DNA (cDNA) copy from single stranded viral RNA is made using reverse transcriptase. This transcription contains the

instructions which HIV need to "hijack" the T cell's genetic machinery to reproduce itself. During the process reverse transcriptase uses nucleotides (DNA) from the host cell to make more viral particles.

- **Integration**

If the infected cell remains in the resting phase, the integration of the viral DNA into the host cell DNA may have no immediate consequences. If the cell is stimulated to divide and proceeds to the second phase of the infective cycle new viral particles and RNA are produced in the human host cell.

- **Viral Latency and Protein Synthesis**

Host cell is latently infected with HIV. This viral DNA is referred to as provirus, which lies latent within the cell. Upon activation of the immune cell the virus is mobilised and instructs the cell machinery to produce the necessary HIV components. Two strands of RNA are constructed from the viral DNA and transported out of the cell nucleus. One strand undergoes translation into subunits of HIV such as protease, reverse transcriptase, integrase and structural proteins whilst the second strand becomes genetic material for new viruses.

- **Cleavage and Viral Assembly**

The molecules initially formed as precursor proteins are cleaved by viral proteases to form functional proteins, which escape through the host cell membrane, by a process of budding.

- **Budding**

Activation of viral synthesis leads to release of new infective particles from the host cell surface by budding. Budding is the final step of the viral life cycle and is a process whereby the genetic material, which is enclosed in the nucleocapsid merges with the deformed cell membrane to form a new viral envelope. The nucleocapsid contains its own genetic material with a new outer coat made from the host's cell's membrane. The HIV pinches off and enters the host's circulation.

During the entire life cycle of the virus, the T cell (host cell) is altered and damaged causing the death of the cell. Host cell death may be mediated via several diverse mechanisms:

- apoptosis (programmed cell death)
- direct viral cytopathic effects
- autoimmune mechanisms
- disruptive interaction of HIV envelope proteins with the cell membrane.

- Natural Killer cells of the immune system recognise the infected cells and destroy the cells.

Apoptosis plays a key role in the decline in T cell numbers during HIV infection. Mechanisms that contribute to HIV-associated lymphocyte apoptosis include chronic immunologic activation gp120/160 ligation of the CD4+ receptor, enhanced production of cytotoxic ligands or viral proteins by monocytes, macrophages, B cells, and CD8+ cells, and direct infection of target cells by HIV resulting in apoptosis. Apoptosis of lymphocytes is increased with progression of HIV disease and diminished with effective antiretroviral therapy (Gougeon *et al.*, 2003).

1.6 Pathophysiology and Course of Infection

After HIV enters the host, the protruding trimeric gp120 glycoproteins on the lipoprotein bilayer envelope bind to CD4 cell surface receptors and CCR5 or CXCR4 chemokine coreceptors. The CD4+ receptors are located on CD4 T lymphocytes, monocytes and macrophages, which are needed for viral infection. The gp120 glycoprotein has a V3 region, which determines cellular tropism. Cellular tropism is required for syncytial formation. The nonsynctial (macrophage-tropic) strains prefer the CCR5 coreceptors and are the primary causes of infection (Frye, 2004; AIDS SA, 1998). It has been determined that the absence of the CCR5 coreceptor in certain Caucasian groups of people appears to confer protection against the infection (O'Brian *et al.*,1996; AIDS SA, 1998). Once gp120 binds to the receptors, the associated gp41 transmembrane glycoprotein is inserted into the cell membrane and initiates cell-membrane fusion. A second chemokine receptor CXCR4 was shown to be a coreceptor for syncytium inducing (SI) variants of HIV-1 predominates in the later stages of infection.

Within the cell the protease enzyme produces the reverse transcriptase and ribonuclease (RNAse) H enzymes. These enzymes are responsible for synthesising the single–stranded DNA (ssDNA) molecules and primers necessary to produce the complementary DNA strand. The high mutation rate, combined with the high reproductive rate is due to the enzyme RT, which lacks proof-reading machinery. This results in significant base to base variability in coding of DNA strands. Thus, a substantial evolution leading to resistance to treatment (Zeichner SL, 2005).

1.7 Heterogeneity and Viral Resistance

The human immunodeficiency virus has an extensive genetic diversity. Heterogeneity in nucleic acid sequences occurs because of:

- The error prone nature of the viral enzyme reverse transcriptase (RT).
- The high rate of viral reproduction.

Viral heterogeneity allows for quick adaptation to antiretroviral drugs and the human immune system. Therefore, heterogeneity may ultimately result in increased viral fitness to environmental, immunological and pharmacological selection pressure (Blackard *et al.*, 2002). Superinfection is a necessary first step for viral recombination to occur. Recombination may produce more viruses with altered cell tropism and viral resistance to drugs. According to Blackard *et al.* (2002) recombinant virus and superinfection are implicated in acceleration towards disease progression.

1.8 Pathogenesis of Human Immunodeficiency Virus

The time interval between primary infection and the development to AIDS is variable and is estimated to take 10 to 11 years (Munoz *et al.*, 1989). It is estimated that approximately 20% of infected individuals will progress to AIDS in less than 5 years, while approximately 5% will maintain normal immunological responses for over 10 years (Munoz *et al*, 1995). The differences have been attributed to differences in viral strains, host immune responses and exposure to cofactors (microbial or environmental).

Human Immunodeficiency Virus infection can be divided into two distinct stages:

Acute Primary Infection Syndrome- Several weeks after HIV-1 infection a non-specific clinical syndrome referred to as primary infection, acute infection syndrome or acute retroviral syndrome is observed (Lyles *et al.*, 2000). This phase can be asymptomatic or may be associated with influenza like illness, with fevers, diarrhoea, headaches, and malaise. Neurologic or gastrointestinal symptoms can predominate in the absence of fever in up to 25% of cases (Vanhems and Beaulieu, 1997). The illness lasts approximately 2-3 weeks with full recovery. Upon presentation these individuals will often be antibody negative but will have detectable viral RNA copies (Aldrovandi GM, 2005). During primary infection extremely high levels of viral load, as much as 10^7 copies/ml are observed. These levels drop to 100 000 copies/ml or less after several weeks (Daar *et al.*, 1991; Clark *et al.*, 1991). During this period of viraemia, the absolute numbers of CD4+ cells decline sometimes to < 200cells/μl. Later as the viral load decreases, the CD4+ count increases to normal levels (Androvandi GM, 2005).

Asymptomatic Infection- After a period of fluctuation, plasma viral levels reach a "set-point" usually between 10 000 to 100 000 copies/ml, remaining fairly constant for many years. This period is referred to the asymptomatic carrier stage, which follows initial infection. A gradual decline in the number of circulating CD4+ T cells over many years is observed during this phase of the infection. An estimated 10 billion virions with an estimated half life of 6 hours are produced each day (Perelson *et al.*, 1996). In a minority of cases, infection does not proceed beyond this asymptomatic

phase and the CD4+ counts remain stable. These are known as long–term non-progressors.

When eventually plasma viral levels increase and CD4+ counts decrease, the disease advances to immunodeficiency and AIDS (Lyles *et al.*, 2000). The point of more rapid decline of CD4+ cells is known as the "inflection point" and symptomatic HIV infection and AIDS–HIV-1 related symptoms eventually begin to develop. In adults, AIDS typically occurs about 10-12 years after initial HIV infection and is defined by more serious AIDS related illnesses with a decline of CD4+ count to below 200 cells/mm^3.

1.9 Pathogenesis of AIDS

Primary Infection: Primary or acute viral infection results within a week of initial infection with HIV 1. The immune system first encounters HIV during primary infection. The virus is transported to regional lymph nodes within 48 hours of exposure. Within the lymph nodes CD4+ T cells become infected through interactions with dendritic cell- associated HIV. Subsequently large numbers of virions are produced which leads to infected T cells and free virus in the peripheral circulatory system and in lymph tissue. This occurs approximately 4 to 11 days after infection (Mc Farland EJ, 2005). The levels of HIV-1 in circulation increase rapidly and viral levels reach a peak at 21 days after infection. As cells leave the blood and enter the tissues, the CD4+ T cell levels drop and reach a stable set point at approximately 6

months after infection. The appearance of cytolytic T-lymphocyte in peripheral blood temporarily correlates with the initial decline in plasma HIV-1 levels and may appear as early as 6 weeks after infection (McMichael *et al.*, 2001). Cell mediated responses as well as innate immune responses contribute to control of plasma HIV-1 levels.

Asymptomatic Phase: Within a few weeks a specific immune response to HIV is mounted and viral replication is greatly reduced. With a decrease in viral load the number of CD 4+ T cells is allowed to return to near normal levels. During this stage the viral load continues to increase slowly. This is accompanied with approximately 1 billion CD4+ T cells destroyed each day. (Perelson *et al.,* 1996).

Although viral loads tend to diminish rapidly after acute infection in adults, the decrease is slower in vertically infected children and may not reach baseline until they are aged 4-5 years (Frye, 2004). In comparison to adults, infants possess a high number of antigen presenting and effector cells. However, their cytokine production, proliferation and cytotoxicity are reduced.

Envelope-specific cytotoxic T lymphocytes are less common in children with vertically acquired HIV as compared to children who acquire the disease by means of blood transfusions. Eventually the CD 4+T lymphocytes drop below a level compatible with effective immune function leading to disease progression and death.

1.10 Viral Dynamics of HIV-1 Infection

Assays for the direct detection and quantification of HIV-1 RNA in plasma have made it possible to determine the viral dynamics of HIV-1 infection (Piatak *et al*, 1993; Ho *et al*, 1998). The average virion generation time was estimated to be 2.6 days. Plasma virions possess a half life of 6 hours and approximately 10 billion viral particles are produced in an untreated HIV infected individual each day (Ho *et al*, 1995; Wei *et al,* 1995). The tendency of the HIV enzyme reverse transcriptase to introduce mutations when it copies the genome and the rapid kinetics of HIV implies that the mutation rate is extraordinarily high (Palumbo P, 2005). It has been postulated that mutation at each end of the nucleic acid base within the HIV genome could be produced within an infected person each day. The findings suggest that ARV's should be able to completely suppress viral replication to avoid the rapid selection of drug – resistant mutant virus (Palumbo P, 2005). When HIV protease inhibitors were introduced the postulation was that short periods of ARV's would be sufficient to eradicate infection. Thereafter, it was demonstrated that in individuals in whom plasma virus was non-detectable for years remained positive for proviral DNA found within circulating mononuclear cells and for culturable virus (Wong *et al,* 1997; Chun *et al.*, 1997, Peraud *et al.*, 2000). These studies reported that CD4+ T lymphocytes were the hosts for persistent proviral genomes with low frequency (1 in 1 - 10 million cells). The calculated decay rates for this cellular reservoir are very slow with elimination time measured in decades (Palumbo P, 2005).

1.11 Assays for Viral RNA and Viral Loads

Assays for the quantitation of viral RNA in plasma include:

- Reverse transcription (RT) polymerase chain reaction (PCR) amplification (RT-PCR). Quantitation is made possible by the use of internal controls (quantitative competitive). The assay is widely used for quantitation of HIV 1 RNA.
- Branched DNA (bDNA) analysis is a technique which relies on a super-sensitive method to probe for the presence of viral RNA, using a highly branched and labeled DNA probe.
- Nucleic acid sequence- based amplification (NASBA) is a methodology involves the amplification of HIV RNA using a bacterial RNA polymerase, in combination with a reverse transcriptase.

1.12 Virus Replication Kinetics

The level of HIV RNA or "viral load" is an important assessment tool in diagnosis and management of HIV infected patients. Whilst CD4+ counts indicate the current immunosuppression status, the viral load indicates the rapidity with which the disease is likely to progress. Higher viral loads have been shown to be associated with more rapid rate of disease progression (HIV/AIDS Bureau, 2000). Accurate quantitation of the amount of virus in infected patients has revealed that much more virus is present then originally thought (www.micro.msb.le.ac.uk, 2004). Using these accurate PCR

quantitative methods it has been shown that replication of HIV occurs in all infected individuals at varying rates. It was initially reported that the average half life of an HIV particle or infected cell in vivo is 2.1 days. Recent reports have suggested:

- A faster turnover of plasma virus, corresponding to a half-life of 28-110 min.
- Up to 10^{9}-10^{10} HIV particles are produced each day
- An average of 2×10^{10} new CD4+ cells are produced each day

Thus, contrary to initial beliefs, the dynamic situation in HIV infected people involves continuous infection and destruction and replacement of CD4+ cells (Douek DC, 2003).

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1.13 Immunological Features of Human Immunodeficiency Virus Infection

Peripheral blood CD4 lymphocytes play a key role in initiating and promoting immune responses both on the cellular and humoral levels. A characteristic feature of HIV/AIDS is a marked depletion of peripheral blood CD4 lymphocytes. The exact mechanism of this phenomenon is as yet unclear but various suggestions include:

- a direct cytopathic effect of HIV
- syncytia or giant cell formation with subsequent cell death
- a form of autoimmunity against lymphocytes using either cytotoxic T cells or other anti-lymphocytic factors.

The major effect of HIV infection appears to be damage to the cell mediated immune response, which results in susceptibility to infections such as virus, fungi and/or protozoa.

1.14 Overview: Immune System

There are two major divisions in the immune system, namely innate inmmunity and adaptive immunity. Innate immunity comprises of all those protective mechanisms an individual is born with, such as mechanical barriers, body secretions, enzymes, complement and phagocytic cells. All these systems are non-specific. They interact with and neutralise a wide variety of foreign materials (antigens). No 'memory' is associated with these mechanisms (Roitt, 2003). Adaptive immunity is specific and shows the property of immunological memory. Due to this property of immunological 'memory', adaptive immunological responses are much stronger to antigenic stimulation. Adaptive immunity is subdivided into:

1. Humoral immune responses which involve the production of antibodies or immunoglobulins with specific emphasis on B lymphocytes.
2. Cell mediated immune responses are directed by the T lymphocytes against antigens.

Other participating cells include macrophages, monocytes and granulocytes (Roitt, 2003).

1.14.1 The Immune System in Relation to Cellular Immunity In Pregnancy

Pregnancy does not alter the number of T cells or the proportion of subsets of T cells in the peripheral blood. Selective suppression of cellular immunity may occur in response to soluble immunosuppressive factors, specific antigens or subtle local shifts in the subset population of T cells (Weinberg, 1984). According to Shridama *et al.* (1982) a decline in proportion of CD+4 during pregnancy may result in immunosupression. The number of Natural Killer (NK) cells in the circulation is apparently normal in pregnancy but Gregory *et al.* (1985) found the cytolytic activity of these cells to have decreased. However, overall evidence suggests that systemic T cell function is generally maintained during pregnancy. Although changes may not be measurable systemically, local regulation of immune events at the maternal-foetal interface is important. Factors of placental origin and molecules secreted by novel forms of maternal lymphocytes within the deciduas appear to inhibit the generation of cytotoxic T cells (Billington, 1992).

Evidence established from in vitro experiments indicates there is a down regulation of maternal immune responses to histocompatibility antigens during pregnancy. One mechanism mediating tolerance during pregnancy seems to be the expression of non-classic HLA-G molecules and complement regulating proteins (Deniz *et al.*, 1994).

According to Laumbacher and Wank in Key *et al.* (1998) identification of virus – derived, HLA – restricted 'pilot' peptides from individuals who are immune to a specific virus has potential for the development of vaccines against many viral diseases

1.14.2 Adult Humoral Response against Human Immunodeficiency Virus

Activation of innate immune cells as well as antibody and cytotoxic T lymphocyte (CTL) responses are induced by primary virus infection. Occurrence of CTL responses is within 7-10 days. This is followed by a decrease in CTL response approximately 2-3 weeks post- infection (Whitton *et al.*, 1996). Antibody responses usually peaks later than CTL (2-4 weeks) but detectable levels may be found for a lifetime (Whitton *et al.*, 1996). The cellular T cell response kills infected cells ensuring that the amount of virus released is minimised. This eases the burden to allow the antibodies to continue the immune response. The humoral antibody response reduces the load of extra cellular infection units. Thereby the number of infected cells that the T cells have to deal with is reduced. This symbiotic relationship within the immune system attempts to contain and control the number of viral particles to some degree. However, the ensuing low-grade persistent infection leads to gradual erosion of the host's immunological system capacity. The hallmark of HIV is a sustained decline in CD4+ T cells, which eventually progresses to irreversible immunodeficiency, the stage, recognised as AIDS.

1.14.3 Natural Killer Cells in Health and Disease

The immune system is a complex system comprised of numerous cells including blood lymphocytes, polymorphonuclear leucocytes, monocytes and various soluble chemical mediators such as growth factors and cytokines. Together with the primary and

secondary lymph organs a network of communication is set up to fight and protect the human body against pathogenic invasions and malignancies. NK cells are a relatively minor group of cells in the peripheral blood. The early realisation that NK cells and T cells are different from one and another in their recognition mechanism generated an interest in understanding the mechanism. The role of NK cells initially focused on their ability to kill tumour cells. However, NK cells have a broader role in the first line of defence against invading pathogens in early phases of host immune responses.

Natural Killer cells recognise specific MHC class I molecules through surface bound receptors but deliver signals that suppress NK cell activation (Kelly, 1999). Thus NK cells are activated by target cells that have lost MHC class I expression and its loss (for e.g. in cancer or virus infected cells) leads to lysis by NK cells. Unlike T cell and B cell amplified responses, prior exposure to pathogens or clonal expansion (*in vivo*) is not required for NK cell activity. Although, NK cells are not effective against all viruses (e.g., influenza and choriomeningitis virus), these cells have been shown to accumulate at sites of viral replication where they produce and release several cytokines (Gersuk *et al.*, 2002). The mechanism by which NK cells mediate their antiviral effects are not clearly understood but studies using mice have demonstrated that there is significant increase of NK cells in circulation after repeated exposure to viral antigens. This expansion is associated with NK cell mediated resistance to the viral infection. The predominant mechanism for this accumulation has not been elucidated but it raises the possibility that NK cell activity can be enhanced by immunization. Viral invasions or strong antigenic stimulus induce both the activation of NK cells and an increase in NK cell number. NK cell activity and absolute NK cell

counts are not the same findings (Gersuk *et al.*, 2002). Although, NK cells may be present in sufficient numbers in the circulation, unless they are activated, they will be ineffective in their job. Decreased NK cell activity has been linked to the development and progression of many diseases and is an important indicator for declining or improving health (Gresuk *et al.*, 2002).

Abnormal NK cell activity has also been reported in patients infected with HIV-1 (De Noon *et al*, 1996). It has been found that women who remain uninfected despite frequent sexual exposure to HIV had high numbers and percentage of NK cells (De Noon *et al.*, 1996).

Naturally occurring biological substances such as cytokines released by NK cells include gamma interferon (IFN-γ), tumour necrotic factor alpha (TNF-α) and the haemopoetic colony stimulating factors (CSFs) granulocyte- macrophage (GM)-CSF and interleukin–3 (IL-3). Studies have shown that IFN and IL-2 act as potent regulators of NK cells and can rapidly enhance the functional activity of NK cells leading to enhanced target killing (Gersuk *et al.*, 2002. IFN has been shown to affect NK cell cytotoxicity by the following mechanisms:

- Increasing the number of NK cells able to bind to their targets and the proportion of cytotoxic cells within the NK cell population.
- The kinetics of lysis is accelerated.
- Increasing the recycling ability of active NK cells.

Natural Killer cells clearly resemble T cells and are developed from a common progenitor cell. However, distinguishable features are apparent from other cells in the lymphocyte lineage. This lineage separation can be made according to differential expression of certain cell surface molecules and NK cells can still be defined by function and by exclusion. NK cells do not express surface immunoglobulin or the TCR-CD3 complex and generally do not require MHC class I expression on target for lysis (Gersuk *et al.*, 2002).

1.14.4 Natural Killer Cells in Antiviral Defence

Natural Killer cells are populations of lymphocytes that can be activated to mediate significant levels of cytotoxic activity in response to different infectious agents. They comprise about 6-21% of the total lymphocyte population (Gersuk *et al.*, 2002). These cells play an important role in the recognition and lysis of pathogen-infected or transformed cells and are important contributors to innate defence against viral infections. MHC class I- specific receptors expressed on cell surfaces partly regulates NK cells. Upon ligand engagement most of these receptors transduce an inhibitory signal. This explains the tendency of NK cells to attack class I deficient target cells while sparing normal cells. Inhibitory and stimulatory signals determine the outcome of NK-target cell interactions.

1.14.5 Identification of Natural Killer Cells

Natural Killer cells are identified by monoclonal antibodies that react with low affinity surface membrane receptor for the Fc portion of the immunoglobulin G (IgG) molecule. This molecule is also known as the FcR or CD16+ antigen. The CD16+-FcR binds to the IgG molecule, which is often found complexed with either soluble or insoluble antigen. The FcR does not bind monomeric IgG. In a population of peripheral blood mononuclear CD16+ remains a relatively good marker for NK cells.

CD56+ antibodies are also used to identify NK cells, usually in combination with CD16+. Antibodies to CD56+ recognise a cell surface protein on NK cells that is related to the neural cell adhesion molecule (NCAM). Various receptors for multiple cytokines that may influence NK cell functioning include IL-2, IL-12 and TNF-α. (Ryder *et al.*, 1989). The CD69 molecule is the earliest activated marker expressed on all NK, T and B cells following stimulation by a variety of mitogenic agents. However, very few CD69 expressing T, B and NK cells are found circulating in peripheral blood. The CD69 cell surface antigen is induced within 4 hours following stimulation by IL-2 and generally peaks within 18 hours following treatment. The presence of CD69 surface antigen reflects the cellular and biochemical events that are germane to the initial stages of immune activation and cell signaling. These properties suggest that CD69 represents a generic marker for lymphocyte activation and is well suited to rapid analysis of discrete subsets of responding cells. (Dunn *et al.*, 1995; Kuhn *et al.*, 1999).

1.14.6 Major Histocompatibility Complex

Major Histocompatibility Complex (MHC) molecules are found on the surface of all cells. T cell receptors recognise and bind to their corresponding antigen if it is bound to an MHC molecule. Unlike an antibody or T cell, MHC molecules are not specific. Their function is to present peptides derived from protein antigens to CD+8 and T cells (MHC class I) and CD4+ (MHC class II) (Coico *et.al.*2003).

There are two classes of MHC molecules, MHC class I and MHC class II (Fig. 3). Class I molecules display peptides from proteins synthesised within the cell. In class II molecules display peptides from phagocytised protein and are found on macrophages, dendritic cells and B cells. The basic structure of an MHC molecule comprises of two polypeptide chains. The first polypeptide is long and consists of:

- An intracellular domain
- A transmembrane domain
- Three extracellular domains

The second short polypeptide chain has one domain.

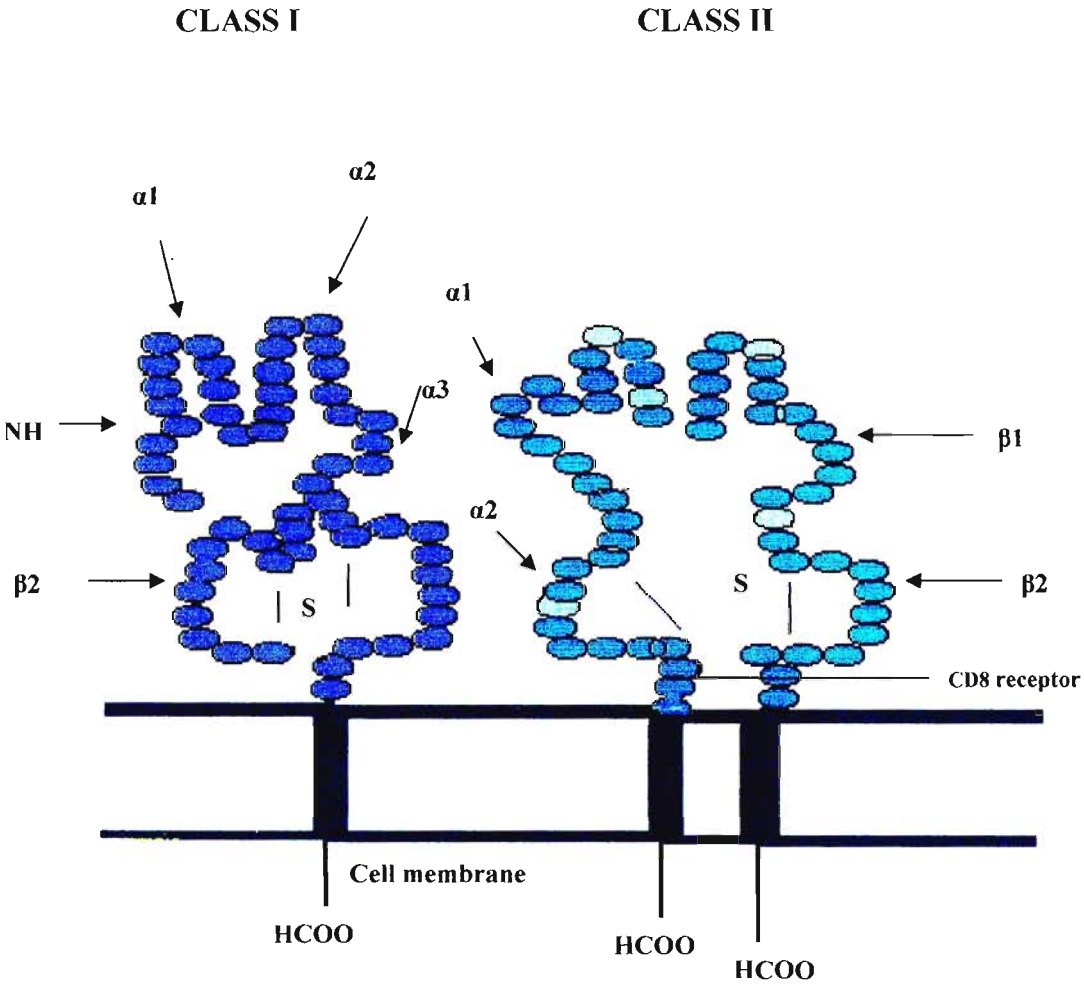


Figure 3: Structure of the Major Histocompatibility Class I and Class II Molecules

1.15 Human Leucocyte Antigen – G Expression in the Human Placenta

The structural uniqueness of the human placenta provides a separation between maternal and foetal tissues and circulation. Maternal immune system does not reject the developing foetus. Amongst the various factors implicated in foetal tolerance, a key factor has been attributed to HLA-G molecules, which are expressed by

trophoblastic cells. In contrast to HLA-A and –B class Ia genes that are down – regulated in human trophoblastic cells, HLA-G class Ib molecules are expressed in the placenta throughout gestation.

The nonclassical class I molecule, HLA-G has been described as a tolerogenic antigen from the MHC. The distinctive feature of HLA-G from the other nonclassical class I antigens is its quasi-monomorphism and restricted tissue distribution. HLA-G is expressed in healthy individuals in trophoblasts, amnion and thymic epithelial cells. The molecule has seven distinct protein isoforms; each encoded by a specific, alternatively spliced transcript. Four isoforms are membrane- bound proteins, HLA-G1,-G2, -G3 and G4. The other three isoforms are soluble proteins HLA-G5, -G6 and -G7 (Carosella *et al.*, 2001).

The general opinion is that the full length HLA-G1 isoform is expressed by extravillous cytotrophoblast cells. The source of soluble HLA-G5 isoform and expression of all other isoforms in the human placenta is still under debate (Dohr *et al.*, 1987). HLA-G has been described as the unique ligand of the killer cell inhibitory receptor KIR2DL4, which is expressed on all NK cells (Rajagopalan and Long, 1999). Furthermore, HLA-G:

- inhibits the transendothelial migration of NK cells.
- shifts the cytokine balance towards Th2 dominance.
- suppresses the proliferation of allogeneic CD4 T lymphocytes.
- in its soluble form induces the apoptosis of activated CD8 T cells.
- inhibits cytolysis by NK cells.

1.16 The Expression of HLA-G and Cytokines

The expression of HLA-G is up regulated by the anti-inflammatory cytokine IL-10 which conversely down- modulates the expression of HLA class I and II molecules. According to Moreau *et al.* (2001) the pro-inflammatory cytokines such as TNFα, IFN-γ or IL-1β enhances the expression of HLA class I molecules which led to the proposal that the expression of HLA-G, induced by glucocorticoids and other cytokines such as IFN-β, might also protect tissues from cytotoxic effector cells.

1.17 Role of HLA-G in Immune Response

Several explanations for the role of HLA-G in creating maternal-foetal tolerance have been considered. The limited polymorphism of HLA-G may allow the recognition tissues as "self" at the site of implantation, preventing activation of maternal alloreactivity and natural killer cells. Alternatively, HLA-G may induce specific immunoregulatory lymphocytes capable of down regulating maternal alloreactivity. Finally the presence of variants of HLA-G suggests that HLA-G may block interactions between T cell receptor polymorphic class I MHC molecules.

1.18 Human Immunodeficiency Virus Infection and Human Leucocyte Antigen

Immune responses to HIV involve manifold interactions and migration of immunoactive cells. According to Malkovsky (1996) the major histocompatibility complex (MHC) gene has a critical role in regulating antiviral immune reactions, it is possible that the MHC–coded molecules influence the course of HIV infection. Studies conducted by Mc Neil *et al.* (1996) reveal that A1-B8-DR3 haplotype is associated with fast progression of HIV disease and rapid loss of CD4+ T cells whilst the presence of the B27 antigen is associated with slow loss of CD4+ T cells and HIV disease progression.

1.19 The Human Placenta

The placenta is a very complex organ that is responsible for maintaining the foetus during pregnancy. Due to this complexity and the nature of the reproductive process there are a wide range of abnormalities and diseases that can create problems for the developing foetus. According to Butlin (2004) the placenta has three main roles:

- To enable the selective exchange of various substances such as nutrients and gases between mother and foetus and also protect the foetus from bacteria and toxic chemicals. The exchange requires a wide range of transport processes including passive diffusion, active transport, phagocytosis and pinocytosis.
- To produce a range of steroid and protein hormones that are thought to play a role in pregnancy and foetal development. Hormonal involvement occurs via

blocking of further ovarian follicular development and ovulation and uterine contractile responses to foreign bodies. Mobilisation of metabolic reserves such as fats within the maternal system and development of mammary growth. Continued secretions of nutrients by the endometrial glands occur (Wooding and Flint, 1985).

- To provide a barrier between the embryo and mother to stop the mother's immune system from attacking the foreign implanting foetus.

The placenta consists of tissues derived from both mother and embryo. The foetal part is derived from the embryonic membranes and the maternal part from the uterine wall. This creates the semiallogeneic environment for the foetus. This area of foetal-maternal apposition is enormously increased during pregnancy as the placenta develops by folding and refolding while it proliferates in parallel with the foetus in order to provide the foetus with nutrients for normal development and removal of waste products.

1.19.1 The Development and Structure of the Human Placenta

After conception the morula enters the uterus and develops into the blastocyst surrounded by a cell monolayer the trophoectoderm. There is no increase in size at this point but only an increase in cell numbers. A glycoprotein coat (zona pellucida) surrounds the oocyte and then the blastocyst. As the blastocyst begins to grow it either ruptures or is lysed by the uterine and /or embryonic proteases (Guillomot *et al.*, 1993). The blastocyst normally implants into the mid-line of the upper part of the posterior wall of the uterus. Implantation occurs in stages as follows:

- Change in structure of trophoblastic cells with a reduction in the number of apical microvilli. This allows a closer association between the trophoblastic cells and the uterus.
- Interdigitation of the uterine microvilli and the trophoblastic cell membrane occurs. The trophoblastic cells have highly invasive properties during implantation, which penetrates the uterine epithelium into the underlying stroma. As the penetration occurs the trophoblastic differentiates into two layers producing the cytotrophoblast and the syncytiotrophoblast (contains maternal and trophoblastic nuclei).
- Syncytiotrophoblast cells continue to proliferate and delineate extracytoplasmic cavities. The cavities increase in size producing a spongy layer and communicate with one another. The process results in the formation of lacunae lined with syncytiocytotrophoblast cells.

- Syncytiotrophoblast cells have lytic activity, which causes the rupture of both maternal arterial and venous blood vessels. The result is a flow of maternal blood from arteries into the lacunae spaces and back into the maternal system via the veins.
- Primary villi are formed by the trophoblastic cells (a core of cytotrophoblast cells with a syncytiotrophoblast covering) which, are then invaded by mesenchyme cells forming secondary villi.
- The vessels within the secondary villi will later join with vessels formed in the embryo thus creating the circulation needed to supply the embryo with the required substances and gases for development and removal of waste products. (Guillomot *et al.*1993, Junqueira *et al.*, 1989; Wooding and Flint, 1985).

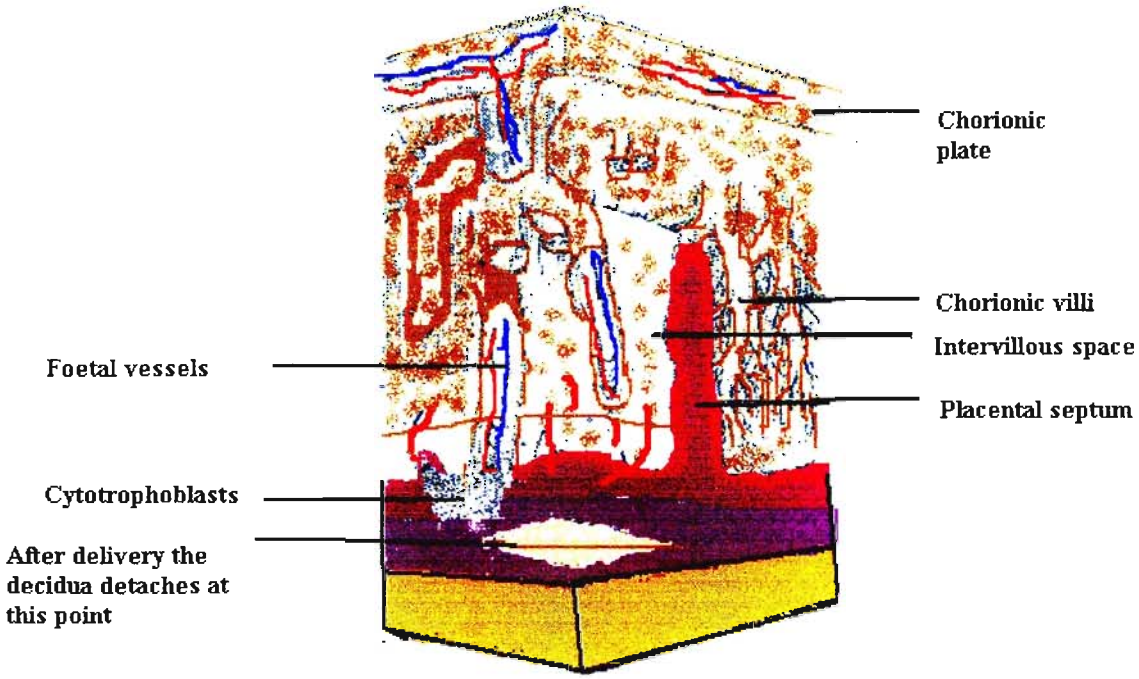


Figure 4: Internal Structure of the Human Placenta

Placental growth and development continues throughout pregnancy. At term the size of the organ is about 20cm in diameter, 3cm thick and 500g in mass.

Exchange area of the chrionic villi is large, reaching $14m^2$ at term. The length of the capillaries within the placenta is about 50 km. Maternal blood flow rate is between 500-700ml/minute of which 80% is destined to supply the placenta (Martal and Cedard, 1993). The final internal structure of the placenta is shown in Figure 4.

1.20 The Immune System in the Foetus and New Born

The intrauterine milieu offers the foetus protection from invasion by pathogens that have entered the maternal host. The placenta and foetal membranes act as a barrier against invading organisms. From birth the newborn is exposed to an array of pathogenic organisms in its new external environment. Therefore, the immune system must achieve competency to deal with infections during foetal and neonatal life. This is achieved partly through the acquisition of preformed maternal IgG antibodies and partially by the maturation of the foetal cellular immunity response.

1.21 Development of Foetal Immune Defense

When compared to adults the immune system of the infant is less efficient. Mucosal barriers are less effective with lower levels of immunoglobulin production. The net immunological status is that the infant is at a greater risk to systemic diseases from colonising organisms. In developing neonates a decrease in cytokine production by the lymphocytes is observed. Dendritic cells display a less effective response to antigen presentation. Therefore, the immature immune system affects the ability of the HIV infected neonate to control viral replication.

1.22 Innate Immunity in the Young

Throughout gestation and at term the epidermal cells increase in layers and thickness. At approximately 2 weeks of age IgA levels are detectable and continue to increase reaching adult levels by 6-8 years of age. The rate of increase is determined by the intensity of antigen exposure (Wilson *et al.*, 1996). The higher the IgA levels the less likely pathogens will adhere to the mucosal epithelium.

1.22.1 Complement Levels

The Complement Cascade is initiated at gestation (6-14 weeks) and progress to birth. According to Smith and Melvin (2005) the levels and activity of some portions of the complement cascade are comparable to adult levels. However, the C_8 and C_9 of the Alternate Pathway are at approximately 20% of adult levels with low C_3b levels (Wilson C, 1996).

1.22.2 Cellular Response

The human immune system developments from gut-associated tissue in early embryonic life. Undifferentiated haematopoetic stem cells (HSCs) first appear in the yolk sac at 4 weeks of gestation and migrate to foetal liver at 6 weeks. Differentiation occurs into various cell lineages (erythroid, megakaryotic, myeloid and lymphoid)

within the microenvironment of the foetus. The yolk sac and primary lymphoid organs such as the liver, spleen and bone marrow contain haemopoetic stem cells (HSCs). After birth the HSCs are found only in bone marrow.

Mature monocytes migrate from the bone marrow to the peripheral blood and circulate for 1-4 days before differentiating into tissue macrophages. Tissue macrophages have an extended half life from 60 days to many years (Smith and Melvin, 2005). The absolute number of monocytes is higher in neonates with a lower adult level reached by early childhood. Lymphokines released by activated T cells determine monocyte function. Neonatal monocytes elicit a lesser response to IFN-γ production by NK and T cells than adult monocytes (Johnson RB, 1998). The deficit is further augmented by reduced production of TNF-α, IL-8, IL-6 and G-CSF in neonatal monocytes and macrophages (Wilson C, 1996; Johnston RB, 1998). According to Bullock (1969) immature macrophages display decreased chemotaxis to sites of inflammation and infection.

Differentiation of neutrophils to granulocytes occurs in the bone marrow. Neutrophil development is dependent upon various cytokines and growth factors such as G-CSF. Detection of mature neutrophils can be as early as 14-16 weeks of gestation and is divided into marginated and circulatory components. Immediately after birth the peripheral circulation neutrophils increase but further expansion of the neutrophil storage pool is limited in neonates (Christensen and Rothstein, 1980). As in neonatal monocytes the chemotaxis of neutrophils to sites of infection is decreased.

The deficit is related to:

- decreased ability to adhere to vascular endothelium
- decreased cytokine production by monocytes
- deficiency to respond to chemotactic stimuli (Johnson RB, 1998).

1.22.3 Natural Killer and Dendritic Cells in Neonates

A significant portion of neonatal NK cells is found in the liver of neonates which can be detected as early as 6 weeks gestation (Smith and Melvin, 2005). The cytolytic activity of neonatal NK cells is approximately 50% of that of adults and only reaches adult levels at about 9-12 months of age.

Neonatal dendritic cells have a reduced ability to present antigens which may the contributing factor to reduced T and B neonatal cells response to antigens (Petty, 1998). Fewer MHC and ICAM-1 molecules are expressed by cord blood dendritic cells. When compared to adult dendritic cells the response to antigenic stimulation is less effective.

1.22.4 Cell Mediated Immune Response

Lymphoid stem cell development and maturation occurs at 8-10 weeks of gestation within the primary lymphoid organs, thymus and bone marrow. Thymus T cells undergo positive and negative selection processes. B cells developing mainly in the foetal liver and bone marrow are subjected to a selection process at the site of their generation. Mature lymphocytes migrate to secondary lymphoid tissue (membrane associated lymphoid tissue) where they respond to antigens. Oligoclonal B cell proliferation, antibody class switching, affinity maturation and the development of immunological memory occur at the site of germinal centres.

Activation of T cells by antigens from antigen presenting cells can occur as early as the 12 week of gestation. Foetal thymocytes (immature T cells) later segregate to form subsets of CD 4/CD8 cells.

The number of circulating T cells increase from mid-gestation until approximately 6 months of age (Smith and Melvin, 2005). The median CD4+ cell count at 6 months is approximately 3000 cells/mm^3 with a gradual decline to 1000 cells/mm^3 by late childhood. The CD4+ to CD8+T cell ratio reaches the adult ratio of 2:1 at approximately 4 years of age (Denny *et al.*, 1992).

1.22.5 Humoral Immune Response

Although the functional capacity of the humoral arm of the immune system is limited the foetus can elicit an immune response by 6-7 months of gestation. B cell maturation within the bone marrow continues throughout life and B cells with non-functional immunoglobulins are continually eliminated (Parslow T, 1994). Neonatal B cells differentiate into plasma cells that secrete IgM and with the help of T cells can effectively switch to produce IgA and IgG (Banchereau *et al.*, 1991).

1.23 Placental Role in Foetal Human Immunodeficiency Virus Infection

Anti-HIV therapies have reduced HIV-1 transmission from mother to foetus (Moodley *et al.*, 2003). However, total eradication of the infection of the newborn has not been achieved using ART. The question still remains as to the reason why 25-35% of HIV positive mothers have babies that are infected (Ehrnst *et al.*, 1991; Kapoor *et al.*, 2004). Growing evidence directs attention to the role of the placenta in controlling the infection of the foetus in utero. Miller *et al.* (2000) states that there are four control points in the placenta that can modulate the infection of the foetus besides the gross rupture of membranes leading to direct infection of the foetus:

- Viral entry
- Infection (DNA)
- Production (RNA)
- Viral transit to the foetal circulation.

Emphasis has been placed on understanding HIV-1 entry, the role of viral strain and chemokine receptor interactions in the maternal-foetal system. However, recent in utero and in vitro investigations have focused on understanding the control of both viral infection and production within the placenta. Both autocrine and paracrine controls of these processes involving various cytokines and placental proteins. Therefore, the human placenta acts as the guardian of the embryo/foetus to viral onslaught and infection and is important as a principal defence mechanism during development.

CHAPTER TWO

CHAPTER 2

AIMS AND OBJECTIVES

2.1 Background Statement

An important feature of the immune system is its ability to discriminate self and non-self antigens. However, there are few regions in the body, which have the capacity to accept foreign grafts. In the past the uterus was thought of as a privileged immune site. Preliminary data demonstrate the presence of apoptic leukocytes at the maternal-foetal interface and the expression of placental trophoblast support the notion that peripheral tolerance to alloantigens allows the foetus to escape rejection by the maternal immune system.

2.2 The Pregnant Uterus as an Immune Privilege Site

During the implantation period of the foetus in the maternal deciduas, the foetus is in direct contact with the maternal tissue of the pregnant uterus. However, foetal rejection by the maternal immune system in the majority of cases is prevented by mechanism(s) yet undefined. One of the mechanisms proposed is the suppression of the maternal immune system during pregnancy. This theory postulates the existence of non-specific immunosuppression during pregnancy and a wide variety of materials in human serum have been found to have profound in vitro immunosuppressive activities.

It has been reported that extravillous cytotrophoblast expresses a class truncated antigen, the HLA-G molecule. This expression is restricted to the placenta and it has been shown to induce resistance to Natural Killer cell media. This suggests that HLA-G might confuse, trap, or diffuse the T-cell response complex. Although, the lack of MCH expression suggests that the pre- implantation embryo is protected from direct immunological attack by the restricted T-cell, the pre-implantation embryo could still be vulnerable to delayed type hypersensitivity (DTH) reaction, as well as to the adversity of the antibodies and cytokines by non-MHC restricted effector cells.

It has been noted that only about 25% of infants born to HIV infected mothers are persistently infected with HIV and progress to AIDS (Blanche *et al.*, 1994). Of the remaining 75% many are uninfected. However investigations of foetuses of HIV positive mothers who electively aborted in early pregnancy have demonstrated the presence of HIV DNA sequences in cells from various organs. It would therefore appear that the majority of infected foetuses are able to overcome and clear the infection. If the immune system of the foetus is immature and ineffective then there is a possibility of an alternative mechanism which helps in clearing the infection. In exploring this mechanism one needs to look at the implication of HLA-G. This nonclassical class I HLA-G gene is primarily expressed in foetal tissue that comes into direct contact with maternal tissue in the pregnant uterus. Natural Killer cells have an affinity for viruses. If HLA-G acts as a control mechanism against NK cell activity then the possibility that decreased NK cell activity at the maternal foetal barrier may result in a decrease in foetal protection against HIV. What needs to be investigated is whether there is a relationship

between the level of NK cells and the concentration of HLA-G at the placental barrier in HIV positive pregnant women.

2.3 Aim of the Study

This study is undertaken to establish a correlation between the level of expression of HLA –G and the presence of NK cells at the placental barrier in HIV positive pregnant women and the significance, if any, of the interaction in influencing vertical transmission .

2.4 Specific Objectives

- To perform viral load tests on plasma and CD4+ cell counts on the blood of pregnant mothers and their babies at birth.
- Establish the presence and level of Natural Killer cells and p24 antigen in placental tissue.
- Determine the level of HLA-G expressed in placental tissue of HIV-1 infected mothers
- To correlate the level of placental HLA-G to placental NK cells and maternal viral load in *in utero* infected and uninfected babies.

CHAPTER THREE

CHAPTER 3

METHODS

SECTION A

3.1 Patient Population

3.1.1 Region of Study

The study was carried out at a Regional Hospital situated in KwaZulu-Natal, South Africa. The hospital services both the local urban and surrounding rural areas. It also acts a regional hospital to the smaller outlying hospitals as well as Primary Health Care clinics within the DC29 area. Approval for the study was obtained from the Ethics Committee, University of KwaZulu-Natal as well as the Hospital Manager.

3.1.2 Patient Recruitment and Selection

The patients attending the antenatal clinic (ANC) for general monthly antenatal examination were recruited into this study between June 2003 and December 2003. Patients were screened, with consent, for the presence of HIV antibodies (Determine, Abbott, USA). Samples of peripheral blood were obtained from the women at 28 weeks of pregnancy by the attending gynaecologist and sent to the laboratory for HIV testing. The HIV status of the patients were established in a routine diagnostic laboratory. Prior to testing, pre- counseling was performed by a

HIV counselor based at the hospital's antenatal clinic. All patients who tested HIV positive at the initial screen were post counseled and signed consent was obtained before inclusion into the study (Appendix I). Eligible participants were women aged 18 years and older and who had confirmed HIV-1 infection.

The prevention of mother-to-child-transmission (PMTCT) program was in the early stages of implementation in KwaZulu-Natal. Therefore, nevirapine was not available to the study population at this point of the study. Subsequently, during the sample collection process nevirapine was made available to HIV-1 positive pregnant women before delivery. In order to have a sample population who were ARV naïve, sample collection ceased. At this point in the study a total of 70 samples were collected from mother-baby pairs for laboratory analysis. However, some samples were not suitable for laboratory analysis. Therefore, the final analysis was undertaken using complete data from 55 mother-baby pairs.

Patients were divided into two groups post delivery:

- HIV positive mothers whose babies had a viral load of >400 copies at birth.
- HIV positive mothers whose babies had a viral load of <400 copies at birth.

Since the timing of HIV infection could not be ascertained the working definition of *in utero* transmission proposed by Bryson *et al.* (1992) was used. Bryson proposed that *in utero* transmission be defined as viral detection < 48 hours. For the purpose of this study all babies who had a viral load of >400 copies/ml at birth were considered infected *in utero.* Those babies with viral RNA copies < 400 copies/ml were

considered uninfected pending further viral load investigations. The rate of MTCT was based on the HIV test result on infants samples collected at birth.

3.2 Collection of Blood and Placental Samples

Blood samples were obtained from mothers at delivery and from babies immediately after birth. All patient blood samples met the following criteria. Approximately 2-5 mls of blood was collected in 2 EDTA vacutainer tubes from the mothers. Smaller volumes of blood samples were collected from babies (±1ml) in 2 separate EDTA tubes. The specimens were processed for CD4+ counts within 48 hours of collection. Samples were transported from the site of collection at room temperature (20-25°C) to the laboratory for testing. Adequate transportation safety measures were taken during transport of samples.

Fifty five placental samples were collected post delivery from the same mothers in whom blood samples were taken. Approximately $5cm^3$ of placental tissue was collected from two sides of the placenta to give an adequate representation of placental cells. Dual sampling of placentas yielded a total of 110 sampling blocks. The placental specimens were immediately placed into 10% formal saline used as preservative. Placental tissue samples were thereafter transported on ice, to the laboratory for testing.

3.3 Transport and Storage of Specimens

All blood specimens were transported as per requirement for CD4+cell counts (Appendix II B). On arrival at the laboratory, specimens for viral loads were immediately centrifuged, plasma removed into sterile, labeled test tubes and frozen at –70°C for batch testing. Placental samples were cut into portions processed and embedded in wax (Appendix III). The paraffin blocks were carefully handled and sectioned in order to avoid cross-contamination. Sections were prepared from the wax embedded samples and placed onto Poly-L-Lysin slides (Sigma,USA) for immunohistochemistry staining.

3.4 Laboratory Methods

3.4.1 CD 4+ Cell Counts

CD4+ cell counts were performed on unlysed peripheral whole blood samples obtained from all mothers and their babies immediately after delivery. The automated FACS Count System instrument and reagent kit (Becton Dickinson, USA) was used to calculate the absolute cell counts (Appendix IVA). Since the babies in the study were neonates the CDC reference range for under 1year was used (Appendix VIII). The normal ranges for CD4+ cell counts were taken as:

Male/Female:	CD4+ 600 - 1500 cells/mm^3
Babies (<1year):	CD4+ $\geq$ 1500 cells/mm^3 ($\geq$25%)

3.4.2 Viral Load

All samples were tested for number of viral RNA copies. Viral load was performed according to manufacturer's protocol (Roché Amplicor Version 1.5, Germany) (Appendix IVB). Roché Amplicor Version 1.5 was used because it displayed a specificity of 100% (Murphy *et al.*, 2000). Additional factors which were taken into consideration were cost of assay, limit detection and volume of sample required for analysis (Appendix IV C). The volume of sample required for the assay was crucial in this study because the study population included neonates. Only small volumes of blood were available from the babies at birth. A sub-sample of uninfected babies were tested using the ultrasensitive technique (Roché Amplicor Version 1.5- usCA MONITOR 1.5). The sub-samples had undetectable levels of viral RNA copies (<50 copies/ml). By extrapolation any sample <400 copies/ml was considered negative.

Controls were set up as follows:

- Negative controls: normal human sera free from hepatitis B and HIV.
- Positive controls: heat inactivated human sera free from hepatitis B but with antibodies to HIV-1 and HIV- 2

3.4.2.1 Interpretation of Results

i) Negative Control: Mean absorbance $_{450nm}$ should be less than 0.250

If the HIV-1 negative control does not meet this criterion, the entire run is invalid. The entire process i.e. specimen and control preparation, reverse transcription, amplification and detection) must be repeated.

ii) Positive Control: Mean absorbance $_{450nm}$ should be 0.400 above the mean of the negative control.

The positive controls (Low and High) are assigned ranges which are specific for each lot of control which, is provided with each batch of HIV-1 Monitor Test (Roché Amplicor Version 1.5). If the HIV-1 positive controls do not meet the criterion, the entire run is invalid. The entire process i.e. specimen and control preparation, reverse transcription, amplification and detection) must be repeated.

If the above two criteria were not met, the test run was considered invalid.

iii) Calculation of Results

Negative Control: Calculate the mean = 0.199

Cut off: 0.2 + negative mean = 0.2 + 0.199

= 0.399

Non Reactive (Negative samples) < 0.399

Reactive (Positive Samples) ≥ 0.399

All samples, which were equal to the cut off values, were repeated.

3.4.2.2 Validity of Test

A test run was considered valid when the positive control mean minus the negative control mean was > 0.400.

3.4.2.3 Limitations of the Viral Load Procedure

Test has been validated for human plasma collected in EDTA anticoagulants. The limit of detection is 400 copies/ml with a linear range of 400 ($Log_{10} = 2.60$) to 750 000 ($Log_{10} = 5.87$) copies/ml. Samples with results greater 750 000 HIV-1 RNA copies/ml can be diluted with HIV negative human plasma and retested.

SECTION B

3.4.3 Immunohistochemistry Staining for Natural Killer Cells (CD56+)

Duplicate sections of all paraffin wax embedded tissue, 4-5 μm thick were placed onto Poly-L-Lysin coated slides (Sigma, USA) and incubated overnight at 40 °C. After dehydration the slides were washed with distilled water 3 times for 2 minutes each, placed in citrate buffer (pH=6; 1l dist water; 2.1g citric acid, 15 ml NaOH-Appendix VA)) and antigen retrieval was performed as follows:

Samples were treated in a microwave oven at 750 Watts for 5 minutes (twice). After cooling for 20 minutes at room temperature the sections were washed in PBS. To remove endogenous peroxidase activity, the sections were kept in 3% hydrogen peroxide (DAKO, Glostrup, Denmark) for 30 minutes and thereafter washed three times in PBS. Sections were incubated for 60 minutes with primary antibodies to CD56+, a marker for uterine NK cells (Zymed, USA, Clone 123C3) diluted 1:300. This was followed by sequential 30 minutes incubations with a biotinylated link antibody and peroxidase labeled streptavidin. The sections were counterstained with Mayer's Haematoxylin and mounted with DPX. Positive controls using commercially available CD56+ tissue slides were included in each staining batch. Normal mouse immunoglobulin (IgG) control antibodies at the same concentration as the primary antibodies provided in the kit as per manufacturer's protocol was used as negative controls. Two sections from uninfected placentas were also stained with each batch run. This was done to assess the presence of CD56+ cells in uninfected placental tissue.

3.4.4 Immunohistochemistry Staining for p24 Antigen in Placental Tissue

Standard immunohistochemical procedures were employed. Duplicate sections of all specimens were immunolabeled. The slides were washed in Tris buffered saline (TBS) and blocked with 3 % bovine serum albumin in TBS for 15 minutes. The specimens were incubated with monoclonal mouse Anti-Human Immunodeficiency Virus, p24 (DAKO, Denmark, Code No. M0857) at a dilution of 1:10 for 20 minutes. After 3 washes in TBS the slides were incubated with secondary biotinylated rabbit anti-mouse antibody (DAKO, Denmark, Code No.M0857) at a dilution of 1:200 for 30 minutes. The slides were then washed in TBS and incubated with avidin-biotin complex (ABC) (1:500) for 30 minutes. The staining procedure was performed according to manufacturer's protocol (Appendix V B). Commercially available positive p24 tissue slides were used as positive controls. Negative controls were performed by omission of the primary antibody. Instead normal mouse immunoglobulin (IGg) control antibodies were used at the same concentration as the primary antibodies. Placental sections from HIV negative women were also stained. All positive cases showed more than one immuno-stained cell.

3.4.5 Haematoxylin and Eosin Staining Method

Tissue sections mounted onto Poly-L-Lysin coated slides (Sigma, USA) were placed in water. Thereafter, the sections were dewaxed in xylene and transferred to alcohol. The slides were washed in water and stained in Haematoxylin for 5 minutes. The stained slides were washed in running water and blued in lithium carbonate. Counter stain of eosin was applied to all sections for 1 minute. Sections were dehydrated,

cleared and mounted in DPX (mounting medium). The nuclei stained blue and the cytoplasm stained pinkish red.

SECTION C

3.4.6 Ribose Nucleic Acid Extraction from Placental Tissue

Samples were homogenized using a modified Trizol method (Perou *et al.*, 1999) followed by a total RNA extraction using the Arum protocol (BIORAD, USA. Cat. No. 732-6820).

To increase the yield of RNA from placental tissue a modified RNA extraction protocol was used. Placental tissue was cut in 100 mg portions and homogenized in 1 ml Trizol reagent. The homogenate was placed into a cryoshredder tube and spun at 12 000 rpm for 2 minutes at 4°C. The tube was removed from the centrifuge and left to stand for 10 minutes at room temperature. This was followed by centrifugation for 10 minutes at 12 000 rpm at 4°C. After centrifugation 200µl of chloroform/1ml Trizol was added to the tube and shaken vigorously for 30 seconds. This was followed by a standing time of 10 minutes at room temperature. Thereafter, the sample was spun for 15 minutes at 12 000 (4°C). The chloroform aqueous layer was removed and added to equal volumes of 60% ethanol in DEPC water. RNA binding column was inserted into a 2ml capless tube. The lysate was transferred into the column and centrifuged for 60 seconds. After centrifugation the filtrate was discarded. Low stringency wash solution (700µl) was added to the

tube and spun for 60 seconds. The filtrate was discarded; 80µl of DNase 1 (1:15 dilution) was added to the tube and incubated at room temperature for 25 minutes. The column was centrifuged for 30 seconds and the filtrate discarded. High stringency wash (700µl) was added to the tube and centrifuged for a further 30 seconds. The filtrate was discarded and 700µl of low stringency wash was added to the binding column. A further 30 seconds centrifugation followed. The filtrate was again discarded and the tube was subjected to an additional 1 minute spin. The RNA binding column was placed into a 1.5ml capped tube and 80µl (70°C) elution solution was placed onto the membrane stack. The tube was incubated for 3 minutes and centrifuged for 2 minutes to elute. The concentration of the total RNA was measured at absorbance 260nm.

3.4.6.1 Determination of Ribose Nucleic Acid Concentration

The concentration of RNA was determined by Nanodrop (ND1000 spectrophotometer). The absorbance was taken at 260 nm and 280 nm and a factor was used (40RNA). Purity of all RNA samples was determined by looking at the 260/280 ratio and a value of 2 was considered acceptable. Sample value <2 was not considered.

3.4.6.2 Confirmation of Ribose Nucleic Acid (RNA) Using Gel Electrophoresis

Confirmation for the presence of RNA was performed on 2% agarose gel using electrophoresis. A 2% w/v agarose gel was prepared by adding 1.4g of agarose to 70ml of Tris-Borate/EDTA (TBE) buffer (1X). The agarose mixture was heated in a microwave oven until the agarose granules had completely dissolved. The gel was cooled to approximately 60°C and 3.5µl of ethidium bromide was added (Appendix VI). The gel mixture was poured into casting trays with the comb inserted into the gel at 10mm from one end of the tray. Polymerisation of gel was achieved at 30-40 minutes at room temperature prior to use. Once the gel polymerized, the comb was removed and the gel was placed in a gel tank containing 1X TBE buffer (TBE must cover the gel). The PCR product (10µl) was prepared by adding 2µl of bromophenol blue loading dye (0.25% and 40% (w/v sucrose). The solution was loaded into an appropriate well in the gel. The DNA molecular marker (Fermentas # SMO373, GeneRuler 50 base pairs (-bp) DNA Ladder- Appendix VIII 3) and 2µl of loading dye and loaded into the well in the

gel. The gel was subjected to electrophoresis for 60 minutes at 80 volts (V). The gel was viewed using the Chemi-Doc XRS UV-transmilluminator (BIORAD, USA) and photographed. RNA was stored at -70 °C for preparation of cDNA.

3.4.7 Complementary Deoxyribose Nucleic Acid (cDNA) Synthesis using Reverse Transcriptase Polymerase Chain Reaction

The BIORAD iScript cDNA (Cat.No.1708891) synthesis kit was used to produce cDNA from the RNA sample. The procedure was performed according to manufacturer's protocol. Complementary Deoxyribose Nucleic Acid was synthesized using 1µg of extracted tissue RNA. The reaction mixture was constituted using the following:

4µl of 5x iScript reaction mix, 1µl of iScript reverse transcriptase, 8µl of nuclease free water and 7µl of RNA template. All the reagents were added to a sterile PCR tube placed on ice. The reaction mixture was incubated for:

- 25 °C for 5 minutes.
- 42 °C for 30 minutes.
- 85 °C for 5 minutes.

The hold cycle was at 4°C for 45 minutes. The reaction was performed in the GeneAmp 9700 PCR system (Applied Biosystems, California, USA)

3.4.7.1 Data Reliability

Reliability of the Real Time - PCR experiment can be improved by including an invariant endogenous control (housekeeping gene) in the assay. This procedure allows for correction of sample to sample variation in Real Time -PCR efficiency and errors in sample quantitation. The house keeping gene used in this assay was GAPDH (glyceraldehydes-3-phosphodehydrogenase).

3.4.7.2 Primers

Quantification of the HLA-G gene was performed using forward and reverse primers. The primers, manufactured by Inqaba (SA) were designed to amplify and detect a 98-bp segment of the non classical class I HLA-G gene. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH was used as internal control (house keeping gene) to normalize data.

Table II: Forward and Reverse Primers of HLA-G and GAPDH Genes

Gene	Primers	Amplicon Size
HLA-G: **Forward (F)**	5'- CTGGTTGTCCTTGCAGCTGTAG - 3'	98- bp
Reverse (R)	5'- CCTTTTCAATCTGAGCTCTTCTTTCT- 3'	
GAPDH: **Forward (F)**	5'- AAGGTCGGAGTCAACGGATT- 3'	257-bp
Reverse (R)	5' - CTCCTGGAAGATGGTGATGG - 3'	

3.4.8 Real Time Polymerase Chain Reaction for Human Leucocyte Antigen-G Quantification

Real Time-Polymerase Chain Reaction (RT- PCR) assays used for quantitative RT-PCR combine accuracy, precision and the capability of high throughputs in the evaluation of available tests.

Amplification is extremely reproducible in the exponential phase. Therefore, for the sake of accuracy and precision, it is an ideal point in which to collect quantitative data. An analysis of reactions during the exponential phase at a given cycle number should theoretically provide several orders of magnitude of dynamic range (http:// www.ambion.com, 2005). In practice, a dynamic range of 2-3 logs can be quantitated during end-point relative to RT-PCR. Therefore, in order to extend this range, replicate reactions may be performed for a greater or lesser number of cycles, so that all of the samples can be analysed in the exponential phase (htpp://www.ambion.com, 2005)

Real Time-PCR automates this process by quantitating reaction products for each sample in every cycle. It has a 107 fold dynamic range, which requires no user intervention or replicates. Data analysis is performed automatically which includes a standard curve generation and copy number calculation.

A standard curve is generated from a dilution series constructed from a reference sample which can be a single RNA sample, pooled RNA, genomic DNA, cDNA or cloned DNA. The units used to describe the dilution series are relative values

based on the dilution factor. It can be expressed in dilution folds (e.g. 1 fold, 10 fold, 1000 folds etc.) or expressed as equivalent mass amounts (e.g. 100ng, 10ng, 1 ng etc.)

3.4.8.1 Optimised Experimental Protocol

A series of experiments were carried out to construct a LightCycler protocol for Real-Time HLA-G quantification. HLA-G quantification was based on a standard curve of known concentration. Karge *et al* (1998) suggested the use of a house-keeping gene (internal control) against which the unknown gene can be normalized). This study used GAPDH as the internal control. The controls were run without reverse transcriptase and PCR grade water. This was performed to ensure that there was no amplification of any contaminating DNA. Normalisation was performed using the equation HLA-G value/GAPDH value. The starting material was 1µg of cDNA for both HLA-G and GAPDH genes.

Following optimisation the PCR procedure was performed as described for a 20µl of standard reaction. Capillaries were handled with gloves. The required number of LightCycler capillaries was placed in pre-cooled centrifuge adapters

PCR mix for one 20µl reaction was prepared in a 1.5ml reaction tube held on ice. The following components were added to the capillaries in the order as mentioned. Water (PCR grade) 5.8µl, $MgCl_2$ 1.2µl, PCR primers forward and reverse 0.5µl (0.5 µM) each, 1.0µl of Fast Start SYBR GREEN I (Roche Diagnostics, Germany) and

the LightCycler Master mix of 9.0µl. Finally, 2.5µl of cDNA (reverse transcribed from 4ng of total RNA) was added to give a total volume of 20.5 µl. The PCR mix was prepared for the required number of reactions by using the volume for one reaction and multiplying it by the number of reactions. The contents of each tube were mixed by pipetting up and down.

Each capillary was sealed with a stopper. The adapters containing the capillaries were placed in a balanced arrangement into a standard microcentrifuge. The samples were spun at 3000 rpm for 5 seconds. Thereafter, the capillaries were transferred into the sample carousel of the LightCycler Instrument and cycled.

One cycle (95 °C for 10 minutes) followed by 40 cycles of (95 °C for 10 seconds, 60 °C for 50 seconds, 72°C for 16 seconds and 85°C for 5 seconds with a single fluorescence measurement). All temperature transition rates were programmed at 20°C/s. After amplification was complete a final melting curve was recorded at 95°C for 30 seconds and 80°C for 15 seconds (20°C/s). Slow heating of the sample was accomplished at 95°C with a ramp rate of 0.1C/s. Fluorescence was measured continuously during the slow temperature ramp to monitor the dissociation of the SYBR Green I. This was followed by a final cooling step to 40°C (Appendix VII). Melting curves were converted to melting peaks by plotting the negative derivative of the fluorescent channel with respect to temperature against temperature [-d (F1)/dT vs T). Excluding preparation time the entire LightCycler process took 2 hours. Melting point (T_m) calculations were performed using the Roche Molecular Biochemical LightCycler Relative Quantification Software, Version 3.5.

To improve the SYBR Green quantification, high temperature fluorescence measurement at 85°C for HLA-G was performed. This step eliminates non-specific PCR products such as primer dimers below the chosen temperature. Also, non-specific fluorescence signals are eliminated thus ensuring accurate quantification of the HLA-G and GAPDH real-time products. It was necessary for the mathematical model (the "Second Derivative Method") to determine the crossing points (CP) for each transcript.

3.4.8.2 Verification of Polymerase Chain Reaction Products

The fragment size of the PCR product obtained from real time-PCR was verified by electrophoresis. Product separation was achieved using the technique described by Maniatis *et al.* (1989). The polymerized agarose gel supported by the casting tray was placed into the electrophoresis tank. A 1x concentration of TBE buffer was poured into the chamber to achieve a level of approximately 1.5 cm above the gel tray. Prepared PCR product (Appendix VI-2D) and a known molecular weight marker (Fermentas# SMO373, GeneRuler 50-bp DNA Ladder) were loaded into the wells. Electrophoresis was performed at 100V for 60 minutes at room temperature.

Visualisation of the separation bands on the agarose gel was performed using the Chemi-Doc XRS UV-transilluminator (BIORAD, USA) capture of image and product size was completed with the aid of Quantity One software (BIORAD, USA). The product size was compared to the molecular weight marker and the fragment size calculated.

3.4.8.3 Standard Curve Construction

A standard curve was constructed using GAPDH. The absorbance was measured at 260 nm after the melt curve and fragment size analysis. A 1:20 dilution was performed on the real-time PCR product in DEPC water and the absorbance was measured at 260nm (GeneQuant) and the concentration of double stranded DNA was recorded (Appendix VI - 4).

The values used to extrapolate the standard curve for HLA-G and GAPDH were calculated using the number of molecules corresponding to the initial standard. The equations were as follows:

- Concentration of double stranded DNA (GM) = [absorbance at 260 nm] x [nanodrop concentration μg/ml] x [dilution factor]
- Average molecular weight (MW) of a DNA base pair: 660 (Da), mm or MW of dsDNA= [number of base pairs] x [660Da]
- Number of moles (n) = Concentration of double stranded DNA (GM)\Average molecular weight (MW) of dsDNA (mm).
- Number of moles = number of moles (n) x [6.022 x 10^{23}] (Avogadro's number)

The calculations were performed using the following values obtained from the quotation procedure:

Absorbance $_{260}$ = 0.787

Concentration = 983.8 μg/ml

Dilution factor is 20

Volume used in Gene Quant = 0.1 ml

Ratio (purity) = 1.948 (1.8 to 2.0)

Ds = [1.123]

Concentration of double stranded (ds) DNA:

Ds [ ] = (0.787) x 983.8μg/ml x (20) x (0.1ml)

= 1548.5012 μg

Molecular weight = 98 bp x 660mm

= 64680

Number of moles (n) = Gm/mm

1548.5012 μg /64680 mm

= 0.0239 μg/mm

Number of moles (Avogadro's number) = 0.0239 x 6.022 x10^{23}

= 1.44 x 10^{23}

A standard curve was generated for HLA-G and GAPDH with 10 fold serial dilution of the DNA standards (Real-Time PCR product) ranging from 10^{20} to 10^{11} copies per PCR reaction. Seven dilutions were chosen 10^{-3}, 10^{-4}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-10}, 10^{-12} (Appendix VI-4). Quantitative analysis of data was done using the LightCycler analysis software (Version 3.5, Roché Diagnostics). Melting curve analysis was used to determine the presence of non-specific amplification products.

After construction of the standard curve unknown samples were run with a calibrator, to ensure that the PCR conditions were controlled. The standard curve was imported at the end of the experiment to calculate the sample concentration.

3.4.8.4 Reproducibility of the Human Leukocyte Antigen-G(HLA-G) Quantification Assay

The reproducibility of the assays were assessed by performing triplicate PCR amplified reactions of a representative sample in the LightCycler and calculating the inter- sample variation.

3.5 Statistical Methods

SPSS statistical software was used for all descriptive analyses in the study. Some variables were unequally distributed therefore; non parametric tests were used. Correlations were assessed using Pearson's correlation test. The Chi Square test was used for assessing probabilities between variables and categories.

SECTION A

CHAPTER FOUR

CHAPTER 4

MATERNAL VIRAEMIA AND MOTHER TO CHILD TRANSMISSION OF HIV/AIDS

4.1 Summary

Background: Mother–to-child transmission of HIV can occur perinatally and postnatally. Mechanisms of antepartum and intrapartum transmission of HIV include transfer of the virus via maternal blood and transplacental exposure. Understanding the immunological dynamics of HIV-1 infection and their association with *in utero* HIV-1 infection in infants can help identify risk factors in vertical transmission.

Methods: This is a cross sectional cohort study. Investigations in this area of the study addressed the relationship between the viral load and CD4+ cell counts of HIV-1 infected mothers and their babies at birth. Peripheral blood samples were obtained from fifty five mothers at delivery and their babies immediately after birth. Quantitation of HIV-1 RNA (Roché Amplicor Version 1.5, Germany) were performed on plasma and CD4+ cell counts blood samples obtained from all mother- baby pairs in the study.

Results: Viral load analyses from each mother-baby pair revealed that 27.3% of babies had viral copies >400 copies/ml and were presumed infected *in utero*. The

remaining 72.7% had undetectable levels of viral RNA (<400 copies/ml) at birth. The pooled rate of transmission was 0.27(95%CI 0.15-0.39). In addition CD4+ lymphocytes defined the immunological status of both mothers and babies at birth. Separate sets of observations demonstrated that high levels of viral RNA copies associated with low CD4+ cell counts in mothers placed them within AIDS defining conditions. Infection status of the infants was classified using the CDC HIV Paediatric Classification (1994) criteria.

Conclusion: The findings suggested that the CD4+cell mediated immune response was low in approximately one third of the babies who had high viral loads. Maternal viral load increased the risk of viral transmission to the foetus.

4.2 Introduction

As human immunodeficiency virus (HIV) infection spreads amongst the adult population, the incidence of perinatally acquired HIV infection continues to be an area of concern. The knowledge and mechanism of mother –to child-transmission (MTCT) of human immunodeficiency virus type 1 (HIV-1) accounts for more than 95% of the cases of paediatric AIDS (Salvatore *et al.*,1997). Accumulating evidence indicated that about one third of these infected infants will develop severe symptoms of the disease coupled with severe immunodepression by the first year of life (Salvatore *et al.*, 1997). Some of the postulations which explain the observed differences to disease progression are the timing of transmission of the virus from mother to baby and the

host's capability to control the growth of the viral population (Pizzo *et al.*, 1995). Although the precise timing of viral transmission to the child cannot be pinpointed it has been proposed that detection of the virus in the infant's plasma at birth might reflect *in-utero* infection (Dunn *et al.*, 1992). Some of the risk factors associated with viral transmission from mother to child include low CD4+ cell counts during pregnancy, maternal viraemia, mode of delivery, disruption of the placenta and feeding practices (Dunn *et al.*, 1992; European Mode of Delivery Collaboration, 1999; Newell *et al.*, 1998; Coutsoudis *et al.*, 1999; Health and Human Services, 2006). It was proposed that evolution of the viral population was linked to the host's selective immune forces and disease outcome (Wolinsky *et al.*, 1996). To obtain better clarity on MTCT we studied the relationship between HIV replication and CD4+ counts in blood samples from pregnant HIV-1 infected women and their babies at birth.

4.3 Patients and Methods

4.3.1 Patient Population

All details on patient population are provided in Chapter 3

4.3.2 Laboratory Methods

Techniques used in assessing absolute CD4+ cell counts and viral loads on all blood and plasma samples are discussed in Chapter 3, Section A.

4.4 Results

Fifty five HIV-1 infected pregnant mothers enrolled in the study. All babies in the study were tested immediately after birth. Data and analyses of data follow.

4.4.1 Maternal Data

Information on all mothers participating in the study was obtained by the attending gynaecologist from the antenatal and labour records.

- Antenatal history.
- Past obstetric history
- Age of mothers
- Parity

Labour and Delivery

- Method of delivery
- Gestational period

Personal Details

- Marital status
- Number of sexual partners
- HIV status of partners

The overall demographic data of the patients studied are presented in Table III. The median age of the mothers was 26 years with an interquartile range (IQR) of 23 to 28 years. There were 49 (89.1%) vaginal deliveries and 6 (10.9 %) caesarian sections. The caesarian deliveries were unplanned. Pregnancies ended in full term deliveries in 52 instances (94.5%). Only 3 (5.5 %) deliveries occurred before 36 weeks of gestation. No deliveries required assistance through instrumentation. According to patient records a positive syphilis serology was found in 4 (7.3%) patients at their initial antenatal clinic attendance. These patients were treated in the first trimester of their pregnancy. Thirty four (61.8%) women had haemoglobin concentrations $\geq$10g/l and 21(38.2%) with < 10g/l. At the time of delivery no maternal deaths occurred.

Evaluation of patients' antenatal and obstetric history revealed that the median parity was 1 (IQR 1-2) and the median gravidity 2 (IQR 2-3). Only 1 (1.8 %) participant was married. A total of 54 (98.2 %) women attested to being single mothers. Thirty seven (67.3%) had one lifetime sexual partner and 18 (32.7%) had two or more sexual partners. Partners HIV status were known by 12 (21.8%) participants and unknown by 43 (78.2%). The combined results and characteristics related to the subjects are represented in the tables which follow.

Table III: Demographic Data of HIV -1 Infected Pregnant Women.
N=55

Parameters		**Data**
Age in years:	Median	26
	Interquartile Range	23 - 28
Gestation Period (weeks):	Median	38
	Interquartile Range	37 - 38
Gravidity:	Median	2
	Interquartile Range	2 - 3
Parity:	Median	1
	Interquartile Range	1 - 2
Mode of Delivery:	Vaginal	49 (89.1%)
	Caesarian section	6 (10.9%)
Haemoglobin:	≥10g/l	34 (61.8%)
	<10g/l	21 (38.2%)
Number of lifetime sexual partners:		
Single		37 (67.3%)
Two		1 (1.8%)
>2		17 (30.9%)
Marital Status:	Single	54 (98.2%)
	Married	1 (1.8%)
HIV status of Partners:	Known Positive	12 (21.8%)
	Unknown	43 (78.2%)

4.4.2 Neonatal Data

Information on babies was established at birth. All neonates were clinically examined at birth by the attending paediatrician. The following parameters were measured and recorded:

- Agar scores at 1 minute and 5 minutes.
- Weight at birth
- Gender
- Occipito-frontal circumference
- Length
- Full physical examination

The median Apgar score was 9.0 (interquartile range 1-9) and 10 (interquartile range 1-10) at 1 minute and 5 minutes respectively. No statistical significance was observed in apgar scores between babies considered infected and uninfected ($p = 0.540$). There were 38 (69.1 %) males and 17 (30.9 %) females born to 55 mothers in the study. The median weight of babies at birth was 3 kg (range 2.0-4.0 kg) (Table IV).

Table IV: Demographic Data of Babies N=55

Apgar:	Median (1 min /5 min)	9.0 /10
	Interquartile Range (1 min/5 min)	1-9 / 1-10
Birth Weight:	Median	3.0 kg
	Interquartile range	2.0-4.0 kg
Gender of Baby:	Male	38 (69.1%)
	Female	17 (30.9%)

4.4.3 Maternal Viraemia and CD4+ Cell Values

Analysis of viral loads and CD4+ cell counts were undertaken in categories (Table V). Among the patients studied, association between viral load categories and CD4+ cell categories were performed to define the immune status of the patients and gauge the level of cellular response (Fig.5). No significant association was observed between the mother's viral loads and CD4+ cell counts ($p = 0.134$). Despite the lack of statistical significance a trend was noted. Mothers (33.3%) with low CD4+ cell counts (<200 cells/mm^3) were most likely to have high viral loads (5-5.99 logs) compared with 7.7% with low CD4+ count and low viral load (3-3.99 logs). Conversely, mothers with high CD4+ counts were most likely to have low viral loads (53.8% in the 3-3.99 log category). Twenty three (41.8%) had high CD4+ counts >600 cells/mm^3 and viral loads which, may indicate recent infections. Mothers with CD4+ <200 cells/mm^3 had a mean log viral load of 2.2 whilst those mothers with > 200 cell/mm^3 had a mean log viral count of 1.86. A difference of 0.4 log is not considered to be significant.

Table V: Maternal Viraemia and CD4+ Cell Values. N = 55

Mothers Viral Load (log)	Mothers CD4+ T Cell Counts (mm^3) Number (%)			
	<200	≥200 - 600	> 600 - 1500	TOTAL
3 - 3.99	1 (7.7)	5 (38.3)	7 (53.8)	13 (100)
4 – 4.99	6 (18.2)	15 (45.5)	12 (36.4)	33 (100)
5 – 5.99	3 (33.3)	2 (22.2)	4 (44.4)	9 (100)
TOTAL	10 (18.2)	22 (40.0)	23 (41.8)	55 (100)

4.4.4 Comparison of Mothers and Infected Babies CD4+ Counts

Overall, 10 (66.7%) of all infected babies were observed in category 1 which demonstrated better preserved immune systems. No statistically significant correlation was observed (p = 0.176). However, there was a tendency for babies to have higher CD4+ counts if mothers did not have AIDS (Table VI).

Table VI: Comparison of Mothers and Infected Babies CD4+ Counts N=15

Mothers CD4+cells/ mm^3	Babies CD4+ cells/mm^3 (%)			
	No suppression Category 1 ≥1500	Moderate suppression Category 2 750-1499	Severe suppression Category 3 <750	Total
<200	3 (50.0)	2 (33.3)	1 (16.7)	6 (100)
≥200	7 (77.8)	0 (0)	2 (22.2)	9 (100)
Total	10 (66.7)	2 (13.3)	3 (20.0)	15 (100)

4.4.5 Maternal CD4+ Count and Haemoglobin Values in HIV-1 Infected Pregnant Women

Using the CDC criteria (1993) 10 mothers who had < 200 CD4+ cells/mm^3 were classified as having AIDS (Table VII). Of these 6(60%) had haemoglobin (Hb) values <10g/l. However, 15 (33.3%) infected mothers with CD4+ counts ≥ 200 cells/mm^3 had Hb values ≥10g/dl. In mothers with Hb values <10g/l there was a difference of 26.7% between those with AIDS and those with CD4+ counts ≥200 cells/mm^3. Although not statistically significant (p = 0.156) there was a trend for mothers with low CD4+ counts to have low Hb values.

Table VII: Comparison between Mothers CD4+ Cell Counts and Haemoglobin Values. **N=55**

Mothers CD4+ cells/mm^3	Mothers Haemoglobin (g/l)		
	No. <10 (%)	No. ≥10 (%)	Total (%)
<200	6 (60.0)	4 (40.0)	10 (100.0)
≥200	15 (33.3)	30 (66.7)	45 (100)
Total	21 (38.2)	34 (61.8)	55 (100.0)

4.4.6 Association between Maternal Viraemia and Maternal Haemoglobin

Mothers (66.7%) with low haemoglobin (Hb) values (<10g/l) reflected high viral loads (log 5-599). Conversely, 84.6% of mothers with Hb ≥10g/l had lower viral loads (log 3-3.99). The association between maternal viral loads and maternal Hb values was statistically significant (p = 0.050). It was noted that mothers with high viral loads had low Hb values and vice versa (Fig. 6).

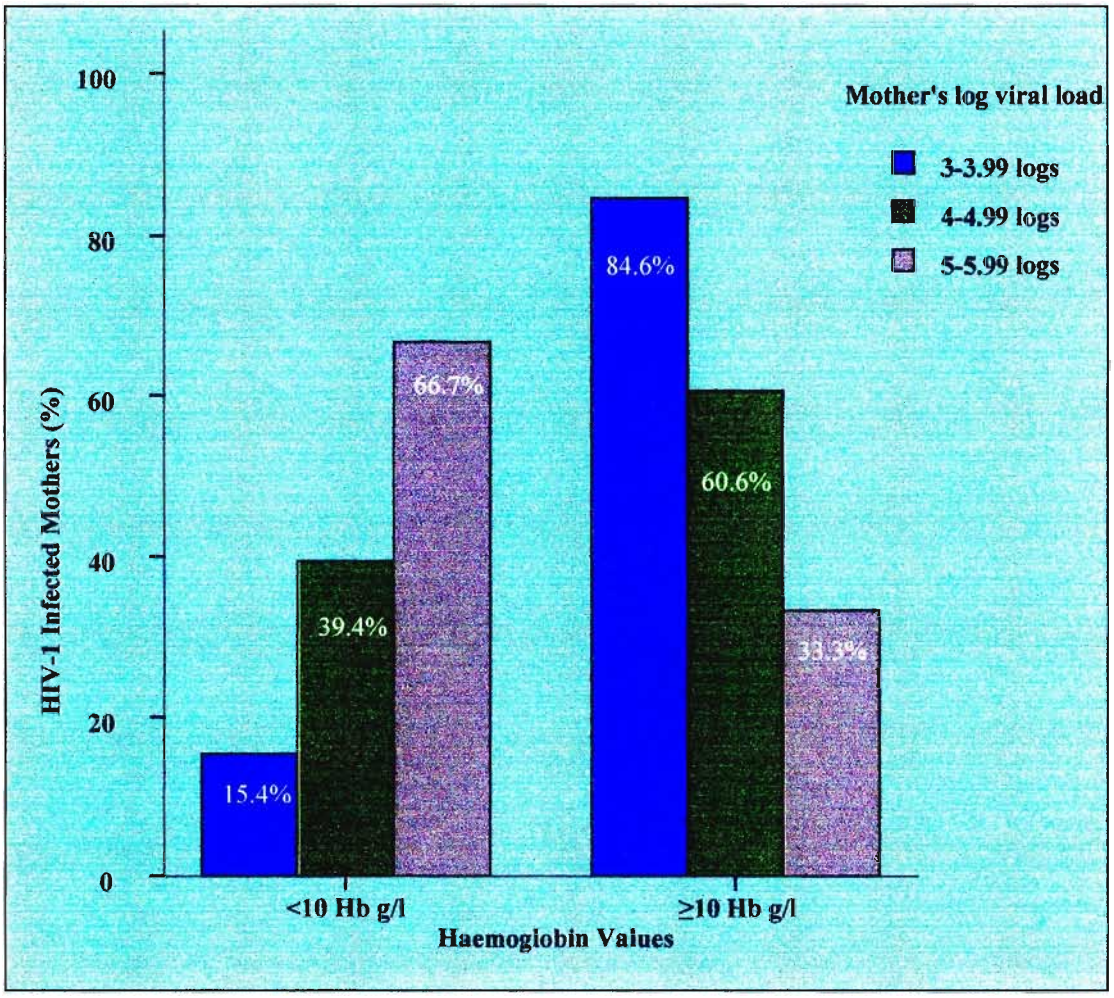


Figure 6: Haemoglobin values compared to viral loads of HIV-1 infected women

4.4.7 Babies Weight and Viral Load

The weight of all infants was recorded at birth. The relationship between the babies viral load and birth weight was investigated (Table VIII). The median weight of all babies in the study was 3 kg (Fig.7). The interquartile range was 2.4 to 3.4 in babies with > 400 copies/ml and 2.5 to 3.1 in babies with <400 viral RNA copies/ml. Collectively 39 (70.9%) babies weighed >2.5 kg and 16 (29.1%) were ≤ 2.5 kg at birth Four (26.7%) of the 15 infected babies had low birth weights (≤2.5 kg). The remaining 11 (73.3%) weighed >2.5kg. There was no significant difference in the median birth weight between infected and uninfected babies (p = 0.457).

Table VIII: Weight at Birth of Infected and Uninfected Babies. N=55

No. of Babies	Weight (kg)		
	≤ 2.5	>2.5	Total
Uninfected	12 (30.0)	28 (70.0)	40 (100.0)
Infected	4 (26.7)	11 (73.3)	15 (100.0)
Total	16 (29.1)	39 (70.9)	55 (100.0)

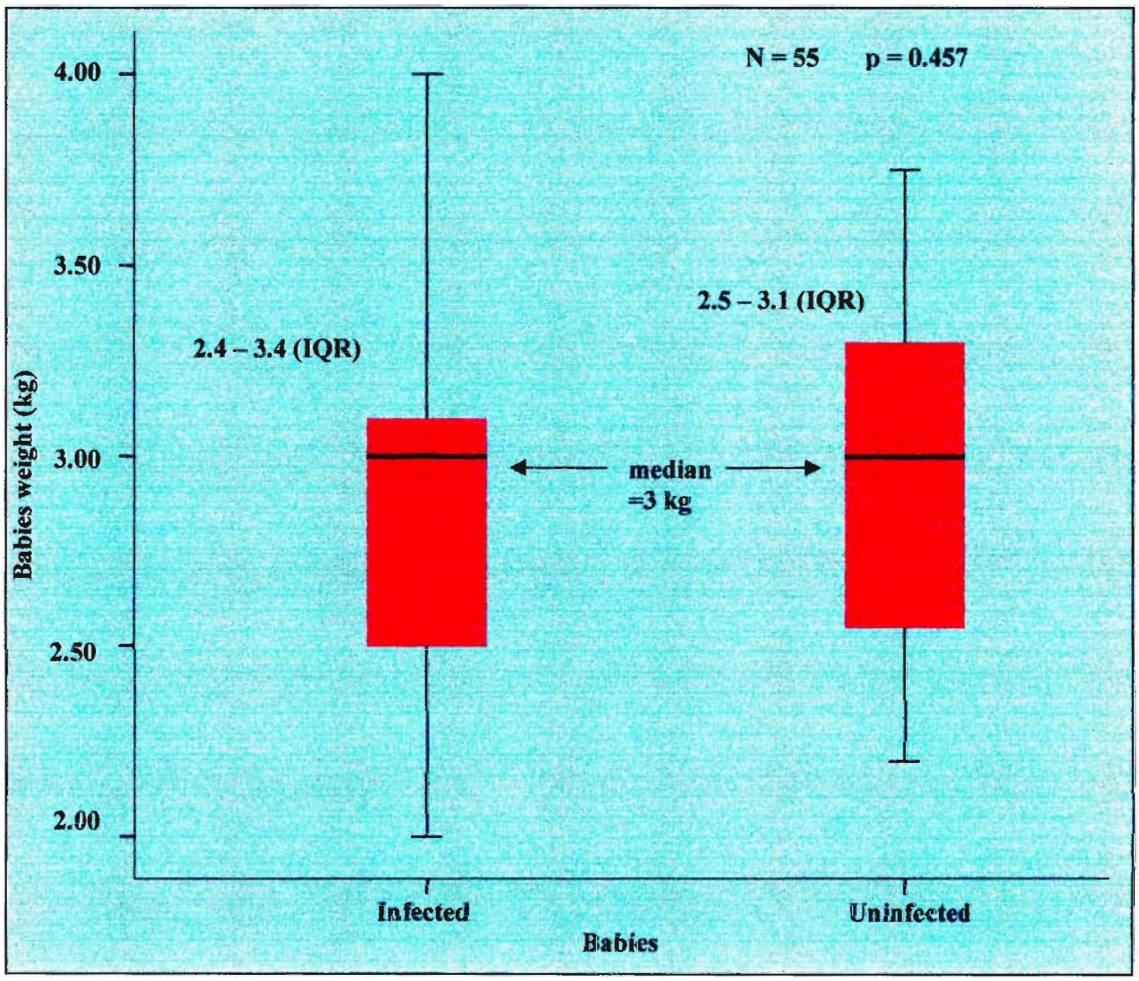


Figure 7: Median weight (3 kg) of all babies born to HIV-1 infected women. Interquartile range (IQR) was 2.5 to 3.1 in uninfected babies and 2.4 to 3.4 in infected babies.

4.4.8 CD4+ Cell Counts of Infected and Uninfected Infants

A comparison of CD4+ counts of infected and uninfected babies was undertaken. A median difference of 500 CD4+ cells was noted between those babies infected (2000 cells/mm^3) and uninfected (2541 cells/ mm^3)(Fig.8). An overlap in the interquartile range indicates no significance difference between the CD4+ cell counts of babies considered infected *in utero* and those uninfected at birth (Table IX). The lack of significant association (p = 0.237) could be due to small sample size.

Table IX: Statistical Values of CD4+ Count in Infected and Uninfected Babies. N=55

	Babies CD4+ Count (cell/mm^3)	
	Infected	**Uninfected**
Median	2000	2541
Percentiles: 25	928	1554
75	2696	2979

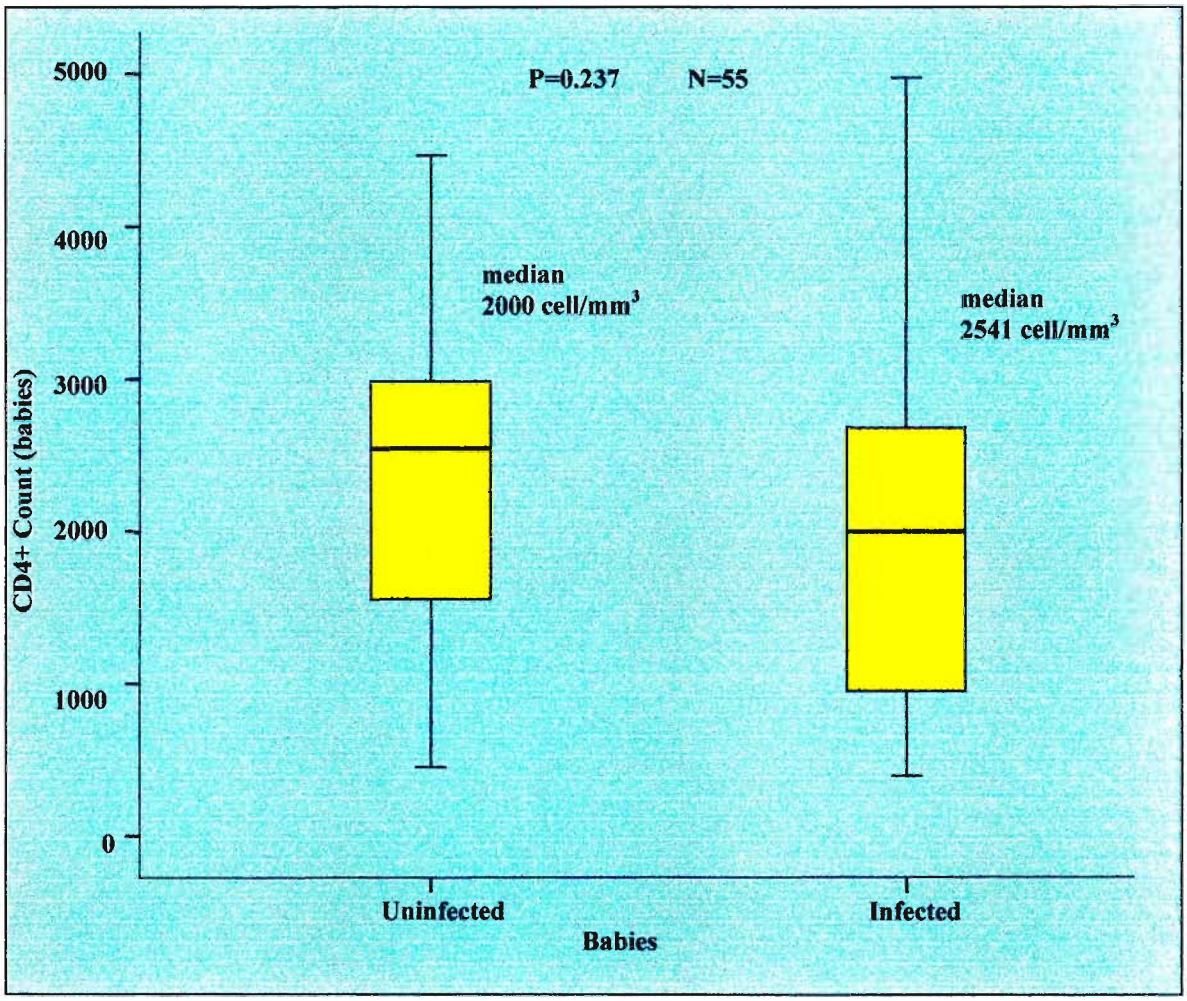


Figure 8: A median difference of 500 CD4+ cells/mm^3 between those babies infected (2000 cells/mm^3) and uninfected (2541 cells/ mm^3).

4.4.9 Viral Load and CD4+ Cell Counts of Infected Infants

Statistical values obtained on CD4+ Counts of all infected infants in the study showed a median value of 2000 cells/mm^3 (IQR 928 – 2696). CD4+ counts ranged from 399 to 4976 cells/mm^3 with log viral loads from 3.3 to 5.0 logs. A median log viral load of 4.18 (IQR 3.75 – 4.5) with a minimum value of 3.3 logs and a maximum of 5.0 logs was recorded for all infected infants (Table X).

Table X: Statistical Values of Infected Babies Log Viral Load and CD4+ Count.

N=15

	Babies log Viral Load	**Babies CD4+ cells/mm^3**
Median	4.18	2000
Minimum	3.3	399
Maximum	5.0	4976
Percentiles: 25	3.75	928
50	4.2	2000
75	4.5	2696

Of the 15 babies considered infected *in utero*, 6 (40.0%) had log viral loads between 3 to 4 logs of which 1(16.7%) had CD4+ counts <750 cells/mm^3 whilst 5 (83.3%) had >1500 CD4+ counts. The remaining 9 (60.0%) infected babies had viral loads of >4 logs. Among the babies with high log viral loads, low CD4+ counts (<750cells/mm$^{3)}$ was observed in 2(22.2%). Two (22.2%) babies were found to have moderate immune suppression (750 to 1499 cells/mm^3) and 5 (55.6%) with high CD4+ counts showed no immune suppression (>1500 cells/mm^3). Overall, 66.7% of infected babies demonstrated a well preserved immune system. The relationship between infected babies CD4+ counts and log viral loads was negatively correlated (r = -0.437) with no observation of a statistical significance (p = 0.103).

Table XI: Comparison of Infected Babies Viral Loads and CD4+ Counts

N=15

Babies	**Babies CD4+ cells/mm^3 (%)**			
Viral load	**No suppression Category 1 ≥1500**	**Moderate suppression Category 2 750-1499**	**Severe suppression Category 3 <750**	**Total**
3-4 logs	5 (83.3)	0 (0)	1 (16.7)	6 (100)
>4-5 logs	5 (55.6)	2 (22.2)	2 (22.2)	9 (100)
Total	10 (66.7)	2 (13.3)	3 (20.0)	15 (100)

4.4.10 Predictive Value of Maternal Viraemia and Babies Viral Load

The predictive value of maternal plasma viral load in vertical transmission was performed on plasma of fifty five HIV positive pregnant mothers. Variables were entered into a backwards selection procedure. The variables included log viral load and CD4+ cell counts of all mothers, gestational period, sex of babies and age. Three variables which were not significant were eliminated from the model (age, gestational period and CD4+ cell counts). Only two variables remained in the model i.e. log viral load of mothers and sex of babies (Table XII). The log viral load of mothers was significantly associated with transmission of infection to babies ($p = 0.047$). The odds ratio (Exp (B) = 3.137 (95%CI, 1.015-9.696) indicated that for every 1 log increase in viral load the risk of babies acquiring the infection increased by 3.1 times.

Table XII: Logistic Regression to Determine the Predictive Value of Maternal Viraemia and Babies Viral Load. N=55

	Wald	df	Sig.	Odds Ratio	95.0% C.I.for EXP(B)	
					Lower	Upper
Log Viral Load of Mothers	3.944	1	0.047	3.137	1.015	9.696
Gender of Babies	2.777	1	0.096	3.110	0.819	11.809
Constant	5.745	1	0.017	0.001		

Variables entered are mothers' age, log viral load and CD4+ cells, Pregnancy weeks and Gender of babies. Only viral load and gender was accepted as variables for evaluation.

The predictive value of gender of babies and risk of infection (95% CI, 0.819-11.809) reflected that 7 (41.2%) of 17 female babies were infected as compared to 8 (21.1%) of 38 male babies born to HIV-1 infected mothers in the study population (Table XIII). Although there was no significant association between gender of babies and mothers viral load (p = 0.096) female babies were more likely to become infected *in utero* than males (3.1 times higher risk). The lack of statistical significance could be due to small sample numbers which leads to a greater likelihood of type II errors.

Table XIII: Association between Gender and Viral Load of Babies. N=55

Babies Gender	Babies Viral RNA Copies		
	Uninfected (RNA copies 400/mm³) No. (%)	**Infected (RNA copies 400/mm³) No. (%)**	**TOTAL No. (%)**
Male	30 (78.9)	8 (21.1)	38 (100)
Female	10 (58.8)	7 (41.2)	17 (100)
TOTAL	40 (72.7)	15 (27.3)	55 (100)

4.4.11 Comparison between Mother's CD4+ Counts and Babies Viral Load

Mothers with CD4+ counts <200 cells/mm^3 were classified as having AIDS. An evaluation between HIV-1 infected mothers with <200 CD4+ cells/mm^3 (AIDS) and those with CD4+ cells/ mm^3) and the viral loads of their babies was undertaken. There was a significant association between mothers with AIDS and the viral loads of their babies at birth (p = 0.018). Sixty percent of infected babies were born to mothers with low CD4+ counts (<200 cells/mm^3) as compared with 20% born to mothers with >200 CD4+ cell counts (Table XIV). If the mothers had AIDS the infants were more likely to be infected. The mean log viral loads of babies born to mothers with <200 cells/mm^3 and >200 cells/mm^3 were 3.6 (SD = 0.9) and 2.9(SD = 0.6) respectively.

Table XIV: Comparison between Mother's CD4+ Counts and Babies Viral Load.

N = 55

Mother's CD4+cells /mm^3	**Babies**		
	No. Uninfected (%)	**No. Infected (%)**	**Total (%)**
<200	4 (40)	6 (60)	10 (100)
≥200	36 (80)	9 (20)	45 (100)
Total (%)	40 (72.7)	15 (27.3)	55 (100)

4.5 Discussion

One of the objectives of this study was to determine the level of viral load and CD4+ cell counts in HIV-1 infected mothers and their babies. In defining the relationship between various immunological parameters in maternal- infant transmission of HIV-1 the factors enhancing transmission of the virus may be elucidated.

Analysis of viral loads and CD4+ cell counts were undertaken in categories. Among the patients studied, association between viral load and CD4+ cell categories were performed to define the immune status of the patients and to gauge the level of cellular response. No significant association was observed between the viral load of mothers and their CD4+ cell counts. Despite the lack of statistical significance there was a trend towards mothers with high viral RNA copies to have low CD4+ cell counts. Conversely, mothers with high CD4+ counts were most likely to have low viral loads. Some authors have found that there is an inverse, but variable correlation between plasma viral RNA levels and the level of CD4+ lymphocytes (Daar *et al.,* 1991; Clark *et al.*, 1991; Aldrovandi, 2006).

Low CD4+ counts are indicators of a waning immune system. HIV-1 infected individuals with CD4+ levels below 200 cells/mm^3 are classified as having AIDS (CDC, 1993). Eighteen percent of the women in this study population had < 200 CD4+ Counts and were classified as having AIDS (CDC, 1993). Ninety percent of the women with AIDS had viral loads greater than 4 logs thereby, increasing the risk

for transmission to the baby. One of many risk factors for MTCT is the amount of virus to which the child is exposed (Fowler *et al,* 2000).

Other maternal factors found to be associated with an increased risk of transmission were vaginal deliveries and low haemoglobin levels during pregnancy. Studies conducted by the European Mode of Delivery Collaboration (1999) have demonstrated that the efficacy of caesarian section before delivery for the prevention of MTCT. Caesarian sections are also emphasized to a greater extent by The British HIV Association and the European Consensus Panel. However, in resource poor settings caesarian sections as prevention mechanism for MTCT is generally not feasible because of the lack of skilled attendants during labour (American College of Obst and Gynae, 2000). The findings that caesarian sections were less likely to transmit the virus cannot be commented on in this study because only 10.9% of the total number of women in the study had the procedure performed. The numbers are too small for an accurate evaluation. As a general comment viral loads in all six babies delivered by caesarian section were at undetectable levels at birth. However, the babies were not available for retesting which renders the final diagnosis inconclusive. Dunn *et al.* (1995) states that most infants with perinatally acquired infection, who test negative at birth, will have a positive PCR test results by 2 weeks of age. Together with the CDCP (1995) he recommends that testing should be performed within 48 hours of birth, at 1-2 months of age and again at 4-6 months of age (Dunn *et al.*, 1995). The babies in this study were not retested due to default in follow up visits.

The implication of low haemoglobin concentrations in HIV-1 infected women and the risk of vertical transmission were evaluated. Sixty percent of the women classified as having AIDS, had low haemoglobin values with a corresponding low CD4+ count. Those with haemoglobin values greater than 10g/l had > 200 CD4+ cell counts. Although not statistically significant in this study, there was a trend for mothers with low CD4+ cell counts to have low haemoglobin values.

We also report here a statistically significant association between maternal viraemia and maternal haemoglobin. Mothers with haemoglobin values less than10g/l reflected high viral loads. Conversely, mothers with haemoglobin values greater than 10g/l had lower viral loads.

This study and many others have established that high maternal viral loads are significant predictors to vertical transmission. A study conducted in Zaire (St Louis; 1993) reported an association between risk of transmission and low haemoglobin concentrations in HIV-1 infected women. Later studies by Bobat *et al.* (1999) found that HIV-1 infected women whose haemoglobin values were <10g/l during pregnancy were at an increased risk of transmitting the virus to their babies. The author further postulated that low haemoglobin values in HIV-1 infected mothers may reflect the effect of advanced sub clinical disease, general poor health or nutrition.

Detection of HIV infection in the neonatal period is important so as to provide appropriate and managed health care to the patient. General birth data such as birth weight and prematurity for surveillance and monitoring purposes can be adjusted to

account for the impact of HIV infections (Bobat *et al.* 1999). In this cross sectional analysis we compared only birth weight and apgar scores. Human immunodeficiency virus in *in-utero* infected and uninfected babies were compared in relation to their weight at birth. The anthropometric index of weight for age showed no significant differences in both groups of babies. Other studies (Bobat *et al*; 1999) have also reported that the weight percentile for infected and uninfected babies was not significant. Furthermore, the apgar scores between babies infected and uninfected were not statistically significant. Reports from Europe and US also found no difference in the growth parameters and low birth weight (Blanche *et al.*, 1998; Lindregren *et al.* 1991; Nesheim, 1994). A different observation by Butlerys (1994) in Rwanda was reported whereby; infected newborns were more likely to have lower birth weights and lengths. The Malawi findings (Taha, 1995) compared only growth and found no differences. Although, these studies were longitudinal studies, the initial paediatric clinical screening procedures for all neonates remain relatively constant to allow for comparisons.

Prematurity was also reported as a risk factor to vertical transmission. In the European Collaborative Study (1992) there was a strong correlation with prematurity and increased risk of transmission. In this study only 3 babies were born before 36 weeks. The numbers are too small to comment on its statistical significance.

The absolute risk of transmission is estimated to be 5% to 6% (Rouzioux, 1995). The mathematical modeling suggests that much of this is *in utero* transmission which occurs relatively late in gestation (Fowler *et al.*, 2000). Bryson *et al* (1992) proposed that viral detection at less than 48 hours demonstrates in utero infection. We applied

this definition to the babies' viral loads in this study and found a transmission rate of 27.3%. Most studies have found that infants which fit this "in utero" definition have a shorter median time to onset of symptoms and death (Mayaux *et al,* 1996; Dickover *et al*, 1994). This pattern of decline was not demonstrated in our study because longitudinal investigations are required to identify disease progression. Mothers in this study population did not comply with scheduled follow up visits after delivery. Others studies have reported transmission rates in the range of 25%-30% (The Working Group on Mother–to-Child Transmission of HIV, 1992). In resource poor settings the values rose to as high as 42% (Read *et al.* 2005). The amount of exposure of the foetus to maternal virus is one of many factors facilitating MTCT.

Infection status of the infants was classified according to the CDC HIV Pediatric Classification (1994). Age specific CD4+ cell count was used to analyse immunologic response in all babies in the study (CDC, 1994). Overlapping of interquartile ranges between CD4+ counts of infected and uninfected babies indicated that there was no significant difference between the two groups of babies. Using the CDC classification (1994) the infected babies were categorized as no evidence of immune suppression, evidence of moderate immune suppression and evidence of severe immune suppression. Overall, 20% of all infected babies were categorised as having evidence of severe immune suppression. In the same category 6.7% of babies were born to mothers with a diagnosis of AIDS. It has been reported that infants born to women with advanced disease and higher viral loads also tend to be rapid progressors (Blanche *et al.*, 1994; Tovo *et al.*, 1994; Lambert *et al.*, 1997). Again, we are unable to substantiate these findings on rapid progressors because no follow up visits occurred.

A better preserved immune system was demonstrated by 66.7% of infected babies who were classified as no evidence of immune suppression. The mothers CD4+ counts of 46.7% of babies in this category were greater than 200 cells/mm^3. An observation that babies born to HIV-1 infected mothers with immune deterioration are more likely to have lower CD4+ counts than babies born to mothers with high CD4+ counts. Finally, it cannot be ascertained whether the babies with no evidence of immune suppression will be able to sustain viral suppression because again, the study population was unavailable for retesting.

Studies have indicated that without ARV treatment, transmission rates ranged from 5% to 15%. If the viral load was over 2 logs the transmission rate rose to 37% (Contopoulos-Ioannidis and Ioannidis, 1998). These statistics are in line with our findings. Antiretroviral treatment was not available to our study population during pregnancy. The infected babies in our study had between 3 to >5 log viral loads with a transmission rate of 27.3%. The possibility of a higher transmission rate cannot be excluded because 72.7% of the babies in our study, with an initial undetectable viral load, were not retested.

The relationship between infected babies CD4+ counts and log viral loads was negatively correlated ($r = -0.437$) and there was no observation of statistical significance ($p = 0.103$). Of the 40% with lower log viral loads 83.3% had CD4+ counts >1500 cells/mm^3. When compared to 55.6% with CD4+ counts >1500 cells/mm^3 and log viral loads >4, we can report that babies with lower viral loads had higher CD4+ counts. These babies who showed a greater CD4+ cell response at birth

may suggest that they may be more capable of mounting a CD4+ cell mediated immune response. Previous studies have elucidated that in the first few months of life the course of disease appears to correlate with patterns of plasma viral dynamics (Palumbo *et al.*, 1995). In contrast, one study found low plasma RNA levels at birth for those with early infection. However, the RNA copies rapidly increased over the next two months to very high levels before slowly declining over the years (Shearer *et al.*, 1997).

The predictive value of maternal viral load and risk of transmission revealed that maternal viraemia was significantly associated with transmission of infection to babies ($p = 0.047$). The odds ratio indicated that for every 1 log increase in maternal viral load the babies were 3.1 times more likely to acquire the infection. Furthermore, the study found that a higher number of female babies were infected than males. Although not statistically significant the odds ratio indicated that female babies were 3.1 times more likely to become infected than males.

In conclusion, several paediatric studies have shown that plasma viral RNA levels can independently predict clinical outcomes (Mofenson *et al.*, 1997; Palumbo *et al.*, 1998; Lindsey *et al.*, 2000). Infants infected by vertical transmission tend to progress more rapidly than those who acquire infections by blood products (Jones *et al.*, 1992; Fredrick *et al.*, 1994; Morris *et al.*, 1996). The clinical outcomes on babies in this study cannot be commented on because there were no follow up visits. The challenges presented in maintaining follow up visits were complex and varied. Some of the challenges were that patients accessed other outpatient clinics or primary health clinics

for treatment when required. Most patients were from low socioeconomic backgrounds, especially from rural areas and could not afford the cost of frequent transport to the hospital. Specific outreach teams were not available to trace mothers and babies who did not adhere to follow up visits. Finally, the observations seen in this study are within a small sample population. Extrapolations of these findings to other population groups and in other geographical settings remain to be determined.

SECTION B

CHAPTER FIVE

CHAPTER 5

NATURAL KILLER CELLS AT THE FOETAL-MATERNAL INTERFACE OF HIV-1 INFECTED PREGNANT WOMEN AND ITS IMPLICATION IN *IN UTERO* TRANSMISSION

5.1 Summary

Background: Several lines of evidence suggest that Natural Killer (NK) cells have an important role in antiviral defence (Stanic *et al.*, 2003; Chehimi *et al.*, 1991). Thus to be effective, NK cells have to recognise and cause lysis of virus-infected cells (Schattner and Rager-Ziman, 1986). The impairment of NK cell function in the course of HIV infection contributes to a decreased resistance against HIV (Stanic *et al.*, 2003). Therefore, the interaction between the presence of NK cells and p24 at the placental interface of HIV-1 infected mothers was investigated.

Methods: Evidence of human immunodeficiency virus (HIV) replication was sought in 55 human placentas obtained from pregnancies complicated by maternal HIV-1 infection. The placentas were examined by immunohistochemical staining techniques using monoclonal anti-p24 antibodies. In addition placental tissue were stained for the presence of CD56+ cells, a subset of NK cells found in uterine and placental tissue.

Results: Both syncyciotrophoblasts and cytotrophoblasts displayed staining to p24 antigens. Majority of the placentas (94.6%) had evidence of p24 antigens. Natural Killer cell activity was observed in 98.3% of samples. The median NK cell count in placentas of mothers with infected and uninfected babies was 4 cells/30hpf) and 10 cells/30hpf respectively. There was no statistically significant correlation between number of NK cells and vertical transmission ($p = 0.145$). However, the risk for vertical transmission was increased 3.4 times more if NK cell values were low [OR= 3.424 (95% CI 0.65-17.89)]. Furthermore, the presence of p24 in placental tissue was not influenced by maternal viral load ($p = 0.448$) and CD4+ cell counts ($p = 0.660$).

Conclusion: The level of maternal viral load or CD4+ counts did not appear to affect the presence or absence of p24 antigens in placental tissue of HIV-1 infected women.

5.2 Introduction

The placenta provides a potential barrier between maternal and foetal circulation. The human placenta is of the villous haemochorial type and consists of foetal villi bathed directly in circulating maternal blood (Tscherring-Casper *et al.*, 1999). The villi outermost layer consists of syncytiotrophoblasts, which forms a multinucleated epithelium maintained by an underlying population of mononuclear cytotrophoblastic cells. The mesenchymal cells of the villous core are separated by a trophoblastic cell basement membrane layer. The villous core houses a significant number of macrophages (Hofbauer cells, some of which express CD4 molecules) and foetal

capillaries Tscherring-Casper *et al.*, 1999). Trophoblastic cells constitute the external layer of chorionic villi and are in direct contact with maternal blood. These cells are primary target for maternal blood-borne infections. However, the placental barrier is not complete, and there is evidence that bidirectional traffic of cells, including leucocytes, may occur in human pregnancy (Papadogiamakis *et al.*, 1997). Infection of human placental tissue cells with HIV-1 requires direct contact with infected leucocytes (Douglas *et al.*, 2001). The mechanisms regulating the transfer of HIV into placental cells are varied and integrated.

Infection of the foetus occurs at various trimesters. The majority of *in utero* infections occur during the final trimester of pregnancy. The infection rate in the second trimester is reported at 2-5% (Sprecher *et al.*, 1986). Transmission during the first trimester appears to be rare (Maury *et al*, 1989). Therefore, understanding the mechanisms or routes involved in intrauterine transmission of HIV during pregnancy and parturition is important for planning intervention strategies.

Human NK cells are a major population of lymphocytes and are innate effectors critical to early host defense. Expressed as subsets CD56+, CD16+ and CD3- in peripheral blood and as CD56+ in uterine tissue, NK cells recognise and kill viral and bacterial infected cells (Nagler *et al.*, 1989).

As critical cells in bridging the gap between innate and adaptive immunity, NK cells display a unique lineage characterized by CD1d recognition and a heavily biased T-cell

receptor (TCR). High levels of immunoregulatory cytokines are produced upon stimulation through their TCR, which is associated with a potent ability to influence immune responses in a wide range of infections (Godfrey *et al.*, 2000) As a discrete subset of leucocytes, distinct from B and T lymphocytes, NK cells mediate spontaneous killing of a wide variety of target cells prior to sensitization. Based on this information it follows that the loss of NKT cells could have a profound negative impact on normal immune functioning.

5.3 Patients and Methods

5.3.1 Collection of Human Placental Specimens

Placental samples from infected HIV-1 mothers were collected from fifty five patients post delivery. Ethical approval for the study was obtained from the Ethics Committee (University of KwaZulu-Natal) along with informed consent from all participating women. The placental specimens were immediately placed into 10% formal saline used as preservative (see Chapter 3 section 3.2).

5.3.2 Transport and Storage of Samples see Chapter 3 section 3.3)

5.3.3 Laboratory Methods

Placentas were each examined by immunoperoxidase immunohistochemistry using p24 and CD56+ monoclonal antibodies for the presence of HIV antigens and NK cells (CD56+) respectively. Experimentally false positive results are seldom observed with the use of monoclonal antibodies (Dictor *et al.*, 2001). However, commercially available CD56+ and p24 positive controls (DAKO) were used to verify test specificity and sensitivity. Duplicate placental sections from HIV negative women were also stained for CD56+. This was done to assess NK cell activity in placentas of HIV negative women. Reagents were prepared prior to staining of slides (Appendix VA). The staining procedure is discussed in Chapter 3, Section B with a detailed discussion in Appendix VB. Controls and placental sections were evaluated by a pathologist for quality assurance. All sections were evaluated for the presence of p24 and CD56+ cells. To maintain consistency, p24 antigen and CD56+ estimation was done according to number of stained cells/30 hpf. Infected placental cells were assigned categories ranging from absent (0 cell/30hpf), category A (20-30 cells/30hpf), category B ((10- 19 cells/30hpf) and category C ($\leq$ 4 cells/30hpf). Sections were regarded as negative in the absence of immunoreactive p24 antigen in distinct cells. Natural Killer (CD56+) cells stained dark brown with monoclonal antibodies. An estimation of the total number of neutrophils and lymphocytes was performed on all tissue sections to gauge the overall inflammatory response in the placenta. A basic Haematoxylin and Eosin staining technique used for the assessment of neutrophils and lymphocytes (inflammatory cells), is outlined in the Chapter 3, Section B.

5.4 Statistical Methods

Both parametric and nonparametric statistical analyses were used to assess the relationship between variables. Parametric statistics was used for variables with normal distribution. Linear regression was used to determine the significance of inter-related interactions and the risk ratio. Confounding was assessed by the backward elimination method. Variables which did not alter the risk ratio were eliminated. Variables such as maternal viral load and gender of babies which changed the risk ratio were maintained and extensively evaluated. The entire analysis was conducted using SPSS statistical software.

5.5 Results

5.5.1 Detection of HIV-1 antigens and CD56+ Cells in Placental Tissue Using Immunohistochemistry Staining

Immunohistochemistry of infected placental cells produced strong ring like granular cytoplasmic staining of HIV- positive cells only when anti- p24 was used (Fig. 9). HIV negative control cells (DAKO) showed no staining with or without anti-p24. Both syncytiotrophoblasts and cytotrophoblasts displayed staining to p24 antigens. Detection of p24 antigens was noted in 52 (94.6%) placentas. The median value was 10 cells/30hpf.

The presence of CD56+ cells were observed using primary antibodies (Zymed, Clone 123C3)) specific for CD 56+ cells (Fig.10). A similar grading system as used for p24 antigen was utilized to quantify the presence of CD56+ cells in placental tissue (5.3.3). The presence of NK cells was observed in 54 (98.2%) placental tissue sections. The median cell count was 10 cells/30hpf.

No significant association was observed between CD56+ cells and the presence of p24 antigens ($p = 0.438$). Overall, low NK (CD56+) cell values were observed with high p24 antigen values and vice versa. Only one sample indicated a low NK (CD56+) cell count with low numbers of p24 antigens (Fig.11). There was evidence of NK cell activity in majority of infected placentas. However, the level or intensity of the activity varied as indicated by the difference in NK cell numbers in placental tissue. Assessment of CD56+ cells in placental tissue of HIV negative women was performed to gauge a general overview of NK activity in uninfected placentas. Overall, CD56+ cell counts in placentas of HIV negative women ranged from 8 to 10 cells/30hpf.

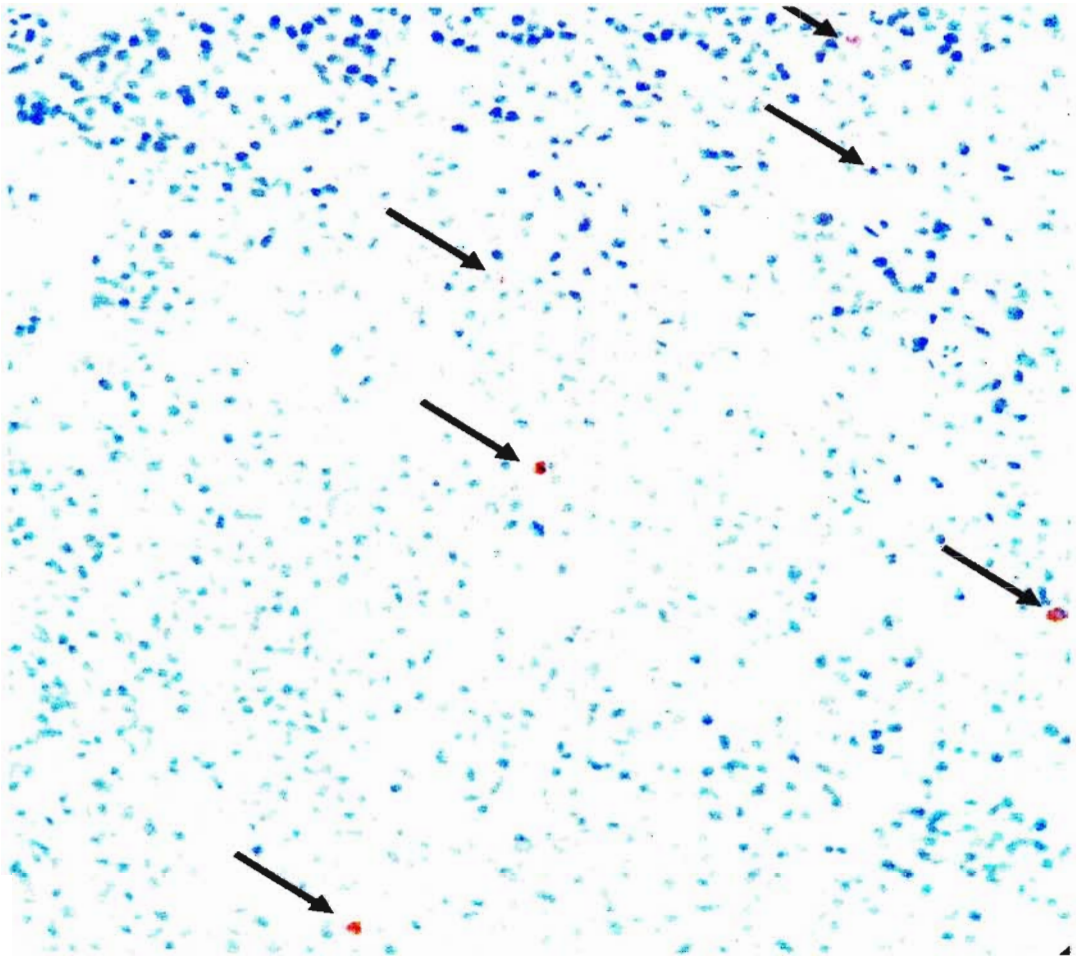


Figure 9: **Immunostaining of p24 antigen in tissue. The tissue section was used as a positive control slide incubated with monoclonal mouse anti-Human Immunodeficiency Virus antibodies (DAKO, Code No. M0857) to detect the presence of p24 antigen in placental tissue. The scattered distribution of p24 antigen is observed as dark brown stained cells infiltrating the surrounding tissue (indicated by arrows).**

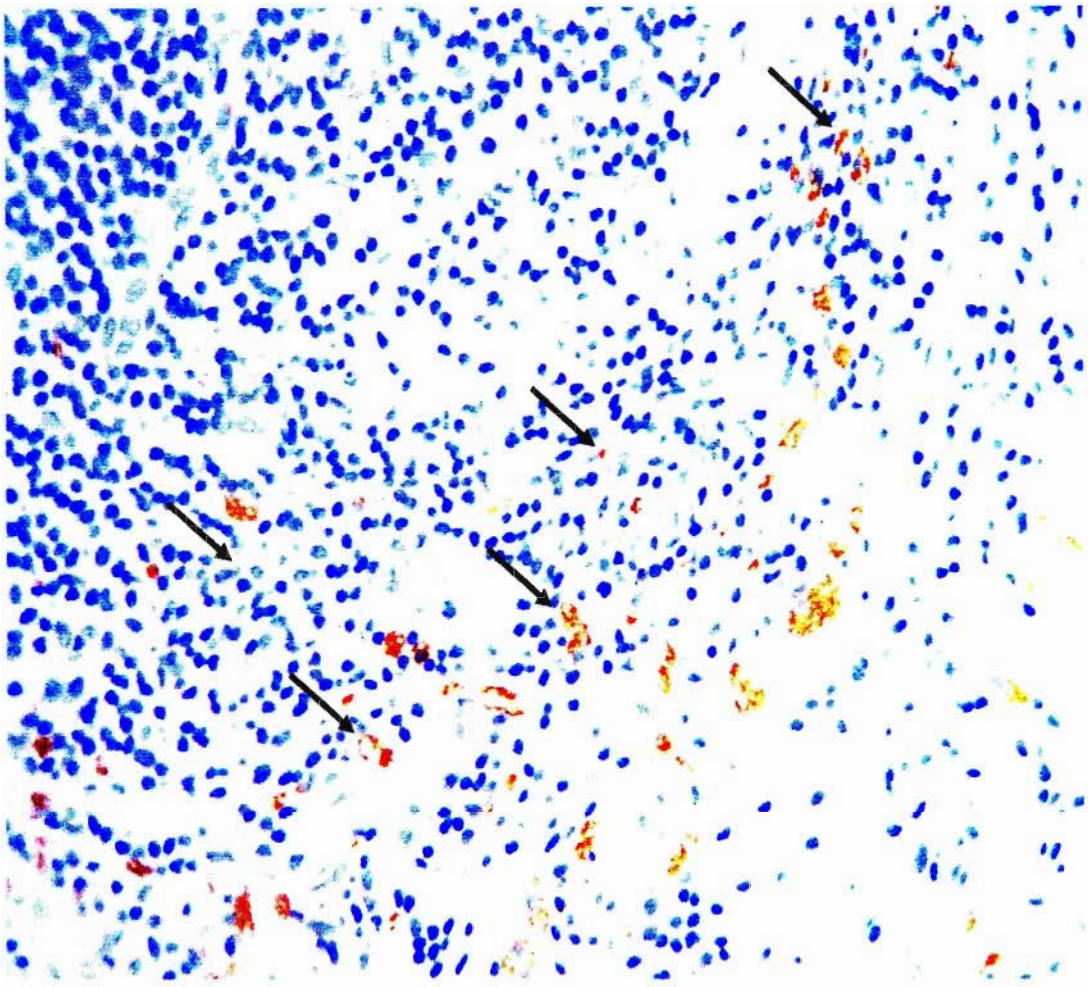


Figure 10: **Positive control tissue section: Immunostaining of CD56+ cells in tissue was performed with primary antibodies to CD56+ (Zymed, Clone 123C3). The distribution of CD56+ cells is seen throughout the tissue as brown stained cells. (indicated by arrows)**

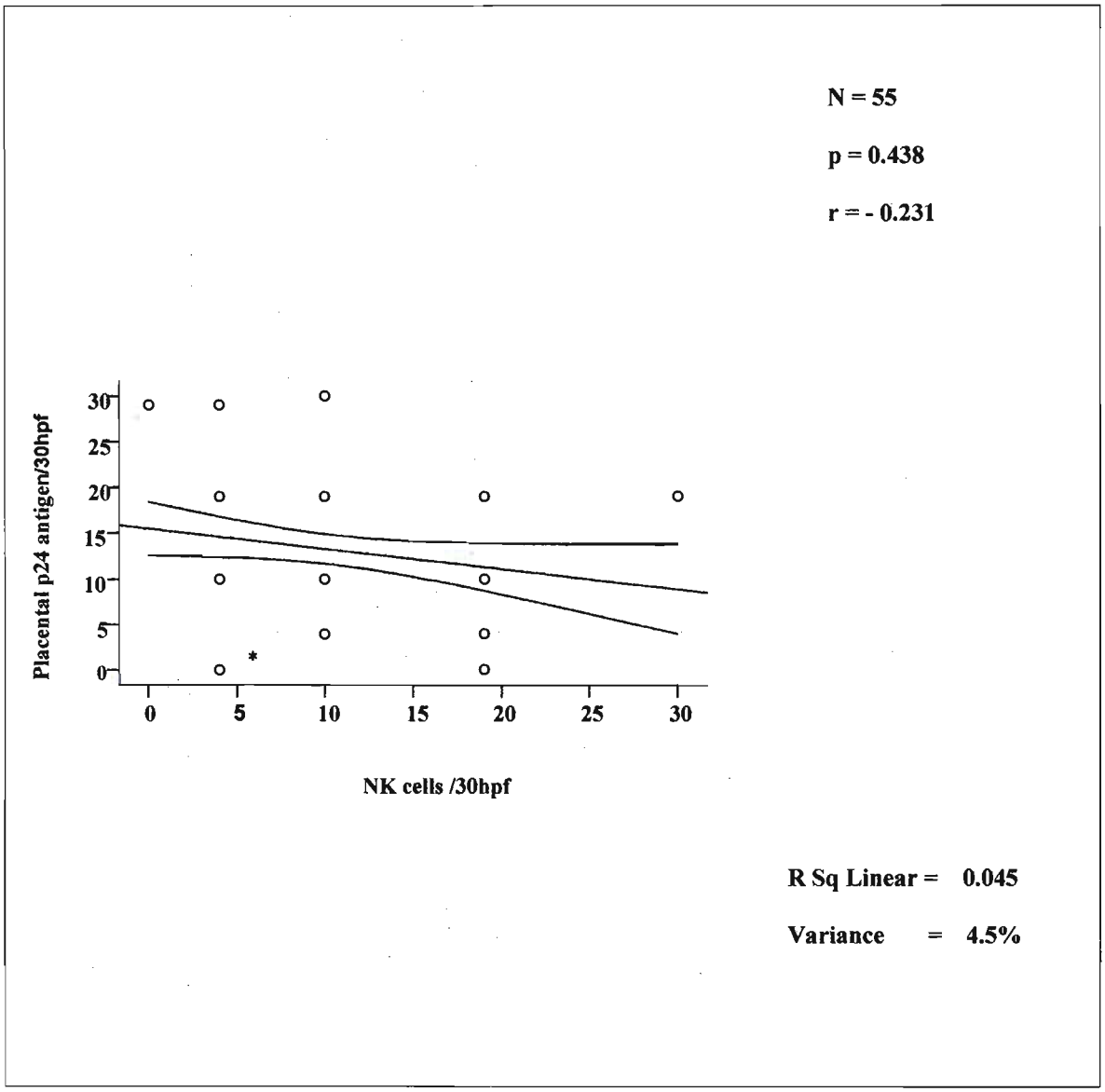


Figure 11: Correlation between the presence of NK cell and p24 antigens in placental tissue of HIV-1 infected pregnant women. NK cell response at the placental interface varied in HIV-1 infected mothers. Low numbers of NK cells and p24 antigen was observed in 1 sample (*)

5.5.2 HIV-1 Detection in Placentas Compared with Maternal Viraemia and CD4 +Cell Counts

Plasma HIV-1 RNA levels of all infected mothers were analysed in conjunction with their CD4+ counts and p24 antigen in the placenta. Parametric statistical analysis between maternal viral load ($p = 0.448$), CD4+ cell counts ($p = 0.660$) and the presence of placental p24 antigens was not significant. High p24 antigens with low or high viral RNA copies were evenly distributed. There was a small variance of 1.1% in p24 antigens (Fig.12). Similarly, high p24 antigen values were observed with both high and low CD4+ cell values. The R Sq linear value 0.002 (0.2% variance) was negligible for any definitive conclusion (Fig.13). The presence of p24 antigens in placental tissue of HIV-1 infected mothers is not influenced by their viral load or CD4+ cell count.

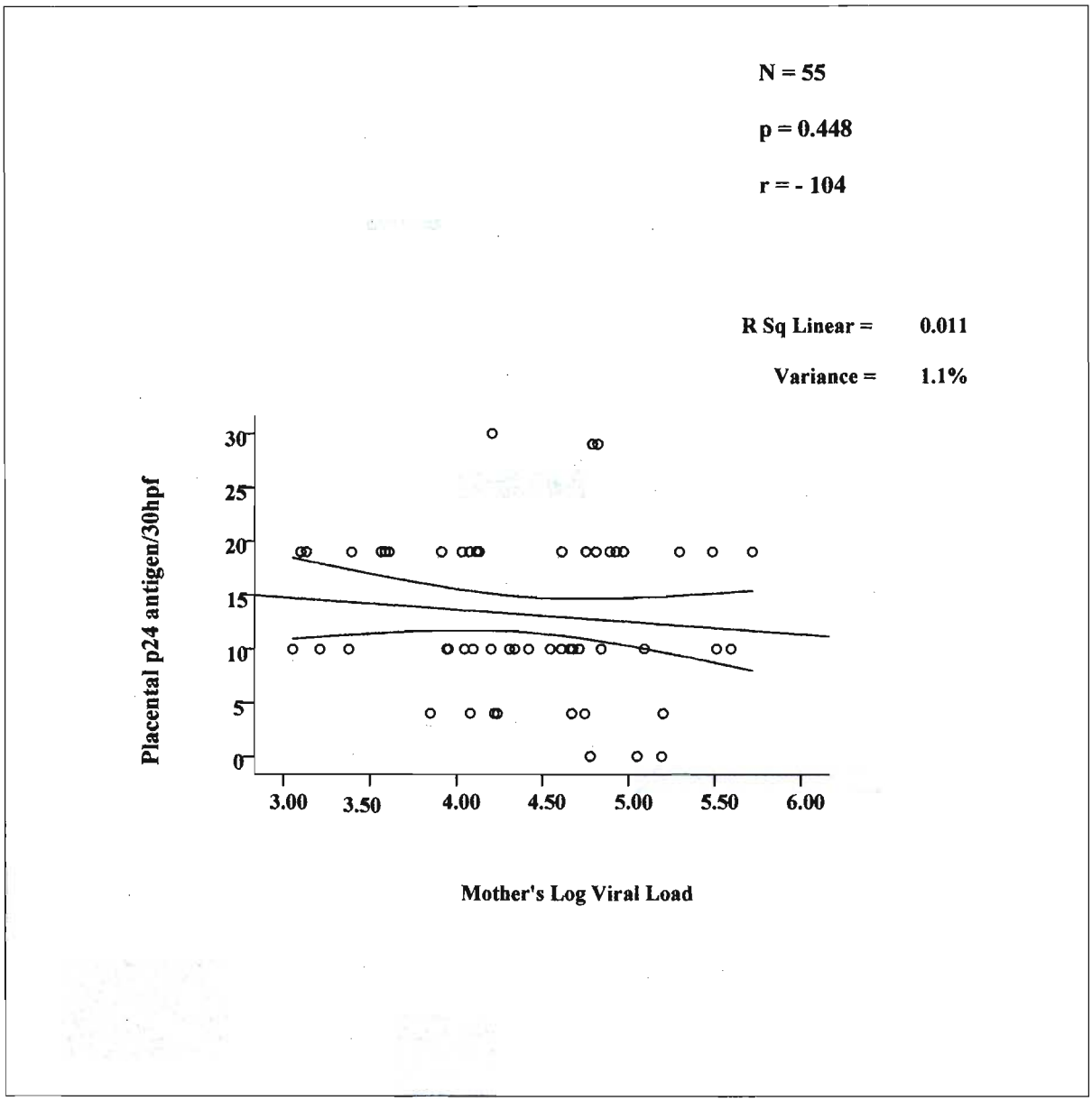


Figure 12: Scatter plot of mothers log viral load compared to the presence of placental p24 antigens.

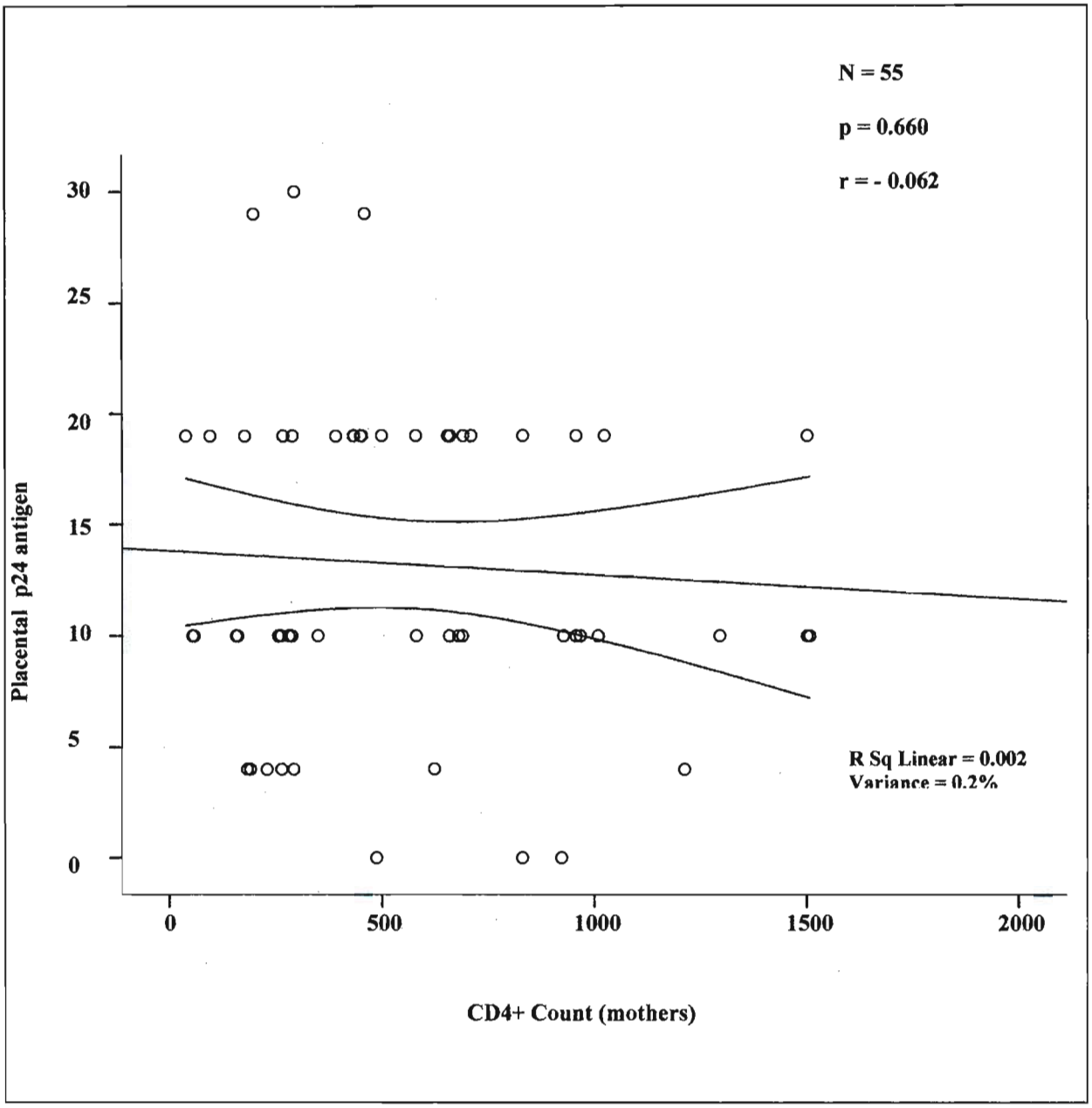
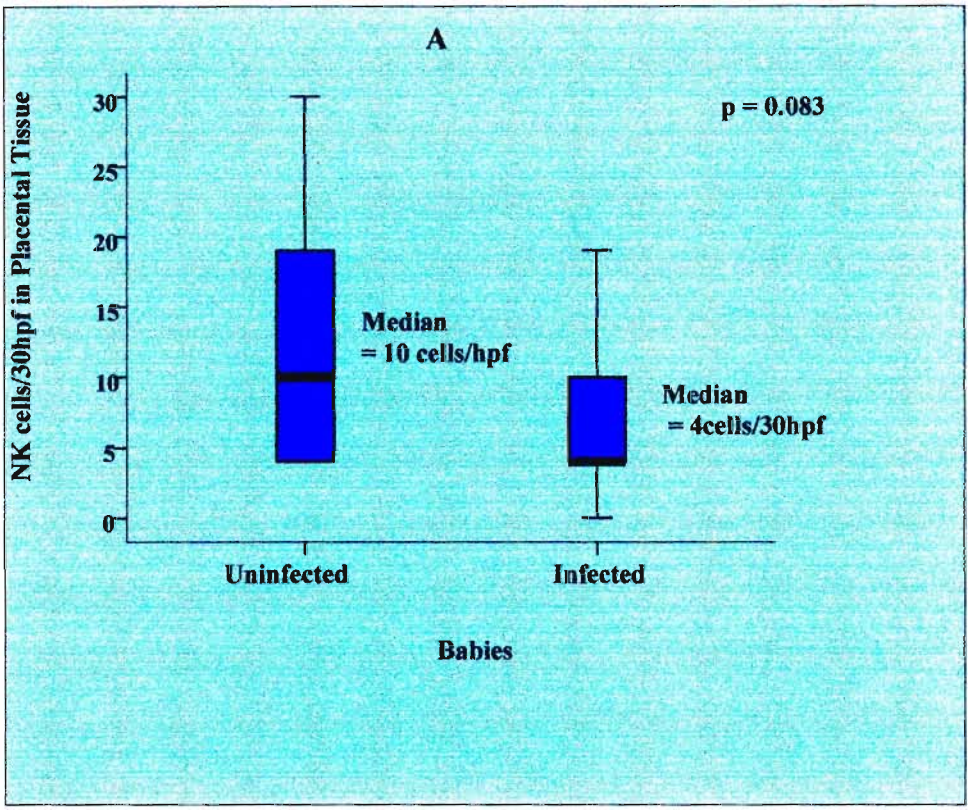


Figure 13: Scatter plot of mothers CD4+ cell count compared to the presence of p24 antigens in placental tissue of mothers.

5.5.3 Relationship between Placental Natural Killer Cells, Maternal CD 4+ Cell Counts and Babies Viral RNA Copies

The level of NK cell activity was examined in both groups i.e. mothers with uninfected and infected babies. The median NK cell counts for the uninfected and infected groups of babies were 10 cells/30hpf (IQR 5-20 cells/hpf) and 4 cells/30 hpf (10-20 cells/30hpf) respectively (Fig.14A). No significant correlation was observed at the 95% confidence level ($p = 0\ .083$). This could be due to a small sample size which, leads to lower statistical power and an increased probability of type II error (failure to reject a clinical null hypothesis). However, it was noted that there was a lower median NK cell value in mothers with infected babies in comparison to mothers with uninfected babies.

The distribution of scores when plotted on a stem and leaf plot showed that most of the data were clustered to the left. Therefore, we observed that the distribution was positively skewed with a median placental NK value of 10 cells/30hpf in mothers with uninfected babies and 4 in the infected group. The data was not normally distributed as indicated by the bimodal pattern (Fig.14B)



B

Placental NK Cell Values of Mothers with Uninfected Babies		Placental NK Cell Values of Mothers with Infected Babies	
Frequency	Stem and Leaf	Frequency	Stem and Leaf
14.00	0 . 44444444444444	9.00	0 . 044444444
.00	0 .	.00	0 .
12.00	1 . 000000000000	3.00	1 . 000
13.00	1 . 9999999999999	3.00	1 . 999
.00	2 .		
.00	2 .	**Median score = 4**	
1.00	3 . 0		
Median score = 10		width:	10
width:	10	each leaf:	1 case
each leaf:	1 case(s)		

Figure 14: **The presence of NK cells at the placental interface of mothers with uninfected (<400 copies/ml) and infected (> 400 copies/ml) babies. The median NK cell count in placental tissue of mothers with uninfected and infected groups of babies was 10 cells/30hpf and 4 cells/hpf respectively (A). The Stem and Leaf Plot represents the distribution of NK cell values found in placenta of mothers in both groups of babies (B).**

A logistic regression equation was performed to evaluate NK immune response at the placental interface and the protection it confers on babies against HIV-1 infection (Table XV). The variables which were statistically accepted were the log viral load of the mother, gender of babies and NK cell values. The protective effect of NK cells was evaluated in category B (≤4 NK cells/30hpf) and category C (10-19 NK cells/30hpf) using category A (20-30 cells/30hpf) as the baseline value. Category A was chosen, in the equation, as the baseline for evaluation, the basis being that having a low NK cell value is associated with the risk of infection when compared with a higher NK cell presence. Therefore, the highest category with the lowest risk was chosen as baseline. There was no statistically significant association in both categories B ($p = 0.145$; 95%CI 0.655 – 17.891) and C ($p= 0.940$; 95%CI 0.138 - 6.237). Data analysis revealed that the risk of vertical transmission was 3.4 times more in placentas which fell in category B (<4 NK cells/30hpf). Further regression analysis revealed that with every 1 log increase in maternal viral load the risk of transmission was increased approximately 3.3 times more. Maternal log viral load emerged as a significant predictor of viral transmission ($p = 0.047$). No statistically significant association was established between the presence of NK cells and gender ($p = 0.069$). However, even after adjustment for placental NK cells and maternal viral load, the male to female risk of acquiring the infection increased by 3.7 in favour of female babies.

Table XV: Logistic Regression to Determine the Predictive Value of NK Response in the Placenta and Risk of Infection in Babies. N=55

	Wald	df	Sig.	Odds Ratio	95.0% C.I.for EXP(B)	
					Lower	Upper
Log Viral Load of Mothers	3.943	1	0.047	3.278	1.016	10.578
Gender of Babies	3.309	1	0.069	3.701	0.904	15.159
NK Overall (20-30 cells/30hpf) Category A	3.386	2	0.184	-	-	-
NK (< 4 cells/30hpf vs* 20- 30 cells/30hpf) Category B	2.129	1	0.145	3.424	0.655	17.891
NK (10-19 cells/30hpf vs* 20-30 cells/30hpf) Category C	0.006	1	0.940	0.929	0.138	6.237
Constant	6.587	1	0.010	0.001	-	-

vs denotes versus

Babies CD4+ cell counts and placental p24 counts were added to the logistic regression model to assess the risk of babies acquiring the infection. Babies CD4+ cell counts (p = 0.582; 95%CI 0.140 to 1.00) and the presence of placental p24 antigen (p = 0.964; 95%CI 0.099 to 1.094) were not predictors to viral transmission. According to the odds ratio, babies CD4+ counts were affected by every 1 log increase in mother's viral load (Table XVI)

Table XVI: Logistic Regression to Determine the Predictive Value of Babies CD4+ Cell Counts and the Risk of Infection in Babies. N=55

	Wald	df	Sig.	Odds Ratio	95.0% C.I.for EXP(B)	
					Lower	Upper
Log Viral Load of Mothers	3.491	1	0.062	3.145	0.945	10.460
Gender of Babies	2.850	1	0.091	3.473	0.818	14.735
CD4+ cells (babies)	0.303	1	0.582	1.000	0.140	1.000
p24 antigens	0.002	1	0.964	0.998	0.999	1.094
Constant	0.464	1	0.035	0.001	-	-

5.5.4 Overall Presence of Inflammatory Cells in Placental Tissue

Distribution of neutrophils and lymphocytes (inflammatory cells) was investigated in all placental cells (Fig.15). Circulating inflammatory cells were differentiated from tissue inflammatory cells so as to adequately assess only the level of tissue immune response to infection (Fig.16). Overall, the median lymphocyte count in placental tissue of all mothers was 9.2% (IQR 0-16) and 90.9% (IQR 83.3-100) for neutrophils (Table XVII).

The cellular response in placental tissue of mothers with uninfected and infected babies was investigated. A median of 90.9% for neutrophils and 9.1% for lymphocytes was observed in placental tissue of mothers with uninfected babies (Fig.17). The median neurtophil and lymphocyte percentage in placental tissue of mothers in the infected group of babies was 91.7% and 8.3% respectively. The difference between placental inflammatory response of mothers with infected and uninfected babies was minimal. Viral transmission to the baby was not related to the presence or absence of neutrophils or lymphocytes. Generally the presence of leucocytes suggests an immunological response and the importance of these cells for foetal survival.

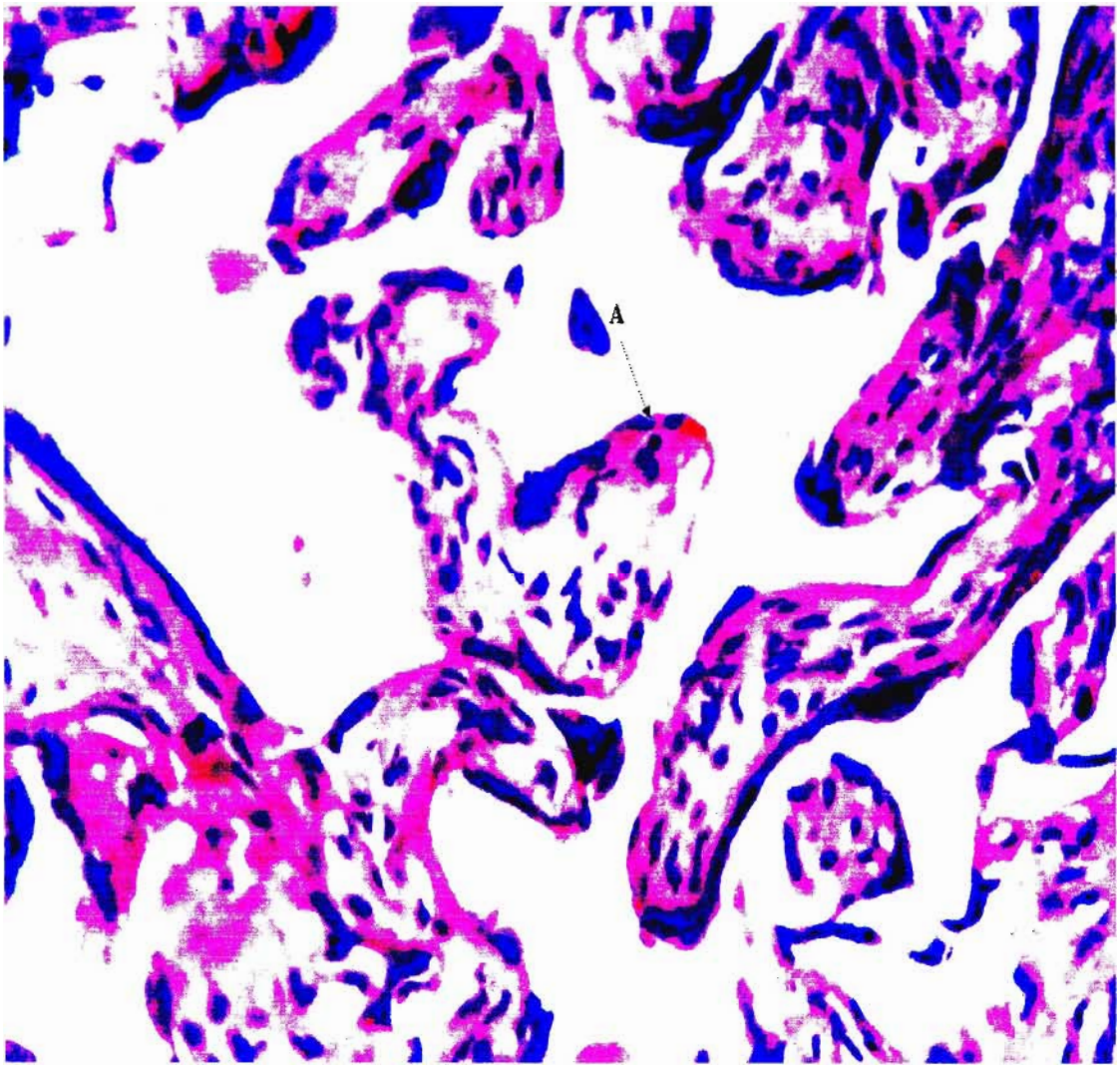


Figure 15: **Presence of lymphocyte in placental tissue (A). The tissue was stained using Haematoxylin and Eosin (HE) stain. Lymphocytes stained a deep blue.**

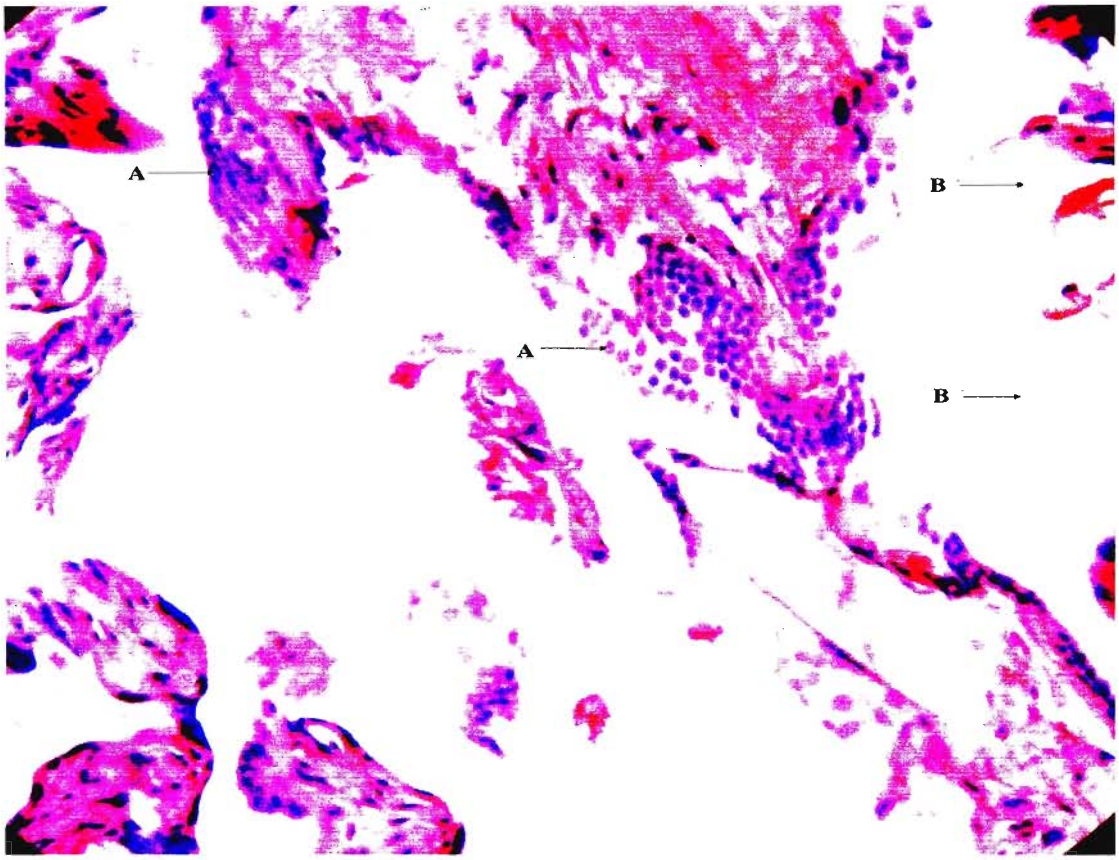


Figure 16: The outer layer of placental cells (syncytiotrophoblasts-A) forms a multinucleated epithelium maintained by an underlying population of cytotrophoblasts. Circulating leucocytes (arrow B) were differentiated from placental inflammatory cells.

Table XVII: Statistical Values of Tissue Lymphocytes and Neutrophils in Placental Tissue. **N=55**

Statistical Frequencies	Percentage Lymphocytes	Percentage Neutrophils
Mean	9.4	90.6
Median	9.1	90.9
Percentiles: 25	0.0	83.3
50	9.19	90.9
75	16.7	100

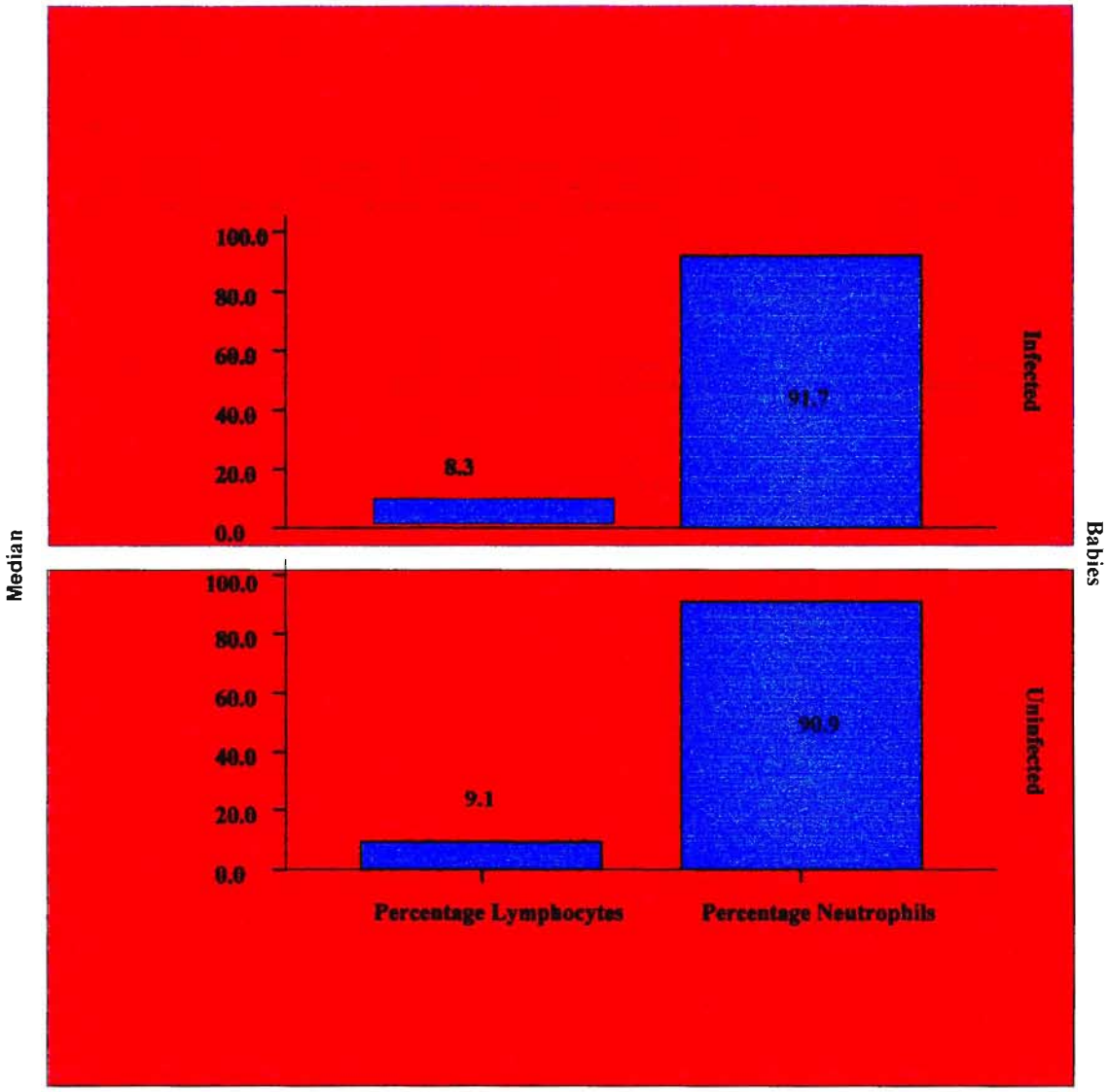


Figure17: Percentage of placental tissue neutrophils and lymphocytes in HIV-1 infected mothers with infected and uninfected babies.

The inflammatory cell response in the placentas of HIV-1 infected mothers was assessed against the viral RNA copies of the babies. The count was performed as number of inflammatory cells/30 hpf. The range between the two groups was above 5 and under 15 cells/30hpf (Fig.18). Only one infected baby ranked in the higher range (*). The uninfected group (A) displayed a slightly higher range of inflammatory cells then the infected group (B) but the median in both groups was the same (10 cells/30hpf).

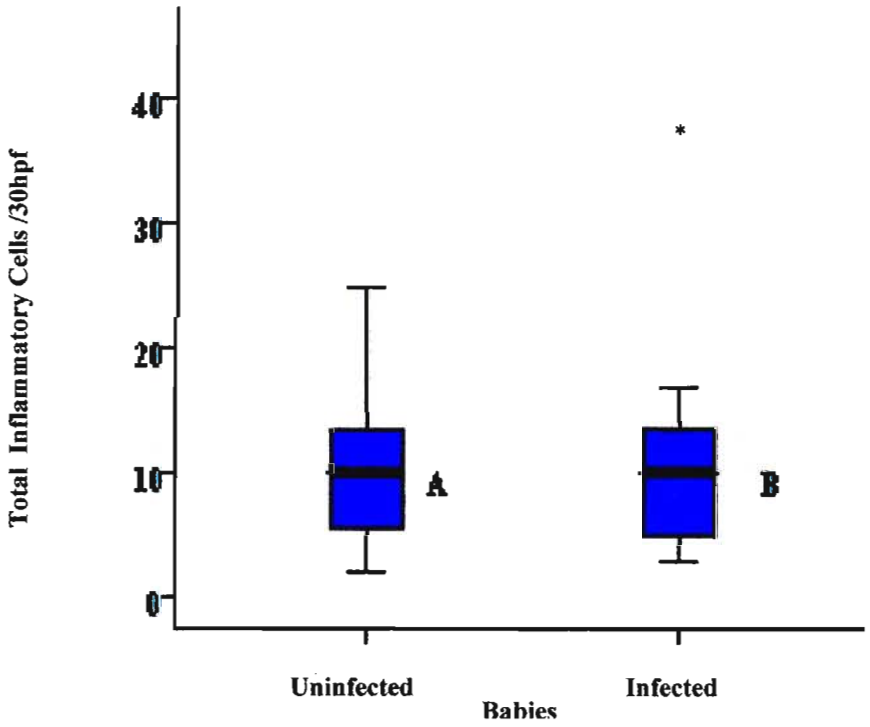


Figure 18: Inflammatory response in placenta of HIV-1 infected mothers with infected and uninfected babies. Median count for groups A and B was 10 cells/hpf.

5.6 Discussion

The purpose of the study in this section was to document the presence of HIV-1 in the placenta and to determine the relationship between the presence of NK cells (CD 56+) and p24 antigens in placental tissue. The presence of p24 in placentas was analysed in relation to immunological and virological factors and its implication in vertical transmission. Investigation into the mechanisms regulating the transmission of placental HIV has inherent difficulties. Quantitation of viral and cellular responses in placental tissue is a challenge because there are insufficient separation techniques to differentiate between trophoblastic cells from nontrophoblastic cells. Further difficulties are encountered in separating maternal cells from foetal cells at the placental interface. Therefore, we chose a staining technique which will allow some visual indication of identification. We used an immunohistochemical technique for quantifying the level of viral invasion. Although the stain is specific for p24 antigen it is unable to differentiate between cells containing viral antigens and those with viral particles, a limitation of evaluation using immunohistochemistry. However, observation of either viral antigens or viral particles indicates viral presence

Placentas were placed into two categories. Those with the presence of p24 antigens (infected) and those without p24 antigens (uninfected). Altogether, 94.6% of placentas in this study bore evidence of HIV-1 infection. A study conducted by Dictor *et al.* (2001) retrieved evidence of HIV infection in 14 of 39 (36%) placental samples from untreated HIV-1 infected women. Backe *et al.* (1992), using immunostaining techniques, found that 43% of 23 therapeutic abortions and 45.5% of 11 term placentas

from HIV-1 infected women had evidence of HIV antigens. These studies suggest that the transmission routes were:

- a haematogenous route from maternal intervillous space to villous stromal cells.
- bidirectional flow between chorion laeve and amniotic fluid. (Backe.*et al.*, 1992).

Further information from the study data partly suggested that transmission may occur:

- during early gestation by direct contact between basal deciduas and budding trophoblastic cells.
- Entry into foetal circulation via the small veins from the capsular deciduas.

Immunohistochemistry staining techniques were used for identification in these studies as well as in our study. The sample numbers were also small. However, there are substantial differences in detection levels in comparison to our study (94.6%) The difference may probably be due to study population from diverse geographical regions. Therefore, it is important to further interrogate contributing factors to HIV activity in placental tissue in different population groups.

Some studies examined placental tissue obtained from HIV-1 infected mothers and concluded that because of the absence of p24 antigens, the placenta forms an efficient barrier to viral transmission (Anderson *et al.*, 1997; Tscherring-Casper *et al.*, 1999). However, cohorts in these studies were undergoing antiretroviral therapy (zidovudine and reverse transcriptase inhibitors) during their pregnancy. In our study the mothers were antiretroviral naïve during pregnancy. Mattern *et al.*, (1992) found that in his

study the HIV antigen containing cells were very sparsely distributed and that evidence of viral replication was found in approximately 25% of 19 placentas. Other studies indicated that cells can be infected by HIV when coming into contact with infected leucocytes (Douglas *et al.*, 1997; Fazely *et al.*, 1997; Xiao *et al*; 1997).

Presence of p24 antigens in placental tissue was evaluated against the number of placental NK cells, maternal viral load and CD4+ cell counts. The analysis revealed that the presence of p24 antigens in placental tissue was not influenced by maternal viral load or CD4+ cell counts. These findings are in line with those of Dictor *et al.* (2001), who reported that there was no relation between placental infection and, either CD4+ counts or plasma viral loads. Furthermore, Maury *et al.* (1989) in her study demonstrated that an inversely proportional relationship exists between the presence of p24 and maternal viraemia. Dictor *et al* (2001) also commented that the inverse relationship may indicate differences in tropism between HIV-1 in placenta and plasma.

Natural Killer cells comprise between 6-21% of the total lymphocyte population (Gesuk *et al.*2002). Amplified responses of the NK (CD56+) cell at sites of viral replication produce and release numerous chemokines which are effective against certain pathogens. In our study plasma HIV-1 RNA levels in infected mothers did not appear to influence NK response in the placentas. Statistical limitations could be related to sample size because small sample size leads to lower statistical power.

In our study there was evidence of NK cell activity in majority of infected placentas but the level and intensity of the activity varied as observed by the differences in NK cell numbers in placental tissue. Welsh e*t al.* (1996) outlined the significant role of NK cells as active participants in immune response to pathogens and Gesuk *et al.* (2003) found that patients' deficient in NK cells and infants where NK cell compartments does not fully appear are immunocompromised. However, in our study CD56+ evaluation in peripheral blood was not established which limits any conclusive comments on the interaction of CD56+ between blood and placenta. Based on findings in pregnant women which show that increased numbers of circulating peripheral blood CD56+ cells results in greater presence of CD56+ cells at the placental interface (Kwak *et al.*,1995; Beer *et al.*,1996) we can make the assumption that placentas which demonstrated low CD56+ counts could possibly be due to low circulating peripheral blood CD56+. Larger studies are required to validate these findings.

Analysis of the functional activity of NK cells at the placental barrier induced by p24 stimulation antigen showed that there were lower median NK cell values in placentas of mothers with infected babies as compared with the uninfected cluster. Although not statistically significant, low placental NK cells were associated with the risk of infection when compared with higher NK cell presence. The risk of vertical transmission was increased 3.4 times more in placentas which had lower NK cell values. However, the association between placental NK cells and vertical transmission has not been conclusively established in other studies. In lieu of the limited information available for comparison with other studies, comments about the

interaction and role of NK cells in vertical transmission can only be made from data in this study. Further studies are required to test the validity of our observations.

Logistic regression analysis revealed that the risk of viral transmission increased in babies with lowered CD4+ cell counts. According to the odds ratio, babies CD4+ counts were affected by every 1 log increase in mother's viral load. However, CD4+ count and presence of p24 antigens did not emerge as predictors to vertical transmission. Given the lack of direct linkage between the detection of p24 in placental tissue and viral RNA copies in infected babies' plasma, other cell mediated immunological responses require investigating. Also, we cannot exclude the possibility that undetectable levels of viral copies in babies in contact with infected placentas will not at a later date emerge seropositive. Overall, maternal viral load emerged as a strong predictor for risk of infection from infected mothers to their infants. Our analysis indicated that female babies were 3.7 times more likely to acquire the infection than males. Biggar *et al* (2006) also reported that girls were at a higher risk of early (*in utero*) HIV infection than boys. These findings remain an interesting avenue of research for future studies.

The overall presence of neutrophils and lymphocytes (inflammatory response) in HIV infected placentas was assessed using a basic Haemotoxylin and Eosin staining technique. The presence of inflammatory cells was evaluated within trophoblastic cells. Trophoblasts have been proposed to play a critical role in modulating virus spread to the foetus ((Vidricaire *et al.*, 2003). To eliminate complications in assessment due to non-trophoblastic cells, circulating inflammatory cells infected with

the virus was not included in the evaluation. According to Tscherring-Casper (1999), p24 antigens in non-trophoblastic cells may be a reflection that the maternal blood in the placenta contains infected CD4+ cells.

The inflammatory cell response in placental tissue of mothers with infected babies and uninfected babies was minimal. No statistical significance was observed. Viral transmission to the babies was not associated with the presence or absence of placental neutrophils or lymphocytes. We report here from collective data that there was no difference in the total number of inflammatory cells between babies considered infected *in utero* and those uninfected at birth. Generally the presence of leucocytes suggests an immunological response and is important of cells for foetal survival (Vidricaire *et al.*2003).

In concluding this section of the investigation we found that 27.3% of babies were considered infected *in utero*. It appears that maternal immune competence in clearing or preventing p24 antigens from the placenta appears to be intact in 72.7% of mothers whose babies were born with undetectable levels of viral RNA copies at birth. We need to consider this result as a tentative estimation, since the babies were not available for retesting. Therefore, the limitation of the study is that it is difficult to comment on how many babies will eventually succumb to the virus.

SECTION C

CHAPTER SIX

CHAPTER 6

HUMAN LEUCOCYTE ANTIGEN -G GENE EXPRESSION AT THE PLACENTAL INTERFACE OF HIV-1 INFECTED MOTHERS AND ITS IMPLICATION IN *IN UTERO* TRANSMISSION OF HIV

6.1 Summary

Background: The ability of HLA-G to inhibit the cytolytic effect of immunocompetent cell types, namely NK cells suggests that HLA-G has a role in immunosurveillance. A high level of transcription and expression was found in foetal-maternal cells at the placental barrier indicating that the molecules confers maternal immunological tolerance of the foetal semi allograft (Menicucci *et al.*,1999). The major histocompatibility complex (MHC) gene products are critical in regulating many antiviral immune reactions (Lafon *et al.* 2005). Therefore, it is possible that the MHC-coded molecule HLA-G influences the course of HIV infection. The identification of gene transcripts associated with favourable or unfavourable outcomes of exposure to HIV may assist in developing and designing protective strategies to viral transmission from mother-to child.

Methods: Evaluation of the effect and level of the membrane–bound HLA-G1 expression was carried out on fifty five placental samples obtained from HIV-1 infected mothers at term. RNA was extracted from placental tissue samples and cDNA

was synthesized using the RNA extract. Amplification of cDNA using specifically designed primers complementary to the full length HLA-G1 isoform was quantified using real time-PCR. Agarose gel electrophoresis was used to confirm successful amplification of the HLA-G PCR product. Comparison between the level of placental CD56+ cells, HLA-G transcription and maternal and babies viral loads was done to evaluate the influence of HLA-G in MTCT.

Results: HLA-G primers detected the full length isoform HLA-G1 PCR product at 86.5°C. The range of transcriptional levels of HLA-G in placental samples was 0.21 to 6.64 with a mean value of 1.92. There was no significant correlation between HLA-G transcriptional levels and the level of NK cells at the placental barrier ($p = 0.841$). However, there was a positive correlation between mothers' log viral load and transmission of infection to the baby ($p = 0.045$; 95%CI 1.029- 11.499). Logistic regression calculations indicated that the risk of babies becoming infected was increased 1.29 with every 1 unit increase in HLA-G expression (molecules). The risk ratio for female babies was 3.40 times higher than males in acquiring the infection. Placental HLA-G expression was upregulated 3.95 times more in placentas of HIV-1 infected mothers with infected babies when compared to uninfected babies.

Conclusion: Collectively the results confirmed the presence of CD56+ human NK cells at the placental interface. However, the level of cytolytic activity was inconclusive. Maternal viral load was the strongest predictor of viral transmission. HLA-G expression was upregulated in placental tissue of HIV-1 infected mothers with infected babies and female babies were at a higher risk than males in acquiring the infection.

6.2 Introduction

At the implantation site, the pregnant uterine mucosa is infiltrated by large granular lymphocytes CD56+ a subset of NK cells. The population of trophoblastic cells that invade into the maternal deciduas express HLA-class I antigen HLA-G. The expression of HLA-G is normally restricted to the placenta during pregnancy specifically to those trophoblasts at the maternal- foetal interface. However, most evidence suggests that it protects the foetus from maternal uterine NK cell lysis (Dorling *et al.*, 2000). The immune response to HIV infection are complex, involving manifold interactions of regulatory and protective immunoactive cells. HLA-G may provide a signal to escape from NK cell- mediated cytotoxicity. Accordingly, once HLA-G is expressed, it becomes the major NK inhibitory ligand (Riteau *et al.* 2001).

HLA-G is produced as four membrane–bound (HLA-G1, G2, G3 and G4) and three soluble (HLA-G5, G6 and G7) isoforms by means of an alternative splicing of a single primary transcript (Carosella *et al.* 2003). Only the membrane bound HLA-G1 and the soluble HLA-G5 isoforms are associated with β_2 microglobulin, the binding site for CD8+ (1.14.6: Fig.3). These isoforms act in diverse inflammatory conditions protecting tissue against NK and T cell infiltration. Inhibition of these inflammatory cells creates an anti-inflammatory cytokine environment (Carosella *et al.*, 2003; Contini *et al.*, 2003; Le Discorde *et al.,* 2003). Destruction of T and NK cells occur when HLA-G binds to the inhibitory receptor KIR3DL4 expressed on NK cells and ILT receptors on both T and NK cells (Colonna *et al.*, 1997). The immunosubversive activity of HLA-G in non-viral inflammatory conditions extends to viral infections. It has been reported that HLA-G

expression in cells is also upregulated following infection with human cytomegalovirus (CMV) and HIV (Cabello *et al.*, 2003; Lozano *et al.*, 2002; Onno *et al.*, 2000). The suggestion is that viruses might use HLA-G to subvert adverse immune responses.

6.3 Participant Recruitment

The HIV in pregnancy study was designed to determine the interaction between NK cells and expression of HLA G. All fifty five HIV-1 infected pregnant women were randomly selected from an antenatal population and were antiretroviral naïve at delivery. Patient selection and recruitment is discussed in Chapter 3.

6.4 Specimen Collection and Storage

Placental tissue samples were collected immediately after delivery from all participants with prior informed consent. As site of collection and site of testing were great distances apart, fixation of samples in 10% formal saline was considered the most feasible method of preservation. Sections were stored at -70°C for RNA extraction and cDNA synthesis

6.5 Laboratory Methods

Extraction of RNA from placental tissue was followed by cDNA synthesis. The synthesized DNA was used to perform a quantitative analysis of HLA-G using real-time PCR. An in-depth explanation on procedures and protocol used to perform the RNA isolation and quantitation of expression of HLA-G is described in Chapter 3 Section C. The optimised parameters are presented in table format in Appendix VII.

6.6 Data Analysis

6.6.1 Quality Assessment of Ribose Nucleic Acid in Placental Tissue Extract

The quality of the RNA for cDNA synthesis was determined using agarose gel electrophoresis. The gel pattern of extracted RNA indicated successful extraction was accomplished. The evaluation was complete by the presence of a 28S and 18S subunit (Fig.19). The 28S and 18S ribosomal subunits show the presence of in tacked RNA. In tacked RNA was required to synthesize cDNA. Therefore, their presence indicated successful RNA extraction.

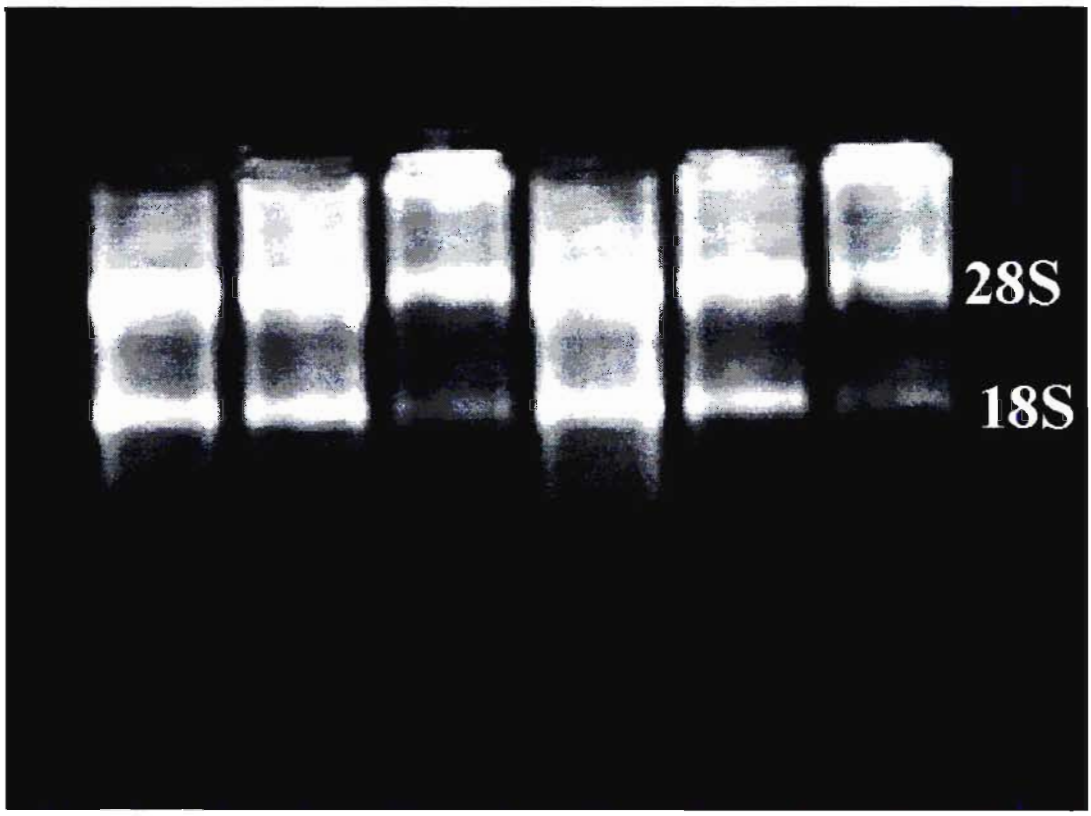


Figure 19: Representative gel electrophoresis of total RNA on 2% agarose gel. Electrophoresis was performed at 100 volts for 1 hour at room temperature.

6.6.2 Evaluation of Primer Specificity and PCR Efficiency

After RT-PCR the amplified HLA-G PCR products were validated for specificity. The RT-PCR products were analysed with a control marker on a 2% agarose gel using standard conditions in order to ensure that the PCR procedure successfully amplified cDNA. It was important to observe a single band on the agarose gel because multiple bands could interfere with distance measurements due to the formation of heteroduplexes between DNA strands of different sizes (Fig.20).

Validation of GAPDH specificity was performed using electrophoresis on 2% agarose gel. The GAPDH gene was successfully amplified as indicated by the presence of 257-bp fragment (Fig.21).

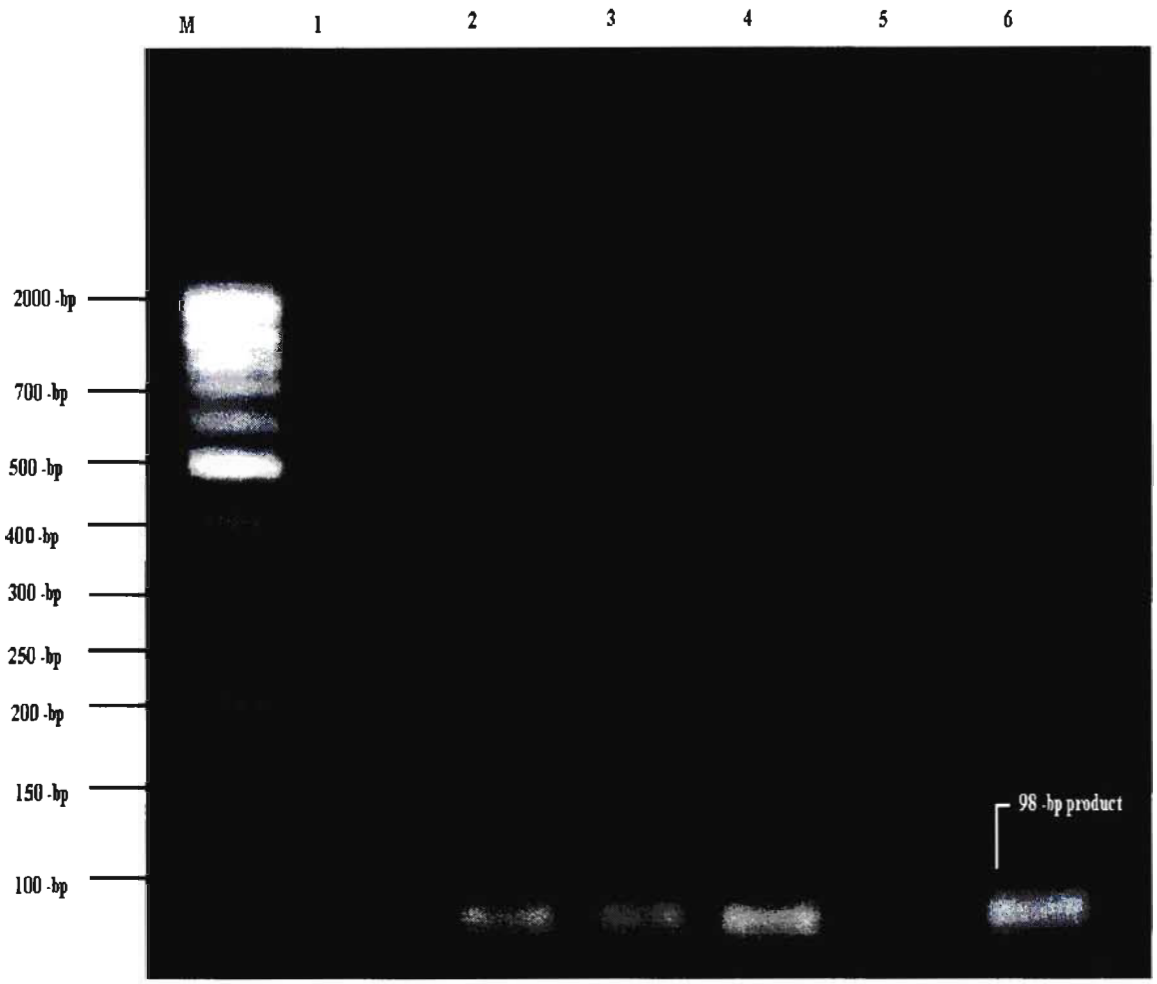


Figure 20: Representative gel electrophoresis (2% agar gel, stained with ethidium bromide) showing the 98 -bp HLA-G PCR product obtained using the LightCycler (lanes 3, 4, 5, 6). Lane (M) identifies the molecular weight marker (Fermentas #SMO373, GeneRuler, 50-bp to 2000-bp Ladder). Lane (1) is the negative control (water); Lane (2) is the standard. Successful amplification of the HLA-G gene is indicated by the presence of a 98-bp product fragment.

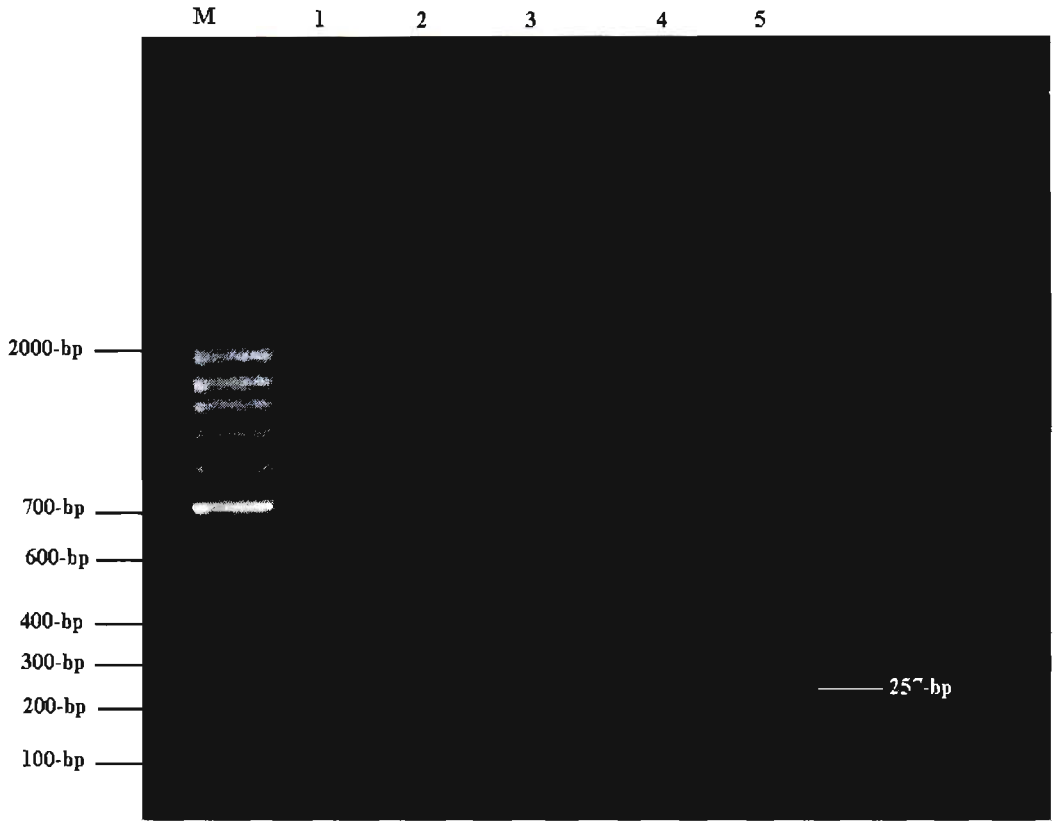


Figure 21: **Validation of GAPDH PCR specificity using electrophoresis on 2% agarose gel. M indicates the DNA marker size (50 -bp to 2000 -bp). Lane 1: negative control (water), Lane 2: real time-PCR standard, Lane 3- 5 GAPDH gene products. GAPDH gene was successfully amplified as indicated by the presence of 257-bp fragment.**

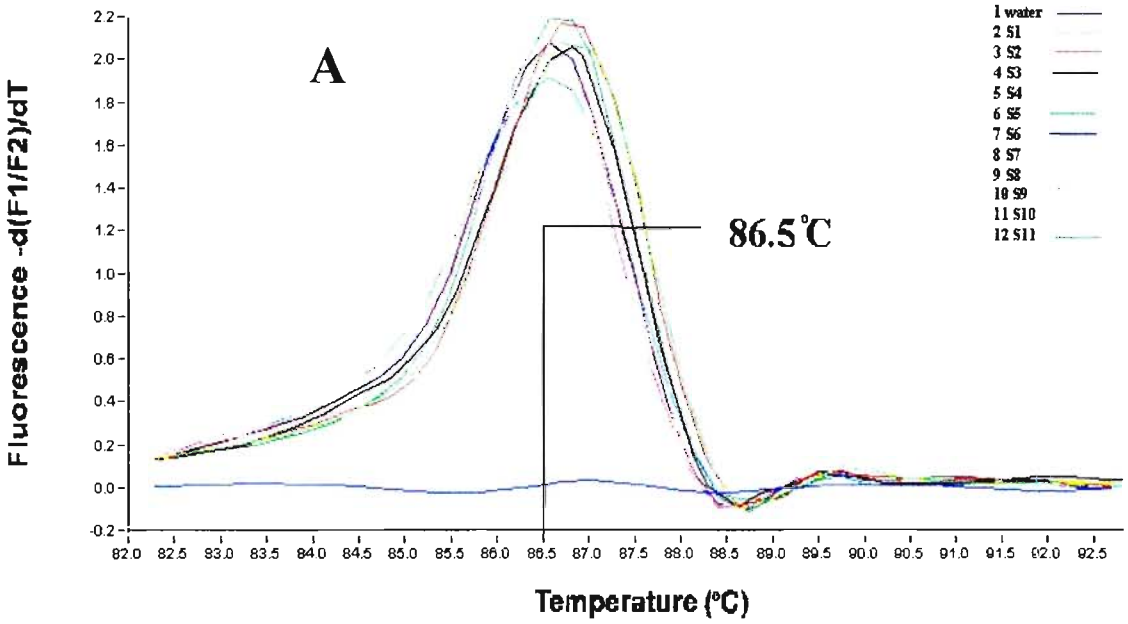
6.6.3 LightCycler Melt Curve Analysis of HLA-G and GAPDH Genes.

The results generated in the HLA-G quantification assay was analysed using the (Roché Molecular Biochemicals LightCycler Relative Quantification Software, Version 3.5). HLA-G positive samples gave a fluorescent signal for the 98-bp PCR product, characterized by a melting peak 86.5°C. As an internal control of amplification the fluorescent signal for the GAPDH 257-bp PCR product was detected, producing a melt peak at 84.5°C (Table XVIII). Despite the lower fragment length, the HLA-G specific PCR product (98-bp) yielded a higher T_m value compared with the GAPDH 257-bp product. This was due to the higher GC content of the HLA-G PCR product.

Melt analysis resulted in a single product-specific melting temperature of 86.5°C. There was no generation of primer dimer formations during the PCR amplification cycles. The HLA-G gene amplified for 40 cycles is represented in Figure 22A. The concentration of the patient samples were calculated, from the crossing points of the standards for HLA-G (Fig.22B) and GAPDH (Fig.23). Calculations were performed using the "second derivative method".

Table XVIII: Melting Curve Analysis of HLA-G and GAPDH Products

PCR Products	T_m of Melting Peaks (°C)	Fragment Length (-bp)
HLA-G	86.5	98
GAPDH	84.5	257



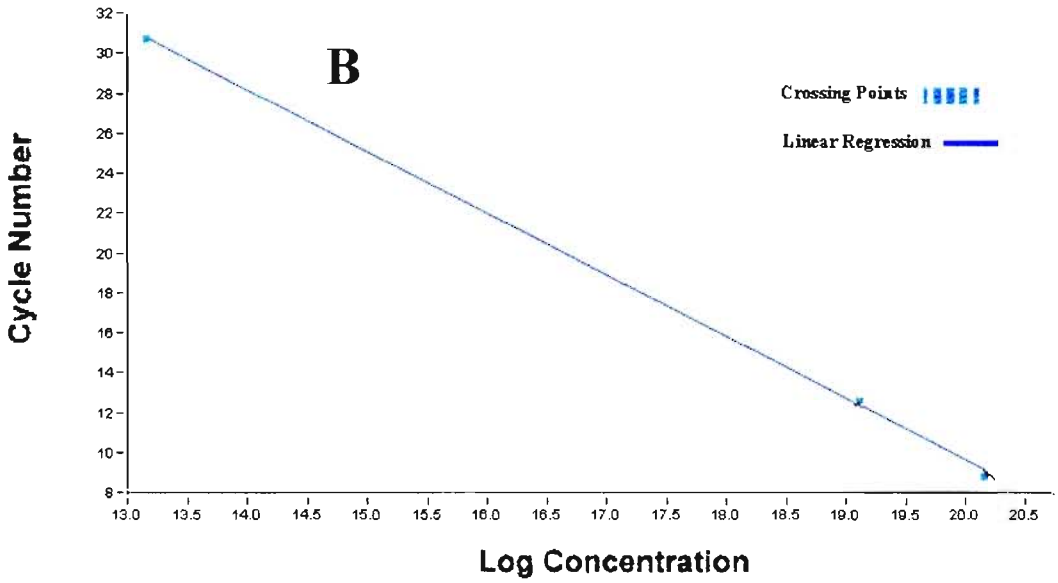


Figure 22: Melting peaks for HLA-G products. Melting curves were converted to melting peaks by plotting the negative derivative of the fluorescence channel with respect to temperature against temperature [-d (F1)/d T vs T]. The melting temperature (T_m) of the HLA-G gene was measured at 86.5°C (A). The calibration curve represents the crossing points (cycle number) (B).

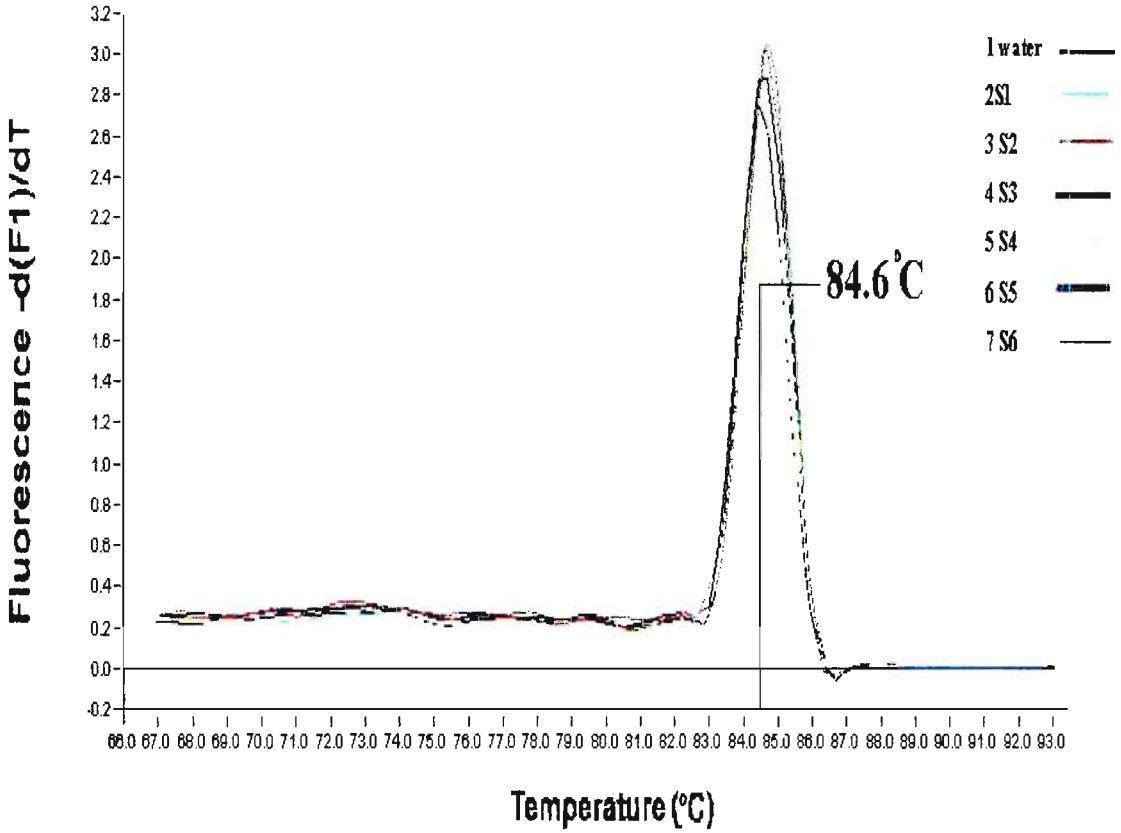


Figure 23: **Melting Peaks for the GAPDH gene. A representative real time PCR profile for the internal control GAPDH which was amplified for 40 cycles with a single product-specific at a T_m of 84.6°C.**

6.6.4 Standard Curve and PCR Efficiency

Use of real-time PCR to amplify cDNA products from mRNA expression is an efficient tool to study low copies of gene expression. It provides the necessary exactness and produces rapid and reliable quantification with the relevant mathematical formulation for data analysis. The relative expression ratio (R) of a target gene in comparison with a reference gene is computed based on the PCR efficiencies (E) and the CD deviation (delta CP). The equation of expression is calculated:

$$R = \frac{(E_{target})^{\Delta CP\ target\ (control-sample)}}{(E_{ref})^{\Delta\ CP\ ref\ (control\ -\ sample)}}$$

with C and Δ representing the crossing points and delta respectively.

A representative real-time PCR standard curve profile for HLA-G which was amplified for 40 cycles is illustrated in Figure 24. This was the initial standard used to extrapolate the standard curve for HLA-G. HLA-G levels were calculated as per equation in Chapter 3 (3.4.8.2). The calculated number of moles was 1.44×10^{23}. A series of dilutions were performed on PCR products starting from 10^{-1} to 10^{-12}. The standard curve was extrapolated using dilutions 10^{-4}, 10^{-6}, 10^{-8}, 10^{-10}, and 10^{-12} (Fig.25). Standard concentrations used ranged from 1.44×10^{15} to 1.44×10^{20} molecules.

Linear regression analysis indicated the slope of the reaction curve in the exponential phase. The slope was -3.452 and the amplification efficiency (E) calculated using the formula E (amplification efficiency) = $10^{-1/slope}$ was 1.94. A theoretical efficiency of 2, suggests that 100% of the template is converted to products.

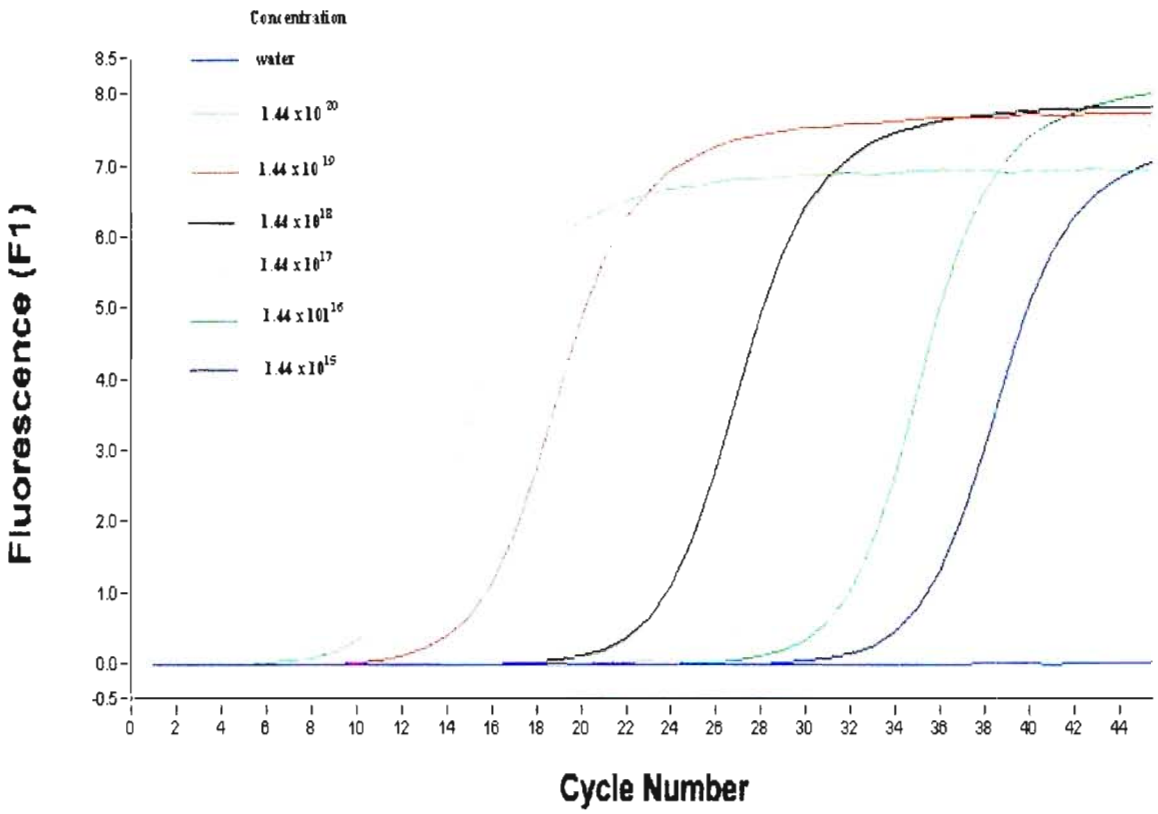


Figure 24: LightCycler amplification curve of HLA-G standards using SYBR Green dye I. Dilution concentrations ranged from11.44 x10^{15} to 1.44 x 10^{20}. Reactions show a series of equidistance curves in order of dilution. The flat line represents the water control. The exponential phase of the PCR corresponds to the log-linear segment of the curves.

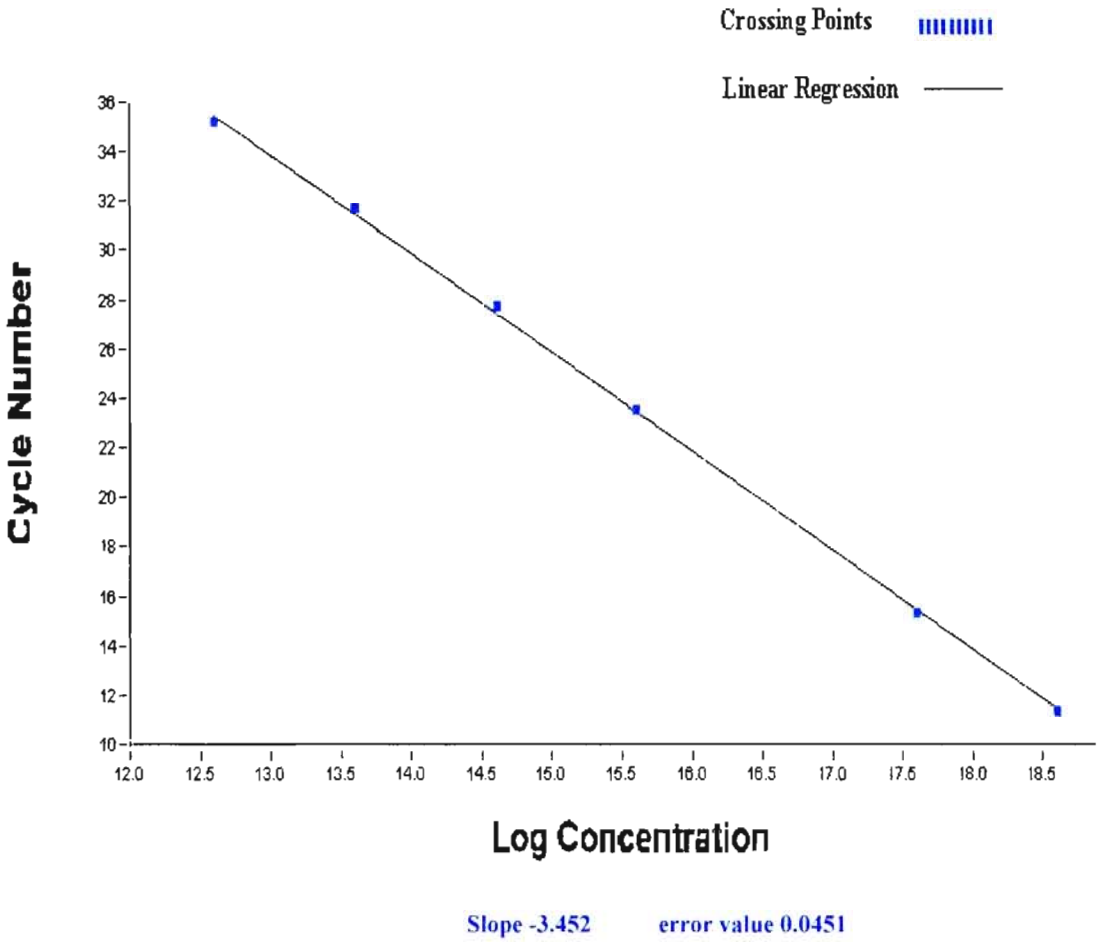
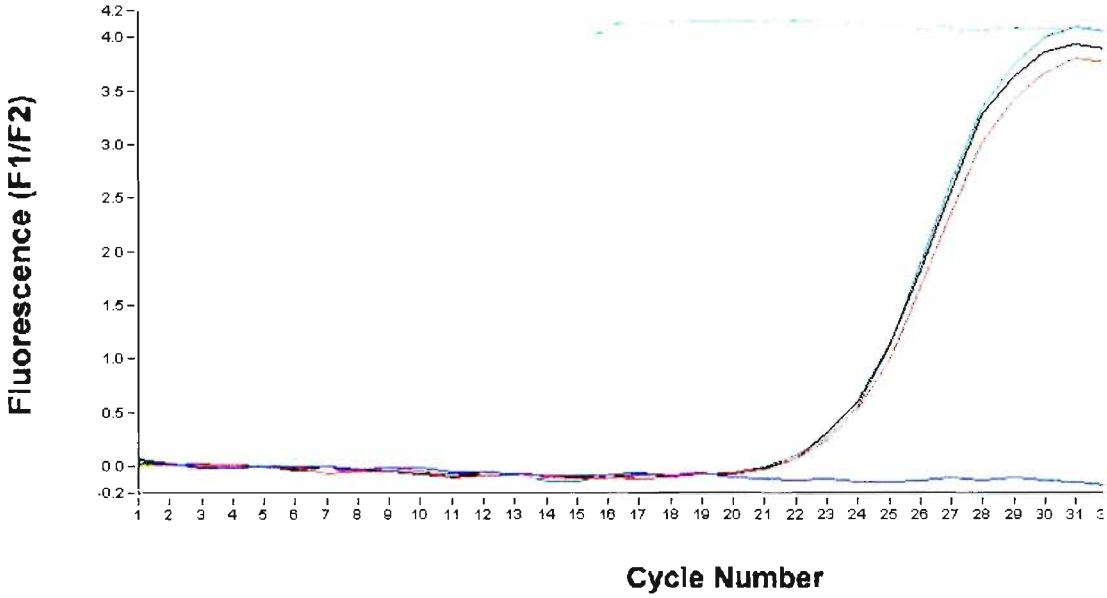


Figure 25: **Linear regression analysis of data generated in LightCycler amplification of HLA-G standards (Figure 24). The calibration curve shows the crossing points (cycle number) of each standard plotted against the logarithmic concentration to produce a standard curve. The error value was 0.0451 with an R value of 1.00 and the efficiency of the standard curve was 1.94. The slope for this analysis was -3.452.**

6.6.5 Quantification of Human Leucocyte Antigen-G in Placental Tissue using Real-Time PCR on the LightCycler

The concentration of unknown samples was calculated from the crossing points of the standards for each gene (HLA-G and GAPDH). The calculation was performed using the "second derivative method'' (Roche Molecular Biochemicals LightCycler Relative Quantification Software, Version 3.5). The method was used for the quantification of both HLA-G and GAPDH genes. Representative graphs showing quantification of HLA-G are shown in Figure 26 A. Figure 26 B represents the calculated concentration of sample values.

A



B

Sample	Concentration	Crossing Point
water	-	-
Std 1	1.44×10^{20}	9.692
Sample 1	2.5×10^{14}	29.85
Sample 4	1.7×10^{14}	30.48
Sample 5	2.330×10^{14}	30.1
Sample 9	2.360×10^{14}	29.99

Figure 26: Representative graph showing the quantification of the HLA-G gene in placental tissue samples. (A) is the logarithmic plot of fluorescence versus cycle numbers. Representative profile of sample data and their calculated concentration is shown in (B)

6.6.6 Placental Expression of Human Leucocyte Antigen-G and its Interaction with NK Cells in HIV-1 Infected Pregnant Women.

HLA-G expression using real-time PCR was calculated as a ratio using HLA-G/GAPDH values. The expression levels of HLA-G detected in placental samples ranged from 0.21 to 6.64 with a mean value of 1.92. The median ratio was 1.33 with an interquartile range from 0.56 to 2.79 (Table XIX). Normal ranges have not been previously established for HLA-G expression in placental tissue. Comments on the reference ranges are restricted. Comparisons can only be made according to fold increases.

The expression of HLA-G in HIV-1 infected placental tissue showed no significant correlation when compared to the presence of NK cells ($p = 0.841$). There were high NK cell counts with lowered HLA-G levels, low NK cell counts with high HLA-G levels and high NK cell numbers with increased expression of HLA-G (Fig.27).

Table XIX: Descriptive Statistical Values of HLA-G Expression in HIV-1 Infected Placental Tissue. N=55

Frequencies	HLA-G/GAPDH Values
Mean	1.92
Median	1.33
Min	0.21
Max	6.64
Percentiles: 25	0.56
50	1.33
75	2.79

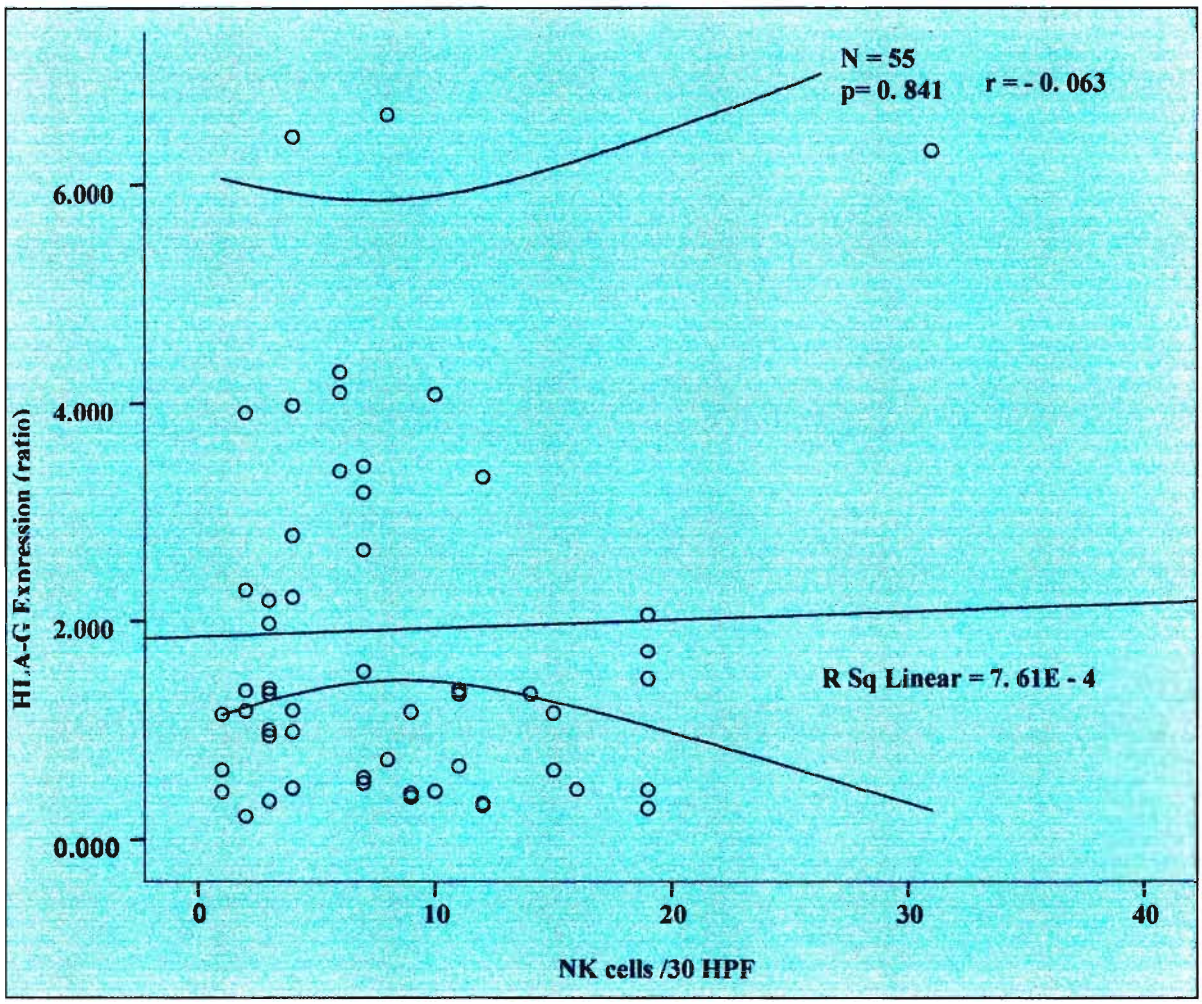


Figure 27: Scatter Plot showing the correlation between NK cells/30hpf and HLA-G expression.

6.6.7 Relationship between Human Leucocyte Antigen-G and Maternal Viraemia in Mother-to-Child Transmission of HIV Infection

A logistic regression analysis was carried out to determine the relationship between maternal viral load and HLA-G expression in vertical transmission of HIV infection (Table XX). There was a positive correlation between mothers log viral load and transmission of infection to the baby (p = 0.045; 95%CI 1.029 - 11.499). No significant correlation was noted with HLA-G (p = 0.066; 95%CI 0.983 - 1.712). However, the odds ratio indicated that the risk of infection increased by 1.3 with every 1 unit increase in HLA-G expression. No statistical significance was observed with gender of babies and vertical transmission (p = 0.088; 95%CI 0.833 - 13.881). According to our regression analysis female babies are 3.4 times more likely to become infected than males.

Table XX: Logistic Regression to Determine the Predictive Value of Maternal Viraemia, HLA-G Expression and Babies Gender.

	Wald	df	Sig.	Odds Ratio	95.0% C.I.for EXP(B)	
					Lower	Upper
Mothers Log Viral Load	4.023	1	0.045	3.439	1.029	11.499
HLA-G	3.376	1	0.066	1.297	0.983	1.712
Gender coded	2.906	1	0.088	3.400	0.833	13.881
Constant	6.743	1	0.009	0.000		

Variables entered: log viral load of mother, HLA-G level of expression, gender of babies.

A threshold value of 1 was used for fold changes. Placental fold increases in HLA-G expression was observed in 37(67.2%) women, whilst 18(32.7%) demonstrated HLA-G placental values <1 fold (Figure 28). A positive correlation between maternal viral load and placental HLA-G was observed ($p = 0.038$). However, there was a wide scatter of points around the central line and some quite strong outliers. The outliers showed a high viral load and low fold changes and vice versa. Although statistically significant, there is a lack of evidence of clinical significance between the two variables. This may be so because of sample size.

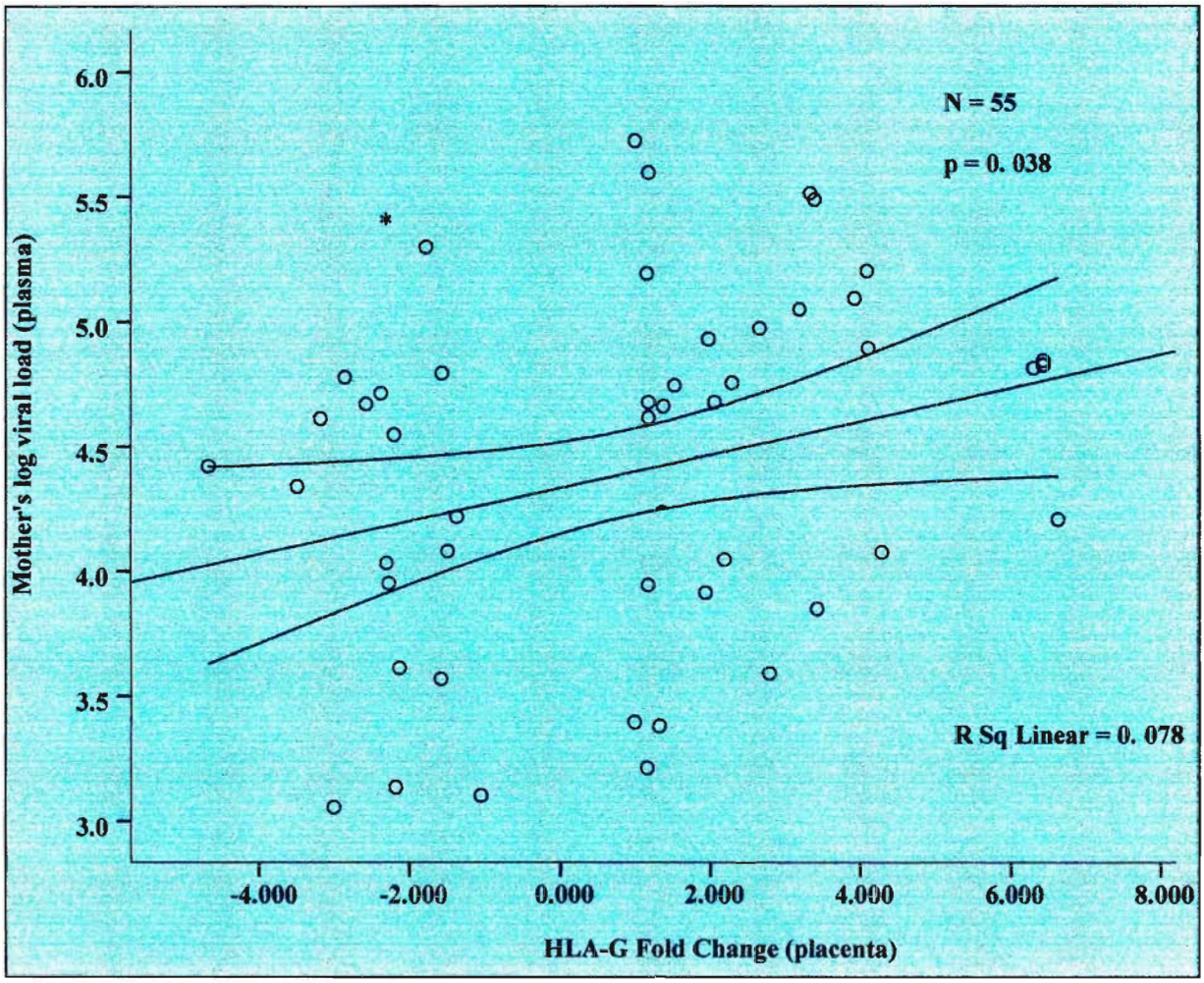


Figure 28: Scatter Plot showing the correlation between mother's log viral load and HLA-G fold changes in placental tissue. Negative values indicate a fold decrease in HLA-G expression and positive values represent a fold increase in HLA-G expression.

6.6.8 Influence of HLA-G on Babies Viral Load

In the following analyses the infants were grouped according to their viral load at birth, those infants with viral RNA copies <400 and those with RNA viral copies >400. The vertical transmission rate was 27.3%. The remaining 72.7% of babies had undetectable viral RNA copies. The correlation of placental HLA-G ratios and plasma viral load of babies in both the infected and uninfected groups just falls on the level of significance ($p = 0.05$). The absolute difference in median values between the infected babies (2.22) and the uninfected babies (1.17) is approximately 1 (Table XXI).

Table XXI: Descriptive Statistical Ratio Values of HLA-G Expression in Placental Tissue of Mothers with Infected and Uninfected Babies. N=55

Frequencies (ratios)	Infected (>400copies)	Uninfected (<400copies)
Mean	2.82	1.58
Median	2.22	1.17
Min	0.31	0.21
Max	6.64	6.31
Std Deviation	2.18	1.42

In the infected group the mean HLA-G fold increase was 2.17 (SD = 3.04) with a median of 2.06. In the uninfected group the mean was 0.55 (SD = 2.57) and median 1.18. The median values were higher in the infected than in the uninfected groups of babies. The minimum and maximum fold increase values ranged from (-3.19)* to 6.64 and (-4.67)* to 6.31 in the infected and uninfected group of babies respectively (Table XXII).

Table XXII: Descriptive Statistical Fold Change Values of HLA-G Expression In Placental Tissue of Mothers with Infected and Uninfected Babies. N=55

Frequencies	Infected (>400copies)	Uninfected (<400copies)
Mean	2.17	0.55
Median	2.06	1.18
Min	-3.19*	-4.67*
Max	6.64	6.31
	3.04	2.57

- ***minus denotes fold change below the threshold value of 1.***

Comparison of placental HLA-G fold changes and babies viral loads revealed that 12 (34.3%) infected babies and 23 (65.7%) of uninfected had increased fold changes. The placental HLA-G fold changes in 3 (16.7 %) and 15 (83.3%) infected and uninfected babies respectively was below the threshold value of 1fold (fold decrease). Increased fold changes in 34.3% of the infected babies' population as compared to fold decrease in 16.7% in the same population revealed an odds ratio of 2.61. Although there was no statistical significance ($p = 0.177$) OR indicates that the baby is more likely to be infected if placental HLA-G is increased. (Table XXIII). However, larger studies are required to confirm findings.

The implication of HLA-G in transmission between males and females was sought (Fig.29). The median HLA-G ratio was higher in infected male babies (2.3) than in uninfected male babies (1.3). A statistically significant association was observed ($p = 0.013$). The median HLA-G values of infected and uninfected female babies were 1.2 and 1.3 respectively. The females showed no difference in values ($p = 0.963$). Overall, HLA-G emerged as a factor for infection in males

Table XXIII: Fold Changes in HLA-G Expression in Placental Tissue of Mothers with Infected and Uninfected Babies. N=55

Placental Tissue of HIV-1 Infected Mothers	Number of Babies (%)		
Fold Changes	**Uninfected (<400viral copies/ml)**	**Infected (>400 viral copies/ml)**	**Total**
Increase	23 (65.7)	12 (34.3)	35 (100)
Decrease	15 (83.3)	3 (16.7)	18 (100)
No Changes	2 (100)	0 (0)	2 (100)
Total	40 (72.7)	15 (27.3)	55 (100)

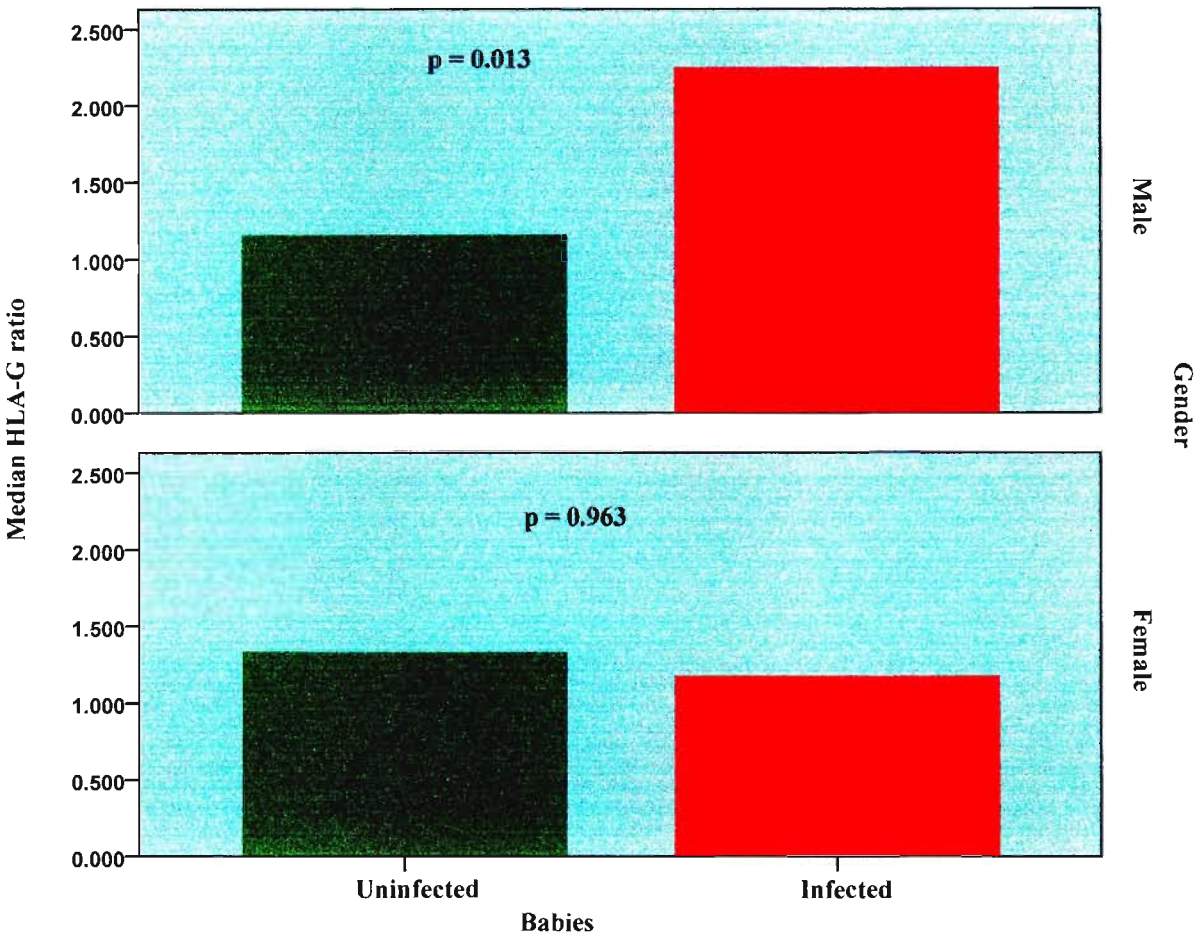


Figure 29: Comparison of HLA-G to gender specificity. The difference in median values between infected and uninfected male babies was significant. Median HLA-G values for female babies in both the infected and uninfected groups were minimal

To assess the upregulation of HLA-G expression the following calculation was used: mean value of HLA-G expression (2.17) in mothers with infected in babies (>400 copies/ml) divided by the mean value of HLA-G (0.55) in mothers with uninfected babies (< 400 copies/ml). HLA-G expression was upregulated 3.95 times more in placental tissue of HIV-1 infected mothers with infected babies (Fig.30).

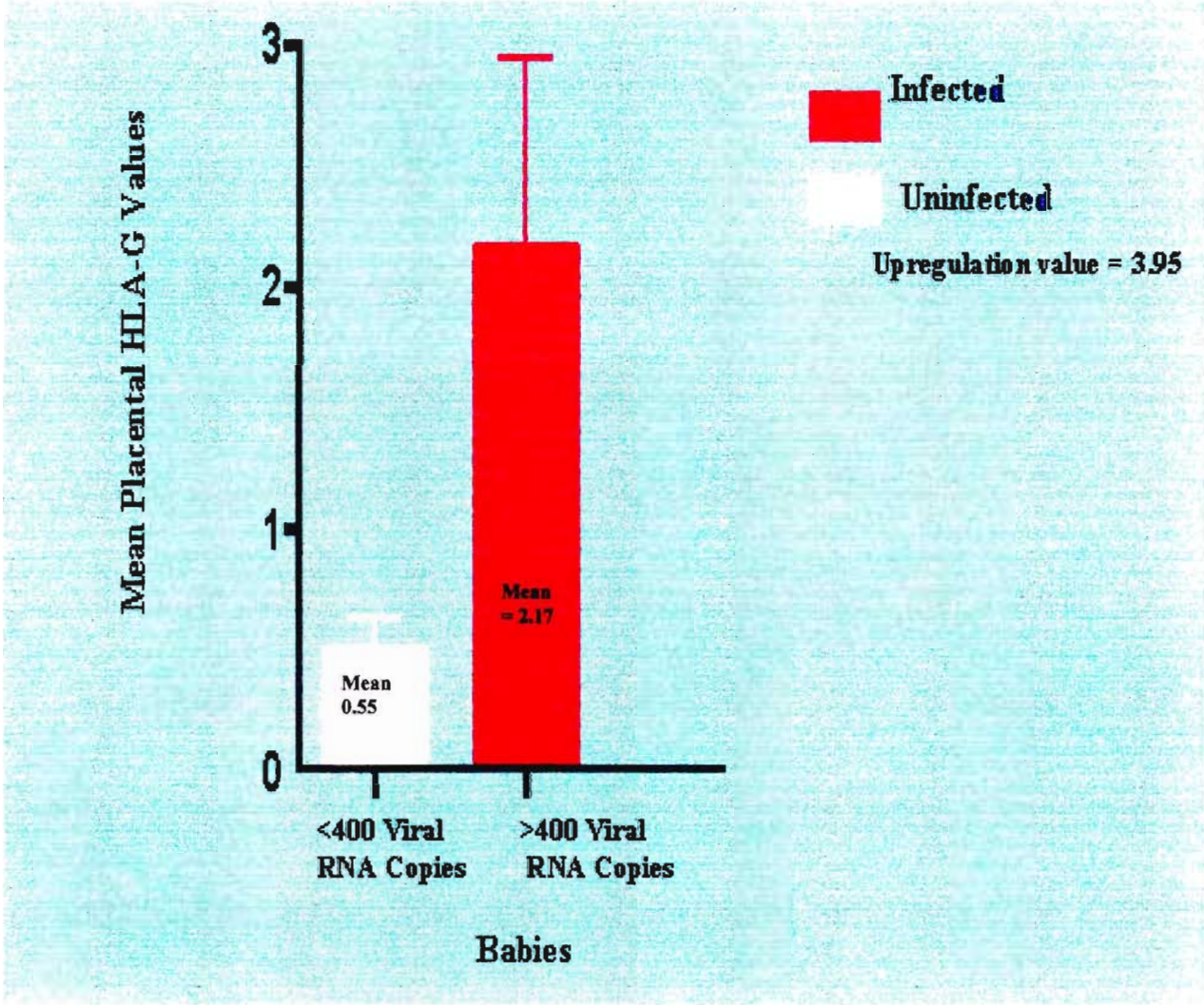


Figure 30: Mean placental HLA-G transcriptional values compared to viral loads of infected (>400 viral RNA copies/ml) and uninfected (<400 viral RNA copies/ml) babies. HLA-G expression was upregulated 3.95 times more in placentas of mothers with infected babies.

6.7 Discussion

The focus of this section of the study was to optimise an assay for the quantification of HLA-G and use the data to assess the influence of HLA-G expression in vertical transmission of HIV from mother to child. A PCR based assay was appropriate because PCR based assays have the ability to amplify low copy gene sequences. Real-Time PCR was used as it quantifies the amount of starting material in samples with specifically designed primers. A further advantage of RT-PCR is its accuracy and reproducibility of sample results. Its performance in a closed system with direct analysis of results reduces the number of manipulations and the risk of contamination within the working environment. The instrument used to perform quantitative evaluation was the LightCycler (Roché Diagnostics, Germany). In order to proceed with quantification RNA was extracted from placental tissue. The purity of the extracted RNA was validated and confirmed with electrophoresis on 2% agarose. Complementary DNA was synthesized from RNA and used as starting material in the LightCycler. Successful optimisation using the LightCycler was achieved and HLA-G was quantified for analysis.

HLA-G transcription was detected in 100% of placental samples. Analysis of HLA-G transcriptional levels was carried out in conjunction with the presence of NK cells and maternal viral load.

The interaction between placental NK (CD56+) cells and HLA-G was investigated. Evidence suggests that HLA-G protects foetal cells from lyses by maternal uterine NK

cells, which are found in large numbers around invading trophoblastic cells (Dorling *et al.*, 2000). Another function of HLA-G is to inhibit NK cell migration (Dorling *et al.*, 2000). Analysis of the interaction between NK activity and HLA-G expression at the placental interface showed no significant correlation. Studies by Masour *et al.* (1990) indicated that HIV-infected patients showed a significant decrease in NK cell subsets. Therefore, if circulating NK cells are depleted by HIV, the NK response in the mother's immune system can range from adequate to suppressed. In studies conducted by other researchers (Henderson AD, 1995; Lucia *et al.*, 1995) findings indicated that there was a selective depletion of NK cells amongst HIV-1 infected individuals. However, in this study NK activity in peripheral circulating blood was not established. Based on the findings of other researchers we can assume that since all the mothers in this study were HIV-1 infected, circulating NK cell levels will be low leading to low NK activity at the placental interface. The ability of HLA-G to inhibit the immune effect of two immunocompetent cells types namely NK and T cells, suggests that HLA-G may assist viral particles to evade the immune system (Lefebvre *et al.*, 1999).

One of the strategies that the human immunodeficiency virus has evolved is regulation of the major histocompatibility complex (MHC) class I molecules to evade recognition by the host immune system (Nattermann *et al.*, 2005). Increased MHC-expression favours inhibition of NK cell cytotoxicity and impairs antiviral activities of NK cells (Nattermann *et al.*, 2005). Evidence implicating the MHC gene in HIV disease progression and loss of CD4+ T cells has been documented (Mc Neil *et al.*, 1997)

Some authors have commented that HLA-G expression occurs in a complex manner by several cytokines, including beta interferon, glucocorticoid treatment and stress (Chu *et al.*, 1999; Lefebvre *et al.*, 2001). However, it has also been reported that HLA-G expression has been up regulated with CMV and human immunodeficiency.

Previous studies investigated the functional expression of HLA-G either in transfected cell lines, anti-HLA class I monoclonal antibodies or western blotting techniques (Rouas-Freiss *et al.*, 1997; Paul *et al.*,1998; Moreau *et al.*, 2003). This study evaluated the level of HLA-G expression using real-time PCR on the LightCycler (Roche Diagnostics, Germany). Using data obtained from real-time PCR we investigated the relationship between maternal viral load and the quantity of HLA-G expression. Logistic regression models revealed that mothers viral load was the strongest risk factor for viral transmission to baby. A positive correlation between mothers log viral load and transmission of infection to the babies was observed. No statistically significant correlation was noted with HLA-G and viral transmission. However, the odds ratio indicated that the risk of infection increased by 1.3 with every 1 fold increase in HLA-G expression. An analysis of mother-to-child transmission rates by gender revealed that the odds ratio for transmission was 3.4 times more in female babies than in males. Similar findings that girls were at a higher risk of *in utero* HIV infection than boys were reported by Biggar *et al.* (2006). The author proposed that minor histocompatibility reactions between maternal lymphocytes and Y chromosome-n derived antigens reduce the risk of HIV transmission in boys.

We then investigated the relationship between maternal viraemia and HLA-G expression. A positive correlation between maternal viral load and placental HLA-G was observed. Although statistically significant, there is a lack of evidence of clinical significance between the two variables. Data consolidated by Lafon *et al.* (2005) indicated that up regulation of HLA-G expression impeded host antiviral responses based on NK and T cells. The author further suggests that defects in surface HLA-G expression at too low a density may maintain immune pressure, leading to latency (Lafon.*et al.*, 2005). According to Emmer *et al* (2002) lower transcriptional levels may directly correlate with decreased protein expression.

Comparison of placental HLA-G fold changes and babies viral loads revealed there was no statistical significance. Increased fold changes in 34.3% of the infected babies as compared to fold decrease in 16.7% in the same group revealed an odds ratio of 2.6. The likelihood of babies becoming infected was 2.6 times more if placental HLA-G was increased. However, larger studies are required to confirm findings.

In the logistic regression analysis, although females were 3.4 times more likely to acquire the infection, there was no statistically significant association between male and female babies and HLA-G expression. However, when gender susceptibility to HLA-G expression was explored graphically the median HLA-G ratio was lower in infected males than in uninfected males. A statistically significant association was observed in placental tissue of mothers with infected and uninfected male babies and HLA-G expression. No difference in HLA-G expression was observed between placental tissue of mothers with infected and uninfected female babies. HLA-G

emerged as a significant risk factor in males than in females. In view of these discrepancies a separate analysis needs to be conducted on male and female babies to establish the risk of infection. Other factors besides the presence of HLA-G could have increased the 3.4 odds ratio in favour of females.

To continue the debate, if the risk factor occurred or presented itself before transmission then it lends evidence towards casualty (Bradford Hills,1965). Existing knowledge about the exact timing of viral transmission has mainly been made based on detectable levels of the virus from birth to seroconversion. According to the mathematical modeling theory (Markov), *in utero* transmission occurs relatively late in gestation (Rouiz, 1995). Several studies have established that HLA-G is expressed at the placental interface early in pregnancy as a protective mechanism for the foetus against maternal NK cells (Nagler *et al.*, 1989; Dorling *et al.*, 2000). Using this as our basis for evidence we can assume that HLA-G was present at the placental interface in early pregnancy. Therefore, if we apply the information generated from the Markov modeling for transmission timing and combine it with the evidence presented in the HLA-G studies, we can conclude that HLA-G is present before transmission of virus. Therefore, HLA-G lends itself as a contributing risk factor. Further experiments are required to conclusively establish casualty. To conclude the analysis in this section we found that HLA-G was up regulated 3.95 times more in placental tissue of mothers with infected babies than in mothers with uninfected babies.

CHAPTER SEVEN

CHAPTER 7

OVERALL DISCUSSION OF RESULTS

7.1 Overview of Study

This study set out to establish the relationship between HIV-1 infected pregnant mother's immune system and an associated immune response in *in- utero* infected infants. Specifically, it investigates the implication of HLA –G expression in *in utero* transmission of the virus in HIV positive pregnant women. A series of immunological investigations were performed on a group of fifty-five HIV-seropositive pregnant women and their babies at birth. The variables included in the evaluation were maternal viral load and CD4+ cell counts and babies' viral RNA copies and CD4+ lymphocyte count at birth. Other parameters in the investigation were the presence of p24 antigens, NK cells and the level of HLA-G expression in placental tissue.

7.2 Maternal Immune Response in HIV-1 Infected Pregnant Women

This study has shown that *in utero* transmission occurred in 27.3% of babies tested at birth. Follow up testing was not possible due to challenges experienced in tracking the study population. Maternal viral load emerged as a strong predictor of MTCT. Mothers with low CD4+ counts had high viral loads and vice versa. We observed that

18.2% of HIV positive mothers had a depressed immune system (CD4+ cell counts <200 cells/mm^3) and were classified as having AIDS. Forty percent demonstrated an adequate immune system (CD4+ cell counts ≥200-600 cells/mm^3). The remaining 41.8% of participants demonstrated good immune responses (CD4+ cell counts of >600 cells/mm^3).

Mothers with AIDS had low haemoglobin levels (<10g/l) as compared with mothers with adequate immune responses. Although not statistically significant, there was a trend for mothers with low CD4+ counts to have low haemoglobin values. Association between maternal viraemia and maternal haemoglobin values were statistically significant ($p = 0.050$). It was noted that mothers with high viral loads had low haemoglobin values.

The weights of the babies were analysed in conjunction with their viral loads. This was done to assess whether the level of viral RNA copies had an impact on the ability of the foetus to thrive and grow *in vivo*. There was no significant difference in the median weight between infected and uninfected babies. Collectively, 29.1% of infants weighed <2.5 kg and 70.9% >2.5 kg. Thirty percent of babies born with low birth weights were uninfected whilst 26.7% were infected *in utero*.

A comparison of CD4+ counts was undertaken between the infected and uninfected babies. A median difference of 500 CD4+ cells was noted between the two groups with an overlapping of the interquartile ranges. The infected babies had CD4+ counts ranging from 399 to 4976 cells/mm^3 with log viral loads from 3.3 to 5.0 logs. A total

AIDS and the viral loads of their babies at birth ($p = 0.018$). Sixty percent of infected babies were born to mothers with AIDS as compared with 20% born to mothers with >200 CD4+ cell counts. If the mothers had AIDS the infants were more likely to be infected.

7.3 Placental Immunopathogenesis in HIV-1 Infected Pregnant Women

To assess whether the presence of placental NK cells had an immune suppressive function in controlling viral replication, the levels of NK (CD56+) cells and p24 antigens in placental tissue were recorded. Using immunohistochemistry staining methods, evidence of p24 antigens was found in 94.6% of placentas. There was evidence of NK cell activity in 98.2% of infected placentas. The level or intensity of NK cell activity varied. The influence of maternal viral load and CD4+ count on the presence of p24 antigens in placental tissue was evaluated. The analysis revealed that maternal plasma viral load or CD4+ counts did not appear to have an influence on the level or presence of p24 antigens in placental cells.

Overall, in our study, evaluation of NK cell activity at the placental interface showed that there were lower median NK cell values in placentas of mothers with infected babies as compared with the uninfected cluster. Although not statistically significant, low placental NK cells were associated with the risk of infection when compared with higher NK cell presence. Data from logistic regression analysis indicated that the risk of vertical transmission was increased 3.4 times more if placentas had lower NK cell

values. However, the association between placental NK cells and vertical transmission has not been conclusively established in other studies. In lieu of the limited information available for comparison with other studies, comments about the interaction and role of NK cells in vertical transmission can only be made from data in this study. Further studies are required to test the validity of our observations.

A risk assessment of viral transmission was performed using babies CD4+ cell counts and the presence of placental tissue p24 antigens. According to the odds ratio, babies CD4+ counts were affected by every 1 log increase in mother's viral load. However, the CD4+ count and presence of p24 antigens were not predictors of vertical transmission. Given the lack of direct linkage between the detection of p24 in placental tissue and viral RNA copies in infected babies' plasma, other cell mediated immunological responses require investigating. Also, the possibility that undetectable levels of viral copies in 72.7% of the babies in contact with infected placentas will not at a later date emerge seropositive cannot be excluded.

The inflammatory cell response in HIV-1 infected placentas was evaluated in babies infected *in-utero* and those uninfected. There was minimal difference in placental inflammatory response of mothers with infected and uninfected babies.

7.4 Role of Human Leucocyte Antigen-G Expression in Vertical Transmission

The presence of HLA-G was sought for in all placental samples of HIV-1 infected mothers. The concentration of HLA-G in placental tissue was calculated using values obtained from real-time PCR assays. HLA-G was detected in 100% of placental samples. Data analysis in this study indicates that there was no significant correlation between the presence of NK cells and the level of expression of HLA-G in viral transmission to the baby.

The data was then used to investigate the relationship between maternal viral load and the quantity of HLA-G expression. A positive correlation between mothers log viral load and transmission of infection to the babies was observed. However, no statistically significant correlation was noted with HLA-G and viral transmission. On further investigations the odds ratio indicated that the risk of infection increased by 1.3 with every 1 fold increase in HLA-G expression.

When an analysis of mother-to-child transmission rates by gender was performed we found that female babies were 3.4 times more likely to become infected than males. We then analysed the fold increases in placental HLA-G and its correlation to the plasma viral load of babies in both the infected and uninfected groups. This was found to be statistically significance ($p = 0.05$).

Comparison of placental HLA-G fold changes and the viral loads in babies revealed that there was no statistical significance. Increased fold changes in 34.3% of the infected babies as compared to fold decrease in 16.7% of infected babies revealed that the likelihood of babies becoming infected was 2.6 times more if placental HLA-G was increased. The absolute difference in median values between the infected babies (2.22) and the uninfected babies (1.17) was approximately 1. Infected babies had higher median fold increases (2.1) than the uninfected babies (1.1). Again, the difference of 1 fold increase in median values between infected and uninfected babies suggest that there was a trend for placentas of mothers with infected babies to have increased fold changes than those mothers with uninfected babies.

There association between HLA-G expression and gender was assessed. Logistic regression analysis revealed that the risk of infection was 3.4 times more in favour of female babies than males. However, when gender susceptibility to HLA-G expression was explored, a statistically significant association was observed between HLA-G expression in placental tissue of mothers with infected and uninfected male babies. No difference in HLA-G expression was observed between placental tissue of mothers with infected and uninfected female babies. HLA-G emerged as a significant risk factor in males than in females. In the final analysis of this section we found that HLA-G was up regulated 3.95 times more in placental tissue of mothers with infected babies than in mothers with uninfected babies.

7.5 Limitations of the Study

Our data are incomplete in certain important aspects such as follow up visits and continued HIV testing and monitoring of the babies. Clinical outcomes and clinical staging of babies were incomplete because mothers and babies were not available for follow up visits. However, the data presented here, although with limitations are from a study population in which ARV's were not administered. Sample size restricted comments to statistical significance in many areas of analyses. However, data presented in this study creates a platform for further investigations and future studies.

7.6 Future Studies

The association of HLA class alleles in vertical transmission and disease progression requires further investigations. We are aware that similar haplotypes between mother and babies increases the risk of disease progression in vertically infected children. This study has reported that there are gender differences in risk of acquiring the infection. Larger studies are required to eliminate some of the unanswered questions. Possible areas for investigations into gender differences and risk of transmission are:

- Differences in immunological responses and thymic expression between the sexes.
- The strength of the immune response and protective mechanisms between male and female infants.

- Histocompatibility reactions between maternal lymphocytes and Y chromosome-n derived antigens in boys.

Despite the fact that the mechanisms that control HLA-G expression during pregnancy are not fully comprehended it is clear that the immunological barrier is not inert.

CHAPTER EIGHT

CHAPTER 8

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CHAPTER NINE

CHAPTER 9

APPENDICES

APPENDIX I

Vertical Transmission HIV Study
Patient Consent Form

IN ENGLISH

A sample of your blood will be taken for viral studies and CD 4+ cell count. This will be tested to determine if any genetic basis exists in the immune system of HIV positive and HIV negative babies born to HIV positive mothers. This testing will not affect you or your baby's condition. There are no harmful effects due to the testing. The testing will be free of charge.

Knowing your viral load and CD-4 count will help you to understand your infection. If you have access to adequate financial resources you may want to start antiretroviral therapy in consultation with your doctor

The blood will be used for research purposes only and your identity will be kept confidential. You are free to refuse the testing and this will not affect any further treatment that you receive from your attending doctor.

Patient's Name.....
Patient's signature.....

Attending doctor's name.....
Attending doctor's signature.....
Date .....

TRANSLATION IN ZULU

UKUTHUMELA OKUMILE NGESANDULELA NGCULAZI
UMVUME YESIGULI

Igazi lakho lizothathwa ukuze kwenziwe izifundo zamagciwane nokubalwa kama-CD4. Lokuhlolwa kuzokhomba ukuthi isizinda sofuzo sikhona yini kuhlelo oluvikela izifo emzimbeni kubantwana abanesandulela ngculazi. Lokhu kuhlolwa ngeke kukulimaze wena okanye kulimaze isimo somntwana wakho. Ayikho imiphumela elimazayo ngalokho kuhlola. Lokhu kuhlola angeke bubize imali.

Ukwazi isilinganiso samagciwane akho nokubalwa kwamaCD-4 akho kuzosiza ukuthi uqonde ngesifo sakho sesandulela ngculazi. Uma unayo imali eyanele ungakwazi ukuqala ukwelapha kwe-Antiretroviral ekubonisanane nodokotela wakho.

Igazi lakho lizosetshenziselwa ucwaningo kuphela futhi ubuwena buzogcinwa bube yimfihlo. Ukhululekile ukunqaba ukuhlolwa futhi ngeke kuthikabeze ukwelashwa okuthola kudokotela wakho.

Igama lesiguli.....

Isiginisha yesiguli.....

Igama ladokotela wakho.....

Isiginisha kadokotela wakho :.....

Usuku .....

APPENDIX II

Laboratory Protocol: Processing of Blood Samples

2 EDTA tubes (purple top) will be received from the participating hospital. Each EDTA tube should have approximately 2-5 mls of blood for the mothers and approximately 1 ml for the babies. All samples must be transported at room temperature and not on ice. This is done to avoid lysis of red cells.

A Viral Load Processing

2 EDTA tubes to be processed as follows:

- Sample must be processed within four (4) hours of taking.
- Centrifuge each EDTA tube for 15 minutes at 8000rpm
- Carefully remove the plasma from each EDTA tube and transfer into a sterile tube containing no preservatives or gel. Refrain from removing any red cells.
- DO NOT discard the red cells after removal of the plasma.
- Centrifuge each plasma sample for 15 minutes at 5000rpm.
- Transfer all of centrifuged plasma into another sterile tube. Do not remove any remaining red cells from the initial plasma tube.
- Discard empty tubes appropriately.
- Store each EDTA tube (2X) with red cells and each plain plasma tube (2X) at -20°C overnight.
- Ensure correct labeling of all tubes. Laboratory assigned numbers on the plasma tubes must correspond to the original EDTA tubes with red cells.

NB. Plasma must be separated from red cells within four (4) hours of collection. Red cells must be excluded as this will inhibit *taq* DNA polymerase.

B CD4+ Processing Procedure

ONE (1) EDTA tube must be processed as follows:

- EDTA tube with 2-5 mls of blood.
- Sample maintained and transported at room temperature.
- Samples should be tested for CD4 within 48 hours of taking.

Consumables for FACS (CD4/8 counts)

- Disposable 200ul filtered tips
- FACS count Controls
- Jik – cleaning agent
- CD4/CD8 KIT – FACS count
- Isoton III
- Gloves – disposable

Equipment

- FACS count
- Pipette – 50ul dispenser
- Laminar Flow Facility
- Vortex mixer
- FACS count work station to hold blood samples and operating samples during preparation.
- Timer
- FACS count coring – device used to open reagents and control tubes.
- Infectious Disposable Unit.

APPENDIX III

A Wax embedding of tissue sections

- Place square piece of tissue to fit cassette, just over perforations.
- Place cover over cassette.
- Fix tissue - processing according to given protocol.
- Embed tissue in wax (wax must be kept at 58°F) use small metal square compressor to level the tissue first, and then dispense wax.
- Place embedded tissue on ice to harden adequately.

B Cutting of wax embedded tissue

- Cut tissue at 4-5 microns (for immuno-staining).
- Place sections in water containing albumin.
- Mount slide on silinated (Poly-L-Lysin) slide.
- Drain for 5 mins.
- Heat on hot plate for 10 min.

APPENDIX IV

A CD4+Cell Count Test Procedure

The tube of one reagent tube pair was labelled with the patient accession number with a permanent label marker. The test reaction tube was vortexed upside down for 5 seconds and then upright for 5 seconds. The reagent tubes were opened with the coring station. Patient whole blood was mixed gently for five times. A volume of 50µl of whole blood specimens were then pipetted into each of the two reagents tubes. The tips were changed between the tubes and discarded into a biohazard container. The tubes were thereafter capped and vortexed upright for 5 seconds and incubated for 60 minutes at room temperature away from direct light to protect the reagents. After an hour the tubes were uncapped and 50ul of fixative solution added into each reagent tube with tip change in between the tubes. New caps were used on the reagent tubes and vortexed upright for 5 seconds. Tubes were run on the FACS count instrument within 48 hours of preparation. All stored samples were kept at room temperature at the workstation until they were read. The programme on the FACS count was as per instruction manual.

B Viral Load Test Procedure

All reagents were brought to room temperature. Test strips were removed from the microwell container. Sample diluent of 100µl was added with a microchannel pipette. Thereafter, 50µl of patient plasma was added to the labelled wells. Three (3) Negative and 1 HIV-1 Positive control (high) and 1 HIV-2 Positive (Low) control were also added to carefully labelled wells. The plate was covered with a lid and placed at 37° C for 60 minutes under humid conditions. After the specified incubation period the plate was washed with an ELISA plate washer for 6 times with 400µl of wash buffer (prepared as per instructions). The plate was turned upside down and dabbed dry on a piece of paper towel to get rid of excess wash solution. A multichannel pipette was used to dispense 100µl of substrate into each well. The wells were covered and incubated at room temperature under humid conditions for 30 minutes. Thereafter, 100µl of stop solution (2M sulphuric acid) was added to stop the reaction. The plate was read within 15 minutes at 450nm with a microwell plate reader.

C Multicentre Comparison of Roche Amplicor Monitor Version 1.5 (usCA MONITOR 1.5) and standard (stCA MONITOR 1.5),Organon Technika NucliSens QT with Extractor.

Test	Viral limits	Specificity	Plasma volume
Roche Amplicor Version 1.5(us)	50-750 000	100	0.5 ml
Roche Amplicor Version 1.5(st)	400-750 000	100	0.2 ml
Nuclisense(Technika Organon)-NASBA	80-8 000 000	100	2 ml
bDNA version 3 (Bayer)	50-500 000	98	1 ml

APPENDIX V A

Procedure I

Immunoperoxidase Reagent Preparation for Immunohistochemistry

Safety Precautions:

Use proper ventilation and wear protective clothing, gloves, and goggles to avoid cortact with the following:

- Hydrogen Peroxide-strong oxidizer, corrosive.
- Glacial Acetic Acid-corrosive.
- Hydrochloric Acid-corrosive.
- N, N, - Dimethylfomamide- irritant, combustible.
- Ethanol- flammable, toxic.

Prepare solutions

A Stock Tris (Trizma Base, Sigma T-1503)

- Place 121.14g 0f Trizma Base in a 2000ml volumetric flask.
- Add roughly 1 litre ultrapure water and 60ml concentrated hydrochloric acid (HCL) (Fisher A114).

- Adjust pH to 7.6 with concentrated HCL (approximately 6ml) at room temperature.
- Bring total volume to 2000ml.
- Label, date, initial and store in refrigerator. Good for 1 year.

B **Working Tris** (Make a 1:10 dilution of Stock Tris with ultrapure water, Example: 100mls Stock Tris and 900mls ultrapure water)

C **0.01 PBS - pH 7.2**

• Sodium 'Phosphate Dibasic(Anhydrous)(Fisher S374)	1.48g
• Potassium Phosphate Monobasic (Fisher P382 or P285)	0.43g
• Sodium Chloride (Fisher S271)	7.2g

- Add chemicals to 950mls of ultrapure water. Stir until dissolved.
- pH solution to 7.2.
- Bring total volume to 1000mls.
- Label, date, initial and store in refrigerator. Good for 1 year.

D **Tris/PBS**

• Working Tris	10mls
• 0.01M PBS, pH 7.2	90mls

- Label, date, initial, and store in refrigerator. Good for 6 months

E **Tris/PBS with 3% BSA 1 Liter Tris/PBS**

- BSA (Bovine Serum Albumin, Sigma A- 7030) 30g
- Stir until BSA is completely dissolved.
- Aliquot and freeze at –20°C
- This is the diluting buffer for blocking solution, primary antibody, secondary antibody and HRP Streptavidin.

F Antigen Retrieval

- **For pH 6**

Dilute I part Antigen Retrieval Citra (BioGenex, HKO86-9K) to 9 parts ultrapure water.

Label, date, initial, and store in refrigerator.

- **For pH 10**

Dissolve 12.12g Trizma Base (Sigma T-1503) in 1 liter ultrapure water.

Label, date, initial, and store in refrigerator.

- **For 1mM EDTA**

Dissolve 0.185g EDTA (Fisher 8311) in 500mls ultrapure water.

Adjust pH to 8.0 using IM sodium hydroxide.

Label, date, initial, and store in refrigerator.

G Enzyme Digestion (make solutions fresh)

I 0.1% Trypsin

- Trypsin (Sigma T-8128) 0.1g
- Calcium Chloride 0.1g
- Working Tris 100mls

II 0.05% Protease

- Protease (Sigma P-5147) 0.05g
- Working Tris 100mls

III 2.5mg/ml Pepsin

- Pepsin (Sigma P-6887) 0.125g
- Working Tris pH 2 50mls

H 10% Blocking Solution (make fresh)

Block with a nonimmune serum from the same animal species that your secondary antibody was produced in. Make a 10% solution (this is also a 1: 10 dilution) of serum in Tris/PBS/BSA.

I 3% Hydrogen Peroxide

(Make a fresh solution and use necessary safety precautions)

- 30%hydrogen peroxide (Fisher H325) 10mls
- ultrapure water unless on frozen tissue, then methanol 90mls

(Frozen sections should be treated with 3% hydrogen peroxide for a longer time)

J Biotinylated secondary antibodies (make a fresh solution)

- Primary antibodies as per manufacturers protocol

K Horseradish Peroxidase-Streptavidin

(Make a fresh solution)

Zymed's HRP-Streptavidin (43-4323) diluted1; 200 in Tris/PBS/BSA.

L Sodium Acetate Buffer

(Use necessary safety precautions)

- 0.1 N Acetic Acid 210mls
- 0.lM Sodium Acetate 790mls

0.IN Acetic Acid

- 5.75mls Glacial Acetic Acid(Fisher A38) 5.75mls

  in 1 liter ultrapure water.

0.IM Sodium Acetate

- Sodium Acetate, Trihydrate (Sigma S-8625) 13.61g

  in 1 liter ultrapure water

- Label, date, initial all solutions and store in refrigerator.

  Good for 6 months.

M 0.4% AEC (3-Amino-9-Ethylcarbazole)

(Use necessary safety precautions)

- AEC (Polysciences Cat No.3019) 0.02g
- N,N,-Dimethylformamide (Fisher Dl19) 5 mls
- Date, label, initial and store in refrigerator. Good for 1 month.
-

N 3% Hydrogen Peroxide

(Use necessary safety precautions)

- 30% hydrogen peroxide (Fisher H325) 0.3ml
- ultrapure water 3 mls
- Date, label and store in refrigerator. Good for 1 month.

Q Scott's Tap Water

- Magnesium Sulfate (Fisher M65) 10g
- Sodium Bicarbonate (Fisher S233) 2g
- Add chemicals to 1 liter ultrapure water and stir until dissolved.
- Label, date, initial, and store a room temperature. Good for 6 months.

R Haematoxylin Counterstain

Mayer's Haematoxylin diluted to 1/4 strength with ultrapure water

APPENDIX V B

Procedure II

Tissue Preparation and Staining Protocol

All 5um thick sections on slides and control slides were appropriately labeled. The slides were placed in the 57°C oven for at least 30 minutes. Tissue sections were deparaffinized and rehydrated 5 minutes in Xylene (Twice), 3 minutes in 100% alcohol (three times), 3 minutes in 95% alcohol (two times), 3 minutes in 70% alcohol (once) and 3 minutes in ultra pure water (once). The slides were thereafter, subjected to the following pre-staining procedure:

A Antigen Retrieval Step

Slides were placed in a glass Coplin jar containing Trisoduim citrate buffer. The retrieval solution was brought to boil in the pressure cooker and the slides were placed on a metal rack in the pressure cooker. Slides were pressured cooked until both the rings on the pressure cooker lid appear. Once this temperature was achieved the slides were left for a further 1min 30 secs. Once the process was completed the slides were removed and allowed to cool for 10 minutes and transferred to Tris buffer.

B Enzyme Digestion Step

The slides were pre-warmed in Tris buffer for 5 minutes at the same temperature that the enzyme digestion. Thereafter, the slides were transferred to the enzyme digestion solution for 30 minutes. The enzyme digestion solution was poured off and squirt rinsed with Tris and placed in Tris for 5 minutes.

C PAP Pen Demarcation

A PAP pen was used (BioGenex Cat. No. XT00I-PP) to draw lines on both sides of the tissue section of each slide. This created a barrier with the proper surface tension to hold solutions and reagents on the tissue section, which helped prevent wasting valuable reagents. The following precautions were observed:

- Only 2-4 slides were removed out of Tris at a time.
- The lines were made on the slides and returned back into the Tris.
- The tissue sections were never allowed to dry out.

D Staining Protocol

1 Peroxidase Blocking Step

Slides were removed from Tris, drained and tapped to remove excess buffer. The slides were laid flat and drops of the 10% blocking solution were applied to the tissue section covering the section completely. Blocking occurred for 10 minutes at room temperature. The blocking solution was tapped off and distilled water gently poured to wash off the blocking solution.

2 Primary Antibody Step

Excess liquid was tapped off and the slides were wiped. Drops of the appropriate primary antibody were applied until the section was completely covered. Thereafter, the slides were placed in a humidified chamber and incubated for the 10 minutes. Slides were gently rinsed with buffer solutions. And placed in a fresh buffer bath.

3 Biotinylated Link Step

Slides were removed from buffer, drained and tapped to remove excess buffer. The slides were laid flat and Link Antibody was applied to the tissue section until the section was completely covered. Incubation was carried out for 10 minutes at room temperature. Buffer was thereafter used to squirt rinse the slides.

4 Streptavidin - HRP Step

The slides were wiped again as before and laid flat. Drops of HRP- Streptavidin were applied to tissue sections until the sections were completely covered. This was followed by incubation of slides for 15 minutes at room temperature. Slides were squirt rinsed as before.

5 Substrate - Chromogen Solution Step

Slides were wiped and DAB substrate-chromogen solution was placed over slides to cover the tissue sections adequately. Further 10 min incubation was performed. Slides were gently rinsed with water from a wash bottle.

6 Haematoxylin Counter stain

Slides were immersed in a bath of Mayer's Haematoxylin and incubated for 3 minutes. Thereafter, the slides were washed by dipping in ammonia water to remove excess stain. Scott's Tap Water was used to blue the slides for 1 minute and rinsed in distilled water for 3 minutes.

7 Mounting and Cover slip

Permanent aqueous mounting medium was used to cover slip slides (Glycelgel, Code No. C0563).

APPENDIX VI

Reagents for RNA Extraction and Gel Electrophoresis

1 Preparation of Reagents for RNA Extraction

A Diethyl Pyrocarbonate water (DEPC water)

- 250µl Diethyl Pyrocarbonate (ICN Biochemicals Inc. 150902) was added to 250ml of distilled water in a fume cupboard, followed by incubating at 37˚C overnight. Solution was autoclaved twice before use.
- Stored solution was kept at room temperature.

B Ethanol (75%)

- Absolute ethanol (Merck Ltd, Poole, England) was diluted to 75% with DEPC water and kept at -20˚C.

2 Reagents for Agarose Gel Electrophoresis

A Agarose Gel (2%)

- Agarose (1.4 g) was dissolved in 250 ml Erhlenmeyer flask with 70 ml of Tris-Borate/EDTA (TBE) buffer (1X).
- The slurry was heated in a microwave oven until the agarose granules had completely dissolved.
- After the gel was cooled to approximately 60°C, ethidium bromide (3.5µl) was added.
- The mixture was poured into the casting tray containing a comb that was positioned 10mm from one end of the tray.
- The gel was allowed to polymerise for 30-60 minutes at room temperature prior to use.

B Tris-borate/EDTA (TBE) (10X)

- Tris base (54g), boric acid (27.5 g) and EDTA (4.65g) was dissolved in 400ml of sterile water and then brought up to 500ml.
- The solution was filtered and stored at room temperature (approximately 25°C).

C Ethidium Bromide Dye

- Ethidium bromide (10mg) was dissolved in 1ml of sterile water.
- The dye was stored in an aluminium foil covered bottle at room temperature.

D Preparation of PCR Product

The PCR product (10µl) was prepared by adding it to 2µl of bromophenol blue loading dye (0.25% and 40% (w/v) sucrose). The DNA molecular weight marker (3.0 µl) was used as control.

3 DNA Marker

Fermentas Gene Ruler

50-bp DNA Ladder

SMO378 10ug (for 20 applications)

Ready to use

Lot 1802

Concentration: 0.1µg/µl

Supplied with: 0.1 6X loading dye solution

Store at room temperature

4 Gene Quant

Dilution of 1:20 was done with the product/DEPC water i.e. 5 µl of product to 95 µl of water. The mixture was vortexed and quantified in the Gene Quant. Ten (10) dilutions were done to calculate the standard curve as follows:

Tube 1 contained 1µl of standard to 999µl DEPC water. The dilution was mixed and 10µl transferred in tube 2 containing 90µl of DEPC water. The process was carried through a further 9 tubes as a serial dilution to tube number 10.

APPENDIX VII:

Optimised LightCycler Experimental Protocol for HLA-G Quantification.

Program	Activation			Type	None	Cycles	1
Segment Number	**Temperature Target (°C)**	**Hold Time (sec)**	**Slope (C°/sec)**	**2° Target Temp (°C)**	**Step Size (C°)**	**Step delay (Cycles)**	**Acquisition Mode**
1	95°C	600s	20	0	0	0	None
Program	PCR			Type	None	Cycles	35
Segment Number	**Temperature Target (°C)**	**Hold Time (sec)**	**Slope (C°/sec)**	**2° Target Temp (°C)**	**Step Size (C°)**	**Step Delay (Cycles)**	**Acquisition Mode**
1	95°C	10s	20	0	0	0	None
2	60°C	50s	20	0	0	0	None
3	72°C	16s	20	0	0	0	None
4	85°C	5s	20	0	0	0	Single
Program	Melt			Type	Melting Curves	Cycles	1
Segment Number	**Temperature Target (°C)**	**Hold Time (sec)**	**Slope (C°/sec)**	**2° Target Temp(°C)**	**Step Size (C°)**	**Step Delay (Cycles)**	**Acquisition Mode**
1	95°C	30s					None
2	80°C	15s					None
3	95°C	0s					Continous
Program	Cooling			Type	None	Cycles	1
Segment Number	**Temperature Target (°C)**	**Hold Time (sec)**	**Slope (C°/sec)**	**2° Target Temp (°C)**	**Step Size (C°)**	**Step Delay (Cycles)**	**Acquisition Mode**
None	40°C	30s	0.5°	0	0	0	None

APPENDIX VIII

CDC HIV Pediatric Classification (1994)

Immunological Categories According to the CDC 1994 Revised Paediatric Classification

Age of child	No evidence of immune suppression		Evidence of moderate immune suppression		Evidence of severe immune suppression	
	CD4+ cells/µl	% CD4+	CD4+ cells/ µl	% CD4+	CD4+ cells/ µl	% CD4+
<1 year	≥ 1500	≥ 25	750-1499	15-24	<750	<15
1-5 years	≥ 1000	≥ 25	500-999	15-24	<500	<15
6-12 years	≥ 500	≥ 25	200-499	15-24	<200	<15

Adapted from CDC (1994)

APPENDIX IX:

Representative Profile of Sample Data and Calculated Values of HLA-G/GAPDH Ratio

Study No.	HLA-G	GAPDH	Ratio
21	3.635	3.842	2.651
22	2.784	2.297	0.730
23	3.303	1.857	0.562
32	4.921	1.200	4.1
34	4.370	1.347	3.18
35	1.865	4.285	0.435
39	8.976	1.351	6.64
40	1.180	2.679	0.44
41	2.468	7.509	0.329
43	1.959	9.136	0.214
46	2.594	1.886	1.375
47	1.901	4.056	0.469
48	1.439	2.270	0.634
49	1.714	4.419	0.388
50	2.188	6.990	0.313
52	2.220	1.885	1.178
53	2.360	5.450	0.459
54	2.600	1.316	1.976