PHARMACO-IMMUNOLOGICAL-VIROLOGICAL DYNAMICS IN INTRAPARTUM HIV-1 TRANSMISSION (PIVD STUDY)

By

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Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Obstetrics and Gynaecology, Faculty of Medicine, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban

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Declaration

The original protocol for the research undertaken in this thesis was written by the candidate. All research described in this thesis was carried out in Durban, Johannesburg and Cape Town (South Africa) and this thesis was supervised by Professor Dhayendre Moodley and Professor Emeritus Jagidesa Moodley from the University of KwaZulu-Natal (UKZN). All study administration, ethics applications, progress reports and resolution of ethical queries were managed by the candidate (Ethics Ref No: H004/05). All the fieldwork in this study; patient accrual, patient retention and sample processing was undertaken solely by the candidate with only sample collection performed by medical registrars in training and midwives from the antenatal, labour, obstetric and nursery wards of King Edward VIII Hospital (Durban). All samples were processed and stored at the Women's Health and HIV-1 Research Unit laboratory (WHHRU) at Doris Duke Medical Research Institute (DDMRI). All laboratory assays were performed by the candidate (under supervision of laboratory mentors) from the Department of Virology (Inkosi Albert Luthuli Chief Hospital), CAPRISA and Hasso Plattner molecular laboratories (UKZN) in Durban [VIROLOGICAL]; Department of Clinical Pharmacology, University of Cape Town [PHARMACOKINETICS]; and the AIDS virus research unit (Cell Biology), National Institute of Communicable Diseases (NICD), Johannesburg [IMMUNOLOGY AND HOST GENETICS]. All data were collected, checked, captured and analyzed by the candidate. A senior statistician from the Biostatistics unit of the Medical Research Council was only consulted for advice and assistance on more complex statistical tests. Funding for this study was acquired by the candidate. Where use was made of the work of others, it has been duly acknowledged in the text.

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Dedication

To all the wonderful mums and sweet babies of the PIVD study who

kindly shared their time & lives in the interest of science

To my mum, Mrs Grace Singh for her unwavering love, encouragement and prayers

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- Singh M, Moodley D, Connolly C and J Moodley. Diagnostic utility of the p24 antigen assay as an inexpensive and reliable tool for early perinatal HIV-1 diagnosis and estimation of timing of MTCT. *XV International AIDS Conference* 10-16th July 2004. Bangkok, Thailand.

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List of abbreviations

ACTG	AIDS Clinical Trial Group
AIDS	Acquired Immune Deficiency Syndrome
ARV	Antiretroviral
CC	Chemotactic cytokines
CDC	Centre for Disease Control
CRF	Case report form
CVF	Cervicovaginal fluid
CVL	Cervicovaginal lavage
DLV	Delavirdine, Rescriptor®
DNA	De-oxy Ribonucleic Acid
EDTA	Ethylene-Diamine Tetra Acetic acid
EFV	Efavirenz, Sustiva® or Stocrin®
ELISA	Enzyme Linked Immuno-Sorbent Assay
FDA	Food and Drug Administration
HAART	Highly Active Antiretroviral Therapy
Hap-1	Haplotype-1
HIV-1	Human Immunodeficiency Virus-type 1
HIVNET	HIV network
HPLC	High Performance Liquid Chromatography
IALCH	Inkosi Albert Luthuli Hospital
IC_{50} WT	50% Inhibitory Concentration against wild-type HIV-1
IP	Intrapartum
IP/ml	Infectious Particles per millilitre
IU	*in utero*
IUD	Intrauterine death
KZN	KwaZulu Natal
LC-MS-MS	Tandem liquid chromatography-Mass spectrophotometry
MTCT	Mother-to-Child Transmission
NICD	National Institute of Communicable Diseases

NNRTI	Non-Nucleotide Reverse Transcriptase Inhibitor
NRTI	Nucleotide Reverse Transcriptase Inhibitor
NVAZ	NVP/AZT
NVD	Normal vaginal delivery
NVP	Nevirapine, Viramune®
NVP^R	Nevirapine drug resistance
PACTG	Pediatric AIDS clinical trial group
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
p.d.g	per diploid genome
PEP	Post-Exposure Prophylaxis
PHPT	Perinatal HIV-1 Prevention Trial
PMTCT	Prevention of Mother-to-Child Transmission
PI	Protease Inhibitor
PIVD	Pharmaco-Immuno-Virological Dynamics
RNA	Ribonucleic Acid
rpm	Revolutions per minute
RT-PCR	Reverse Transcriptase - Polymerase Chain Reaction
SAINT	South African Intrapartum Nevirapine Study
sdNVP	Single-dose Nevirapine
SNP	Single Nucleotide Polymorphism
STI	Sexually Transmitted Infection
TLC	Thin Layer Chromatography
TOPS	Treatment Options Prevention study
UKZN	University of KwaZulu-Natal
UNAIDS	The Joint United Nations programme on HIV/AIDS
VCT	Voluntary Counseling and Testing
WHHRU	Women's Health & HIV Research Unit
WHO	World Health Organisation
ZDV	Zidovudine, Retrovir®
3TC	Lamivudine, Zeffix®

Abstract

Background:

Multiple factors contribute to mother-to-child transmission (MTCT) of HIV-1, including virological, obstetric and biological factors. Other possible contributory determinants for high MTCT rates include immunological factors such as host genetics and viral genetic variations. Despite several therapeutic, prophylactic and obstetric interventions to reduce the proportion of infants infected during labour and delivery, mechanisms for intrapartum HIV-1 transmission remain elusive and current interventions, could, therefore remain sub-optimal. Much controversy has surrounded the correlation of HIV-1 RNA (viral load) in the systemic and genital compartments of women.

The influence of short-term antiretroviral (ARV) drugs on genital tract HIV-1 is also unclear. At the time the present study was initiated, a regimen of maternal intrapartum and neonatal postpartum single-dose Nevirapine (sdNVP) was the standard of care for the prevention of mother-to-child transmission (PMTCT). In most low and middle-income countries, including South Africa, sdNVP has been documented as effective intrapartum HIV-1 prevention based on plasma pharmacokinetic levels, decreased viral loads (HIV-1 RNA) and reduced rates of intrapartum transmission, yet operational studies continue to report high intrapartum transmission rates despite the administration of sdNVP. As a result perinatal HIV-1 transmission remains a significant public health concern in several African countries.

Aim:

The primary aim of this study was to describe the pharmacological dynamics of Nevirapine in association with virological and immunological risk factors for intrapartum HIV-1 transmission in a South African PMTCT programme where sdNVP was the standard of care.

Methods:

Following regulatory approval from the Biomedical Research Ethics Committee at the University of KwaZulu-Natal (UKZN), one hundred and twenty pregnant HIV-infected women who received the sdNVP regimen for prevention of mother-to-child HIV-1 transmission were enrolled between April-December 2006 at King Edward VIII Hospital (KEH) in Durban. Blood and cervicovaginal lavage (CVL) samples were collected from women at pre-NVP (during pregnancy) and post-NVP dosing (during labour/delivery). In addition to infant blood sampling at birth (post-NVP), postnatal infants were assessed at four and six weeks postnatally. Pharmacological laboratory investigations involved measurement of NVP drug concentration by Tandem Mass spectrophotometry. Virological investigations comprised HIV-1 RNA (viral load) quantitation, HIV-1 drug resistance testing (HIV-1 transmitting women only) and HIV-1 DNA PCR testing (infants only). Immunological investigations were only undertaken in a selected case-control subset of HIV-1 transmitting women and their infants. In this component, laboratory investigations included the determination of CCL3 and CCL3-L1 gene copy numbers, identification of single nucleotide polymorphisms (SNP's) and haplotype characterisation of the CCL3 gene. All women were also screened for the presence of sexually transmitted infections (STI's) during pregnancy.

Results:

One hundred and twenty women were enrolled onto this study. Of these, 110 women delivered 117 live infants (103 singletons and 7 twin pairs). Twelve (10.9%) women transmitted HIV-1 to their infants, while 95 (86.0%) were classified as non-transmitters. As a result of seven twin deliveries, the infant cohort comprised of 117 infants in total. Following two separate DNA PCR tests, HIV-1 infection was identified in 14 (11.9%) of study infants while the remaining 90 (76.9%) were exposed-uninfected. HIV infection status remained unknown for 13 infants due to infant demise (1.7%), lost to follow-up (7.7%) or study withdrawal (1.7%).

During active labour (sampling that was best representative of the intrapartum phase) and within 20 hours of dosing, the median NVP concentration of 1070 ng/ml in the maternal systemic compartment was almost 44 times higher than the NVP levels detected in the genital compartment [24.5 ng/ml] ($p < 0.001$). NVP drug levels were below the 100 ng/ml therapeutic target in seven (13.7%) of 51 plasma and in all 39 CVL samples. While no significant association was found between NVP concentration in the systemic compartment and HIV-1 transmission ($p = 0.4$), this association was statistically significant in the genital compartment ($p = 0.02$). The median plasma NVP level detected among infants at birth was 83 times above the IC_{50} WT (10 ng/ml) and eight times higher than the 100 ng/ml therapeutic target for NVP. More than 71.0% of the infants achieved NVP drug levels above the therapeutic target.

In general, higher levels of HIV-1 RNA (viral load) were observed in maternal plasma when compared to CVL. Following intrapartum sdNVP dosing, reduction in HIV-1 RNA levels did occur, however ≥80.0% of the women experienced no change to their HIV-1 RNA levels in both systemic and genital compartments during active labour. These findings were further

supported by the strong correlation observed when comparing pre and post-NVP HIV-1 RNA levels in both maternal systemic [$r = 0.81$, $p < 0.0001$] and genital compartments [$r = 0.80$, $p < 0.0001$] during active labour. HIV-1 transmitting women had significantly higher viral loads than their non-transmitting counterparts in systemic and genital compartments, before and after intrapartum sdNVP administration. In terms of perinatal transmission this observation was only statistically significant for plasma ($p = 0.02$) and not CVL ($p = 0.7$). Maternal viral load was inversely correlated with maternal CD4 cell counts in both systemic and genital compartments. Almost 40.0% of women in this study had at least one type of STI detected during pregnancy. Maternal STI's were detected in four (66.6%) intrapartum transmitting women and in 38 (38.8%) of non-transmitting women. No significant association was observed between the presence of maternal STI's and the risk for intrapartum MTCT ($p = 0.2$, RR: 2.90, 95% CI: 0.60-15.40). The presence of maternal STI's was associated with higher median viral loads in both systemic and genital compartments of all women, independent of intrapartum HIV-1 transmission.

Despite trial-like conditions and optimal sdNVP dosing, the overall MTCT rate in this exclusively formula-fed cohort was 11.9%, of which 50.0% were *in utero* and 50.0% were intrapartum HIV-1 transmissions. *In utero* and intrapartum MTCT rates were 5.9% and 5.9% respectively.

Discussion/Conclusion:

Detectable CVL HIV-1 RNA that correlated well with plasma HIV-1 RNA, in conjunction with sub-optimal NVP drug concentration in maternal CVL during active labour, suggests that intrapartum HIV-1 infected women continue to act as reservoirs for both vertical and

horizontal HIV-1 transmission throughout the duration of pregnancy. These findings confirm that the role of sdNVP in PMTCT was primarily one of infant prophylaxis. This was further supported by relatively unchanged maternal HIV-1 RNA (viral load) during active labour, in both systemic and genital compartments.

Early identification of women who need highly active antiretroviral therapy (HAART), and initiation of such therapy as early as possible during pregnancy, not only benefits maternal health but remains the best prophylaxis against mother-to-child HIV-1 transmission. Universal access to HAART and improving strategies to optimize coverage of the current dual ARV regimen sdNVP and Zidovudine for PMTCT remain urgent research priorities in several resource-limited settings. Ongoing STI counseling, intensive screening/testing of women and their partners together promotion of condom usage, safer sex practices and aggressive STI treatment are simple interventions with tremendous impact for PMTCT in resource-limited settings.

CHAPTER ONE: REVIEW OF LITERATURE

1.1 HIV/AIDS: A global and local perspective

According to the latest global summary of the Acquired Immune Deficiency Syndrome (AIDS) epidemic by the United Nations, an estimated 33 million (30.3 - 36.1 million) people were living with Human Immunodeficiency Virus (HIV-1) by the end of 2007 (UNAIDS, 2008a). Sub-Saharan Africa remains one of the most seriously affected regions of the world, accounting for 67.0% of all people living with HIV-1 and 72.0% of AIDS-related deaths in 2007 (Fig 1). This region along with several of the world's HIV-1 epicenters (India, China, Nepal) has been dominated by a clade C viral HIV-1 subtype (Fig 2) [Spira *et al*, 2003].

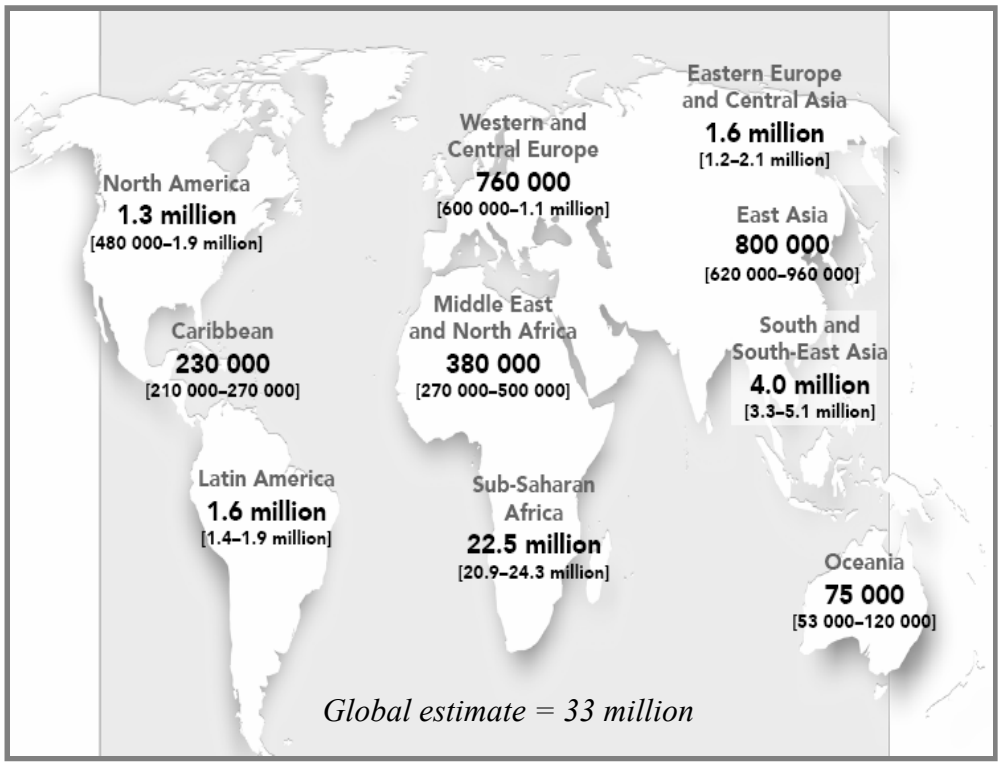


Fig 1: Adults and children estimated to be living with HIV-1 (UNAIDS, 2008a)

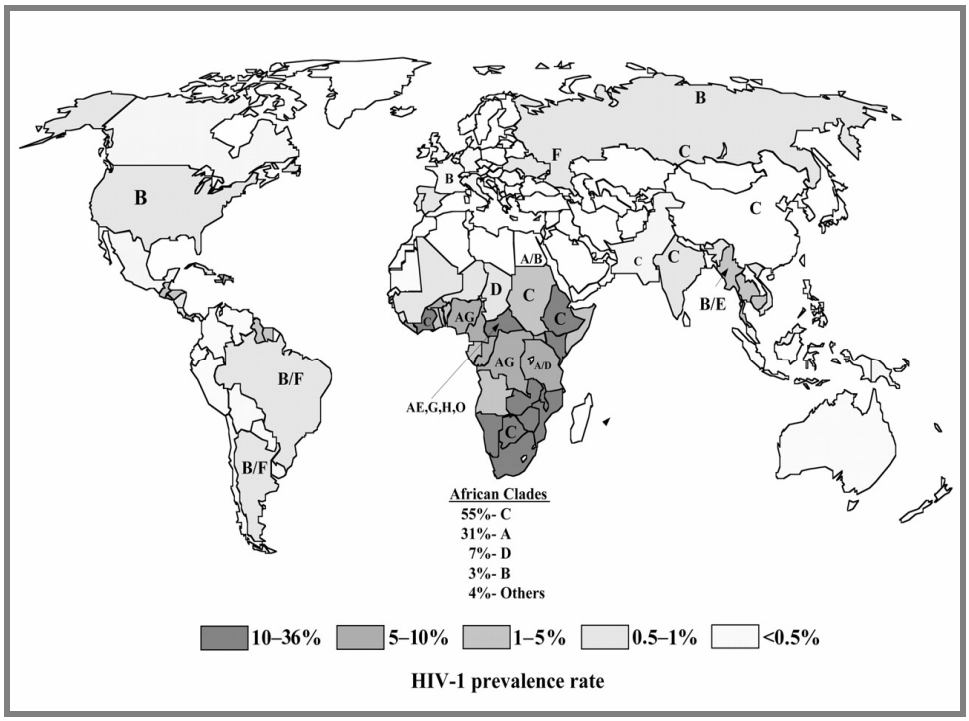


Fig 2: Subtype diversity of HIV-1 infections prevalent worldwide (Spira *et al*, 2003)

Women, in particular (68.0%), continue to endure an extremely disproportionate burden of the HIV/AIDS crisis all across this continent (UNAIDS, 2008a). The South African HIV-1 experience appears to be similar in its disproportionate and devastating impact upon women, particularly those of child-bearing potential. In a survey by the Human Sciences Research Council, women aged between 15-24 years were four times more likely to be HIV-1 infected than their male counterparts (Shisana *et al*, 2005). From inception in the early 1990's, the annual national survey of HIV-1 and Syphilis prevalence (Fig 3), undertaken by the National Department of Health, has continued to indicate that HIV-1 prevalence among antenatal clinic attendees is no longer increasing exponentially but stabilizing at a high value (Department of Health, 2007).

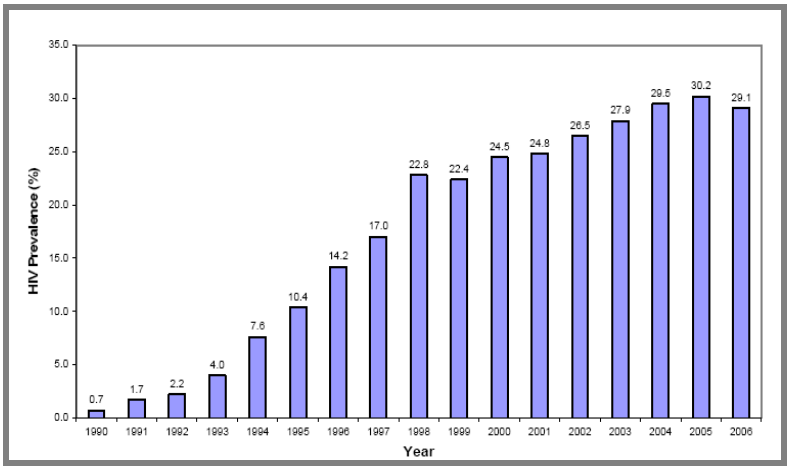


Fig 3: National HIV-1 prevalence among antenatal attendees in South Africa from 1990-2006 (Department of Health, 2007)

While the national antenatal HIV-1 prevalence rate was ~30.2% in 2005, considerable inter-provincial discrepancy was observed (Fig 4) with some provinces being more badly affected than others. In 2005, KwaZulu Natal (KZN) province had the highest antenatal prevalence while the Western Cape (15.7%) province demonstrated the lowest HIV-1 prevalence (Department of Health, 2007).

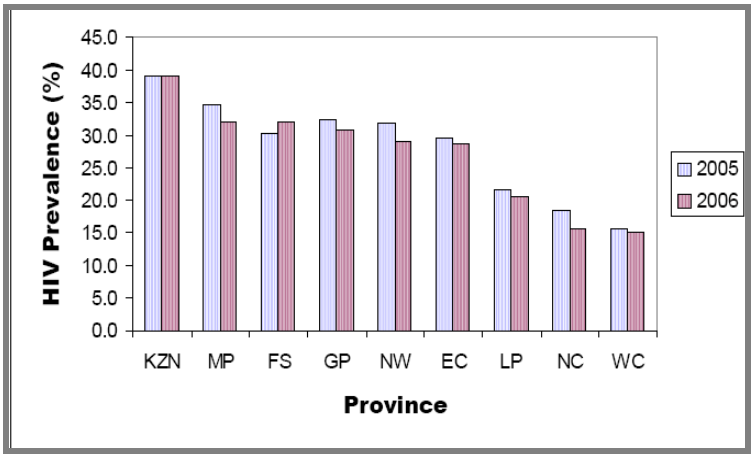


Fig 4: Provincial HIV-1 prevalence among antenatal attendees in South Africa (Department of Health, 2007)

HIV-1 prevalence among antenatal attendees ranged from 13.0% (< 20 years); 28.0% (20-24 years); 38.7% (25-29 years); 37.0% (30-34 years); 29.6% (35-39 years) to 21.3% among women aged 40 years and above (Department of Health, 2007).

1.2 HIV-1 and pregnancy

Worldwide more than 3.28 million pregnant women infected with HIV-1 are estimated to give birth annually. While studies have shown that pregnancy does not adversely affect HIV-1 disease progression or survival, observational studies have strongly implicated HIV-1 in the reduction of fertility and a variable rate of adverse pregnancy outcomes such as increased spontaneous miscarriage, low birth weight and still-birth (Langston *et al*, 1995; Rollins *et al*, 2007).

1.3 Mother-to-child transmission of HIV-1

As the number of HIV-1 infected women has dramatically increased over the years, so has the number of perinatally-acquired infections, with the majority of paediatric infections being attributable primarily to mother-to-child transmission (MTCT). MTCT occurs when a pregnant HIV-1 infected woman transmits or passes the virus onto her baby; either during the course of pregnancy, at the time of labour/delivery or postnatally through breastfeeding.

Each year an estimated 700 000 infants become HIV-1 infected, mainly through MTCT. Around 90.0% of these infections take place in Sub-Saharan Africa, South Asia or South East Asia. In sharp contrast, MTCT has been almost eradicated in resource-rich countries through the effective implementation of evidence-based interventions. These interventions have encompassed; accessibility to antiretroviral (ARV) therapy, safe obstetric practices, effective

voluntary testing and counseling (VCT) and the widespread availability of safe breast-milk substitutes (European collaborative study, 2005; UNAIDS, 2005). The use of evidence-based interventions has resulted in vertical transmission rates that vary from less than 5.0% in resource-rich countries to greater than 30.0% in resource-limited countries without access to ARV interventions and with prolonged breastfeeding practices (Chersich *et al*, 2006; McIntyre and Gray, 2002). Access to HAART, modified obstetric practices and the use of exclusive formula feeding are some of the improved interventions which have further reduced MTCT rates to less than 2.0% (Fig 5) in resource-rich countries (Mofenson and McIntyre, 2000; Coovadia, 2004; McIntyre and Gray, 2002).

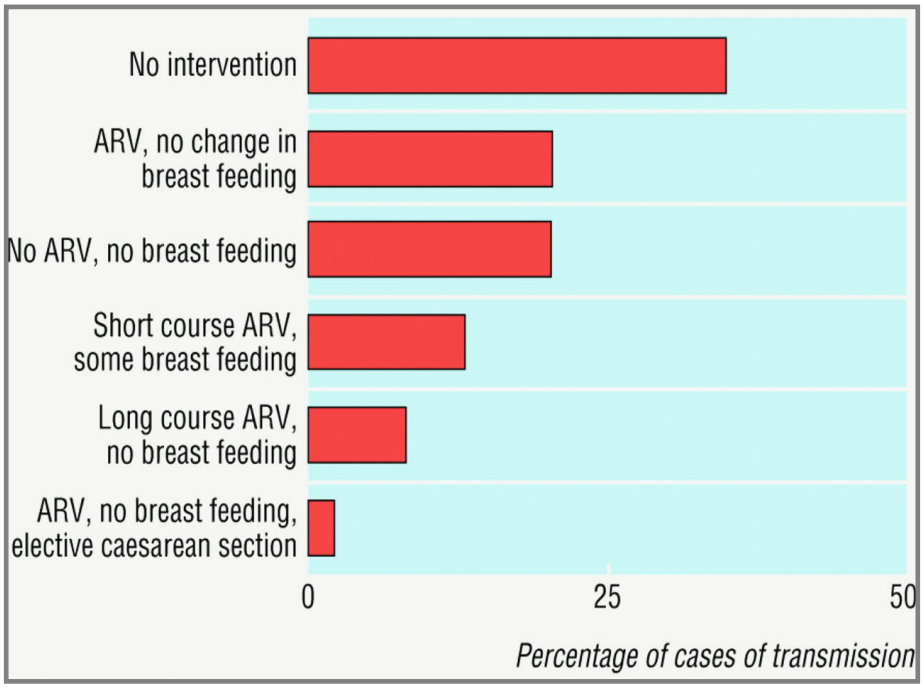


Fig 5: Effect of evidence-based interventions on MTCT rates (McIntyre and Gray, 2002)

1.3.1 Timing and pathogenesis of MTCT

MTCT is known to occur *in utero* (Brossard *et al*, 1995; Lewis *et al*, 1990), intrapartum (Mofenson, 1997) and postpartum through breastfeeding (Leroy *et al*, 1998). In the absence of any evidence-based intervention, 15.0%-45.0% of women will transmit HIV-1 to their children; (5.0-10.0%) *in utero*, (10.0-20.0%) intrapartum and (5.0-20.0%) postpartum [De Cock *et al*, 2000]. MTCT rates can vary from 15-30% without breastfeeding and can reach 30-45% with prolonged breastfeeding depending on population and regional diversities (Moodley and Coovadia, 2008).

The precise mechanism and timing of *in utero* transmission is presently unknown, however studies have shown that these infections may occur in the last two months of pregnancy, hence the administration of ARV drugs during the last trimester of pregnancy may reduce *in utero* infections (Luzuriaga, 2007). Similarly the mechanism for intrapartum HIV-1 transmission is not well understood but may occur due to transplacental microtransfusions (Kwiek *et al*, 2006) or by infection via mucosal exposure to maternal blood or cervicovaginal secretions (Van de Perre, 1999). While multiple factors contribute to MTCT of HIV-1, viraemia in plasma and genital fluids of HIV-1 infected pregnant women has been documented to correlate strongly with intrapartum HIV-1 transmission (Gaillard *et al*, 2000). Observations such as the differential rate of infection for vaginally delivered twins, protective effects offered by caesarean sections, the association between perinatal transmission and prolonged rupture of membranes all suggest that a newborns exposure to HIV-1 present in cervicovaginal secretions and blood while passing through the birth canal may be a very important determinant of intrapartum transmission (Chuachoowang *et al*, 2000). While use of elective caesarean sections prior to the onset of labour is known to reduce the risk of intrapartum transmission,

factors such as prolonged rupture of membranes and chorioamnionitis increase the risk of intrapartum transmission (Landesman *et al*, 1996). Postnatally, the most significant risk factor for MTCT is breastfeeding (McIntyre and Gray, 2002). Studies have shown that breastfeeding transmission may occur within the first few months of life (Miotti *et al*, 1999; Nduati *et al*, 2000). Specific risk factors during breastfeeding include: cracked nipples, mastitis (breast inflammation), high breastmilk viral load (John *et al*, 2001); low CD4 cell count and high plasma viral load (John-Stewart *et al*, 2004). Local studies by Coutsoudis and others have found that mixed infant feeding (breastmilk and replacement feeds) also increases the risk of HIV-1 transmission (Coutsoudis *et al*, 1999). The ZVITAMBO study group confirmed the observations by Coutsoudis and others (1999) and also provided additional data distinguishing the magnitude of risk of HIV transmission or death associated with different breastfeeding patterns (Illiff *et al*, 2005).

1.3.2 Risk factors for MTCT

MTCT may be influenced by a multitude of maternal risk factors that consist of virological, immunological, obstetric and biological factors.

Virological factors include; plasma viral load (HIV-1 RNA), one of the strongest predictors of MTCT, HIV-1 drug resistance, increased genital HIV-1 secretion, viral genotype and phenotype (European collaborative study, 1992; Mofenson, 1997).

Immunological factors ; A wide range of novel and fascinating studies on immune factors such as; maternal autologous neutralizing antibodies (Dickover *et al*, 2006); antibodies to HIV-1 gp120 epitopes including V3 loop (Scarlatti *et al*, 1993; Rossi *et al*, 1989); Leukemia inhibitory factors (Patterson *et al*, 2001; Tjernlund *et al*, 2003); CC chemokines [CCL3, CCL4

and CCL5] (Cocchi *et al*, 1995); CXCL12 (Farquhar *et al*, 2005); Lewis X component found in human breast milk (Naarding *et al*, 2005) and human secretory leukocyte protease inhibitors (Doumas *et al*, 2005) suggests that these innate immune factors appear to be involved in the protection of HIV-1 exposed, uninfected infants (Gray *et al*, 2007).

Obstetric factors include; gestational age, rupture of membranes for greater than four hours prior to labour, placental abruption, amniocentesis, episiotomy, chorioamnionitis and mode of delivery [caesarean section (C/S) vs. normal vaginal delivery (NVD)] (Mandelbrot *et al*, 1996). An elective caesarean section before the onset of labour reduces the risk of perinatal transmission (European Mode of Delivery Collaboration, 1999), while an emergency caesarean section performed in labour for prolonged or difficult labour has been associated with increased HIV-1 transmission rates (Kind *et al*, 1998). Presence of vaginal lacerations during labour or preterm delivery has been associated with prolonged membrane rupture and may increase fetal exposure to maternal blood and body fluids, thereby increasing the risk for HIV-1 transmission (Landesman *et al*, 1996; Kuhn *et al*, 1999). Twin pregnancy has been associated with an increased risk of pregnancy complications such as premature rupture of membranes and preterm labour, which are also well known risk factors for MTCT (Mandelbrot *et al*, 1996). While there have only been a few studies on the influence of multiple pregnancies on perinatal HIV-1 transmission, current available data has suggested that MTCT occurs most often among first-born twins (Park *et al*, 1987; Goedert *et al*, 1991; Duliège *et al*, 1995).

Biological risk factors such as high antenatal prevalence of sexually transmitted infections (STI's) have been linked to adverse pregnancy outcomes (Moodley and Sturm, 2000) and also been implicated in facilitating HIV-1 transmission (Pham-Kanter *et al*, 1996; Taha *et al*, 1998;

Moodley *et al*, 2002). An estimated 340 million new cases of curable STI's occur globally each year in women aged between 15-49 years (WHO, 2001). The various sites of commonly occurring STI's are illustrated in Fig 6, while Table I highlights local and global prevalence rates of these infections. Since 1995, STI's have been managed syndromically in South Africa through the aid of standardized clinical algorithms (Department of Health, 1999).

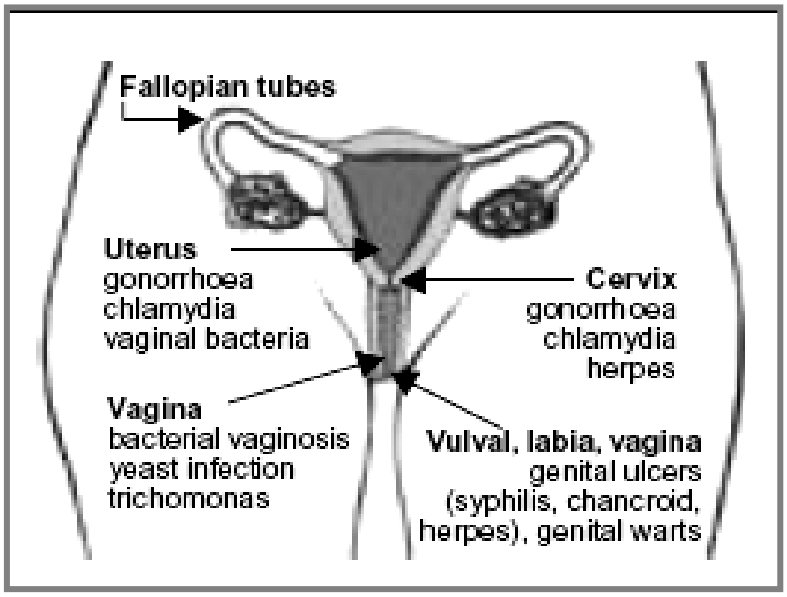


Fig 6: STI infection sites in the female genital tract (WHO, 2005)

Table I: Global and local prevalence of STI's

Type of STI	Global prevalence (WHO, 2001)	South African prevalence rates** (antenatal)
Chlamydia trachomatis	10-25%	3-5%
Bacterial vaginosis	20-40%	15-50%
Treponema pallidum	0-3%	10%
Neisseria gonorrhoeae	3-18%	3-5%
Trichomonas vaginalis	8-16%	15-50%
Candida albicans	_	20-40%
Human papillomavirus	20-60%	No data
Herpes simplex virus	2-12%	No data

** *(Pham-kanter et al, 1996; Kharsany et al, 1997; Johnson et al, 2005b)*

Other factors associated with increasing the risk of MTCT include; acquisition of STI's or new HIV-1 infections due to unprotected sexual intercourse (Bulterys *et al*, 1997), advanced maternal disease (Blanche *et al*, 1994); advanced maternal age (Mayaux *et al*, 1995); infant gender (European collaborative study, 2004), breastfeeding (European collaborative study, 1992), low birth weight and prematurity (Landesman *et al*, 1996). Maternal cigarette smoking during pregnancy has been associated with a three-fold increased risk of MTCT among women with low CD4 cell counts (Burns *et al*, 1994). Similarly, the use of various illicit drugs such as cocaine, heroin, opiates or methadone lowers maternal immunity and increases the risk for preterm delivery and MTCT (Bulterys *et al*, 1997; Rodriguez *et al*, 1996).

1.3.2.1 Viral burden in various body compartments

The amount of *free-virus* (HIV-1) in body fluids such as saliva, tears, breast milk and urine has been estimated to be 10-100 times lower than the amount of virus typically found in blood samples (Table II). While some body fluids may contain only small amounts of *free-virus*, all body fluids contain *virus-infected cells.* In genital secretions, these *virus-infected cells* have been shown to be a major source of HIV-1 transmission, with the risk of sexual transmission of HIV-1 being dependant upon the quantity of *virus-infected cells* present in genital secretions (Levy, 2007).

Table II: HIV-1 (Viral burden) in various bodily fluids (Levy, 2007)

Bodily fluids (cell-free)	Viral burden estimates (IP/ml)
Blood plasma	10-50
Serum	10-50
Saliva	< 1
Semen	10-50
Breast milk	< 1
Vaginal/cervical secretions	< 1
Tears	< 1
Ear secretions	1-10
Urine	< 1

*Footnote: *IP/ml: number of Infectious Particles per millilitre*

Pilcher and others conducted a study wherein they compared HIV-1 RNA levels in various bodily fluids (Fig 7) during primary versus established infection. HIV-1 RNA in blood and cerebrospinal fluid were significantly higher during primary infection, while cervicovaginal lavage and saliva were not significantly different among both groups (Pilcher *et al*, 2001). One

of their major study findings was that primary HIV-1 infection was a dynamic process of dissemination to diverse tissue compartments, which resulted in the early establishment of viral reservoirs and shedding into oral and genital fluids. These investigators confirmed that HIV-1 was shed into genital fluids early during primary infection and have suggested that like blood, dissemination into the oral cavity and female genital tract most probably occurs by the time of onset of symptoms. Those authors also state that while ARV's may be effective in viral suppression in these compartments, it was unlikely to limit the initial dissemination to these respective tissues.

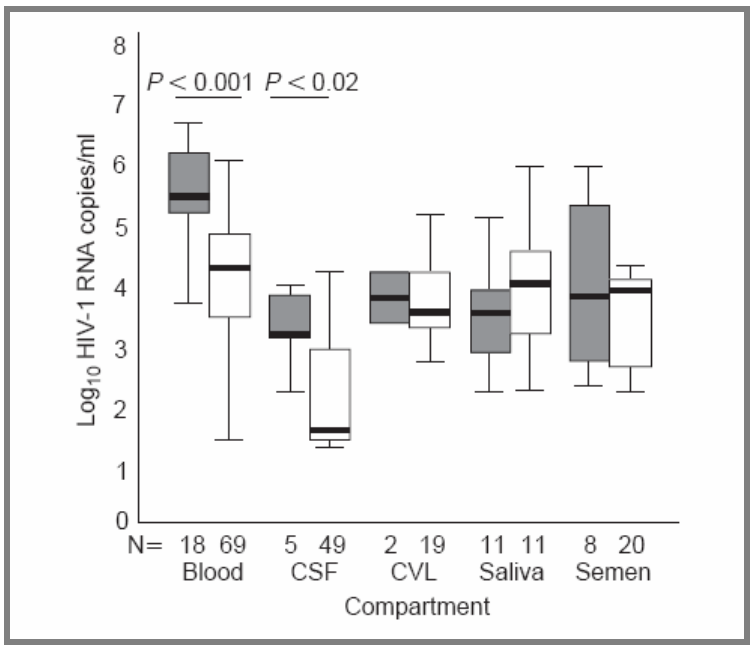


Fig 7: HIV-1 RNA levels in various body compartments/ fluids during primary infection (shaded-boxes) versus established infection (open-boxes) (Pilcher *et al*, 2001)

1.4 The female genital tract: an independent reservoir for HIV -1

HIV-1 was first isolated from the genital tract of women more than two decades ago (Wolfsy *et al*, 1986; Vogt *et al*, 1986). Anatomical sources, the hormonal cycle and a wide array of sampling techniques have made evaluation of the female genital tract quite complex (Coombs *et al*, 2003; Galvin and Cohen, 2006).

1.4.1 Sites of HIV-1 shedding

Genital tract HIV-1 has been detected as cell-free in addition to cell-associated forms (Wolfsy *et al*, 1986; Vogt *et al*, 1986; Hart *et al*, 1999) in the vagina, cervix and upper genital tract (Farrar *et al*, 1997; Johnstone *et al*, 1994). The primary targets for male-female HIV-1 sexual transmission are immune cells of the lower female genital tract. These include dendritic cells, macrophages, T cells and epithelial cells (Coombs *et al*, 2003; Nicol and Nuovo, 2005; Ellerbrock *et al*, 2001).

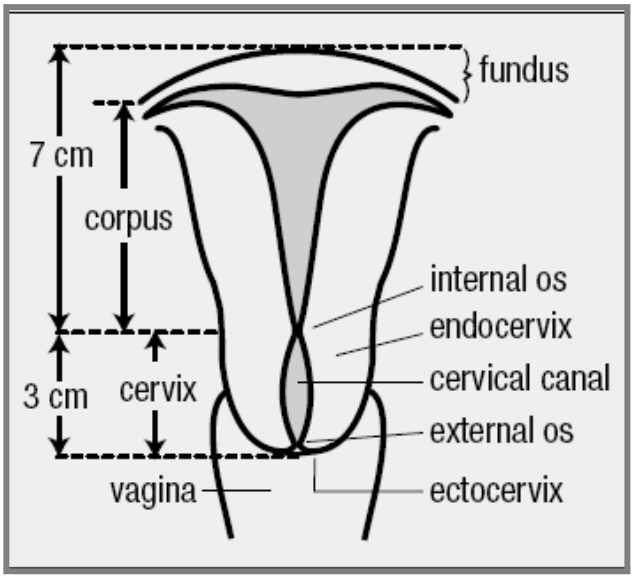


Fig 8a: Uterus of a woman of reproductive age (WHO, 2006a)

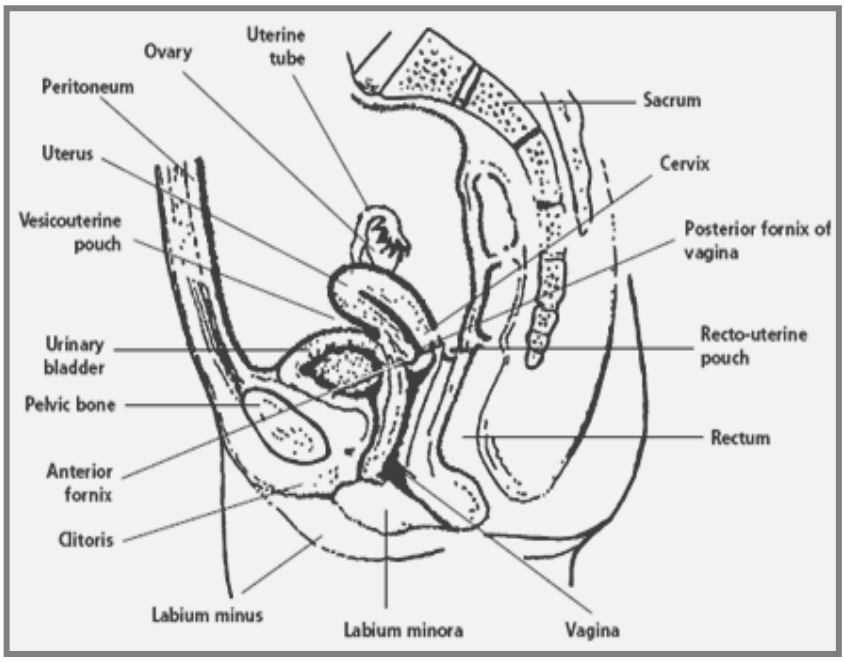


Fig 8b: Median section of the female pelvis

1.4.2 Sampling methods

Sampling methods for the female genital tract remain un-standardised at the present time and range from conventional swabbing techniques, cervicovaginal lavage (CVL) collection to more recent techniques of direct aspirates of cervical mucous, cytobrush, tampons and Sno-strip wicking (Coombs *et al*, 2003).

While direct aspirates, CVL and swabbing techniques collect cell-free and cell-associated virus, Sno-strip wicking collects cell-free virus and cytobrushes collect mainly cell-associated virus (Coombs *et al*, 2003).

There are several advantages and disadvantages to consider when selecting a genital sampling collection method. Variability of assay results is a function of; sampling method, assay type and biological variation within the compartment being sampled (Brambilla *et al*, 1999). The

general approach to "sampling choice" should always proceed from the least intrusive to the most intrusive method (Coombs *et al*, 2001).

CVL sampling can be performed in any clinical setting and involves flushing the cervical os (Figs 8a; 8b) with 10 ml of sterile non-bacteriostatic saline and aspirating the resulting fluids (Kovacs *et al*, 1999; 2001; Hart *et al*, 1999; John *et al*, 2001). Compared to other methods, CVL yields a greater sample volume allowing more assays to be performed on a single sample. The introduction of a dilution factor when using CVL samples in various assays and interpretation of such data has traditionally attracted much criticism as it results in virus or antibody levels becoming more dilute, hence making standardization of the sample volume problematic. Several researchers have reported their results by use of a standardized denominator. The use of "per milliliter of lavage fluid" has been a widely accepted method utilized in several publications (John *et al*, 2001; Kovacs *et al*, 1999; Cu-Uvin *et al*, 2006). A few researchers have proposed the addition of 10 mmol/l lithium chloride to the CVL wash buffer as a means of standardizing sample volume (Bélec *et al*, 1995; Mohamed *et al*, 1997). Whilst such methods have been tested with success on spiked samples in a research laboratory setting, such methodology is yet to be routinely performed on clinical samples. In general, studies have shown that CVL samples are representative of cells shed from the cervix, vagina and uterus (Fig 8a; 8b). While epithelial cells and granulocytes are frequently found, lymphocytes and macrophages are found less often (Hill and Anderson, 1992; Howell *et al*, 1997). Cells known to be permissive for HIV-1 infection include epithelial, stromal and Langerhans cells (Hill and Anderson, 1992; Howell *et al*, 1997; Collins *et al*, 2000).

Sno-strip wicking is another commonly employed sampling technique. Such sampling allows for localization and collection of a defined volume of genital fluid from a specific anatomical

location such as the vaginal fornix, endocervix or exocervix (Fig 8a; 8b) with little or no disruption of the epithelial surface, while permitting concentration and detection of a lower level of virus or antibody (Coombs *et al*, 2003). While this technique may be an optimum method for collecting a known volume of sample, the volume of sample is relatively small, thereby restricting sample usage to just a single assay only (Coombs *et al*, 2001). With specific reference to HIV-1 RNA detection in the female genital tract, the greatest assay variability has been mainly observed with CVL samples while the least assay variability was noted with Sno-strip wicking (Coombs *et al*, 2001). Detection and quantitation of HIV-1 RNA was greatest in the cervix (Nuovo *et al*, 1993) and least in the vagina (John *et al*, 2001; Coombs *et al*, 2001; Iversen *et al*, 1998).

1.4.3 Factors influencing genital HIV-1 RNA (viral load)

Plasma HIV-1 RNA concentration both quantitatively and qualitatively is known to be the most important determinant for the prediction of genital HIV-1 shedding, even among women receiving ARV therapy. Other factors that influence HIV-1 shedding in this compartment include, genital viral discordance (lack of correlation between plasma and genital HIV-1 RNA), sexually transmitted infections (Wang *et al*, 2001), micronutrient supplementation (Mostad *et al*, 1997; McClelland *et al*, 2004), subtype (John-Stewart *et al*, 2005), oral contraceptive use (Mostad *et al*, 1997) and pregnancy (Clemetson *et al*, 1993). Several studies have demonstrated that genital HIV-1 shedding increases incrementally with plasma HIV-1 RNA concentration (Cu-Uvin and Caliendo, 1997; Hart *et al*, 1999; Iversen *et al*, 1998; Kovacs *et al*, 1999; 2001; Goulston *et al*, 1998). Various clinical trials have reported an association between genital HIV-1 shedding and micronutrient supplementation. Mostad and others found that reduced vitamin A levels were associated with increased vaginal HIV-1

shedding (Mostad *et al*, 1997). Similarly a randomized placebo-controlled trial undertaken in Kenya found that selenium and multivitamins resulted in a higher prevalence of quantifiable HIV-infected cells in vaginal (but not cervical) swab samples, compared with the placebo arm (McClelland *et al*, 2004)**.** Prevalence of HIV-1 infected cells, HIV-1 RNA in genital secretions; breast milk and the risk of MTCT were compared among subtypes A, C and D in a cohort of HIV-1 infected pregnant women in Kenya. In this study, (41.0%) of pregnant women infected with subtype C were more likely to shed HIV-1 infected vaginal cells than those women infected with subtypes A (19.0%) and D (8.0%). These findings have suggested that HIV-1 subtypes could also influence mucosal shedding of HIV-1 (John-Stewart *et al*, 2005).

1.4.4 Detection and quantitation of genital HIV-1 RNA

Several studies have detected *cell-free* and/or *cell-associated* HIV-1 RNA in the female genital tract with detection rates ranging from 9.0%-80.0% (Coombs *et al*, 2001; Kovacs *et al*, 2001; Tuomala *et al*, 2003; Chuachoowang *et al*, 2000; Quinn *et al*, 2000).

HIV-1 RNA was detected in most female genital samples with incomplete plasma viral load suppression (Kovacs *et al*, 1999; 2001) and in 20.0% of samples from women with complete plasma viral HIV-1 RNA suppression (Coombs *et al*, 2001). Several studies have found association between detectable whole CVL HIV-1 RNA, *cell- free* HIV-1 RNA and plasma HIV-1 RNA (Clemetson *et al*, 1993; John *et al*, 1997; Hart *et al*, 1999; Goulston *et al*, 1998; Iversen *et al*, 1998; Mostad *et al*, 1997; Cu-Uvin *et al*, 1998). Kovacs and others demonstrated that HIV-1 RNA was detected more often and at significantly higher levels in *cell-associated* compared to the *cell-free* component of CVL (Kovacs *et al*, 1999). Whole unfractionated CVL

comprising of mainly *cell-associated* HIV-1 RNA correlated with plasma HIV-1 RNA in studies by Cu-Uvin and Caliendo (1997). In contrast, Hart and co-workers showed that most of the HIV-1 RNA was accounted for in the *cell-free* fraction and not in the *cell-associated* fraction. Both components appeared to correlate with plasma HIV-1 RNA (Hart *et al*, 1999).

Two studies found no association between plasma HIV-1 RNA levels and the ability to detect HIV-1 in the genital tract (Mbopi-Kéou *et al*, 2000; Rasheed *et al*, 1996). The dilution factor in *cell-free* HIV-1 CVL RNA was proposed as a possible reason for the lack of association between these two compartments.

1.4.4.1 Plasma vs. genital HIV-1 RNA

Fiore and others reported that plasma HIV-1 RNA failed as markers of infectivity of genital secretions after they observed cervicovaginal shedding occurring in 25.0% of women who had undetectable plasma virus (Fiore *et al*, 2003). Similar observations were reported by (Coombs *et al*, 1998; Mbopi-Kéou *et al* 2000; Spinillo *et al*, 2001; Debiaggi *et al*, 2001 and Günthard *et al*, 2001).

A Spanish study undertaken by Garcia-Bujalance and others reported a lack of correlation between vaginal and plasma HIV-1 RNA of pregnant women, therefore women with undetectable plasma HIV-1 RNA were still at risk of transmitting the virus vertically during a normal vaginal delivery (Garcia-Bujalance *et al* 2004). Chuachoowang and co-workers reported that HIV-1 RNA level in the genital tract of pregnant women close to term, increased the risk of perinatal transmission even though the plasma HIV-1 RNA level was low (Chuachoowang *et al* 2000). Studies have shown that the higher the HIV-1 RNA levels were

in maternal plasma, the greater the likehood that HIV-1 will also be found in maternal cervicovaginal secretions.

Several studies reported positive correlations between systemic and genital tract HIV-1 RNA levels (Mostad *et al*, 1997; Iversen *et al*, 1998; Goulston *et al,* 1998; Kovacs *et al*, 1999; Hart *et al*, 1999; Cu-Uvin and Caliendo, 1997; 2006; Andréoletti *et al*, 2003; Si-Mohamed *et al*, 2000).

A longitudinal study to determine the patterns of HIV-1 RNA in paired plasma and CVL sample measurements of non-pregnant women over a 36 month period found that CVL HIV-1 RNA levels were generally lower than plasma levels. The authors confirmed an occasional discordance (5.0%) when CVL HIV-1 RNA exceeded plasma HIV-1 RNA. They suggest that it may be possible that women with undetectable plasma HIV-1 RNA were less likely to express genital tract virus and therefore less likely to transmit HIV-1. Where plasma HIV-1 RNA correlated strongly with CVL RNA levels, it may be concluded that genital tract *cell-free* HIV-1 RNA may be more dependent on changes in plasma HIV-1 RNA than on changes in the local genital environment as compared with *cell-associated* HIV-1 DNA that may derived from local genital reservoirs (Cu-Uvin *et al*, 2006).

The mucosal hypothesis for intrapartum HIV-1 transmission has been supported by an increased risk of transmission with higher levels of *cell-free* and *cell-associated* HIV-1 RNA in cevicovaginal secretions independent from maternal plasma HIV-1 RNA (Chuachoowang *et al*, 2000; John *et al*, 2001; Tuomala *et al*, 2003). A significant relationship was observed between cervical or vaginal HIV-1 RNA levels and perinatal infection. Infant exposure to HIV-1 infected cells in maternal genital secretions has been shown to be an important

determinant for intrapartum HIV-1 transmission (John *et al*, 2001). This was also confirmed in a study undertaken in Thailand in which researchers investigated plasma and CVL HIV-1 RNA levels in relation to MTCT in a randomized placebo-controlled trial of short-course Zidovudine therapy. In this study, a perinatal HIV-1 transmission rate of 28.7% was observed among those women with quantifiable CVL HIV-1 RNA and high plasma HIV-1 RNA. Those women without quantifiable CVL HIV-1 RNA (*cell-free* and *cell-associated*) and low plasma HIV-1 RNA had a perinatal transmission rate of 1.0% (Chuachoowang *et al*, 2000). Both studies have shown that high CVL HIV-1 RNA increases the risk of perinatal transmission at both high and low levels of virus in plasma (Chuachoowang *et al*, 2000; John *et al*, 2001).

1.5 Interventions for the prevention of MTCT

The United Nations (UNAIDS, 2005) currently recommends a three-pronged strategy for the reduction of pediatric HIV-1 infection and disease:

- *prevention of HIV-1 infection among women of child-bearing potential*
- *prevention of unwanted and unplanned pregnancies among HIV-1 positive women*
- *prevention of MTCT during pregnancy, labour and delivery, and breastfeeding*

ARV drug therapy forms only a single component in a broader spectrum of evidence-based interventions proposed for PMTCT (UNAIDS, 2005). Other interventions include:

- improved availability, quality, and use of maternal and child health services
- HIV-1 voluntary counseling and testing (VCT)
- infant feeding options
- caesarean sections
- maternal care and support

1.5.1 Antiretroviral (ARV) therapy

Several large, randomised, controlled clinical trials performed to date have successfully demonstrated the effectiveness of ARV's in the prevention of MTCT as long-term treatment or short-term prophylaxis in breastfed (Appendix 1) and non-breastfed populations (Appendix 2). Most of these ARV regimens have been based on drugs such as Zidovudine (ZDV), Lamivudine (3TC), Nevirapine (NVP) or a combination of these drugs in either short or long-course regimens.

1.5.1.1 Zidovudine (ZDV) monotherapy

Almost 15 years ago, a randomized, placebo-controlled clinical drug trial (PACTG 076) successfully demonstrated the efficacy of ARV drug therapy for PMTCT. In this study, a long-course ZDV regimen initiated from 14 weeks gestation until delivery in the mother and for six weeks in the infant significantly reduced MTCT by 67.0% in a non-breastfed population (Connor *et al*, 1994). Despite the PACTG 076 regimen being hailed as one of the most noteworthy advances in PMTCT, successful implementation of this regimen has been confined to resource-rich countries due to its complexity and cost. The Perinatal HIV Prevention Trial (PHPT-1) conducted in Thailand examined different durations of antenatal and neonatal ZDV regimens in a non-breastfed population (Lallemant *et al*, 2000). These investigators concluded that shorter *antenatal* therapy with ZDV was much more efficacious than longer *antenatal* therapy. Investigators of the CDC Côte d'Ivoire trial reported a 37.0% reduction in MTCT following their assessment of a short regimen of antenatal (from 36 weeks gestation) and intrapartum ZDV against a placebo in a breastfed cohort (Wiktor *et al*, 1999). In this study, MTCT rates (at three months) of 16.5% and 26.0% were noted in the ZDV and placebo groups respectively. Researchers of the ANRS 049 DITRAME study (Côte d'Ivoire/Burkino Faso)

evaluated a short-course regimen of antenatal (from 36 weeks), intrapartum and postpartum (one week) ZDV versus a placebo in a breastfed cohort. On the whole, efficacy in reducing MTCT was reported as 38.0%. At six months, MTCT rates were 18.0% and 27.0% in the ZDV and placebo arms respectively (Dabis *et al*, 1999).

1.5.1.2 Combination therapy

Mandelbrot and others investigated a regimen of (ZDV) and Lamivudine (3TC) from 32 weeks (in pregnancy) and continued in infants until six weeks postpartum in the French ANRS 075 study. They concluded that the addition of 3TC reduced MTCT rates from 6.8% to 1.6% at 18 months (Mandelbrot *et al*, 2001). Similarly, the PETRA trial was a four-armed study undertaken in South Africa, Tanzania and Uganda wherein investigators reported a 67.0% reduction in MTCT at six weeks among breastfeeding women when a regimen of ZDV and 3TC was commenced from 36 weeks and continued until one week post-delivery in both women and their infants (PETRA study team, 2002).

1.5.1.3 Nevirapine (NVP) vs. ZDV or ZDV and 3TC

An effective, easily administered and cost-effective ARV regimen encompassing maternal and infant safety, tolerability and optimal protection from HIV-1 transmission was sought as a suitable alternative intervention for reduction of MTCT in resource-constrained countries.

Several properties such as potent antiviral activity, excellent bioavailability, rapid oral absorption, long half-life and the ability to rapidly cross the placental barrier into the fetus (Cheeseman *et al*, 1993; 1995; Havlir *et al*, 1995) have made the intrapartum and neonatal sdNVP regimen an extremely attractive therapeutic option for reducing MTCT in resource-

poor settings. Kinetic analysis has shown that NVP was able to penetrate *cell-free* virions and inactivate virion-associated reverse transcriptase *in situ*; properties useful in the inactivation of *cell-free* virions in the genital tract and breast milk (Zhang *et al*, 1996).

Mirochnick and co-workers conducted the ACTG 250 trial, the first study that evaluated the safety, toxicity and pharmacokinetics of sdNVP during pregnancy (Mirochnick *et al*, 1998). They demonstrated that an oral 200 mg dose administered to women during labour, followed by a single 2 mg/kg dose to the infant (48-72 hours) was well-tolerated, non-toxic, had a long half-life (36.8-65.7 hours) and high bioavailability. In addition, NVP drug concentration exceeding the 100 ng/ml therapeutic target was achieved in the newborn. These findings were thereafter also confirmed by the HIVNET 006 trial. Researchers reported that high plasma concentration that was ten times the NVP concentration required to inhibit viral replication by 50.0% (IC_{50}) was attainable with sdNVP. In addition, this Ugandan study performed on a predominantly breastfed cohort also reported good drug penetration into colostrum and breast milk with sdNVP prophylaxis (Musoke *et al*, 1999).

The benchmark HIVNET 012 study undertaken in Uganda was an extremely successful perinatal HIV-1 prevention clinical trial which further substantiated the safety and efficacy of intrapartum and neonatal sdNVP when compared with a short-course of ZDV (Guay *et al*, 1999). Similar to HIVNET 006, the potent sdNVP regimen used in HIVNET 012 trial successfully maintained NVP serum concentrations above 100 ng/ml therapeutic target in infants. This regimen also reduced the risk of perinatal HIV-1 transmission in this breastfeeding population by 50.0% during the first 14-16 weeks of life (Guay *et al*, 1999). The sdNVP regimen was found to be simple, well tolerated and cost effective (Marseille *et al*, 1999). Apart from the low occurrence of adverse side-effects, subsequent follow-up data (18

months) from this study provided concrete evidence of its sustained efficacy with a continued HIV-1 free survival benefit maintained well into infancy (Jackson *et al*, 2003). The South African Intrapartum Nevirapine Trial (SAINT) demonstrated similar safety and efficacy when investigators compared a short-course sdNVP regimen to a short-course combination regimen of intrapartum/postpartum (seven days) ZDV and 3TC. Overall efficacy in preventing HIV-1 infection was estimated as 50.6% for the sdNVP regimen and 58.8% for the ZDV/3TC combination. The SAINT trial was particularly relevant for MTCT reduction in developing countries as this study further substantiated the findings of the HIVNET 012 study by extending similar observations across populations, regions and clades (Moodley *et al*, 2003). The SAINT trial encompassed analysis of both formula and breastfed infants and verified the safety, affordability and efficacy of the short-course intrapartum and neonatal sdNVP regimen in a resource-constrained setting. The timing of maternal NVP dosing during labour was another very significant issue addressed by this trial, with the odds of an intrapartum HIV-1 infection found to increase by three-fold when the maternal sdNVP dose was ingested less than two hours prior to delivery (Moodley *et al*, 2003). Findings from the HIVNET 012 and SAINT trials provided compelling evidence in support of the sdNVP regimen for reduction of vertical HIV-1 transmission. These significant research findings have since been successfully translated into the World Health Organisation's (WHO) endorsement of this regimen as one of several recommended ARV regimens for PMTCT in resource-limited settings (WHO, 2006b).

1.5.1.4 Addition of sdNVP to ZDV regimens

The PHPT-2 trial conducted among non-breastfeeding women in Thailand determined the effect of adding NVP (intrapartum or sdNVP intrapartum plus neonatal sdNVP) to a ZDV

regimen (from 28 weeks, with one week ZDV). Compared to ZDV alone (6.3%), transmission rates were significantly reduced (2.8% in sdNVP intrapartum and 1.9% for intrapartum and neonatal NVP) by addition of sdNVP to the ZDV regimen (Lallemant *et al*, 2004). In Malawi, an open-labelled trial (Taha *et al*, 2004) examined the efficacy of adding one week of neonatal ZDV to the HIVNET 012 regimen. Investigators found that additional ZDV did not confer any significant benefit at six weeks and reported MTCT rates of 14.1% (sdNVP only) and 16.3% (sdNVP and ZDV) [Taha *et al*, 2004]. The ANRS 1201 DITRAME PLUS (Ivory Coast and Burkino Faso) was an open-labeled study that compared (ZDV + sdNVP) with (ZDV + 3TC + sdNVP). The MTCT rate at six weeks was 6.5% in the group receiving (ZDV + sdNVP), while the group receiving (ZDV + 3TC + sdNVP) had an MTCT rate of 4.7%. No difference was noted between transmission rates of breastfed and non-breastfed infants at six weeks of age (Dabis *et al*, 2005).

1.5.1.5 *Infant-only* ARV prophylaxis

The benefit of neonatal ZDV was first demonstrated by Wade and others (Wade *et al*, 1998). In this study, the transmission rate was 6.1% in women who received the full ACTG 076 regimen, 10.0% in those who only received ZDV during the intra-and postpartum periods was 10.0% and 26.6% in women who did not receive any ZDV.

A few African studies successfully demonstrated that it was still beneficial to provide ARV treatment to HIV-1 exposed-infants in the form of post-exposure prophylaxis or PEP in the following situations ; there was no access to antenatal care, infants were born at home, women were missed by PMTCT and when maternal intrapartum ARV therapy was missed or not possible. In Malawi, investigators of the NVAZ trial (Taha *et al*, 2003) evaluated a PEP

regimen of sdNVP and one week of ZDV administered to the infants of untreated mothers in a breastfeeding population. The MTCT rate observed at 6-8 weeks was 15.3% with combination therapy and 20.8% with sdNVP alone. This study indicated that dual therapy of sdNVP coupled with one week of ZDV given to the infant was far superior to providing sdNVP alone (Taha *et al*, 2003). In the South African PEP study, investigators compared neonatal sdNVP with six weeks of ZDV among infants whose mothers received no ARV therapy (Gray *et al*, 2005). PEP study investigators reported an overall MTCT rate of 16.3% by 12 weeks of age and concluded that an *infant-only* sdNVP regimen was as efficacious as six weeks of ZDV therapy (Gray *et al*, 2005). The Mashi study performed in Botswana demonstrated that maternal sdNVP may not be needed to reduce MTCT in the setting of maternal ZDV and infant ZDV plus sdNVP. Investigators observed similar infant HIV infection rates at age six weeks irrespective of whether the women received sdNVP (4.3%) or placebo (3.7%) (Shapiro et al, 2006).

1.5.1.6 Current standard of care for PMTCT in low-income countries

The current standard of care for PMTCT of HIV-1 involves a dual regimen of maternal ZDV starting from 28 weeks and intrapartum sdNVP in women. Infant prophylaxis presently entails sdNVP at birth, followed by a seven day short-course of ZDV therapy (Appendix 3a). In the absence of maternal ARV therapy during labour (Appendix 3b), infant ARV regimens of either sdNVP alone or (sdNVP + four weeks of ZDV) are recommended by the WHO as alternate therapeutic options for PMTCT (WHO, 2006b).

Some of the key MTCT lessons learnt to date include: (Dao *et al*, 2007)

- combination ARV regimens are more effective than monotherapy
- longer duration of ARV prophylaxis is usually more effective
- efficacy of intrapartum ARV regimens is diminished over time in breastfeeding populations
- addition of sdNVP improves the efficacy of other short-course ARV regimens
- relative importance of maternal sdNVP in the context of short-course ZDV regimens remains unclear. *Infant only* post-exposure prophylaxis is only a valid alternate therapeutic option for PMTCT in instances when maternal ZDV is received for an inadequate duration or when infants receive ZDV for four weeks

1.5.1.7 ARV coverage for PMTCT

In 2007, ~33.0% of pregnant HIV-1 infected women received ARV drugs for PMTCT. Figure 9a illustrates the estimated ARV drug coverage for PMTCT observed in low and middle-income countries in 2007 (UNAIDS/WHO/UNICEF, 2008b). Variable ARV coverage was noted among the ten countries (including South Africa) with the largest number of HIV-1 infected pregnant women. While ARV coverage in Thailand has been very encouraging (92.0%), some African countries including South Africa (57.0%), Mozambique (46.0%); Kenya (69.0%); Guyana (43.0%) have indicated fair to reasonable coverage. However, countries like India, Democratic Republic of Congo, Nigeria and Ethiopia were able to achieve 10.0-15.0% ARV coverage (UNAIDS/WHO/UNICEF, 2008b). In 2007, ARV drug coverage among infants born to HIV-1 infected women in low and middle-income countries was estimated to be less than 20.0% (UNAIDS/WHO/UNICEF, 2008b).

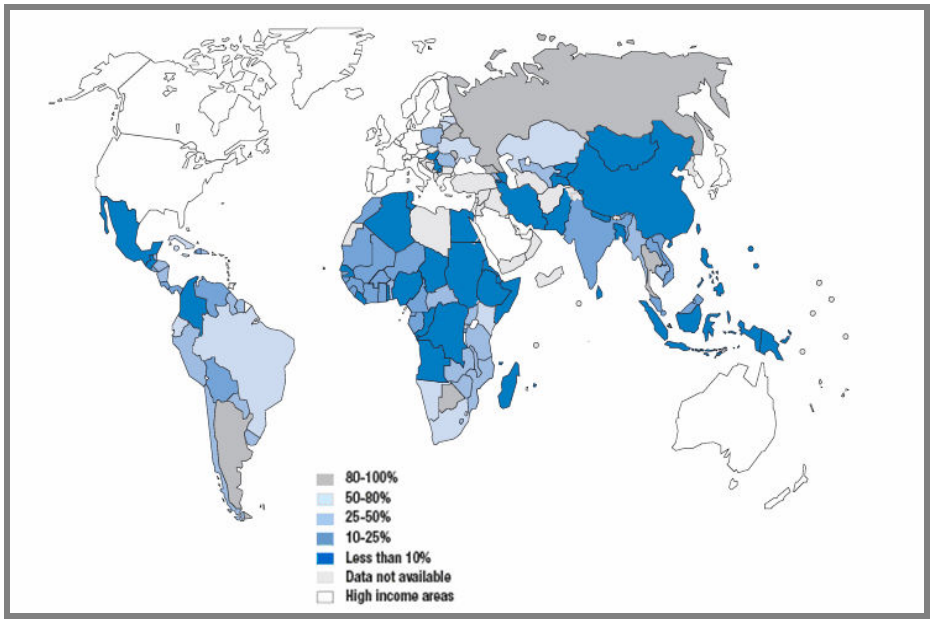


Fig 9a: ARV coverage for PMTCT in low and middle-income countries (UNAIDS/WHO/UNICEF, 2008b)

In 2007, data on ARV drug regimens for PMTCT (provided by 60 countries) revealed that almost 50.0% of women received NVP monotherapy; 26.0% received a dual ARV regimen; and 8.0% received a triple ARV combination (UNAIDS/WHO/UNICEF, 2008b). Data from Sub-Saharan African (provided by 44 countries) indicated that women on treatment received NVP monotherapy (50.0%); dual regimen (27.0%) and a triple ARV combination (6.0%) (UNAIDS/WHO/UNICEF, 2008b).

Despite a host of successful ARV drug regimens, uuniversal access to HAART is still not a reality in most resource-limited countries where the rates of MTCT continue to escalate (Chersich *et al*, 2006). The use of NVP alone or in combination with ZDV (Fig 9b), therefore remains the mainstay of PMTCT programmes in several low-resource settings (Fowler *et al*, 2007).

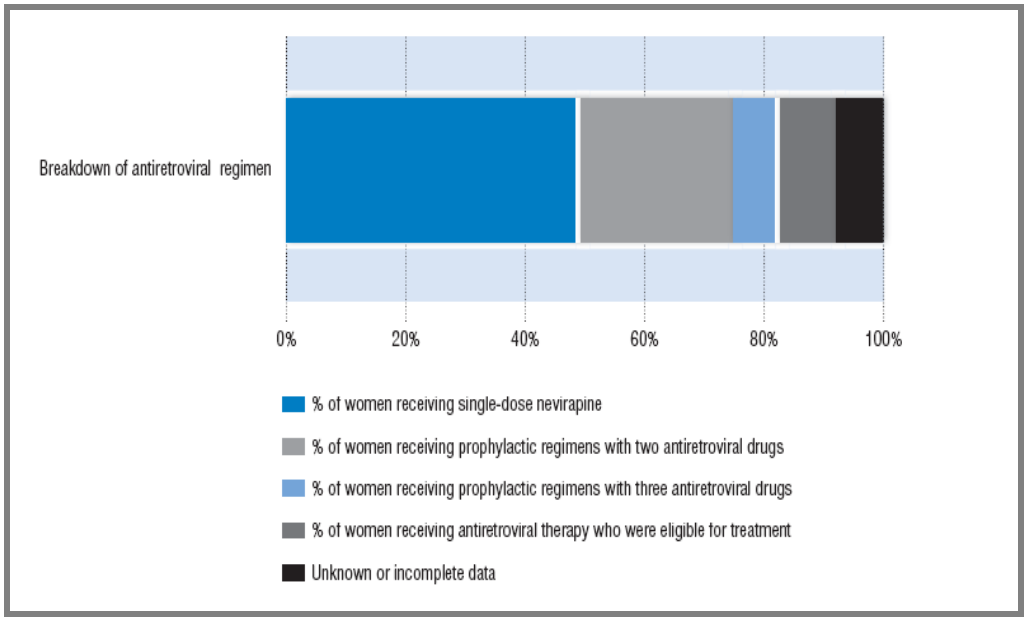


Fig 9b: Distribution of ARV regimens received by pregnant HIV-1 infected women in low and middle-income countries (UNAIDS/WHO/UNICEF, 2008b**)**

1.5.1.8 Pharmacology of NVP

Structurally, NVP (Viramune®) is a member of the dipyrido-diazepinone group of chemical compounds. This potent non-nucleoside reverse transcriptase inhibitor or NNRTI was developed by Boehringer Ingelheim Pharmaceuticals (Viramune®, 2005). NNRTI's are a class of ARV drugs that bind directly to the HIV-1 reverse transcriptase enzyme and prevent RNA from changing into DNA (Richman *et al*, 1991; Merluzzi *et al*, 1990; Hargrave *et al*, 1991).

NNRTI's have shown a high degree of specificity for the reverse transcriptase of HIV-1, but are not effective against that of HIV-2, even with their high homology (Wu *et al*, 1991). Four NNRTI's are currently approved by the Food and Drug Administration (FDA) for use in the treatment of HIV-1 infection. These include NVP, Efavirenz (EFV), Delavirdine (DLV) and Etravirine (FDA, 2009). Figure 10 illustrates the mechanism of action for the NNRTI class of

compounds. An NNRTI such as NVP attaches directly to the reverse transcriptase enzyme at a specific location, near the active site and causes a structural change that disrupts the formation of the active site and leads to weakened polymerisation activity (Immunopaedia, 2008).

NVP is a potent ARV that is readily absorbed after oral administration (> 90.0%) and has a half-life of ~45 hours (Riska *et al*, 1999). It is non-ionised at physiologic pH, is distributed widely and evenly throughout the body, and is highly lipophilic. It is approximately 60.0% bound to plasma proteins. More than 80.0% of the NVP dose is bio-transformed via P450 oxidation to hydroxylated metabolites that are subsequently largely excreted in urine as glucuronides. Only a small fraction of the dose (< 3.0%) is excreted unchanged in urine (Sabo *et al*, 2000). NVP has been shown (*in vitro*) to block an early event in the viral replication cycle, completely inhibiting replication if administered within 24 hours of infection, but having no effect on protein production or viral release (Koup *et al*, 1991).

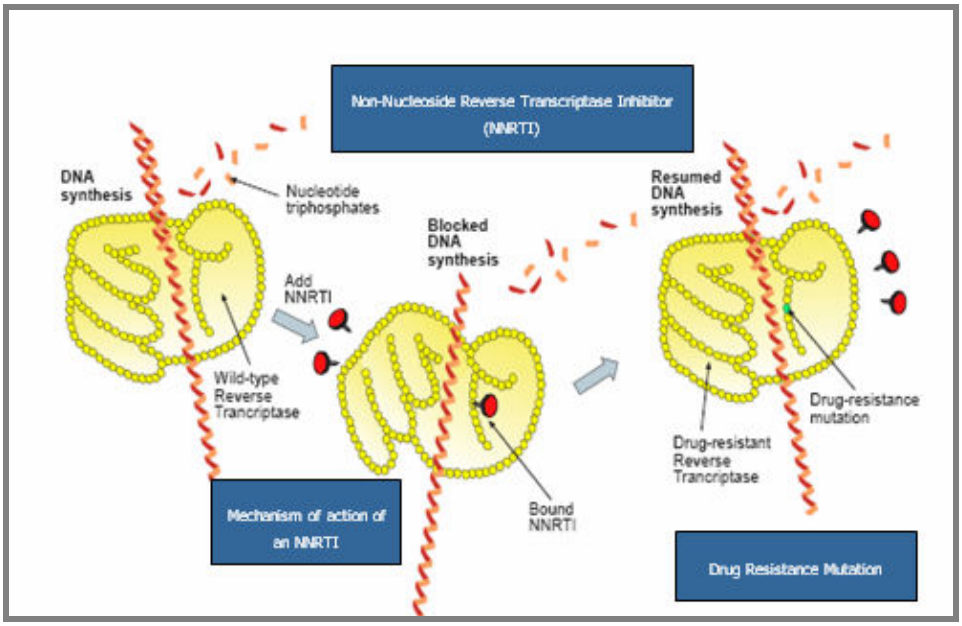


Fig 10: Mechanism of action of NNRTI drugs (Immunopaedia, 2008)

1.5.1.9 NVP detection methods

a) Qualitative Methods

Thin layer chromatography:

Thin layer chromatography (TLC) is a fast, inexpensive method for the qualitative measurement of NVP in plasma. Studies have shown that TLC methods were able to reliably detect the presence of NVP concentration of greater than or equal to 100 ng/ml in human plasma (Dubuisson *et al*, 2004). This study made use of spot detection by TLC plates and was able to detect NVP concentrations as low as 60 ng/ml. The 100 ng/ml concentration was selected as the limit of detection due to the robustness of the spot detection at this concentration, compared to a lower concentration.

b) *Quantitative Methods*

High performance liquid chromatography with ultraviolet absorbance:

The use of high performance liquid chromatography (HPLC) with ultraviolet (UV) absorbance detection was validated for the simultaneous quantitative determination of three currently prescribed NNRTI's (EFV, DLV and NVP). Studies have found HPLC with UV absorbance to be a very accurate, precise, simple and highly reproducible method for NNRTI quantitation in human plasma (Rezk *et al*, 2002). A study comparing protease inhibitors (PI's) and NNRTI's has shown that HPLC with UV absorbance can be successfully applied for reliable and accurate ARV quantitation in samples of maternal plasma and direct aspirates of cervicovaginal fluid (Min *et al*, 2004).

High performance liquid chromatography and solid phase extraction:

The majority of the published methods for NVP quantitation in plasma have relied upon solid phase extraction of ~200-600 µl of sample, followed by HPLC (Pav *et al*, 1999; Marchei *et al*, 2002; Marzolini *et al*, 2002; Rezk *et al*, 2004). Recent modification to this method to increase detection sensitivity has permitted the accurate quantitation of smaller sample volumes such as clinical neonatal samples (Silverthorn and Parsons, 2006). Shorter run times and lower levels of detection have made this method an attractive cost and time-efficient option for application in several research settings (Kagaayi *et al*, 2005).

Liquid chromatography Tandem Mass spectrophotometry:

An overall NVP recovery of 92.4% was achieved by the use of Liquid chromatography Tandem Mass spectrophotometry (LC-MS-MS). This sensitive and rapid method was developed for the measurement of NNRTI (NVP) levels in HIV-1 infected human plasma. In this method, the analyte and internal standard was isolated from plasma by a simple perchloric acid precipitation of plasma proteins followed by centrifugation. Two linear calibration curves were then used to determine NVP concentration (Chi *et al*, 2003). Laurito and others (2002) found the application of the LC-MS-MS method in sdNVP bioequivalence studies to be reliable and adequate. Lowest limit of quantification, inexpensive liquid-liquid extraction, faster run times and high specificity were some of the advantages cited in favour of LC-MS-MS over other HPLC methods. In addition, the LC-MS-MS method was applied with much success for NVP quantitation in samples of paediatric saliva (Rakhmanina *et al*, 2007), oropharyngeal and cervicovaginal secretions (Harms *et al*, 2005; Kunz *et al*, 2007) and dried blood spots (Koal *et al*, 2005).

1.5.1.10 Quantitation of NVP in the female genital tract

While quantitation of NVP drug concentration (following single dosing) has been widely reported in breast milk and plasma studies (Mirochnick *et al*, 1998; 2000; 2003, Muro *et al*, 2005; Musoke *et al*, 1999), limited data were currently available for NVP drug measurements in cervicovaginal secretions from HIV-1 infected pregnant women. The mucosal hypothesis for intrapartum HIV-1 transmission is supported by a higher risk of transmission with higher levels of *cell-free* and *cell-associated* HIV-1 in cervicovaginal secretions independent from maternal plasma HIV-1 RNA (Chuachoowang *et al*, 2000; John *et al*, 2001; Tuomala *et al*, 2003). In their study to analyze the extent of intrapartum transmission after mucosal exposure of the fetus to HIV-1 in the context of sdNVP, Kunz and others quantified NVP concentration in infant oropharyngeal secretions using liquid chromatography/Tandem-Mass spectrophotometry methods (Kunz *et al*, 2007). In this study, no intrapartum HIV-1 transmission was observed after mucosal exposure of the fetus to HIV-1. Investigators found that a high concentration of NVP was rapidly achieved in the oropharyngeal secretions of babies after a single dose which also contributed to the drug's effectiveness (Kunz *et al*, 2007).

In a study carried out in Uganda, a high concentration of NVP was detected in maternal cervicovaginal secretions (median = 742 ng/ml) and oropharyngeal secretions of babies after sdNVP administration to the mother (Harms *et al*, 2005). Sno-strips were used to collect maternal cervicovaginal secretions and NVP concentration was determined by LC-MS-MS methods. Significant positive correlation was noted between maternal plasma concentration (median = 1742 ng/ml) and infant plasma concentration (1291 ng/ml) (Harms *et al*, 2005).

Studies have also found that differential penetration of ARV drugs in the female genital tract could alter the rates of vertical and heterosexual transmission and influence the efficacy of post-exposure prophylaxis. In a study of non-pregnant HIV-1 infected women by Min and others, cervicovaginal fluid samples were collected by direct aspiration methods (Min *et al*, 2004). ARV drug concentration (NNRTI and PI) were assayed at two collection points using HPLC/UV detection. Measurable drug concentration for all ARV drugs, with the exception of Ritonavir and Saqinavir were found in samples of cervicovaginal fluid (CVF). Of the NNRTI drugs assayed, CVF samples had the highest concentration of NVP (Min *et al*, 2004). In contrast, findings of poor NNRTI drug penetration into CVF were previously cited by Ellerbrock *et al*, 2001 and Tirado *et al*, 2004. Cohen and co-workers reported that the penetration of antiviral agents into the female genital tract was dictated by the degree of protein binding and a drug's affinity for albumin and α_1-acid glycoprotein. High protein-bound drugs (> 80.0%) were recovered at a lower concentration in genital tract secretions compared to plasma drug levels (Cohen *et al*, 2007).

Kashuba and others profiled the concentration of ARV drugs based on extracellular and intracellular genital tract exposure relative to blood plasma concentration (Fig 11). Using novel pharmacokinetic methods, these researchers found that despite high plasma concentration, the corresponding concentration of high-protein bound drugs such as the NNRTI's remained remarkably low in the genital tract (Kashuba, 2006; Cohen *et al*, 2007). When compared to blood plasma, the NNRTI's were also detected at a lower concentration in CVF samples using LC-MS-MS quantitation methods (Kwara *et al*, 2008).

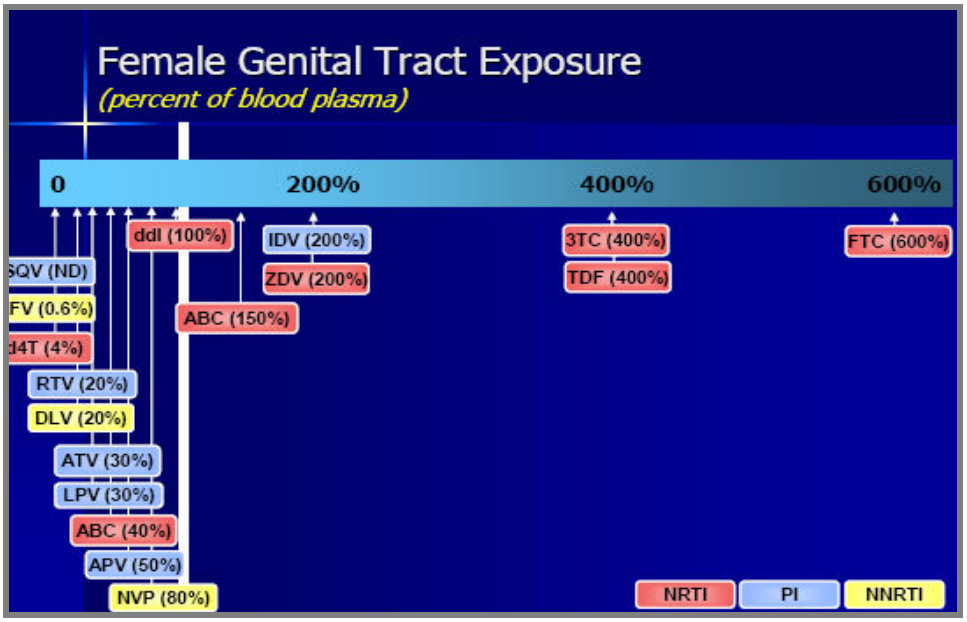


Fig 11: ARV drug concentration in the female genital tract relative to blood plasma concentration [ratio of genital fluid to plasma]. NNRTI drugs: EFV, DLV and NVP. (Kashuba, 2006; Cohen *et al*, 2007)

1.5.2 Other interventions for PMTCT

1.5.2.1 Modified obstetric practice

MTCT rates of 2.0% with ARV and 10.4% without ARV were observed among women who delivered by elective C/S. In contrast, MTCT rates of 7.3% with ARV and 19.0% without ARV were noted among women who delivered vaginally (European mode of delivery collaboration, 1999).

While the use of an elective C/S prior to the onset of labour and rupture of membranes has been widely advocated as a highly effective evidence-based intervention against intrapartum HIV-1 transmission (European mode of delivery collaboration, 1999), there have also been conflicting reports regarding its clinical utility among HIV-1 infected women. In a meta-analysis conducted by Read and others, the use of the elective C/S over the NVD translated

into a 50.0% reduction of risk among women who were not on HAART or had high plasma viral load (Read and Newell, 2006). Data from the WITS study reported an association between maternal morbidity and use of elective C/S (Read *et al*, 1999) among HIV-1 infected women. Similarly the ACTG 185 trial also found an increased rate of infectious complications among HIV-1 infected women who had surgical deliveries (Mofenson *et al*, 1999a). Despite its potential risks, an elective C/S negates the possibility of premature rupture of membranes and also minimizes an infant's exposure to contaminated vaginal fluids during a normal vaginal delivery. Guidelines from the Centre for Disease Control (CDC) have stated that these risks were not of significant frequency or severity to outweigh the potential benefits of reduced transmission. The CDC recommends that an elective C/S be offered to all women provided they were made aware and understood the risks and benefits of such procedures (CDC 2002).

1.5.2.2 Infant feeding methods

Breastfeeding is considered the most ideal food for infants due to its age appropriate nutritive value and protective immune factors. However, the transmission of HIV-1 from breast milk was found to account for a 14.0% risk of transmission (Dunn *et al*, 1992). Breastfeeding continues to remain one of the leading contributing factors to high HIV-1 transmission rates in Africa, with increasing concerns over its direct influence on the efficacy of ARV prophylactic regimens and ultimately infant survival.

A longer duration of breastfeeding has been shown to enhance HIV-1 transmission; however early weaning has been associated with adverse impact on growth, morbidity and mortality. While breastfeeding may be a key determinant for the discrepancy in MTCT rates between

resource-rich and resource-limited countries; safer replacement feeding options are not always available in resource-limited settings. In many of these poverty-stricken regions, the increase in infant morbidity and mortality associated with formula-feeding may far outweigh any reduction in the risk of HIV-1 transmission. A randomized controlled trial that compared breastfeeding and replacement feeding in Nairobi reported that 44.0% of the HIV-1 infection in the breastfed group was attributable to breastmilk. In addition, study investigators observed that HIV-free survival was significantly reduced among those infants who were breastfed (Nduati *et al*, 2000). A local vitamin A supplementation study undertaken by Coutsoudis and others reported an increased rate of HIV-1 infection in their mixed-fed group while similar rates of transmission at 3-6 months was observed between the breastfed and formula-fed infants (Coutsoudis *et al*, 2001). Researchers of the ZVITAMBO study group reported that early exclusive breastfeeding substantially reduced breastfeeding-associated HIV transmission (Illiff *et al*, 2005). International guidelines have recommended that HIV-1 infected pregnant women be provided with all the necessary information to enable an informed decision pertaining to the risks and benefits of breastfeeding vs. replacement feeding (McIntyre and Gray, 2002). The WHO recommends "*exclusive breastfeeding for six months*" which has been defined as only providing breastmilk from birth and the avoidance of all other liquids and solids except drops or syrups, drugs, vitamins and mineral supplements (WHO, 2002). Potential modification of infant feeding practices includes; complete avoidance of breastfeeding, early cessation, pasteurization of breastmilk and refraining from breastfeeding in the presence of a breast abscess, mastitis or cracked nipples (Coutsoudis *et al*, 1999).

1.6 Other MTCT determinants

1.6.1 NVP: timing of maternal and neonatal dosing

The presence of a protective concentration of NVP at the time of delivery and during the first few days of an infant's life are most likely crucial to the efficacy of the two-dose regimen in the absence of other ARV therapy. While the sdNVP regimen is easily administered, wide variability in the timing of the dose relative to delivery is a known occurrence. While no disparity was observed in the pharmacokinetics of NVP between pregnant and non-pregnant women (Mirochnick *et al*, 2001), studies have reported reduced maternal NVP absorption during the time of labour and delivery (Mirochnick *et al*, 1998; 2000; Musoke *et al*, 1999).

Cases of reduced NVP plasma levels during pregnancy were reported (Haberl *et al*, 2004) and more recently from a comparison study of steady-state pharmacokinetics of NVP in men, non-pregnant women and women in late pregnancy. In this study Von Hentig and others observed that pregnant women exhibited an increased NVP clearance and lower plasma concentration than non-pregnant women (Von Hentig *et al*, 2006). In contrast, findings from a small clinical drug trial (PACTG 1022) confirmed that steady-state plasma NVP concentration during the second and third trimester of expectant mothers was equivalent to those outside of pregnancy (Aweeka *et al*, 2004).

Mirochnick and others have previously recommended that the maternal NVP dose be administered as early in labour as possible to maximize the time available for drug absorption and distribution across the placenta (Mirochnick *et al*, 2001). In a Zambian study of HIV-1 infected women, the rate of perinatal HIV-1 transmission was higher when the period between maternal NVP ingestion and delivery was less than one hour (Stringer *et al*, 2003b).

Investigators of the SAINT trial also reported similar observations. They found that the odds of an intrapartum HIV-1 infection increased three-fold when the maternal sdNVP dose was ingested less than two hours prior to delivery (Moodley *et al*, 2003). In analysis of cord blood samples collected during the HIVNET 012 trial, Jackson and others observed a pattern of increased perinatal HIV-1 transmission in cases where the maternal NVP dose was ingested within two hours before delivery (Jackson *et al*, 2006). The authors did, however, acknowledge that their finding was not statistically significant due to the small number of patients in this particular subset. In a similar cord blood analysis, investigators of PACTG 316 trial recommended the inclusion of an additional NVP dose to the infant (48-72 hours) after the initial birth NVP dose, in the event of the infant being born less than two hours after maternal intrapartum NVP dosing (Mirochnick *et al*, 2003). In contrast, an observational analysis by the HIVNET 024 trial performed at four sites in Africa demonstrated that variation in the timing of maternal and neonatal NVP dosing did not influence the risk for MTCT, provided dosing occurred within reasonable proximity to delivery. Investigators also reported that repeat dosing of infants as previously suggested by Mirochnick *et al* (2003) may not be necessary as long as the maternal dose was administered within 48 hours of delivery and the infant dose was given within 72 hours of birth (Chi *et al*, 2005).

While several local and international studies have successfully demonstrated the efficacy of the intrapartum and neonatal postpartum sdNVP regimen for PMTCT, data regarding operational efficacy has been limited. Findings of poor sdNVP efficacy in reducing or preventing MTCT in a real-life setting were reported by a group of Kenyan researchers (Quaghebeur *et al*, 2004). In contrast, Colvin and others concluded that the sdNVP regimen was effective following their evaluation of three PMTCT sites in South Africa. Despite their

findings, the authors also emphasised that implementation could be sub-optimal if the regimen was introduced into an already weakened health care system (Colvin *et al*, 2007).

The prolonged elimination half-life of sdNVP has been established to be ~61.3 hours [range 27-90 hours] from pharmacokinetic studies on HIV-1 infected pregnant women (Mirochnick *et al*, 1998; Musoke *et al*, 1999), implying that plasma NVP may still be detectable in mothers several days after delivery. Muro and others reported a median half-life of 56.7 hours in their cohort of healthy non-pregnant HIV-1 uninfected female volunteers. Although this study was conducted in the Netherlands, their finding of NVP being associated with persistent drug level detection beyond three weeks after single dose administration was extremely valuable in the context of NVP drug resistance (Muro *et al*, 2005). The PHPT-2 trial undertaken in Thailand reported a significant NVP concentration in maternal plasma samples during the postpartum period, following intrapartum sdNVP in addition to ZDV prophylaxis. This trial demonstrated that plasma NVP concentration (around the IC_{50}) may persist in some women for as long as four weeks after intrapartum sdNVP dosing (Cressey *et al*, 2005).

1.6.2 NVP drug resistance

Notwithstanding its significant impact and proven success, use of NVP monotherapy or in combination with other ARV drugs remains shrouded in much controversy due to the development and emergence of NVP-induced drug resistance (NVP^R) mutations.

A drug resistance mutation against an NNRTI is an amino-acid change that reduces the binding affinity of a drug such as NVP to the enzyme so that the enzyme activity resumes (Immunopaedia, 2008). A single point mutation as illustrated earlier (Fig 10) in the viral codon may result in high-level resistance to one or more NNRTI's (Cressey *et al*, 2005).

Plasma is the main source of virus when investigating HIV-1 drug resistance in clinical settings. As the half-life of HIV-1 in plasma is ~ six hours, only actively replicating virus can be isolated from this source. The sequence of plasma virus therefore represents the quasispecies most recently selected by the ARV therapy (Perelson *et al*, 1996). The presence of drug mutations is normally detected through population-based or clonal sequencing. Complementary analysis has recently been provided by several research teams through the use of more sensitive drug resistance assays. Methods have included; Sensitive real-time PCR for K103N and Y181C (Johnson *et al,* 2005a; Loubser *et al*, 2005), Allele-specific RT-PCR for K103N (Palmer *et al*, 2005), LigAmp and TyHRT (Flys *et al*, 2006).

The evidence for the emergence of NNRTI-associated drug resistance dates back to the 1990's (Merluzzi *et al*, 1990; Richman *et al*, 1991; Dueweke *et al*, 1993). Transmission of drug resistant HIV-1 variants occurs through sexual contact and from mother-to-child (Yerly *et al*, 1999; Colgrove *et al*, 1998; Neihues *et al*, 1999). Development of NNRTI drug resistance has been shown to decrease the effectiveness of this class of ARV drug (Richman *et al*, 1991) and this type of drug resistance is also relatively transmissible (Leigh-Brown *et al*, 2003). Effects on the mother include a decreased rate of virologic suppression (Jourdain *et al*, 2004). The archiving of resistant variants in biological reservoirs may have a detrimental impact on mother or infant by impairing future responses to NNRTI-containing regimens (Lockman *et al*, 2007; Jourdain *et al*, 2004).

NVP^R has been detected in a considerable proportion of women and infants following sdNVP intake for PMTCT. As a consequence, trials like HIVNET 012 have been subjected to critical medical review (Cohen *et al*, 2004; Hammer, 2005). Appendix 4a highlights drug resistance

findings from a selection of African studies and clinical drug trials designed with a regimen of intrapartum and neonatal sdNVP. A meta-analysis of published studies and conference proceedings (between 1997 and 2006), estimated the prevalence of NVP^R as ~ 35.7% in women and 52.6% in infants following sdNVP intake for PMTCT (Arrivé *et al*, 2007). This analysis also found that rates of NVP^R were further reduced to ~ 4.5% in women and ~16.5% in children with the inclusion of a short-course postpartum ARV regimen (Arrivé *et al*, 2007). Appendix 4b tabulates prevalence of NVP^R among mothers and infants participating in trials containing sdNVP treatment arms in addition to other ARV treatment arms. Worth mentioning are the extremely high rates of NVP^R observed among infants in the Treatment Options Preservation Study (TOPS) [77.8%] and SAINT (52.5%) trials. In contrast to the high NVP^R rate in their sdNVP treatment arm, the TOPS study investigators reported a drastically reduced rate (12.5%) in their treatment arm that contained a regimen of sdNVP and four days of Combivir (McIntyre *et al*, 2005).

A host of virological, pharmacological and immune factors have been implicated in playing significant roles in the development of NVP^R including; plasma HIV-1 RNA (Eshleman *et al*, 2001), NVP drug concentration, metabolism and clearance (Chaix *et al*, 2007), CD4 cell count, clade diversity, viral subtype, viral fitness, and immune selective pressure (Spira *et al*, 2003; Eshleman *et al*, 2005a; Page-Shafer *et al*, 2006).

NVP resistant HIV-1 was selected independently in infants following sdNVP dosing. Emergence of drug resistance among infants has been attributed to a longer length of NVP exposure by the infants compared to their mothers. In the intrapartum and neonatal sdNVP regimen, infants were theoretically dosed twice (once through placental transfer of maternally

administered NVP and again in a dose following birth) (Eshleman *et al*, 2001). While the risk of maternal and infant toxicity with intrapartum and neonatal sdNVP has been shown to be minimal, data from HIVNET 012 has provided evidence for the development of NVP^R mutations among women with active viral replication during the period in which sdNVP was taken (Eshleman *et al*, 2001). Similarly, findings from the PACTG 316 sub-study concluded that NVP^R mutations could be detected after sdNVP in women receiving standard ARV therapy as well as in patients who had virus with no detectable mutations prior to receiving the NVP dose. While their study found a low rate (14.7%) of NVP^R prevalence (new mutations) among women, their study served to confirm that mutations could also be found among women receiving combination therapy with HAART (CD4 > 400 µl/ml) as well as at any level of replicating virus (Cunningham *et al*, 2002; Chaix *et al*, 2004). Studies in South Africa, Thailand and Botswana have evaluated virologic treatment response following sdNVP. These studies found no significant difference in virologic response for women who commenced treatment more than six months after sdNVP exposure. Two studies found poor response rates in women when treatment was initiated within 6 months of sdNVP exposure (McConnell *et al*, 2007). While most genotypic assessments were performed at 6-8 weeks (Appendix 4a and 4b), some studies have shown successful detection of drug resistance mutations as early as four weeks (Chaix *et al*, 2004; 2006) and at ten days post sdNVP exposure (Jourdain *et al*, 2004).

Light at the end of the dim NVP^R tunnel comes in the form of reports of fading drug resistance observed in studies on women and infants in Africa (Eshleman *et al*, 2001; Eure *et al*, 2006). In a Ugandan study, women with drug resistance at 6-8 weeks lacked detectable drug resistance when re-assessed at 12-24 months (Eshleman *et al*, 2001). Similar reports from South African and Cote d'I'voire studies corroborated the fading of NVP-induced resistance

mutations over time, with no significant impact on MTCT after twelve months (Kantor *et al*, 2003; Martinson *et al*, 2004; 2006; Eure *et al*, 2006). The TOPS conducted in SA reported a reduction in the development of NVP^R when both the mother and infant received a short course of [CBV] or Combivir (ZDV plus 3TC) in addition to sdNVP (McIntyre *et al*, 2005). Sequence analysis of maternal samples collected at six weeks revealed reduced rates of NVP^R; 11.9 % (sdNVP and 4 days CBV) and 10.3% (sdNVP and 7 days CBV) while prevalence of NVP^R was more than 60.3% their sdNVP containing arm. A similar outcome was observed among the infant cohort; 12.5% with (sdNVP and 4 days CBV) and no NVP^R with (sdNVP and 7 days CBV) and 77.8% with sdNVP use only (McIntyre *et al*, 2005). In another study by Eshleman *et al* (2006), the development of NVP^R in infants was lowest (27.0%) when infants received (sdNVP and ZDV) and maternal sdNVP was avoided. When both mothers and infants received sdNVP, NVP^R among infants was (87.0%) and when infants received (sdNVP and ZDV) a rate of 57.0% NVP^R was observed (Eshleman *et al*, 2006).

The most common drug resistant mutation associated with sdNVP has been K103N in mothers and Y181C in infants (Chaix *et al*, 2006; Eshleman *et al*, 2001; Gordon *et al*, 2004; Martinson *et al*, 2004; Sullivan, 2002). HIVNET 012 and PACTG 316 found K103N to be the most frequently detected mutation (Cunningham *et al*, 2002; Eshleman *et al*, 2001). The PHPT-2 trial in Thailand reported the K103N, G190A and Y181C as the most frequently detected drug mutations in their trial (Jourdain *et al*, 2004). The NVP Resistance study undertaken by Martinson and others involved genotypic analysis of samples collected from PMTCT cohorts in Soweto and KZN. Investigators reported maternal drug mutations in their study to include K103N (31.0%), Y181C (12.0%), and Y188C (8.1%) (Martinson *et al*, 2004).

1.6.3 Chemokines and HIV-1

The connection between HIV-1 and the chemokine system over a decade has greatly advanced our knowledge and understanding of the biology, pathogenesis and disease progression of HIV-1. Chemokines are a small group of chemotactic cytokines (CC) which play a key role in the recruitment, activation and trafficking of leukocytes to the sites of infection, resulting in an enhanced local inflammatory response. In addition, these inflammatory proteins also modulate cytokine production by T helper cells (Menten et al, 2002).

The three chemokines: MIP-1α (CCL3); MIP-1 β (CCL4) and RANTES (CCL5) were identified as the major HIV-1 suppressive factors produced by CD8+ T cells (Cocchi *et al*, 1995). The identity of a second HIV-1 cellular co-receptor, CXCR4 was shown to be a chemokine receptor-like molecule (Feng *et al*, 1996). Samson and others (1996a) cloned a second receptor (CC-CK45) for CCL3. This clone CC-CK45, was later renamed CCR5 in new nomenclature. Soon after its discovery, CCR5 was shown to be essential for entry of HIV-1 strains into permissive cells (Murphy *et al*, 2000).

Genetic studies of exposed, uninfected participants by Samson *et al* (1996b) showed the first conclusive evidence of genetically based resistance to HIV-1 infection by the discovery of a 32-bp deletion within the coding sequence of the CCR5 gene (CCR5-Δ32). This allele exhibited a higher frequency in Europe and was extremely rare among East Asians, Native Americans and Africans. This so-called *lucky defect* was shown to confer a high degree of protection against HIV-1 in homozygotes. Although no resistance to HIV-1 was seen in CCR5-Δ32 heterozygotes, the rate of disease progression was significantly delayed. Quillent

and others identified a polymorphism in exposed uninfected subjects (m303, which introduced a premature stop codon in the CCR5 gene), reaffirming the central role played by CCR5 in HIV-1 physiology (Quillent *et al*, 1998).

1.6.3.1 Role of chemokines in MTCT

The inflammatory chemokines; CCL3, CCL4 and CCL5 are the natural ligands for CCR5, a chemokine receptor used by (R5) HIV-1 other than CD4 to gain entry into leukocytes (Deng *et al*, 1996; Dragic *et al*, 1996). By virtue of binding to CCR5 these chemokines have the ability to block replication of macrophage-trophic (R5) viruses' *in vitro* (Cocchi *et al*, 1995). This discovery raised the probability that these molecules could contribute to protective immunity against HIV-1 *in vivo*. Evidence for this hypothesis was shown in prototype HIV-1 vaccine studies performed in rhesus macaques, where CC production by CD8 T cells was correlated with protective immunity (Lehner *et al*, 1996; Heeney *et al*, 1998). Elevated production of these chemokines was also observed in persons who remained uninfected despite multiple high risk sexual encounters (Paxton *et al*, 1996; Zagury *et al*, 1998) Apart from regulating cell-mediated immunity, these chemokines also play a role in chemotaxis (Matsukawa *et al*, 2000).

Naturally occurring host genetic variants of chemokine and chemokine receptor genes have demonstrated the crucial role of these molecules in altering the host immune response to HIV-1 (O'Brien and Nelson, 2004). Investigators have, therefore speculated that the setting present in infants at birth was conducive to providing protection from HIV-1 and could explain why majority of HIV-1 exposed infants somehow escape infection despite the absence of ARV

intervention. This was supported by a study demonstrating the over expression of CCL3, CCL4 and CCL5 in a small number of exposed-uninfected infants, suggesting that these chemokines may mediate non-cytolytic inhibition of infection during perinatal HIV-1 exposure (Wasik *et al*, 1999).

Genome duplication is an important mechanism in human evolution and genetic adaptation. Identification of various polymorphisms or duplications of chemokine or chemokine receptor genes that exert variable effect on HIV-1 disease progression demonstrated that the rate of disease in each individual was not necessarily determined by a single genetic polymorphism, but rather by the combined effects of divergent or multiple genes, which are further modulated by the genetic background of the racial group (Lusso, 2006). As segmental duplications over-represent genes involved in immunity, duplications are thought to benefit host immunity (Bailey *et al*, 2002).

CCL3-L1 is the most potent ligand for CCR5 and provides an excellent example of the benefits of gene duplication. CC chemokine ligand 3-like or CCL3-L1 is one of the two active genes (CCL3/LD78α) and (CCL3-L1/ LD78 β) that code for CCL3. Although CCL3 and CCL3-L1 share a 94.0% sequence similarity (Nakao *et al*, 1990), they encode functional proteins that are biologically different. While CCL3 occurs in two copies per diploid genome (p.d.g), CCL3-L1 is found in variable copies (p.d.g) in different individuals (Menten *et al*, 2002). By binding to CCR5 and promoting its sequestration (Fig 12), CCL3-L1 can block HIV-1 replication. As a CCR5 agonist, CCL3-L1 can also promote T cell activation and migration, enhancing both cellular immunity and generalized immune activation. Genetic polymorphisms thought to determine the amount of CCR5 and CCL3-L1 have complex effects

on HIV-1 pathogenesis that include control of viraemia and cellular immunity, as well as effects that are independent of both (Lederman and Sieg, 2007).

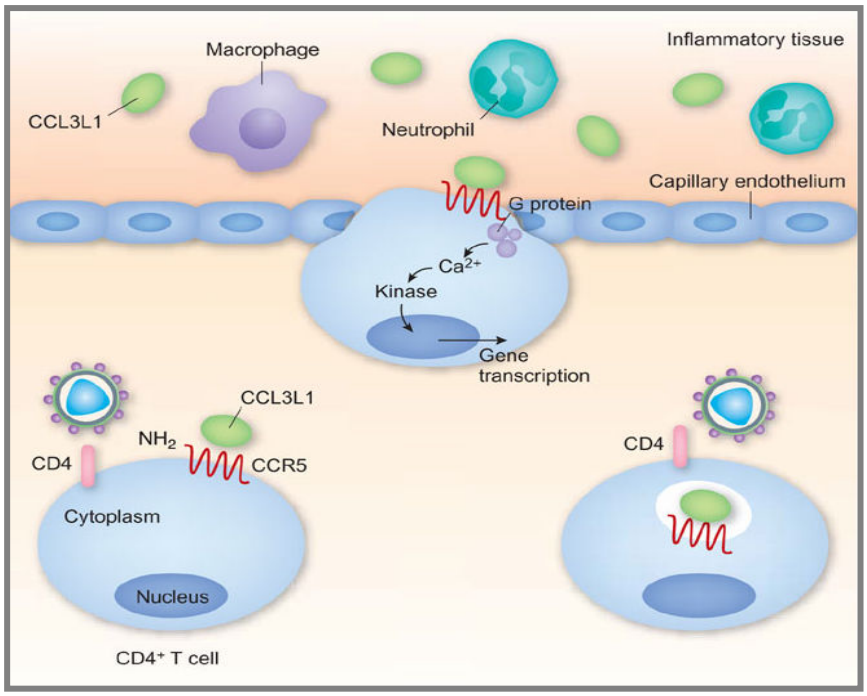


Fig 12: CCL3-L1 HIV-1 blocking mechanism. By binding to CCR5 and promoting its sequestration, CCL3-L1 can block HIV-1 replication. As CCL3-L1 levels rise, more CCR5 receptors are filled, thereby blocking the virus's ability to infect a host cell (Lederman and Sieg, 2007).

The CCL3-L1 gene dose, therefore affects the risk for HIV-1 acquisition and disease progression because as levels of CCL3-L1 rise (Fig 12), more CCR5 receptors are filled, thereby blocking the virus's ability to infect a host cell. In addition, an increase in the number of CCL3-L1 copies was associated with a decrease in the proportion of CD4 cells that express CCR5 and a lower expression of CCR5 decreases the likelihood of HIV-1 infection (Lederman and Sieg, 2007). Individuals with CCL3-L1 gene copy numbers above the population median were less susceptible to HIV-1 acquisition (Townson *et al*, 2002). In a

landmark study that investigated the influence of CCL3-L1 gene-containing segmental duplications on HIV/AIDS susceptibility, Gonzalez and others observed wide variability in the average number of CCL3-L1 gene copies (Fig 13) across diverse human populations that extended across a range of continents (Gonzalez *et al*, 2005).

Compared to non-African individuals, African individuals had significantly higher CCL3-L1 gene copy numbers. Gene copy medians, determined among various African populations ranged from five gene copies p.d.g in the (Bantu Pedi, Bantu Zulu and Bantu Sotho) to six gene copies p.d.g in the Bantu Tswana population (Gonzalez *et al*, 2005). Gonzalez and others (2005) also observed significant inter-individual and inter-population differences in the copy number of a segmental duplication encompassing the gene encoding CCL3-L1. While adults and children bearing a higher copy number of CCL3-L1 gene relative to their population median were at a reduced risk of HIV-1 infection, those possessing a gene copy number lower than the population median were associated with enhanced HIV/AIDS susceptibility (Gonzalez *et al*, 2005).

The relationship between CCL3-L1 gene dose and altered HIV/AIDS susceptibility points to a central role for CCL3-L1 in HIV/AIDS pathogenesis. However, the association between CCL3-L1 gene dose and HIV/AIDS susceptibility exists only when viewed in the context of an individual's ethnicity and geographic ancestry. It also indicates that the differences in the dose of immune response genes may constitute a genetic basis for the variable responses to infections (Gonzalez *et al*, 2005).

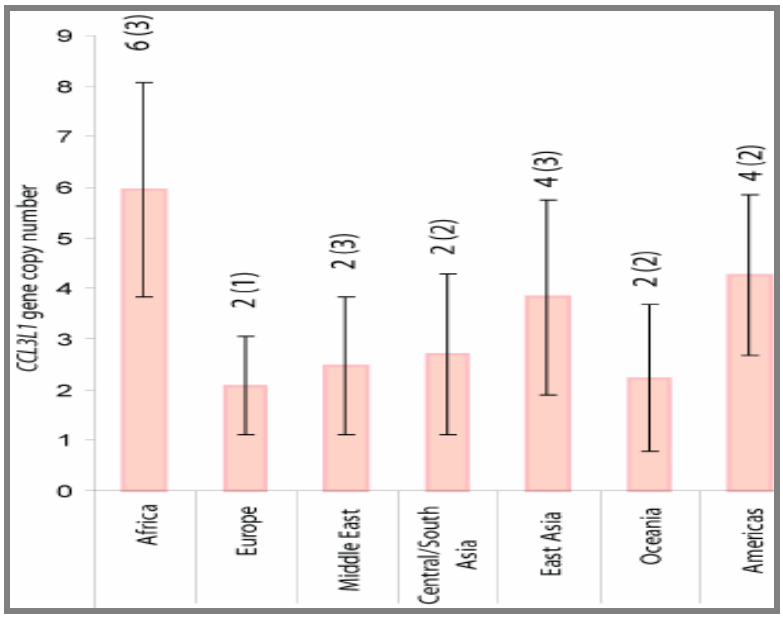


Fig 13: Distribution of CCL3-L1 gene copy numbers among human populations (Gonzalez *et al*, 2005)

A significant discovery by Gonzalez and others was that Argentinean children with CCL3-L1 gene copy numbers less than their population median had a higher risk of acquiring HIV-1 vertically whereas those with copy numbers greater than the population median had a lower risk (Gonzalez *et al*, 2005). Local studies in SA have since confirmed these findings. Infants who displayed a deficient production phenotype were at an increased risk of acquiring HIV-1; hence CCL3-L1 plays an important role as an "*HIV-1 susceptibility factor"* in the South African population (Kuhn *et al*, 2007; Meddows-Taylor *et al*, 2006).

The influence of sdNVP on the relationship between maternal-infant CCL3-L1 gene copies and perinatal HIV-1 transmission was investigated by Kuhn and co-workers. They confirmed the dose dependent relationship between duplications of the CCL3-L1 gene and perinatal HIV-1 transmission (Kuhn *et al*, 2007). In their study, the median number of CCL3-L1 gene copy numbers was reported to be four to five p.d.g in the context of the southern African black

population. Infants with fewer gene copies were more likely to acquire HIV-1 from their mothers and conversely those infants with more gene copies were less susceptible. This finding was however restricted to detection *only in the absence of maternal NVP* or with high maternal viral load (Kuhn *et al*, 2007). No association was found between CCL3-L1 gene copies and perinatal transmission if mothers took sdNVP. NVP could therefore possess anti-viral and immunomodulatory properties which partially change the role of CCR5 and its ligands in HIV-1 transmission (Kuhn *et al*, 2007).

Local NICD researchers performed a detailed single nucleotide polymorphism (SNP) and haplotype characterization of the CCL3 and CCL3-L1 genes (Paximadis *et al,* 2007). SNP (Fig 14) refers to a DNA sequence variation that occurs when a single nucleotide (A, C, T, G) in the genome (or other shared sequence) differs between members of a species (or between paired chromosomes in an individual) [Hall, 2007].

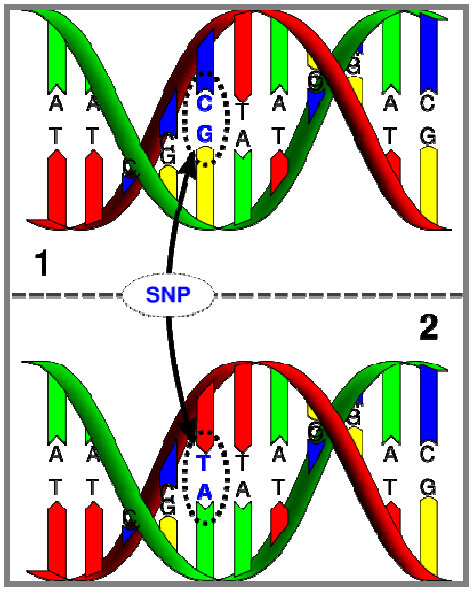


Fig 14: Single nucleotide (C/T) polymorphism (Hall, 2007)

Paximadis and others investigated the role of a unique CCL3 haplotype (CCL3-Hap-1) in respect to HIV-1 susceptibility and MTCT (Paximadis *et al*, 2007). They achieved this by targeting a seven-SNP haplotype (Hap-1) identified in CCL3. This haplotype known to harbour a SNP haplotype in the core promoter region along with two downstream SNP's has been previously associated with a reduced risk of HIV-1 acquisition in African Americans (Gonzalez *et al*, 2001). Paximadis and co-workers investigated this relationship further in their study which included four South African MTCT transmission cohorts (Paximadis *et al*, 2007). They found that CCL3-Hap-1 was significantly associated with a high CCL3-L1 gene copy number only in infants and not in the corresponding mothers. A crucial outcome from their study was the low Hap-1 representation found among infants who were infected *in utero* as opposed to intrapartum. These extremely novel findings suggest that the role of CCL3 in protection could vary for different modes of HIV-1 transmission.

1.6.4 CD4 cell counts and MTCT

Maternal immune depletion has been shown to correlate with vertical transmission (Luzuriaga, 2007; Mofenson *et al*, 1999b). Decreased CD4 T cell counts are a measure of worsening immune deficiency and have been associated with enhanced perinatal HIV-1 transmission. A low CD4 T cell count may result from a high viral load and may therefore not be an independent risk factor for vertical acquisition of HIV-1 (Mofenson *et al*, 1999b). Revised guidelines from the WHO currently recommends that HAART be considered for those patients (clinical stage I and II) with a CD4 cell count below 350 µl/ml, especially if closer to 200-250 µl/ml (WHO, 2006b).

1.7 Study rationale, hypotheses, aims and objectives

Despite therapeutic, prophylactic and obstetric interventions to reduce the proportion of infants infected during labour and delivery, mechanisms for intrapartum HIV-1 transmission remain elusive and current interventions could therefore remain sub-optimal. Much controversy has surrounded the correlation of HIV-1 RNA (viral load) in the systemic and genital compartments. The influence of short-term ARV drugs on genital tract HIV-1 is also unclear.

At the time the present study was initiated, a regimen of maternal intrapartum and neonatal postpartum sdNVP was the standard of care for the prevention of mother-to-child transmission of HIV-1. In most low and middle-income countries, including South Africa, sdNVP has been documented as effective intrapartum HIV-1 prevention based on plasma pharmacokinetic levels, decreased viral loads (HIV-1 RNA) and reduced rates of intrapartum transmission, yet operational studies continue to report high intrapartum transmission rates despite the administration of sdNVP. As a result perinatal HIV-1 transmission remains a significant public health concern in several African countries.

The hypotheses for the PIVD study included the following:

1) The pharmacokinetics of sdNVP when used routinely for the indication of prevention of intrapartum HIV-1 transmission in a public health setting is comparable to Phase I and II controlled studies.

2) The mechanism of action of sdNVP in potentially preventing intrapartum transmission is through infant postexposure prophylaxis and reduction of viral load (HIV-1 RNA) in maternal plasma and cervical secretions.
3) Expression of specific CCL3 and CCL31 "Susceptibility genes" in mothers serve as "early warning" markers for intrapartum HIV-1 transmission despite the provision of sdNVP.

The primary aim of this study was to describe the pharmacological dynamics of Nevirapine in association with virological and immunological risk factors for intrapartum HIV-1 transmission in a South African PMTCT programme where sdNVP was the standard of care.

This was accomplished through the following objectives:

Primary:

1) Determination of NVP drug concentration in maternal plasma and CVL samples in the peripartum period
2) Determination of neonatal drug concentration following dosing at birth
3) Quantitation and correlation of maternal HIV-1 RNA (viral load) in plasma and cervicovaginal lavage (CVL) samples at pre and post-NVP dosing

Secondary:

1) Influence of sexually transmitted infections on maternal viral load and intrapartum MTCT

2) Investigation of host genetics in a subset of confirmed HIV-1 infected infants and their mothers by determination of CCL3 and CCL3-L1 gene copy numbers, identification of single nucleotide polymorphisms and haplotype characteristation of the CCL3 gene
3) Investigation of unique clinical characteristics that determine the presence of NNRTI drug resistant mutations among intrapartum HIV-1 transmitting women

CHAPTER TWO: METHODOLOGY

2.1 Regulatory and institutional approvals

Prior to commencement, this present received regulatory approval from the Provincial Department of Health (KZN) and permission to conduct this study at KEH was obtained from hospital management. Full ethical approval from the Biomedical Research Ethics Committee at Nelson R Mandela School of Medicine (UKZN) was received on 15th February 2006 and patient screening officially commenced in April 2006. The ethics reference number for this study is H004/05.

2.2 Study description

2.2.1 Study design and setting

The PIVD study was a laboratory based study of a cohort of pregnant women attending the maternity and child health services at KEH. This public sector, tertiary care referral hospital is located in Durban, KZN (South Africa). Samples were processed and stored at the Women's Health and HIV Research Unit (WHHRU study laboratory), Doris Duke Medical Research Institute (DDMRI), UKZN. All laboratory assays were performed at the Department of Virology (IALCH), CAPRISA and Hasso Plattner Molecular laboratories in Durban [Virological]; Department of Clinical Pharmacology, University of Cape Town [Pharmacokinetics]; and the AIDS VIRUS UNIT (Cell Biology), National Institute of Communicable Diseases (NICD), Johannesburg [Immunology and Host Genetics].

2.2.2 Standard of care for PMTCT at KEH

PMTCT refers to the prevention of mother to child HIV-1 transmission programme. Fig 15 provides a brief overview of the comprehensive package of services offered at KEH. At the time the present study (2006-2007), all pregnant women were offered voluntary counselling and testing. If confirmed HIV-1 positive by HIV-1 antibody testing (HIV-1 ELISA), a CD4 cell count was performed. Women with lower CD4 cell counts (< 200 cells/µl) were screened for eligibility for HAART (drug adherence training, pill buddies or partners etc.) and commenced on a regimen of Lamivudine (3TC), Stavudine (d4T) and NVP during pregnancy. HIV-1 infected women with a CD4 cell count of ≥ 200 cells/µl were offered a prophylactic ARV drug regimen consisting of single dose (200 mg tablet) NVP issued at 28 weeks gestational age for self-administration during labour to prevent MTCT. A single dose (0.6 ml) of NVP syrup was administered to all their infants within 72 hours of delivery.

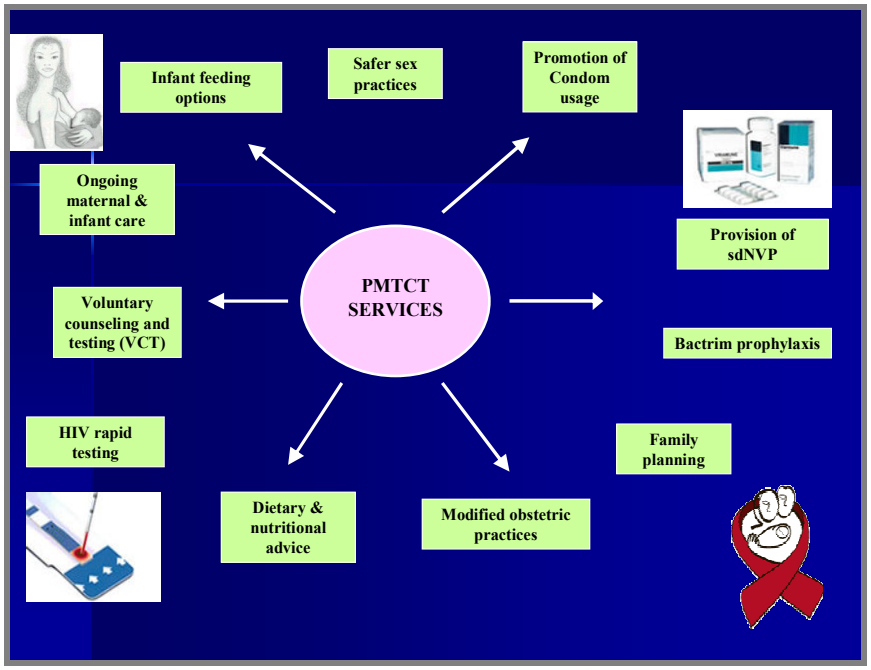


Fig 15: Overview of PMTCT services (adapted from PMTCT site manual, 2005)

2.2.3 Study population

Pregnant women (between the ages of 18-39 years) who tested HIV positive by rapid tests and who decided to only formula feed their infants were screened for their study eligibility during the period April-December 2006. The total sample size comprised of one hundred and twenty mother-infant pairs. The PIVD study was designed with the primary objective of describing the pharmacological and virological dynamics of a single dose of NVP for the prevention of mother-to-child transmission of HIV-1 within a routine PMTCT program setting. Previously described controlled pharmacokinetic studies with sample sizes that ranged from 50 -150 participants served as a guide for sample size determination in the present study (Mirochnick et al 1998, Musoke et al, 1999). Likewise similar virological studies investigating CVL HIV-1 RNA levels (viral loads) also assessed cohorts of 40-100 participants (Kovacs et al, 1999, Hart et al, 1999, Garcia-Bujalance et al, 2004, Kunz et al, 2007). Where the size of sample subsets were not adequately powered to address statistical relationships e.g. CCL3-L1 gene copy expression and intrapartum HIV-1 transmission, this has been duly mentioned in the text under study limitations (page 187). In anticipation of the small number of HIV-1 infected infants, we intended providing a description of our unique observations to steer the way to further investigation in larger cohorts e.g. CCL3-L1 gene copy expression and HIV-1 susceptibility.

2.2.4 Inclusion and exclusion criteria

All patients had to fulfil the following inclusion and exclusion criteria prior to enrolment onto this study:

Inclusion criteria:

- Confirmed HIV-1 positive (HIV-1 antibody testing)
- Decided to only formula feed infant (i.e. to exclude postnatal exposure to HIV-1)
- Willing to attend post-delivery infant assessments (i.e. to continue follow up until six weeks post-delivery)
- Must be ARV naïve (i.e. no previous exposure to NVP or other ARV drugs) and not require HAART
- Must be ≥ 18 and ≤ 39 years of age (i.e. to minimize the risk of pregnancy-related complications)

Exclusion criteria:

- HIV-1 negative
- Refusal to participate
- Decided to only breastfeed infant
- Unable to return for post-delivery infant assessments
- Previous or current ARV exposure OR require HAART
- High risk patients [TB, cardiac, other]
- < 18 and > 39 years of age

2.3 Screening and enrollment procedures

2.3.1 Confirmation of maternal infection status

Antenatal records of potential participants were screened for HIV-1 status. As part of PMTCT standard of care, maternal HIV-1 status was confirmed by routine antibody testing (HIV-1) through the use of a 4th generation HIV-1 and p24 antigen combo ELISA kit (Elecsys HIV combi cobas®, Roche Diagnostics, Mannheim) at the Department of Virology (IALCH, Durban).

2.3.2 Maternal CD4 cell counts

Similarly antenatal records of potential participants were examined for maternal CD4 cell count results. As part of PMTCT standard of care, all CD4 cell testing was performed routinely at a central laboratory service (Department of Virology, IALCH, Durban).

2.3.3 Maternal socio-demographic information

Study participants were interviewed on their socio-demographic characteristics such as maternal age, gestational age, marital status, level of education and employment status. Antenatal records also served as a tool to verify pertinent inclusion criteria such as age, residence within a 10 km radius, no current or previous ARV exposure etc.

2.3.4 Informed consent

Study counseling and informed consent was supervised by the candidate (study investigator) and undertaken by two isi-Zulu speaking research assistants. Counseling was carried out in the language preferred by the participants, with either the ethics-approved isi-Zulu or English versions of the informed consent document being signed by all participants. One copy of the

informed consent document was retained by the study investigator and one copy was given to the study participant. All participants were given a patient information leaflet and made aware of their option to withdraw consent at any time during the course of the study if they so desired.

2.3.5 Data collection

Specific data pertaining to the medical history of a participant (antenatal, obstetric and paediatric clinical chart notes) was documented into a patient case report form or CRF (Appendix 5). Confirmation of maternal NVP intake (during labour) was self-reported by all study participants and verified through the use of PMTCT NVP registers and clinical records. Infant NVP dosing (at birth) was verified through review of clinical records and PMTCT NVP registers. All maternal data was collected from study enrolment up to maternal labour/delivery. All infant data was collected from birth to six weeks of age (completion of all study endpoints).

2.4 Sample collection, processing and storage

Blood and cervicovaginal lavage samples (CVL) were collected from women at pre and post-NVP dosing, while blood samples were obtained from infants (post-NVP dosing) by either nurses or medical officers/registrars stationed at the antenatal clinic, labour ward facility or paediatric nursery of KEH (during the study period). These sampling points were defined as follows:

- ***Pre-NVP dose sampling***: maternal sampling performed during pregnancy (≥ 28 weeks gestational age), prior to single NVP dosing in labour.

- ***Post-NVP dose sampling***: maternal sampling performed during active labour or after delivery and after administration of single dose NVP. Samples from infants were also collected after infant NVP administration.

Table III: Study event schedule

Study procedures and laboratory investigations		Maternal Screening (< 28 wks GA)	Maternal 28-39 wks GA [Pre-NVP]	Maternal Active labour or post-delivery [Post-NVP]	Infant birth [Post NVP]	Infant 4 weeks [Post-NVP]	Infant 6 week [Post NVP]
STANDARD OF CARE	HIV-1 ELISA	x [1]					
	CD4 cell count	x [1]					
STUDY PROCEDURES	Study Counseling	x	x	X	x	x	x
	Informed Consent	x					
	Demographics	x					
	Blood sampling		x	X	x	x	x
	CVL sampling		x	X			
LABORATORY INVESTIGATIONS VIROLOGICAL	Plasma HIV-1 RNA (viral load)		x	X	x	x	x
	CVL HIV-1 RNA (viral load)		x	X			
	HIV-1 DNA PCR				x	x	x [1]
	HIV-1 Drug Resistance			X [2]			
	STI screening		x [4]				
PHARMACOLOGICAL	CVL NVP concentration		x	X			
	Plasma NVP concentration		x	X	x		
IMMUNOLOGICAL	CCL3-L1 gene copy number		x [3]				x [3]
	Haplotype-1 characterization		x				x

Footnotes: GA: gestational age; PMTCT standard of care testing [1]; HIV-1 transmitting women only [2]; case-controlled subset [3] ; testing by Global clinical & viral laboratory [4]

2.4.1 Blood: (maternal and infant)

Maternal and infant whole blood samples were collected by venipuncture in sterile vacutainer tubes (4 ml) which contained EDTA as the anticoagulant (Beckton Dickinson, UK). After transport to the WHHRU study laboratory (DDMRI), whole blood was pipetted into appropriately labeled cryovials (Greiner, Germany) as 200 µl (infant) and 1000 µl (maternal) sample aliquots. Whole blood aliquots were stored in a -70^{o}C freezer at the WHHRU study laboratory (DDMRI) until required for genotyping investigations as per study event schedule (Table III).

All remaining whole blood samples were centrifuged at 800 - 1600 rpm for 20 minutes at room temperature. Plasma was carefully removed and transferred into appropriately labeled cryovials (Greiner, Germany) as 200 µl (infant) and 1000 µl (maternal) sample aliquots. All plasma aliquots were stored in a -70^{o}C freezer at the WHHRU study laboratory (DDMRI) until required for virological and pharmacokinetic laboratory investigations listed in Table III.

2.4.2 Cervicovaginal lavage (CVL)

The purpose of this collection procedure was to obtain a washing of virus and cells from the ectocervix and fluid from the posterior vaginal fornix for virological and pharmacokinetic assessments.

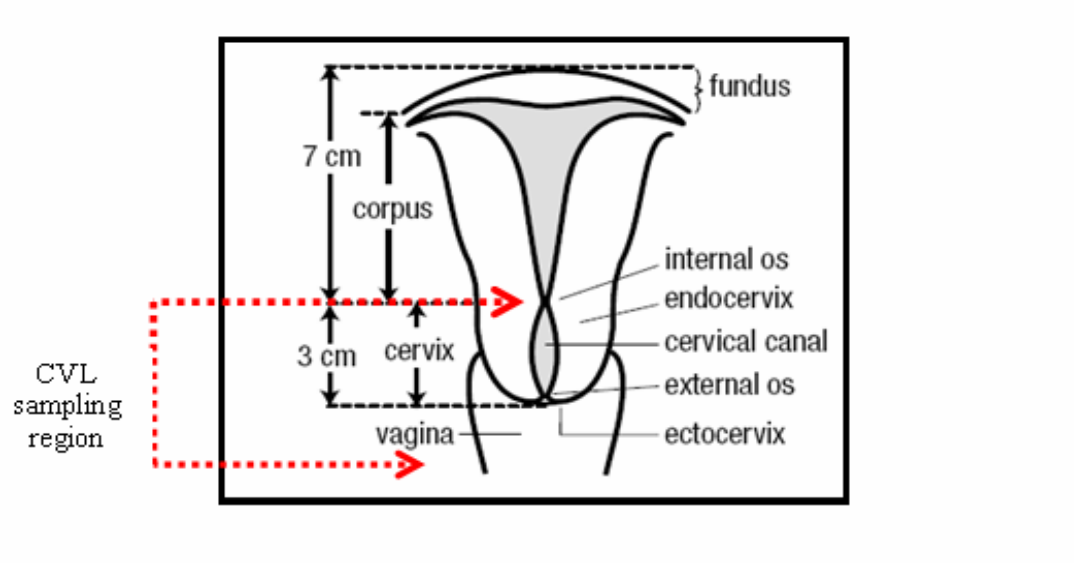


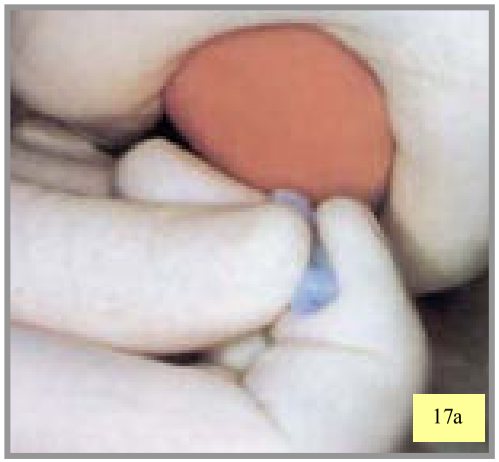
Fig 16: CVL sampling region (adapted from WHO, 2006a)

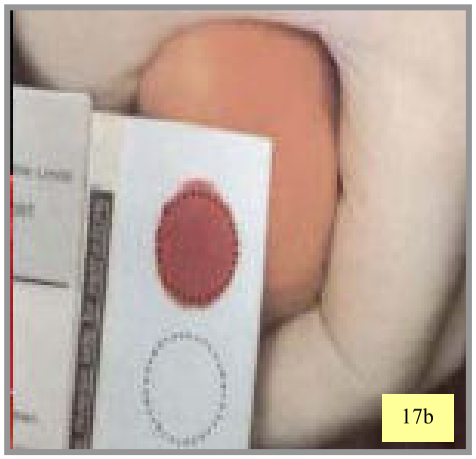
Ten millilitres of sterile pH 7.2 Phosphate Buffered Saline (PBS) [Gibco©, Invitrogen™, UK] was drawn into a sterile medical syringe (Becton Dickinson, UK). After careful insertion of a lubricated speculum into the vagina, the PBS-filled syringe was slowly introduced through the speculum at the opening of the cervical os (Fig 16). The syringe was not inserted into the cervical os. A continuous stream of PBS was aimed directly at and onto the cervical os to bathe the cervix and ectocervix. Once the fluid pooled into the posterior fornix, it was aspirated back into the same syringe. This wash procedure was repeated a further two times using the same fluid. No additional PBS was added to the sample. The pooled fluid (cervicovaginal lavage or CVL) was finally aspirated and transferred to a sterile 15 ml

polypropylene tube (Delta labs, Spain). Samples were transported on ice to the WHHRU study laboratory (DDMRI) within 4 - 6 hours of collection (ACTG, 2004a). The total sample volume and presence of blood contamination was recorded in the patients CRF. Dipstick analyses using urine dipsticks (Uricheck, RapiMED diagnostics, SA) was performed to measure pH and estimate the level of blood contamination. CVL samples were briefly vortexed to ensure equal sample distribution. CVL was stored as whole unfractionated samples in 1000 µl aliquots in a -70^{o}C freezer in appropriately labelled cryovials (Greiner, Germany) until required for laboratory evaluations as detailed in Table III.

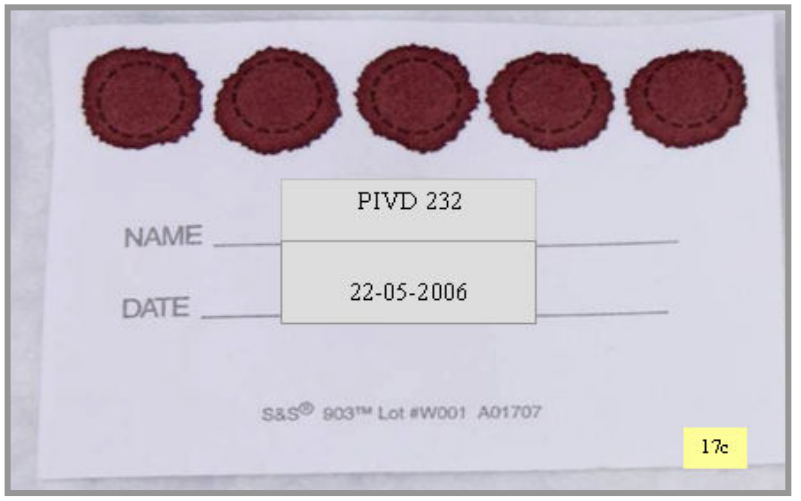
2.4.3 Dried blood spots: (infants only)

Blood was collected onto the filter cards either from a syringe or directly from the venepuncture site. The skin on an infant's heel was first cleaned with an alcohol swab. Once all residual alcohol had evaporated, a heel-prick was performed by a PMTCT medical officer by carefully puncturing the infant heel with a sterile lancet (Fig 17a). A filter collection card (Schleicher & Schuell Bioscience, Dassel, Germany) was held gently against a large drop of blood and a sufficient amount of blood was allowed to soak through to fill a single pre-printed circle on the filter card (Fig 17b). This process was repeated to completely fill as many of the pre-printed circles as possible. An example of a valid dried blood spot sample is shown in Fig 17c. After filter collection cards were appropriately labeled with the infant's study identification (PIVD number) and date of sample collection, they were carefully placed onto a rack and allowed to air-dry completely (Fig 17d). Once thoroughly dried, each filter card was placed into separate zip-lock plastic bags to prevent cross-contamination (Fig 17e). Dried blood spot samples were transported to the WHHRU study laboratory (DDMRI) and stored at -20^{o}C until required for HIV DNA PCR testing as per Table III.

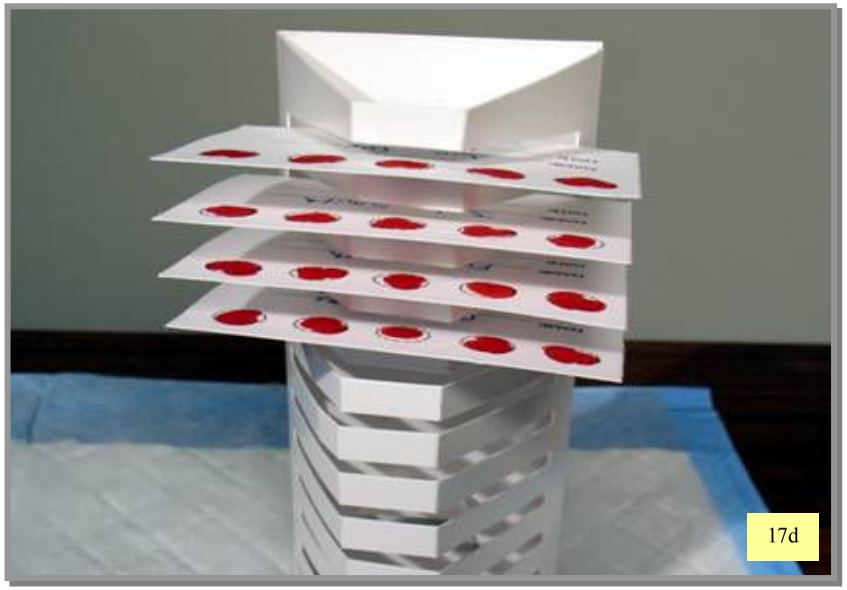




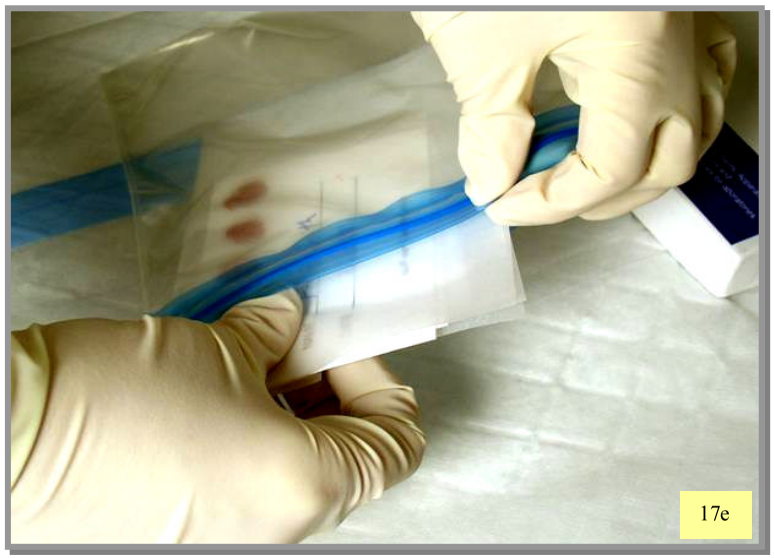
Infant heel prick (17a) and blood spot collection onto filter card (17b)



(17c) Dried blood spots on filter collection card (valid sample)



(17d) Drying of blood spot samples



(17e) Packaging of samples

Fig 17a-e: Procedures for collection and storage of infant dried blood spots *(adapted from CDC, 2005)*

2.5 Laboratory investigations

2.5.1 Infant HIV-1 infection status and prediction of timing of infection

Six weeks of age:

At six weeks of age, all HIV-1 exposed infants received standard of care HIV-1 DNA PCR testing to establish their infection status. Samples were sent to the Virology Laboratory (IALCH, Durban) where they were batch-tested. The typical turn-around time for obtaining laboratory results ranged from 6 - 14 weeks.

Birth and Four weeks of age:

Confirmation of all HIV-exposed uninfected infants at four weeks and prediction of timing of infection in HIV-infected infants at birth and four weeks was performed by the author using HIV-1 DNA PCR under supervision at the Department of Virology (IALCH). Samples were batch tested approximately 8 - 12 months after sample collection.

Principle of the HIV DNA PCR test:

The MagNA Pure LC DNA Isolation kit III (Roche Diagnostic Systems, Branchburg, NJ, USA) was utilized for automated DNA extraction from infant dried blood spot samples. After DNA extraction, a slightly modified AMPLICOR HIV-DNA test, version 1.5 (Roche Diagnostic Systems, Branchburg, NJ, USA) was used for amplification and detection of proviral HIV-1 DNA. The Amplicor HIV-1 DNA test is a qualitative test that involves sample preparation, PCR amplification of target DNA using HIV-1 specific complementary primers, and nucleic acid hybridization of amplified products and detection of probe-bound amplified DNA products by colorimetric determination.

Sample preparation:

A 0.5 cm diameter circle (50 µl sample volume) was cut from each filter paper spot (dried blood spot) using a sterile fine-point scissors. A new pair of sterile scissors was used for each infant sample. The dried blood spot was further cut into smaller slices and then placed into an appropriately labeled 1.5 ml eppendorf tube.

DNA extraction:

An automated protocol [MagNA Pure LC DNA Isolation kit III (Bacteria, Fungi), Roche Diagnostic Systems, Branchburg, NJ, USA] was used for extraction of proviral DNA from dried blood spot samples. A bottle of Proteinase K was first reconstituted with 1200 µl of Elution buffer. A stock reagent was prepared by dispensing 9100 µl Bacterial Lysis buffer, 1400 µl of Proteinase K and 35 µl of Internal control into a 50 ml blue capped centrifuge tube (Greiner, Germany). This tube was inverted ~25 times to mix, and then allowed to stand on a bench for ~ 5 minutes until all air bubbles had dissolved. A 300 µl volume of Stock Reagent was dispensed into each eppendorf tube containing dried blood spot samples. Once dried blood spots were completely immersed in the stock reagent, all tubes were thoroughly vortexed for 10 seconds. Samples were placed onto a heating block for 10 minutes at 65^{o}C. Following incubation, samples were removed from the heating block and vortexed. Samples were placed back onto the heating block for further ten minute incubation at 65^{o}C. After vortexing, samples were placed directly onto a 95^{o}C heating block for ten minutes. All tubes were then centrifuged at 5000 rpm for one minute at room temperature. After centrifugation, 150 µl of sample was transferred into a MagNA pure sample cartridge. Sample cartridges were loaded onto the MagNA Pure LC instrument (Roche Diagnostic Systems, Branchburg, NJ, USA) and the batch run commenced.

DNA amplification and detection:

DNA extracts (50 µl) obtained from the above protocol were then amplified and detected by using the Amplicor HIV-1 DNA test, version 1.5 (Roche Diagnostic Systems, Branchburg, NJ, USA) according to the manufacturer's instructions. Fig 18 illustrates the process of DNA amplification using PCR.

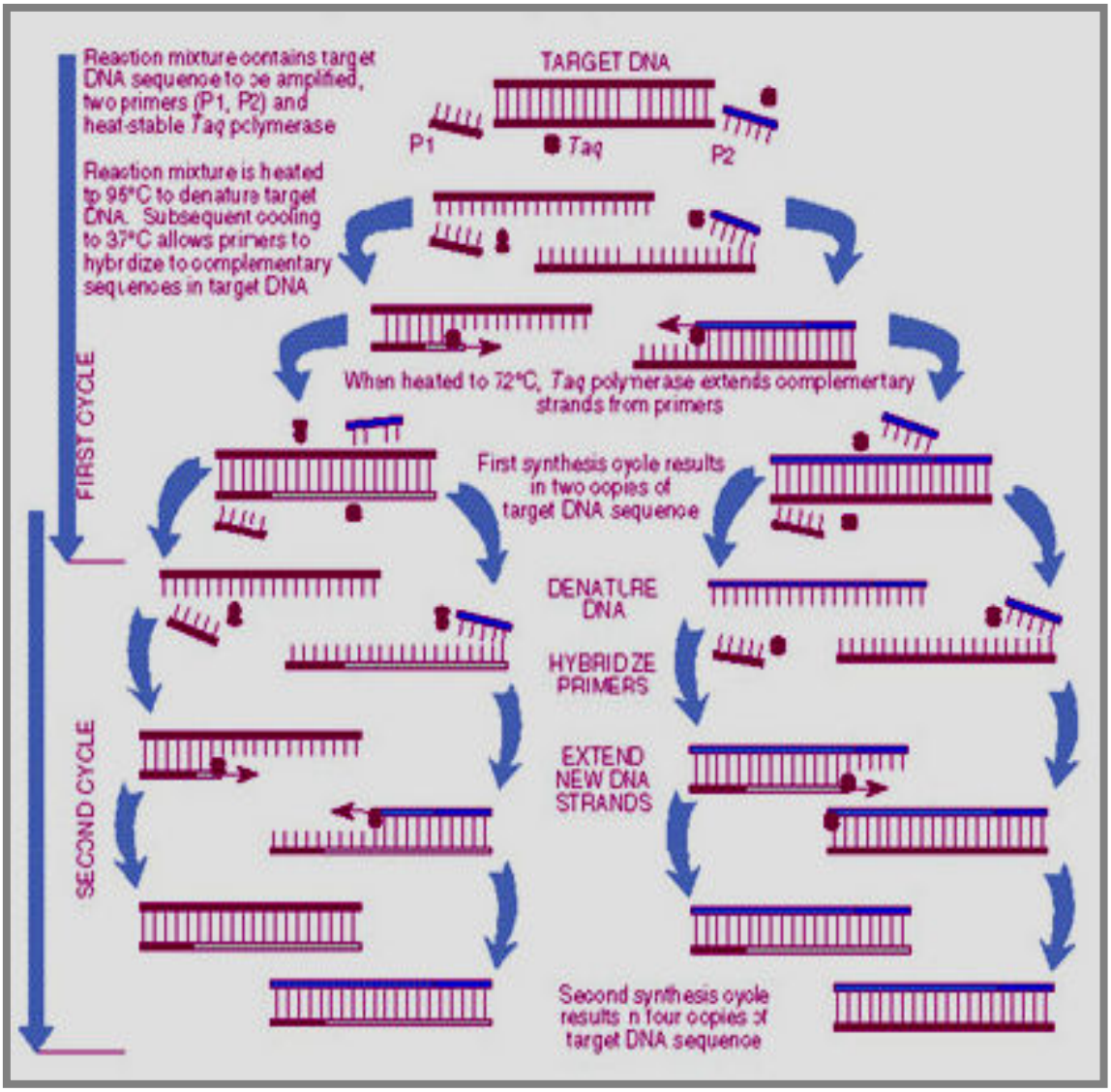


Fig 18: DNA amplification using polymerase chain reaction (NIH, 2009)

Interpretation of results:

HIV DNA PCR testing was performed on all HIV-1 exposed infants at age six weeks according to the standard of care at KEH during the study period April – December 2006. Using CDC guidelines, all study infants were confirmed to be HIV-1 infected only after they tested PCR positive for HIV DNA on two separate occasions (CDC, 1995). Similarly the absence of HIV infection among HIV-1 exposed uninfected infants was confirmed with two separate DNA PCR tests i.e birth and 6 weeks or 4 and 6 weeks. The various permutations listed in Table V were used as a guide to estimate the timing of HIV-1 transmission from mother-to-child.

Table IV: Estimating the timing of HIV-1 infection

	Infant blood sampling (Dried blood spots)				
Test	Birth	4 weeks	6 weeks*	Repeat *	HIV-1 transmission route
HIV-1 DNA PCR	Positive	Positive	Positive		*In utero*
	Positive		Positive		*In utero*
	Negative	Positive	Positive		Intrapartum
	Negative	Negative	Positive	Positive	Intrapartum

** PMTCT standard of care*

On receipt of the six week DNA PCR results, the attending PMTCT clinician proceeded as follows:

- *If infants were HIV-1 negative (after two separate DNA PCR tests), they were discharged from the PMTCT clinic at KEH and mothers were advised to continue with regular infant check-ups at their local district health clinics.*

- *If infants were HIV-1 positive, they were re-tested using DNA PCR. If results were confirmed positive by the second test (two consecutive results), infants were referred to the ARV rollout programme for management and treatment.*

2.5.2 HIV-1 RNA quantitation (viral load)

Testing was performed by the candidate under supervision of medical technologists at the Caprisa Research laboratory based at the DDMRI (Durban).

Principle:

A commercially available nucleic acid amplification COBAS AmpliPrep/COBAS TaqMan HIV-1 test (Roche Diagnostic Systems, Branchburg, NJ, USA) was used for the quantitation of HIV-1 RNA. The COBAS AmpliPrep instrument enabled automated sample preparation and COBAS TaqMan analyzer allowed automated amplification and detection.

The COBAS system involved three major steps:

- Sample preparation for HIV-1 RNA isolation
- Reverse transcription of the target RNA to generate complementary DNA (cDNA)
- Simultaneous PCR amplification of target cDNA and detection of cleaved dual-labelled oligonucleotide detection probe specific to the target

Frozen aliquots of plasma (maternal and infant) and whole unfractionated cervicovaginal lavage were allowed to thaw to room temperature. Samples were thoroughly vortexed and a sample volume of 1000 µl was used for each patient. The high positive, low positive and negative external controls included in the kit were also run for every batch of samples assayed. Plasma and genital HIV-1 RNA levels were quantified in several batch runs according to the manufacturer's instructions (COBAS AmpliPrep/COBAS TaqMan HIV-1 test, Roche Diagnostic Systems, Branchburg, NJ, USA).

Interpretation of results:

The limit of quantitation for this assay ranged from 40 - 10 000 000 copies/ml. HIV-1 RNA concentration for samples, and controls were determined automatically by the COBAS TaqMan analyzer. Samples with "Target not detected" were reported as HIV-1 RNA undetectable. Samples with HIV-1 RNA values below the assay limit were reported as < 40 copies/ml. Samples with HIV-1 RNA values above the specified assay range were diluted (using the original sample) accordingly with kit provided HIV-1 negative human EDTA-plasma and the test was repeated. The reported result was then multiplied by the dilution factor.

HIV-1 RNA concentration was expressed as the number of HIV-1 RNA copies per millilitre (copies/ml) and was $\log_{10}$ transformed for all analysis.

2.5.2.1 Estimation of blood contamination in CVL samples

Pre and post-NVP dose maternal CVL samples were visually examined for blood contamination and by the use of urine reagent strips (Uricheck-9, RapiMED Diagnostics, SA) for measurement of whole blood and haemoglobin. This was performed to determine blood derived HIV-1 RNA in CVL. Estimation of the amount of blood plasma present in CVL was determined using a previously described method by Hart *et al* (1999). Haemoglobin (Hb) concentrations were used to calculate blood volumes in CVL samples. Four Hb levels were defined on the Uricheck-9 reagent strips: 10, 25, 50 and 250 erythrocytes/microlitre. Test sensitivity ranged from 0.015 - 0.062 mg Hb/100 ml. The amount of plasma virus in a CVL sample was estimated by using the following series of calculations:

a) Total amount of Hb in a CVL sample (mg Hb $_{lavage}$) was determined using the equation:

mg Hb $_{lavage}$ = (Hb signal $_{lavage}$ x 0.01) x (volume $_{lavage}$)

The Hb signal $_{lavage}$ was the Hb level (mg Hb/100 ml) estimated from the Uricheck-9 reagent strip, and volume $_{lavage}$ was the total volume of CVL sample in milliliters.

b) As the Hb concentration in peripheral blood (mg Hb $_{blood}$ /100 ml $_{blood}$) was known and the amount of Hb in CVL (mg Hb $_{lavage}$) was determined using the above calculation, the blood volume in CVL samples was then calculated using the equation:

Volume $_{Lblood}$ = (mg Hb $_{lavage}$) ÷ (mg Hb $_{blood}$/100 ml blood)

c) Blood plasma volume (plasma $_{lavage}$) in CVL samples was calculated using the volume of blood in CVL samples (vol $_{Lblood}$) and the peripheral blood heamatocrit in the equation:

(Plasma $_{lavage}$) = (1- haematocrit) x (vol $_{Lblood}$)

d) Blood plasma virus in CVL samples (plasma HIV$_{lavage}$) was calculated using the volume of plasma in CVL samples (plasma vol $_{lavage}$) and the virus concentration in plasma (HIV RNA copies $_{plasma}$/plasma vol) in the equation:

Plasma HIV $_{lavage}$ = plasma vol lavage x (HIV RNA copies $_{plasma}$/plasma vol)

2.5.3 NVP quantitation (pharmacokinetic assessments)

Principle: LC-MS-MS

Tandem Mass spectrophotometry (MS-MS) is a sensitive analytical method used for the identification of organic compounds in mixtures. The first mass spectrophotometer serves to ionize all components of the mixture and selects a major ion (molecular ion of the compound of interest). These ions were fragmented and the resulting fragment ions were separated and mass analyzed in the second mass spectrophotometer (Biemann, 1993). Use of liquid chromatography with Tandem Mass spectrophotometry (LC-MS-MS) improves the rate of sample analysis, assay sensitivity and data quality. Figure 19 illustrates the mode of operation of a mass spectrophotometer (Clark, 2000). A sample was introduced into the ionization source of the instrument. Once inside the ionization source, the sample molecules were ionized. These ions were extracted into the analyzer region of the mass spectrophotometer where they were separated according to their mass (m)-to-charge (z) ratios (m/z). The separated ions were detected and a signal was then sent to a data system where the m/z ratios were stored together with their relative abundance for presentation in the form of an m/z spectrum (Ashcroft, 2008).

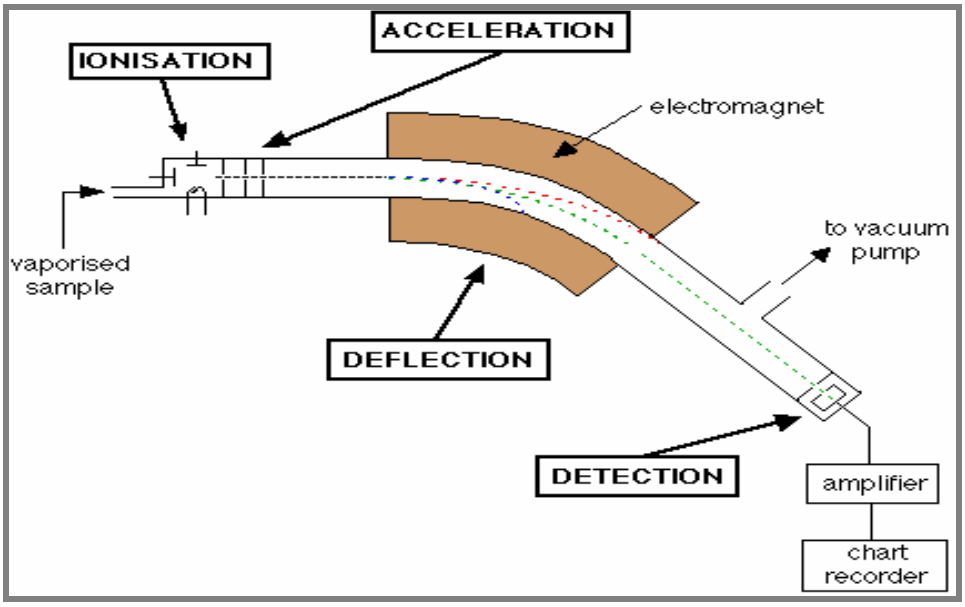


Fig 19: Mode of operation of a mass spectrophotometer (Clark, 2000)

Procedure: NVP quantitation assay

NVP drug concentration levels were quantified by the candidate using a validated Tandem Mass spectrophotometry method (LC-MS-MS), under the supervision of laboratory staff at the Division of Clinical Pharmacology, Department of Medicine, Groote Schuur Hospital/University of Cape Town. This assay procedure was performed with minor modifications as per previously described method (Cohen *et al*, 2008).

a) Reagents:

NVP reference drug was obtained from Aspen Pharmacare (SA). Aspen NVP (generic) was formulated in SA under license of the Boehringer-Ingelheim group of companies (Aspen Pharmacare (Pty) Limited, Sandton, SA).

Using the reference drug above, NVP stock solution of [1 mg/ml] was freshly prepared as per procedures described in Appendix 6. NVP stock solution was used to prepare NVP working standard solutions of 10 µg/ml and 100 µg/ml respectively (Appendix 6). NVP calibration standards (S1 - S10) were prepared by spiking drug-free human plasma with an appropriate amount of NVP working standard solution to obtain final concentrations of 100, 200, 500, 800, 1000, 1300, 1500, 2000, 5000 and 10 000 ng/ml respectively (Appendix 6).

Reserpine (Sigma, St Louis, MO) served as the internal standard. Low (C1), medium (C2) and high (C3) quality control samples of NVP were also prepared by spiking drug-free human plasma with 300, 1800 and 9000 ng/ml of NVP working standard solution respectively (Appendix 6). These quality controls covered the range of the standard curve and were randomly interspersed among unknown patient samples during each sample run.

b) NVP precipitation method:

Frozen patient samples [maternal plasma, maternal CVL and infant plasma] were allowed to thaw at ambient temperature. A 50 µl volume of NVP calibration standard (S1 - S10), quality control sample (C1, C2, and C3) and patient sample were each pipetted into appropriately labelled 2 ml eppendorf tubes. A calibrated Eppendorf Multipette was used to accurately dispense 200 µl of Protein Precipitation reagent [80% Methanol (MERCK, Germany): 20% 0.2 M Zinc Sulphate (Sigma, St Louis, MO), Appendix 6], containing 150 µl Reserpine (Sigma, St Louis, MO)] into each eppendorf tube. All eppendorf tubes containing (patient samples, NVP calibration standards and quality controls) were vortexed (Vortex Genie, Scientific Industries) thoroughly for 10 seconds each. This was followed by a five minute sonnication step (Instrulab, Integral systems, SA). All tubes were centrifuged (Biofuge 13,

Sepatech) at 13 000 rpm for five minutes at room temperature. A 200 µl volume of the resulting supernatant was carefully transferred into a clean glass insert contained within clean glass vial (Agilent technology, Germany). Care was taken to avoid disturbing or dislodging the pellet during this transfer step. All glass vials were carefully capped, placed into an autosampler rack (Agilent technology, Germany) and then finally loaded onto the LC/MS/MS System.

c) Instrument conditions:

The LC/MS/MS System consisted of a linear Ion Trap Quadropole API 3200 Q-trap Mass Spectrophotometer (Applied Biosystems, Germany) attached to an HPLC 1200 Series (Agilent Technologies, Germany) using a 1200 binary pump (Agilent, Germany), Gemini C18-RP 3 µm particle size and a [50 x 2.00 mm 4 micron] Phenomenex fusion column. The mobile phase consisted of; Bottle A: 10% Methanol (MERCK, Germany) and Bottle B: 97% Methanol (MERCK, Germany) + 3% 10 mM Ammonium Acetate (MERCK, Germany) in Glacial Acetic acid (analytical grade, Pal Chemicals) [Appendix 6]. The run time was three minutes at an injection volume of 2 µl and a flow rate 600 µl/minute.

Interpretation of results:

On completion of each sample run, a standard curve was constructed by plotting peak height ratios of NVP to internal standard versus NVP concentration. The standard curve (Fig 20) was assessed for linearity [r value as close to 1 as possible]. Chromatograms of maternal (plasma and CVL) and infant (plasma) samples are shown in Figs 21 a-e. NVP concentrations were determined relative to the internal standard (Reserpine, Sigma, and St Louis, MO) and all results were reported in units of ng/ml. Patient samples with NVP levels above the assay range

were diluted, and then re-assayed to maintain assay specificity. NVP values of (< 10 ng/ml) for CVL samples and (< 100 ng/ml) for plasma samples were regarded as below the limit of quantification for this assay.

Assay modifications:

All maternal CVL samples were first assayed using the same set of calibration standards as used for blood plasma [range: 100 – 10 000 ng/ml]. NVP detection and quantitation in a vast majority of the CVL samples was below the limit of assay quantification (< 100 ng/ml). The range of the standard curve was therefore lowered to [10 ng/ml – 100 ng/ml] to improve the detection limits of the assay. Using the NVP reference drug from Aspen Pharmacare (SA), NVP stock solution of [1 mg/ml] was freshly prepared as per (Appendix 6). NVP working standard solutions of 1 µg/ml and 10 µg/ml were then prepared using the NVP stock solution. NVP calibration standards (S1 – S8) were prepared by spiking drug-free human plasma with an appropriate amount of NVP working standard solution to obtain final concentrations of 10, 20, 30, 40, 50, 60, 80 and 100 ng/ml respectively. Low (C1), medium (C2) and high (C3) quality control samples of NVP were made up by spiking drug-free human plasma with 25, 55 and 90 ng/ml of NVP working standard solution respectively. The final sample injection volume was 20 µl. All other assay parameters in respect of NVP precipitation, internal standards and instrument conditions were performed as per blood plasma methodology.

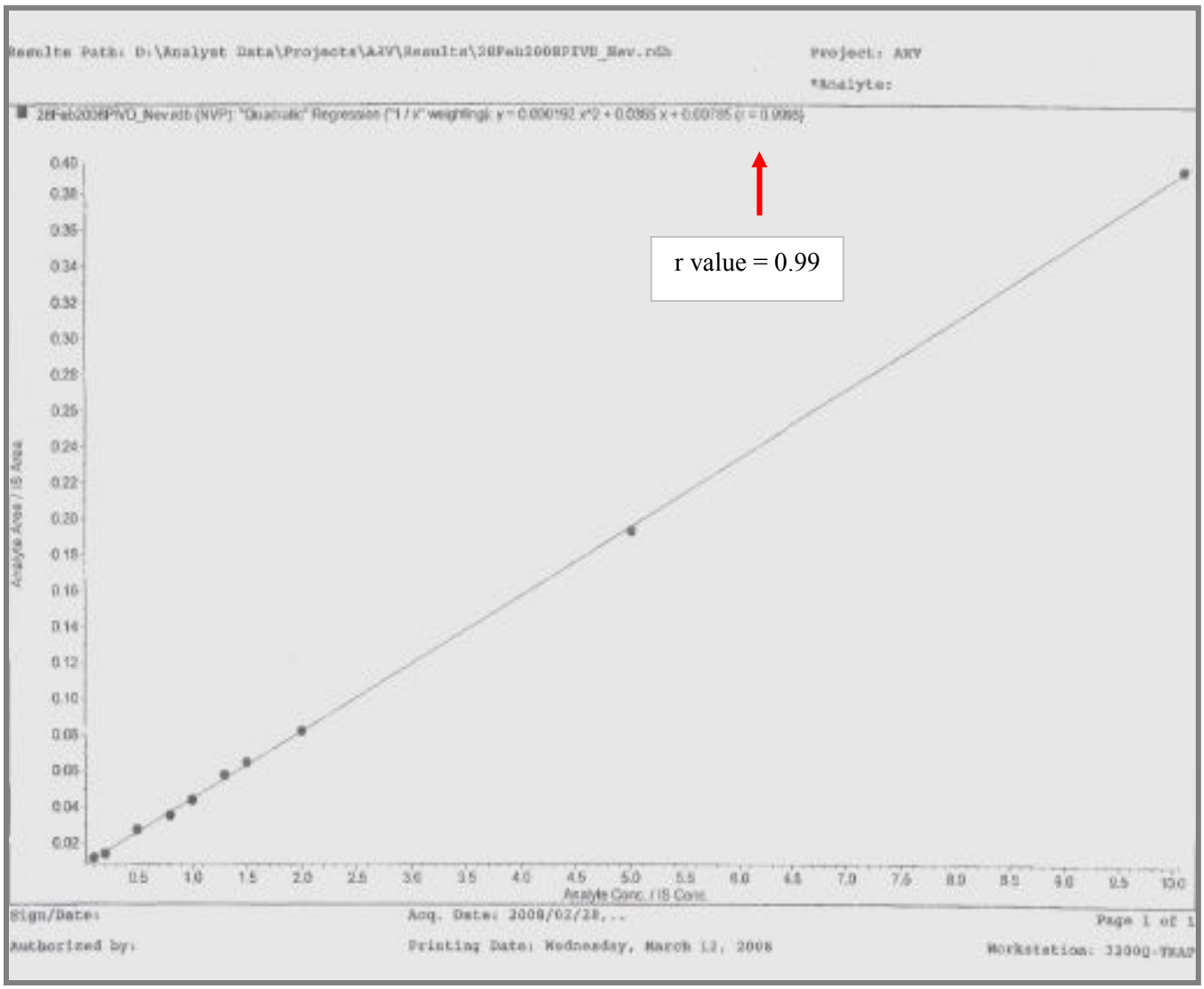
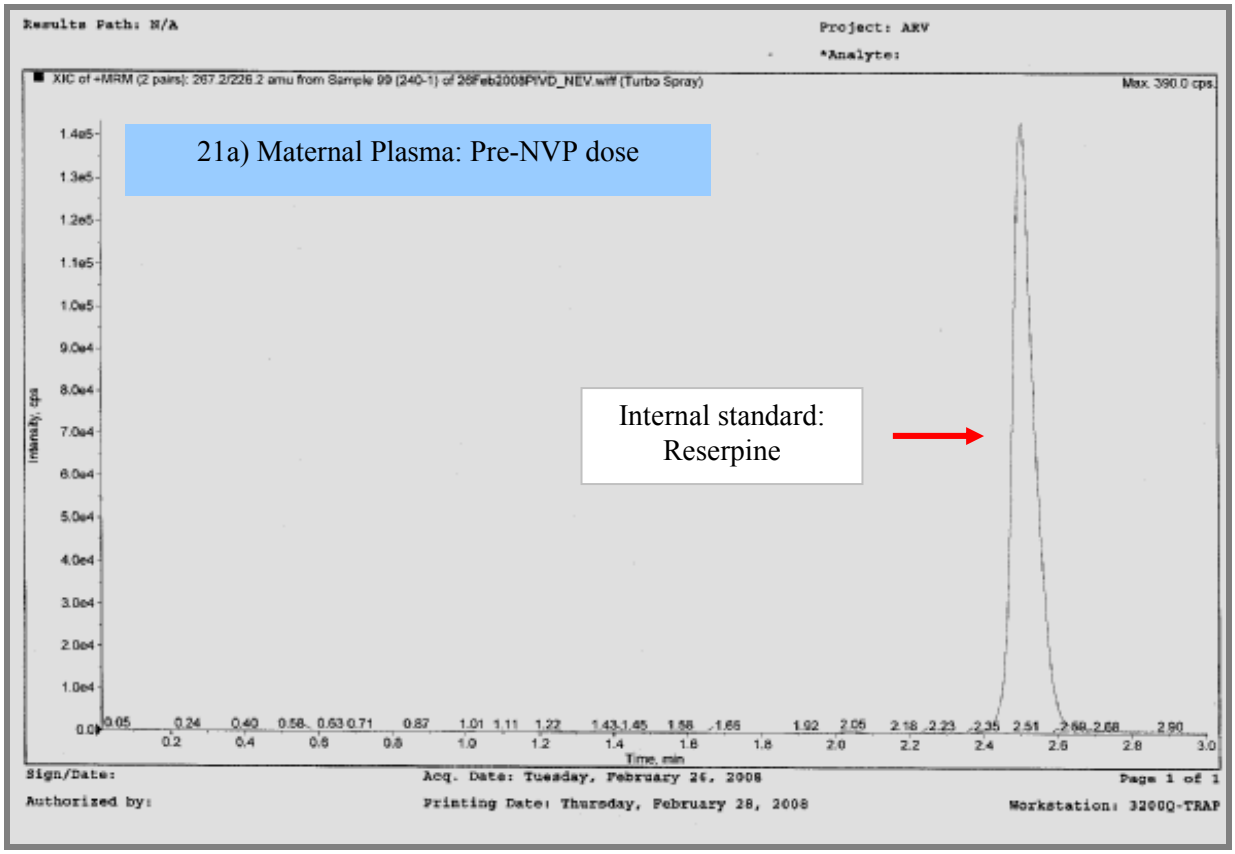


Fig 20: NVP standard curve



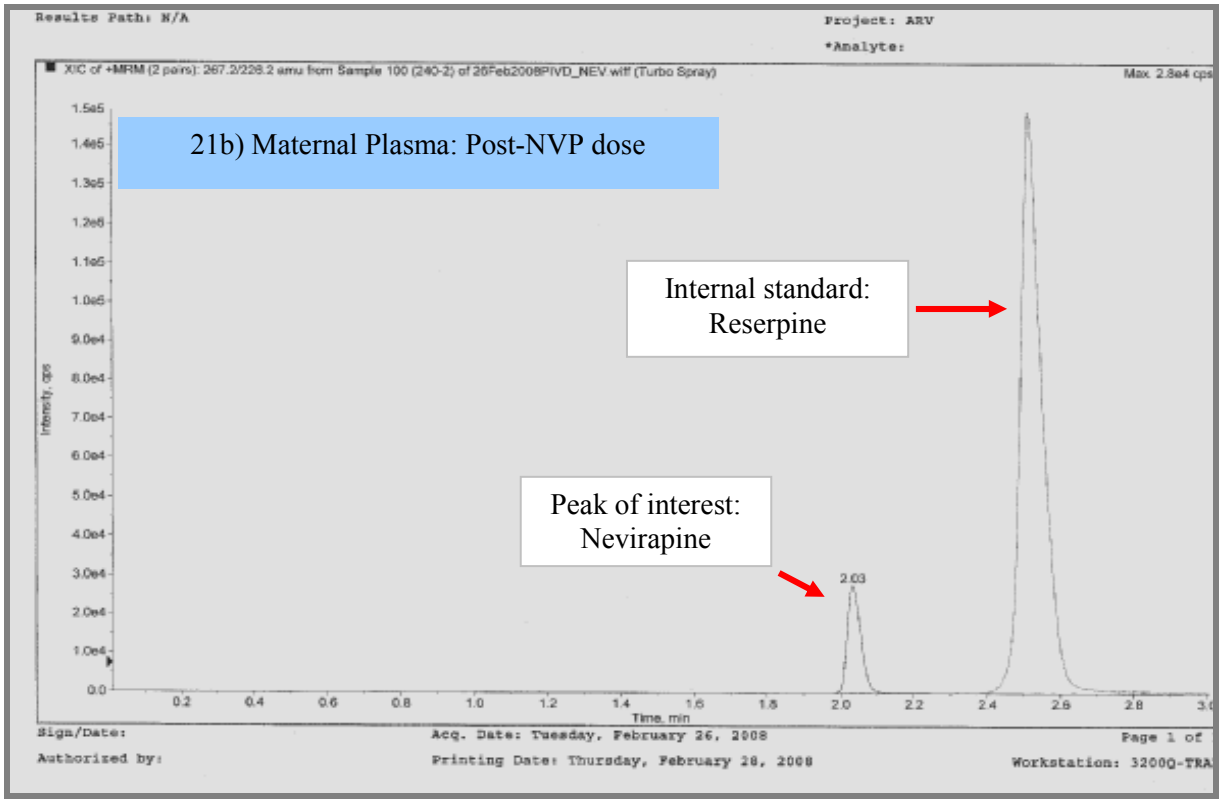
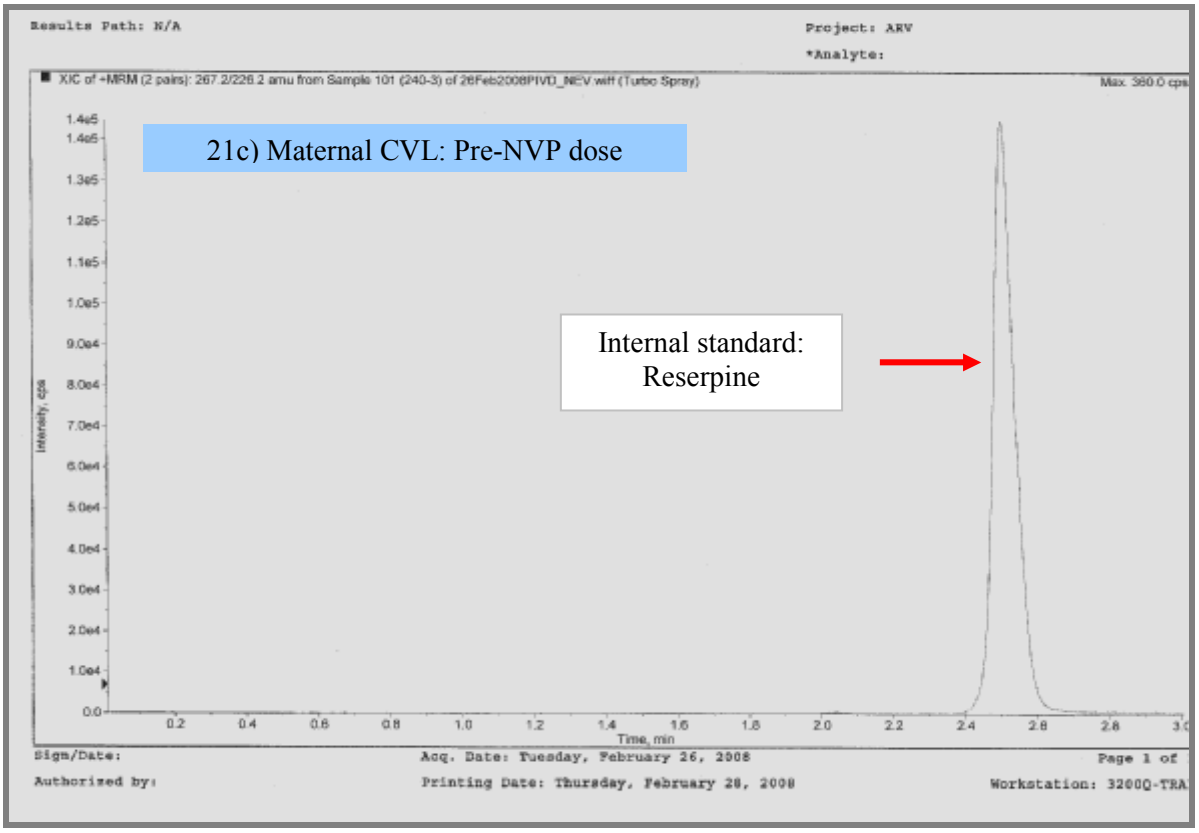


Fig 21 (a-b): Chromatograms of Maternal Plasma at Pre-NVP (a) and Post-NVP (b) dosing



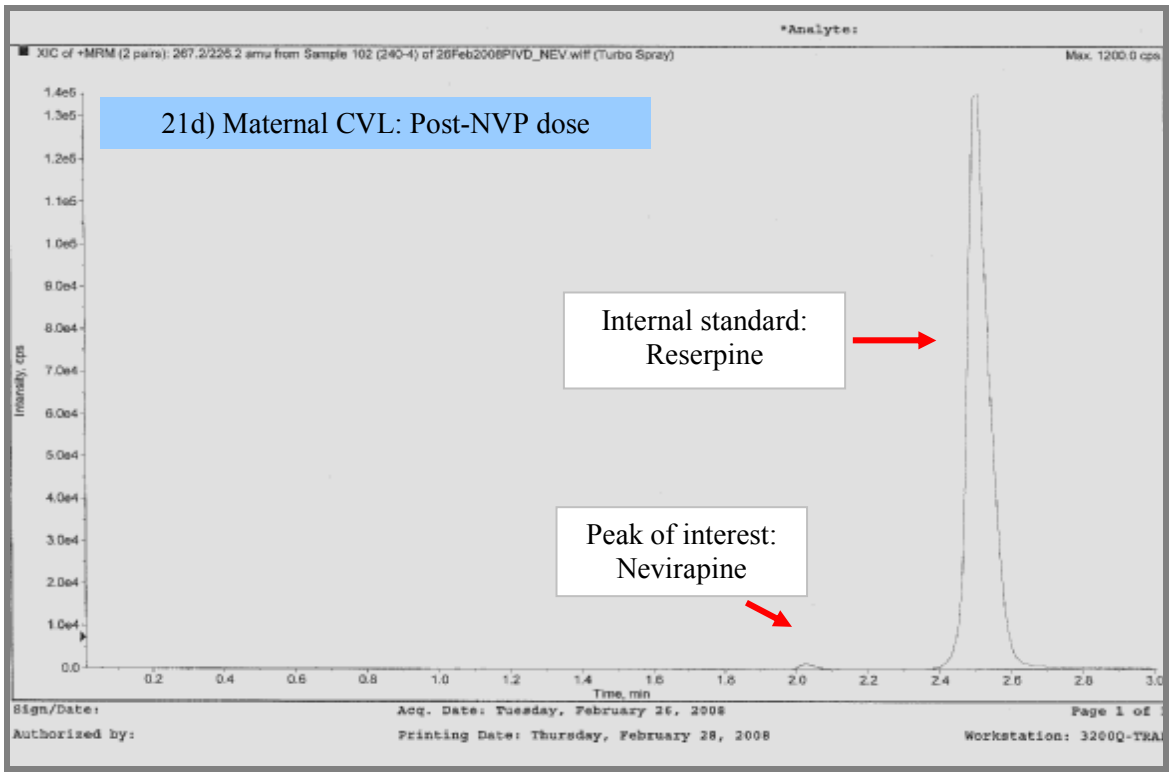


Fig 21(c-d): Chromatograms of Maternal CVL at Pre-NVP (c) and post-NVP (d) dosing

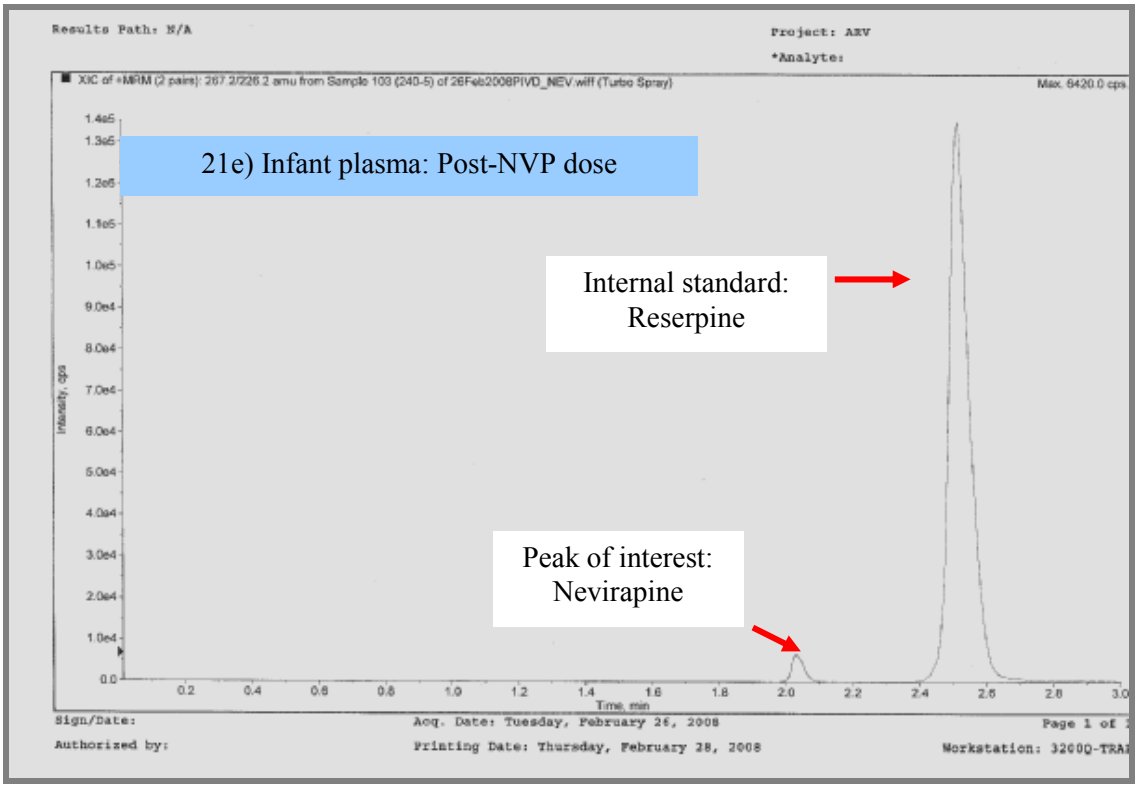


Fig 21e: Chromatogram of Infant plasma at post-NVP (e) dosing

2.5.4 Screening for STI's

Principle of test:

Wet mount microscopy was used for the direct microscopic examination of a wet preparation of cervicovaginal discharge. This WHO recommended method provided simple and rapid diagnosis of *Trichomonas vaginalis*, *Bacterial vaginosis* and *Candida albicans* infections (WHO, 2005). STI screening was performed on all study women (n = 120) during pregnancy at pre-NVP dosing. Following CVL collection, ~500 µl of sample was aliquoted into a clean eppendorf tube and transported immediately to the Global Clinical and Viral Laboratory (Durban) for wet mount microscopy assessments as per WHO guidelines (Appendix 7).

Interpretation of Results:

Laboratory reports were received on a weekly basis. If CVL samples indicated the presence of any STI (*Trichomonas vaginalis, Candida albicans or Bacterial vaginosis*), study participants were immediately contacted by telephone and urged to visit the antenatal clinic at KEH as soon as possible to commence with standard of care treatment (antibiotics) prior to their labour and delivery.

Standard of care for STI management at KEH:

- *Erythromycin (500 mg) x 7 days*
- *Flagyl (400 mg) stat dose*

2.5.5 HIV-1 drug resistance testing

Testing was performed by the candidate under supervision of a medical research scientist at the Hasso Plattner Molecular Research laboratory (DDMRI), Durban.

Sample, processing and storage

HIV-1 drug resistance testing was only conducted on HIV-1 transmitting women [refers specifically to study participants who transmitted or passed the virus onto their unborn infant either during the course of pregnancy or at the time of delivery). Testing was performed on post-NVP dose maternal plasma samples.

Principle:

The procedure described below was adapted from the ACTG protocol, 2004b. The Viroseq™ HIV-1 Genotyping system (Celera Diagnostics, USA) was used to identify mutations in the *pol* gene of HIV-1. In this system, the entire Protease gene and ~ two-thirds of the Reverse Transcriptase (RT) gene in the *pol* open reading frame were amplified. The amplicons were used as a sequencing template to generate sequence data. Genotypic analysis of this region of HIV-1 allows the study of the relationship between mutations and viral resistance to ARV drugs.

The genotyping process involved:

- *isolation of HIV from plasma and purification of viral RNA*
- *reverse transcription of the HIV genome using a single primer*
- *PCR amplification of the protease and RT genes from the cDNA made during the reverse transcription reactions*
- *direct sequencing of the PCR amplification product using custom sequencing mixes*
- *identification of mutations in the protease and RT genes using the Viroseq™ HIV-1 software*

Sample Preparation:

Maternal plasma samples and RNA diluents (kit) were allowed to thaw. A 500 µl volume of plasma was transferred into a pre-labeled tube (with orientation marks on one side of tube). Tubes were placed in a pre-cooled centrifuge (4^{o}C) such that the orientation mark was facing the outside rim of the rotor. Samples were centrifuged for one hour at 25 000 rpm at 4^{o}C. During centrifugation, 70% Ethanol was freshly prepared and viral lysis buffer (from kit) was thawed to room temperature. On completion of centrifugation, a fine-tip transfer Pasteur pipette was used to remove as much supernatant as was possible without disturbing the pellet. Thereafter 600 µl of viral lysis buffer was added to each tube and followed by thorough vortexing. Tubes were briefly centrifuged to sediment contents to the bottom of the tube. To ensure complete viral lysis, samples were allowed to stand at room temperature for ten minutes. This was followed by the addition of 600 µl of Isopropanol to each sample. Each tube was vortexed for 3-5 seconds, followed by 15 minutes of centrifugation with the orientation mark facing out, at 12500 - 16000 rpm at room temperature. On completion of centrifugation, a fine-tip transfer pipette was used to remove as much supernatant without disturbing the pellet. A 1000 µl volume of cold 70% Ethanol was added to each tube, followed by gentle vortexing for 3-5 seconds. All samples were centrifuged, with the orientation mark facing outwards, at 12500-15000 rpm for 5 minutes at room temperature. On completion of centrifugation, a fine-tip transfer pipette was used to remove as much supernatant without disturbing the pellet. Residual 70% Ethanol was removed and all tubes were allowed to air-dry. Each pellet was re-suspended with cold RNA diluent. This step was dependant upon the specific viral load (HIV-1 RNA) of each maternal sample. If HIV-1 RNA was > 15000 copies/ml, 100 µl of RNA diluent was added. Alternatively if HIV-1 RNA of the sample ranged between 2000-15000 copies/ml, a 50 µl volume of RNA diluent was added. Pellets

were then re-suspended by vortexing vigorously for 10 seconds. Samples were then quick spun allowing liquids to settle at the base of each tube. Samples were stored at -80^{o}C until needed for RT-PCR.

RT-PCR:

Using reagents provided in the kit, a PCR master mix (per reaction) was first prepared by combining PCR mix (29.5 µl), AmpliTaq Gold (0.5 µl) and UNG (1.0 µl). Positive and negative controls were included in each batch of extractions. RT master mix (volume/sample) was prepared by combining HIV RT Mix (8 µl), RNAse Inhibitor (1 µl), MuLV RT (1 µl) and 0.4 µl (DTT). A 10 µl volume of cold sample RNA was aliquoted to a 0.2 ml PCR tube. All tubes were placed into a thermal cycler and the instrument was run on pre-set conditions under "viroseq-rt program". The instrument was paused after the 42^{o}C/5min step and tubes were removed from the thermal cycler. RT mix (10 µl) was added into each reaction tube. Samples were returned to the thermal cycler and "RT reaction" was resumed. Samples were held at 4^{o}C for at least 10 minutes, thereafter PCR master mix (30 µl) was added to each sample. Samples were reloaded into the Thermal Cycler and run according to the pre-set instrument conditions under the "viroseq-pcr" program. Samples were stored at -20^{o}C until analyzed.

Purification of Viroseq products:

Microcon-100 Spin columns were inserted into the Microcon 1.5 ml collection tubes (kit provided) for each maternal sample. A 300 µl of KCL (200mM) was carefully added to the top of each column without touching the membrane. The entire 50 µl PCR reaction was loaded to the centre of the KCL-filled column. All caps were secured and columns were centrifuged for 15 minutes at 450 – 550 rpm. Thereafter 300 µl of sterile filtered deionised water was added to

the top of each column without touching the membrane. Caps were secured and the columns were centrifuged for 15 minutes at 450 – 550 rpm. After caps were removed, 35 µl of sterile filtered deionised water was added to the centre of each column. The columns were inverted in new tubes and spun for 5 minutes at 450 – 550 rpm. All columns were removed and discarded. Microtubes now contained approximately 40 -50 µl of purified PCR product. A mini agarose gel (1%) containing 0.5 µg/ml of Ethidium Bromide was prepared (Appendix 9). Five microlitres of the purified PCR product was mixed with 1 µl of Gel Loading Buffer (supplied with the kit) in a tube. A volume of 6 µl of the DNA Mass Ladder (supplied with the kit) was loaded into lane 1 of the agarose gel. Thereafter the entire 6 µl of the sample [purified PCR product and gel loading buffer] was loaded into the remaining wells of the agarose gel. The agarose gel was electrophoresed at 10 V/cm until the loading dye migrated at least 5 cm into the gel. The gel (Fig 22) was examined under UV light and the quantity of PCR products in each sample were estimated by comparing the intensity of each band to the intensities of the DNA Mass Ladder. Thereafter PCR products were diluted to an optimal concentration (as per manufacturer's instructions and briefly vortexed for 3-5 seconds.

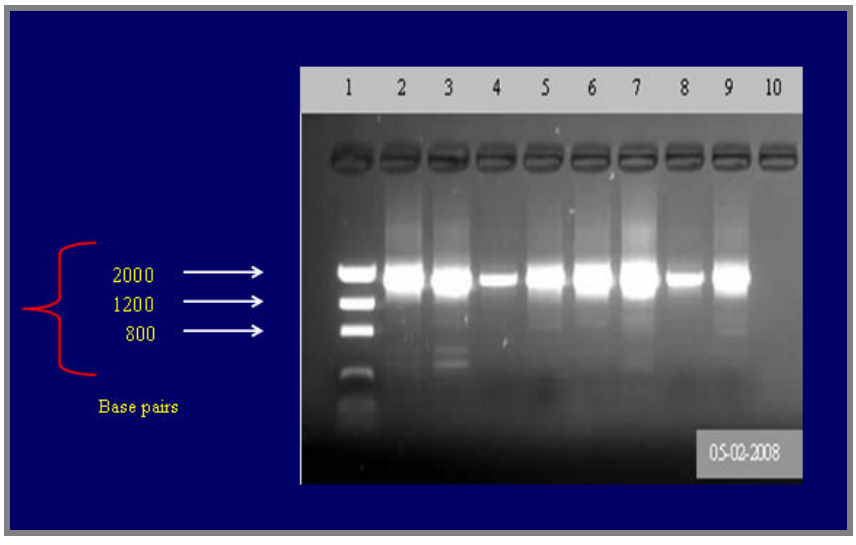


Fig 22: Gel image after electrophoresis; DNA Mass Ladder (lane 1), maternal samples (lanes 2-9)

Sequencing Reactions:

Primer mix reactions were set up in an optical 96-well reaction plate (Applied Biosystems, USA). A 12 µl volume of each primer mix (A, B, C, F, G and H) was added to an empty well. Thereafter 8 µl of the diluted purified PCR product was also added into each of the 6 wells from above step. The reaction plate was vortexed 3-5 seconds, and then quick spun for 5 – 10 seconds. Samples were loaded onto a thermal cycler and run on max mode using the program "seq" on the GeneAmp 9700. A thermal profile of 25 cycles permitted: denaturation of DNA at 96^{o}C (10 seconds), primer annealing 50 ^{o}C (5 seconds) and primer extension 60^{o}C (4 minutes).

Purification of sequencing products:

Stock solution (per reaction) was made by combining 2 µl of 3 M Sodium acetate pH 4.6 and 50 µl of 100% Ethanol (MERCK, Germany). A volume of 55 µl of Stock solution was added to each sequencing reaction. An adhesive cover was used to seal the reaction plate before vortexing. The reaction plate was centrifuged at 3000 rpm for 20 minutes. Thereafter the adhesive cover was removed and the reaction plate was inverted onto folded paper towel. The reaction plate and paper towel was centrifuged at 150 rpm for 5 minutes. A 150 µl volume of freshly prepared 70% Ethanol was immediately added into each well to prevent drying out of the well. The reaction plate was centrifuged at 3000 rpm for 5 minutes. The reaction plate was once again inverted onto folded paper towel. The reaction plate and paper towel was centrifuged at 150 rpm for one minute. Thereafter the reaction plate was allowed to dry at room temperature (~5 minutes). Dried pellets were then re-suspended with 10 µl of Formamide (from kit) and allowed to denature at 95^{o}C for 3 minutes on the GeneAmp 9700

under program “denature”. Samples were then loaded onto the ABI 3100 automatic sequencer (Applied Biosystems, USA) and run according to manufacturer’s instructions.

Interpretation of results:

All resulting sequences were assembled using Sequencher software (Gene Codes Corporation, USA) and examined in a multiple alignment with standard subtype references (Clustal X) available from (http://www.clustal.org). Aligned sequences were uploaded to the University of Stanford drug resistance database website available from (http://hivdb.stanford.edu/) for identification of drug resistance mutations. Drug resistance reports from two study patients indicating the presence and absence of NNRTI drug resistance mutations are shown in appendices 8a and 8b respectively. Phylogenetic analysis was then conducted using the Neighbour-joining method with Kimura's two-parameter model of distance calculation. Bootstrap analysis was performed with 100 replicas to rule out mislabeling of samples and to also confirm the absence of contamination. The virus subtype was determined by neighbour-joining reconstruction using reference sequences of RT and Protease *pol* genes from the Los Alamos HIV sequence database available from (http://www.hiv.lanl.gov).

2.5.6 Host genetics and immunology

All genotyping assays were conducted by the candidate under supervision of research scientists at the AIDS VIRUS UNIT (Cell Biology), National Institute of Communicable Diseases (NICD, Johannesburg).

Sample collection, processing and storage

Whole blood samples were collected from women during pregnancy (pre-NVP dosing) and from infants during their six week postnatal assessment. Maternal and infant whole blood was transferred into appropriately labeled cryovials and stored as 1000 µl (maternal) and 200 µl (infant) aliquots. All whole blood aliquots were stored at -70^{o}C. Of these stored aliquots, a case-controlled matched subset of patients was then selected for genotyping. The subset included all HIV-1 transmitting mothers (n = 12) and their infected infants (n = 14) in conjunction with a matched control group of non-transmitting women (n = 28) and their HIV-1 exposed-uninfected infants (n = 28). HIV-1 transmitting women were paired with HIV-1 non-transmitting women (controls) according to demographic and clinical characteristics that included; CD4 cell count, HIV-1 RNA (viral load), maternal age and gestational age.

Genotyping investigations included:

- *Determination of CCL3 and CCL3L1 gene copy number*
- *Identification of Single nucleotide polymorphisms (SNP's) and Haplotype characterisation of the CCL3 gene*
- *Investigation of a unique CCL3 Haplotype with respect to MTCT*

Genomic DNA extraction and quantitation

Genomic DNA was extracted from whole blood samples (maternal and infant) using the Qiagen QIAamp® DNA mini kit (Qiagen, USA) according to the manufacturer's instructions. Once extracted, purified DNA was stored in 2 ml eppendorf tubes at -20^{o}C until required. DNA purity and concentration was measured using the NanoDrop (ND-1000) spectrophotometer. Absorbance's were read at the following wavelengths (A_{230}, A_{260} and A_{280}) and DNA concentration was expressed in units of ng/µl. Fig 23 shows an example of a DNA concentration report for a study participant.

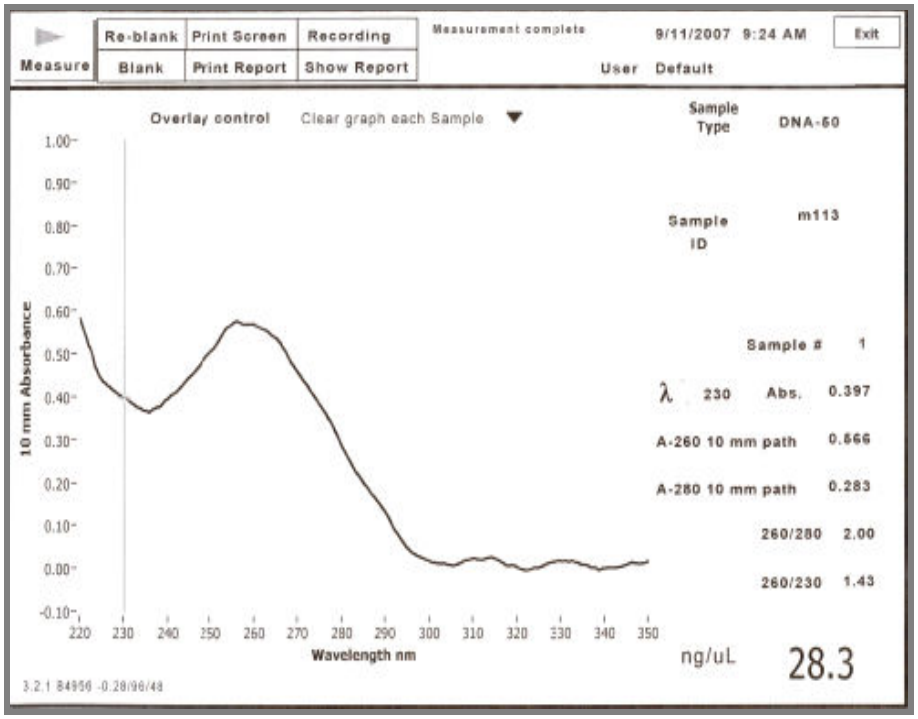


Fig 23: DNA quantitation report for a maternal sample using the NanoDrop spectrophotometer

CCL3 and CCL3-L1 gene copy number determination

CCL3 and CCL3-L1 gene copy numbers were determined by a real-time PCR assay as described by researchers at the NICD (Meddows-Taylor *et al*, 2006). This real time PCR assay made use of the following primers and probes which were synthesized at the University of Cape Town (UCT, SA):

- β-Globin gene upstream primer
- β-Globin gene downstream primer
- β-Globin gene probe
- CCL3-L1 gene upstream primer
- CCL3 and CCL3-L1 genes downstream primer
- CCL3 gene upstream and CCL3 probes

Fig 24 illustrates a summary of the assay procedure. Frozen DNA (extracted) samples and aliquots of primers, probes, internal control (Flu-4, AIDS Virus unit, NICD, SA) and TaqMan Universal PCR Master Mix (Applied Biosystems, USA) were allowed to thaw on ice. Three eppendorf tubes were labelled as CCL3, CCL3-L1 and β-Globin respectively. A bulk mix of each primer/probe was prepared as shown in Table VI and then kept on ice until required.

Table V: Preparation of Bulk Mix

	CCL3*	CCL3-L1*	β-Globin*
CCL3 primer (forward)	31.5 µl	31.5 µl	31.5 µl
CCL3-L1 primer (reverse)	31.5 µl	31.5 µl	31.5 µl
Baxter water	59.5 µl	59.5 µl	59.5 µl
CCL3 probe	17.5 µl	17.5 µl	17.5 µl

** For 30 extracted samples*

PCR microtubes were placed onto a rack and appropriately labelled for DNA samples and two assay controls. FLU-4 was used as the internal control as it has a known CCL3 copy of two gene copies per diploid genome. Water was used as the negative control. TaqMan (17.5 µl) Universal PCR Master Mix [Applied Biosystems, USA] was added into each PCR tube and vortexed thoroughly. Extracted genomic DNA (3.5 µl) was added into each TaqMan containing tube. Thereafter 3.5 µl of FLU-4 was added into the control tube (internal control). Lastly 3.5 µl of water was added into the tube labeled negative control.

A MicroAmp™ optical 96-well Reaction Plate with barcode (Applied Biosystems, USA) was prepared for PCR and placed onto a rack. Three reaction wells were run per DNA sample. Four microlitres of each primer and probe (CCL3, CCL3-L1 and β-Globin) was added into each allocated well respectively. Thereafter 6 µl of DNA/TaqMan mix was added into each of the three wells containing (CCL3, CCL3-L1 and β-Globin). Assay controls (internal and negative) were loaded onto the plate at the end. Fig 24 also illustrates the specific loading orientation of a single patient sample into a PCR reaction plate. Each row of the reaction plate

was then carefully capped with optical caps (Applied Biosystems, USA). The MicroAmp™ reaction plate was gently tapped to combine the primer/probe with patient DNA. The plate was thereafter loaded onto the ABI PRISM 7500 sequence detection system (Applied Biosystems, Netherlands) where Real-time PCR was performed according to the manufacturer's protocol.

Interpretation of results:

For each sample; β-Globin, CCL3 and CCL3-L1 genes were amplified in duplicate, using ~ 20 ng of genomic DNA. Using the relative quantification method and using B-Globin as the endogenous control, CCL3 gene copy number was confirmed at two copies per diploid genome (p.d.g) for each sample. CCL3 was then used as the endogenous control to calculate the absolute CCL3-L1 gene copy number (using the relative quantification method) against a known copy control (Meddows-Taylor *et al*, 2006).

The respective population median of CCL3-L1 gene copy among both maternal and infant samples was then calculated. Comparison of the absolute CCL3-L1 gene copy number relative to the population median was used to determine an association with HIV-1 susceptibility.

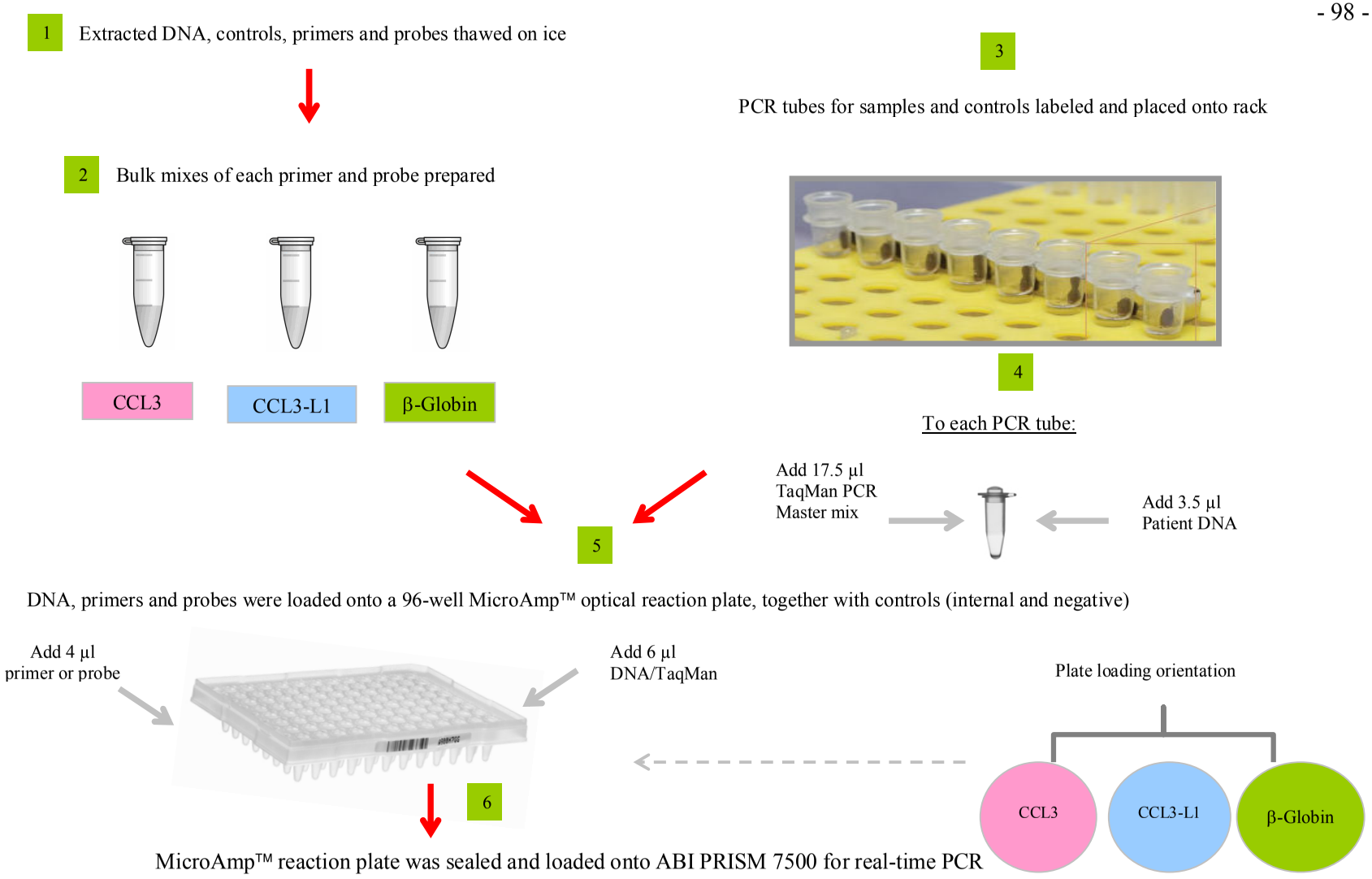


Fig 24: Assay procedure for CCL3-L1 gene copy number determination

Single nucleotide polymorphism and Haplotype characterisation of CCL3 and CCL3-L1

DNA sequencing of CCL3 and CCL3-L1

One hundred nanograms of extracted genomic DNA was used in a PCR amplification designed to co-amplify the region spanning the core promoter, exon 1 and most of intron 1 of both the CCL3 and CCL3-L1 genes. The upstream primer was designed to bind a consensus region flanking the *Alu* element, which is present only in CCL3-L1, and the downstream primer was designed to bind the consensus region in intron 1, that is ~50 bp upstream from the start of exon 2. The primers thereby amplified two fragments, a CCL3 and CCL3-L1 specific amplicons (Meddows-Taylor *et al*, 2006). PCR was performed as per methods described by (Meddows-Taylor *et al*, 2006) using the Expand High Fidelity PCR System (Roche Diagnostic Systems, Branchburg, NJ, USA).

After gel electrophoresis (Fig 25), amplicons were purified using the QIAquick® gel extraction kit (using a microcentrifuge kit protocol) [QIAquick® PCR Purification kit, Qiagen, USA] as per manufacturer's instructions. Sequencing was carried out using four sequence-specific primers, designed to selectively sequence either the forward or reverse sequence of CCL3 and CCL3-L1 from the purified amplicon mixture. Sequencing reactions were set up using the Big Dye Terminator chemistry version 3.1 (Applied Biosystems, USA) and run on a 3100 genetic Analyser (Applied Biosystems, USA) according to the manufacturer's instructions. Resulting sequences were assembled and analysed for the presence of single-nucleotide polymorphisms (SNP's) by using SEQUENCHER software version 4.14 (Gene Codes Corporation, USA) by alignment with published sequences by (Nakao *et al*, 1990). Sequence data has been included in Appendix 10.

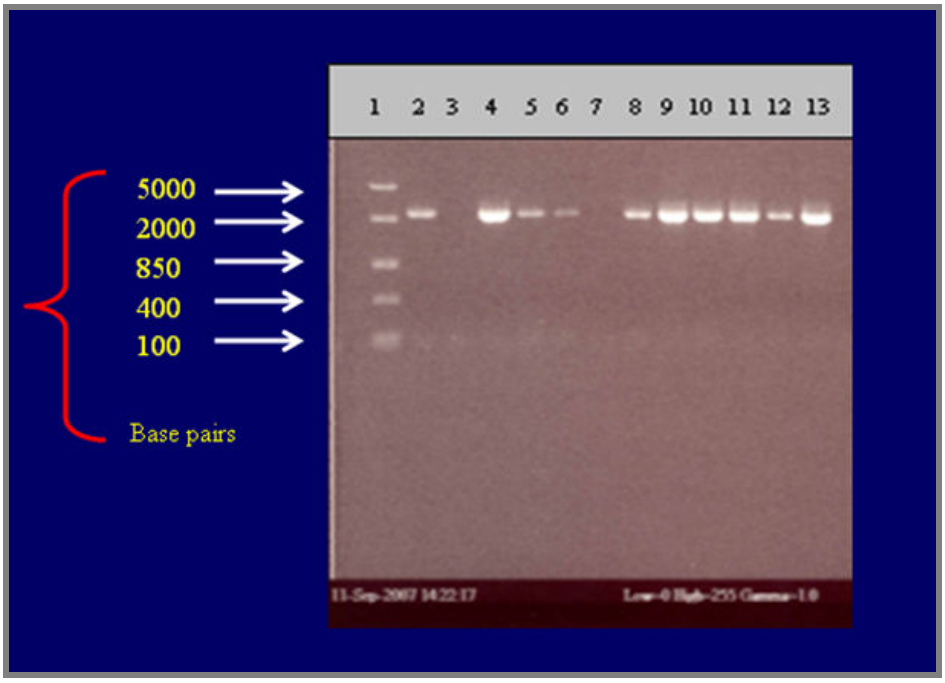


Fig 25: Gel image after electrophoresis; DNA ladder (lane 1); patient samples (lanes 2-13)

Investigation of a unique CCL3 Haplotype with respect to mother-to-child transmission

A SYBR-Green Real-time PCR assay by (Paximadis *et al*, 2008) was used to detect CCL3 C/T promoter SNP at position -86 (Haplotype-1 representation). Samples were thawed and PCR reaction mixes (C and T) were prepared. The C mix was made up of [225 µl SYBR Green PCR master mix (Applied Biosystems, UK), 45 µl C-forward primer, 45 µl Reverse-2 and 45 µl water]. T reaction mix was similar to the C reaction mix, with exception of 45 µl T-forward primer (instead of C-forward primer). All reaction mixes were vortexed, centrifuged then dispensed (8 µl per well) into C and T rows of a PCR reaction plate. Two microlitres of DNA was dispensed into each well. The PCR reaction plate was sealed with optical lids (MicroAmp, Applied Biosystems, USA). The reaction plate was briefly centrifuged before it was loaded onto a thermacycler using the following thermal profile [1 cycle, 10mins at 95^{o}C, 50 cycles;

95^{o}C (15 sec), 60^{o}C (20 sec), 72 ^{o}C (1 min) and 1 dissociation cycle: 95^{o}C (15 sec), 60^{o}C (1 min), 95^{o}C (15 sec)].

A real-time PCR assay developed by (Paximadis *et al*, 2008) using primers with 3′-end locked nucleic acids (LNA's) designed over the SNP position [one primer with 3′-end C LNA and one with a 3′-end T LNA was used for identification of Homozygotes (C/C) and Heterozygotes (C/T) at position -86 in CCL3. All primers were manufactured at (UCT, SA) and included; Seq-alpha-IF, reverse-2, CCL3-Forward, Seq P1, Seq P2, Seq P3 and Seq-P4). While setup reactions were similar to conventional PCR, this real-time PCR measured the accumulation of PCR product via the fluorophore during the exponential stages of the PCR, rather than at the end point (as in conventional PCR). A real-time PCR thermal cycler (Applied Biosystems, USA) was used to measure the fluorescence in the reaction tubes. Fluorescence intensities were logged and data stored for each PCR cycle was then used to create amplification plots of (fluorescent signal detected - background) vs. cycle number to identify the threshold cycle or C_T. The exponential increase of the product was used to determine the C_T, i.e. the number of PCR cycles at which a significant exponential increase in fluorescence was detected and which was directly correlated with the number of copies of DNA template present in the reaction.

Interpretation of results:

Homozygote (Fig 26a) wild-types and mutants were detected by amplification plot shifts in threshold cycle C_T values of approximately ten cycles whereas heterozygotes (Fig 26b) were detected by similar threshold values for each primer.

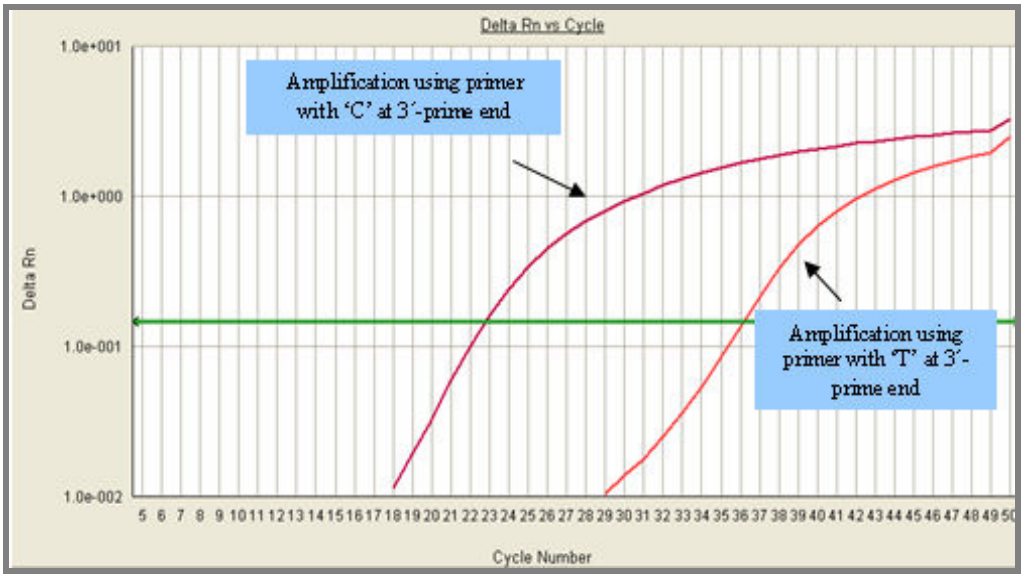


Fig 26a: Homozygote (C/C) at position -86 in CCL3 (A SYBR-Green real-time assay was used to detect -86 SNP in CCL3) in an infant sample (C338)

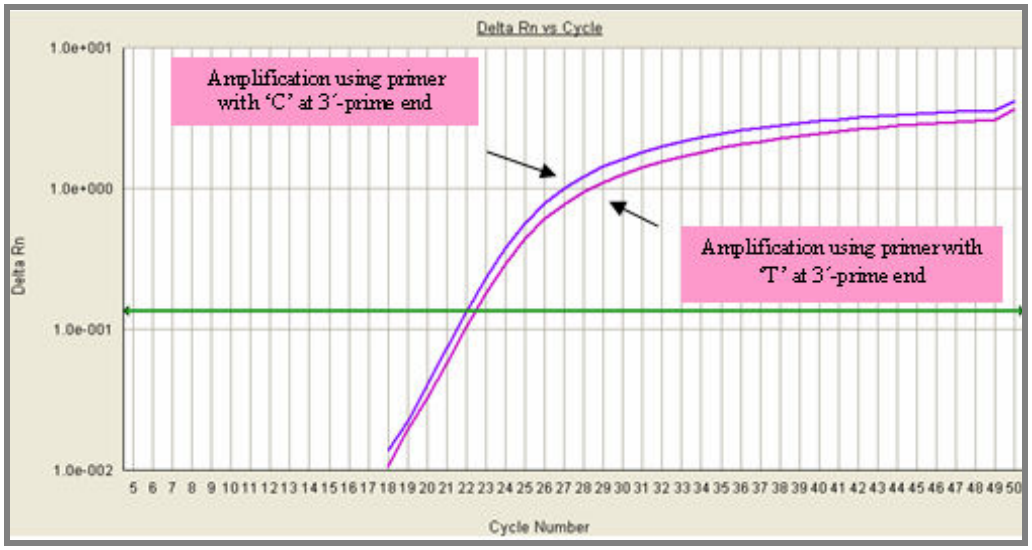


Fig 26b: Heterozygote (C/T) at position -86 in CCL3 (A SYBR-Green real time assay was used to detect -86 SNP in CCL3) in an infant sample (C351)

2.6 Laboratory data: collation and capture

All data collection was undertaken by the candidate. Prior to data capture, all patient CRF's were thoroughly checked for accuracy and completeness by comparison with patient clinical records and laboratory reports. Clinical chart records of study participants were requested from the Medical Registry of KEH, photocopied and used to update and verify all patient data.

Laboratory generated data were collated and filed into respective patient CRF's as sample batches were assayed. Conversions and calculations were performed where necessary e.g. dilution factors, log transformations for HIV-1 RNA, calculations for CVL blood contamination etc. All transcribed laboratory results were cross-checked against original laboratory print-outs and reports. All clinical and laboratory data was captured by the candidate into a study database using Microsoft ® Office Excel 2003 and updated as the study progressed.

2.7 Statistical analysis

Analysis was undertaken by the candidate with assistance from a senior Biostatistician (Dr CA Connolly at the Medical Research Council (Biostatistics unit, MRC, Durban). The following statistical software packages were utilized; Microsoft ® Excel 2003, InStat (GraphPad Software Inc, San Diego, USA) and Stata V9 (Stata Corporation, Texas, USA).

To address the specific objectives of this study, data pertaining to *in utero* transmission were excluded from statistical analysis.

Descriptive statistics such as proportions and medians were used to summarize continuous data (e.g. patient demography, maternal and infant characteristics etc). Weight for age z-scores were calculated using WHO reference tables for child growth standards (WHO, 2009). The unpaired t test was used for all continuous variable comparisons [normal distribution of data]. Categorical data (e.g. comparison of various CD4 categories among HIV-1 transmitting vs. non-transmitting women) were summarized by the use of contingency tables, and then analyzed with the Fisher's exact test. The Mann-Whitney U test was used for analysis of all non-parametric data because of the unsymmetrical distribution of the data and small sample size (e.g. genotyping subset).

Undetectable HIV-1 RNA (viral loads) were assigned a value of 10 copies/ml (1 $\log_{10}$ copies/ml) for CVL samples (Kovacs *et al*, 1999) and a value of 40 copies/ml (1.6 $\log_{10}$ copies/ml) for plasma samples (Cobas AmpliPrep/Cobas TaqMan, 2006). All HIV-1 RNA (viral loads) were $\log_{10}$ transformed to obtain a more symmetrical distribution. Pearson's correlation coefficient (r) was used to determine pair-wise linear associations between

variables (e.g. viral loads and CD4 cell counts). The strength of the correlation was interpreted as: $r < 0.3$ = weak correlation, $r \geq 0.3 - 0.7$ = moderate correlation and $r \geq 0.7 - 1$ = strong correlation.

Statistical tests used during various data comparisons are shown in Table VI. All statistical tests were two-tailed and p values ≤ 0.05 were considered to be statistically significant. Interquartile ranges (IQR), odds ratio's (OR), relative risk (RR) and 95% confidence interval's (CI) were calculated where applicable.

Table VI: Statistical tests

Data comparisons	Statistical test used
Maternal age, gestational age, mode of delivery vs. perinatal transmission	Unpaired t test
Rupture of membranes, infant weight, infant gender, STI vs. perinatal transmission	Fisher's exact test
**CCL3-L1 gene copy number in relation to maternal parameters such as CD4 cell count, HIV-1 RNA (viral load) etc	Mann-Whitney U test
**CCL3-L1 gene copy no vs. perinatal transmission	Unpaired t test; Mann-Whitney U test Pearson correlation coefficient
**CCL3-Hap 1 allelic representation in relation to maternal parameters such as CD4 cell count, HIV-1 RNA etc	Mann-Whitney U test
**CCL3-Hap 1 vs. perinatal transmission	Mann-Whitney U test
HIV-1 RNA (viral load) correlation: CVL vs. plasma Pre-NVP vs. Post NVP sampling	Pearson's correlation coefficient (r)
NVP drug concentration (correlation) : CVL vs plasma Pre-NVP vs. Post-NVP sampling	Pearson's correlation coefficient (r)
Maternal CD4 cell count (pre and post NVP) in relation with maternal HIV-1 RNA	Pearson's correlation coefficient (r)
STI vs. HIV-1 RNA	Pearson's correlation coefficient (r)
STI vs. NVP concentration	Pearson's correlation coefficient (r)
Maternal CD4 cell count and perinatal transmission	Mann-Whitney U test
NVP and perinatal transmission	Fisher's exact
STI detection and perinatal transmission	Fisher's exact test
HIV-1 RNA and perinatal transmission	Unpaired t test, Mann Whitney U test

*Footnote: ** (case-control subset of maternal-infant pairs)*

CHAPTER THREE: RESULTS

3.1 Introduction

In the present chapter, findings from all the laboratory investigations will be described and compared within the context of intrapartum HIV-1 transmission.

3.2 Cascade of study events

Six hundred pregnant women, who tested HIV-1 positive by rapid tests and received the single dose NVP regimen for the prevention of mother-to-child HIV-1 transmission (PMTCT) according to the standard of care at KEH (Durban, SA) were screened for study eligibility during the period April-December 2006. Of the total women screened, 120 (20.0%) were eligible for study participation. A flow diagram detailing all study events is shown in Fig 27.

Fig 27 also highlights the various reasons that accounted for the large number of screening failure which occurred among 480 (80.0%) of pregnant women during the enrolment phase of this study.

HIV-1 antibody testing (HIV-1 ELISA) of all study entrants provided confirmation of their HIV-1 infection status and no discrepant results were observed (n = 120).

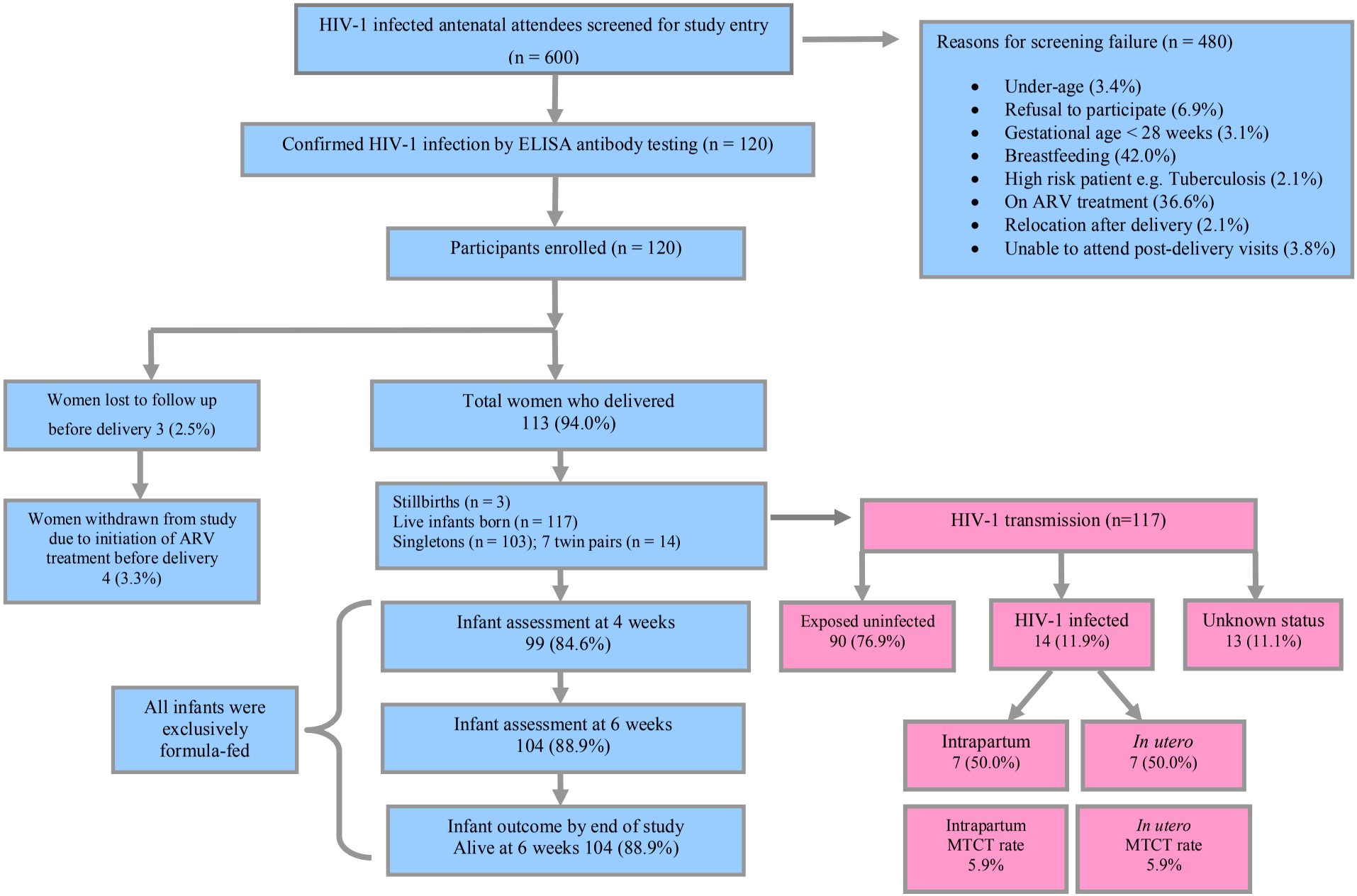


Fig 27: Flow diagram of study events

Footnote: All 113 women who delivered were exposed to intrapartum sdNVP and were therefore included in all analysis

3.3 Study population

3.3.1 Demography

Isi-Zulu speaking participants (66.7%) made up the majority of the cohort (Table VII). The median age of study participants was 29 years (IQR: 18-39). More than 70.0% of participants received a secondary school education and 25.0% of all women were employed (Table VII). The majority of women (78.3%) were unmarried, among whom 33 (35.0%) were living with their partners. While 22 (18.3%) women were able to support themselves, others were dependant on either their husbands/partners (54.2%) or families (27.5%) for financial assistance.

Table VII: Patient demography (n = 120)

Patient characteristics:	**Results n (%)**
Maternal age (years)*	
• < 20	5 (4.2)
• 20-24	31 (25.8)
• 25-29	35 (29.2)
• 30-34	34 (28.3)
• 35-39	15 (12.5)
Ethnic sub-grouping:	
• Zulu	80 (66.7)
• Xhosa	32 (26.7)
• Sotho	3 (2.5)
• Other	5 (4.1)
Level of education:	
• Primary	33 (27.5)
• Secondary	87 (72.5)
Marital status:	
• Single	61 (50.8)
• Married	26 (21.7)
• Living with a partner	33 (27.5)
Employment:	
• Employed	30 (25.0)
• Unemployed	90 (75.0)
Financial support:	
• Self-supported	22 (18.3)
• Family	33 (27.5)
• Boyfriend/husband/partner	65 (54.2)

**Maternal age classification performed according to national survey (Department of Health, 2007)*

3.3.2 Maternal clinical, laboratory and obstetric characteristics

The median gestational age at study entry was 34 weeks (range: 28-39) with more than 100 (80.0%) of the women having had their first antenatal consultation during their second trimester of pregnancy.

Forty-seven (39.2%) of the total 120 women who were screened for the presence of asymptomatic STI's had at least one type of STI detected during pregnancy (24-39 weeks gestational age). Three women (2.5%) presented with two concurrent types of STI. *Candida albicans* was the most frequently detected STI; 25 (20.8%) among women in this cohort. *Trichomonas vaginalis* was detected in 18 (15.0%) women while three (2.5%) women were positive for *Bacterial vaginosis* infections. One case (0.8%) of reactive Syphilis serology (*Treponema pallidum*) was observed among study women. Among the 47 women with detectable STI's who were contacted to return for appropriate clinical management, 19 (40.6%) sought treatment before delivery.

Classification of maternal CD4 cell counts (Table VII) was performed according to the WHO CD4 criteria for initiation of ARV treatment in adults and adolescents (WHO, 2006c). The median and interquartile range (IQR) of CD4 cell counts among the 120 pregnant participants at study entry was 530 (cells/µl) [IQR: 246-497]. Nineteen (15.8%) of these women had a CD4 count of < 200 cells/µl and were eligible for antiretroviral therapy in admission into the ARV rollout programme at KEH. The median CD4 cell count among these women was 142 cells/µl [range: 21-198]. All nineteen women were referred to the ARV rollout clinic (Philani Family Clinic, KEH) and commenced on a comprehensive eight-week ARV drug adherence training programme prior to labour and delivery. As at the final postnatal study visit at six

weeks, ARV treatment (3TC, d4T and NVP) was reported to have been initiated in four (21.0%) of the women prior to delivery, while the remaining 15 (78.9%) women had not commenced ARV treatment. The four women on ARV treatment withdrew from the study prior to delivery and are therefore excluded from the analysis. Reasons for not commencing therapy included various logistical constraints, advanced gestational age or some women were still in the process of completing their drug adherence training.

Of the 120 women enrolled during pregnancy, 113 (94.2%) delivered at the study site for whom obstetric data were available. Women excluded from the study since baseline include three (2.5%) lost to follow-up and four (3.3%) who commenced ARV therapy before delivery.

The median gestational age of women at delivery was 40 weeks [range: 31-43] and the median number of antenatal visits was nine visits prior to delivery as per patient clinical records. There was an almost equal distribution of normal vaginal deliveries (NVD) [n = 57] vs. caesarean section deliveries (n = 56). Of those delivering by caesarean section (C/S), 32.8% were elective and 16.8% were emergency (Table VIII). Duration of membrane rupture of greater than four hours prior to delivery occurred in 13 (18.0%) of women. Fewer women delivered their infants at pre-term (11.8%) compared to women delivering at full-term (86.4%) (Table VIII).

Table VIII: Maternal clinical, laboratory and obstetric characteristics

Previous obstetric history:	**n=120**
• Median Parity	1 (range:0-5)
• Median Gravidity	2 (range: 1-6)
• Miscarriages	10 (8.3%)
• Termination of pregnancy	1 (0.8%)
Current obstetric characteristics:	Median (Range)
Gestational age at first antenatal booking (weeks)	23 (8-36)
Gestational age at study entry (weeks)	34 (24-39)
Weight at first antenatal visit (kg)	69.6 (46.9-122.6)
Weight at last antenatal visit (kg)	74.4 (49.1-128.4)
Detection of maternal STI [n = 120]	**n (%)**
Treponema pallidum	1 (0.83)
Bacterial vaginosis	3 (2.5)
Candida albicans	25 (20.8)
Trichomonas vaginalis	18 (15.0)
***CD4 cell count (cells/µl) (**pre-NVP dosing) [n = 120]	**n (%)**
• ≤ 200	19 (15.8)
• 200-350	35 (29.2)
• 350-500	37 (30.8)
• ≥ 500	29 (24.2)
Maternal outcomes [n = 120]	
• Total women who delivered at facility	113 (94.2%)
• Withdrawn due to ARV treatment	4 (3.3%)
• Lost to follow up (before delivery)	3 (2.5%)
Gestational age at labour (weeks)	40 (31-43)
Mode of delivery: [n = 113][#]	**n (%)**
NVD	57 (50.4)
Emergency (C/S)	19 (16.8)
Elective (C/S)	37 (32.8)

Table VIII continued	
Duration of membrane rupture prior to delivery [n = 73]**	
• 0-3 hours	45 (62.0)
• ≥ 4 hours	13 (18.0)
• Unknown	15 (20.0)
Term of delivery: [n = 110]	**n (%)**
• Full-term (37- 41 weeks)	95 (86.4)
• Pre-term (< 37 weeks)	13 (11.8)
• Post-term (> 42 weeks)	2 (1.8)

Footnotes:

** As per WHO CD4 criteria for initiation of ART in adults and adolescents,* (WHO, 2006c)

[#] *includes three IUD's that occurred at 33[+], 38 and 40 weeks gestational age*

[**] *Includes NVD and emergency deliveries*

3.3.3 Paediatric clinical characteristics and outcomes

Of the 120 women enrolled between June-December 2006, 110 women delivered 117 live infants comprising 103 singletons (93.6%) and seven pairs of twins (6.4%). Multiple pregnancies as risk factors for MTCT will be presented under ancillary study findings in section 3.5. Gender was almost equally distributed among the infant cohort; male (48.7%) and female (51.3%). The median infant birth weight was 3.1 kg and ranged from 1.2-4.1 kg. Table IX details all clinical characteristics and outcomes of the infant population. All infants in this study were exclusively formula-fed. One hundred and four (88.0%) of the infants were alive at study completion (6 weeks).

3.3.4 HIV-1 infection status and timing of MTCT

Of the 110 women who delivered in this study, twelve (10.9%) transmitted HIV-1 to their infants, while 95 (86.4%) were classified as non-transmitters. The transmission status of three women remained unknown. As a result of seven twin births, the infant cohort comprised of 117 infants in total. Following DNA PCR testing, HIV-1 infection was identified in 14 (11.9%) infants while the remaining 90 (76.9%) were exposed-uninfected (Table IX). Infection status remained unknown for thirteen infants as a result of infant demise (1.7%), lost to follow-up (7.7%) or study withdrawal (1.7%) after delivery (Table IX). After confirmation of their infection status by two separate DNA PCR results, all HIV-1 infected infants were immediately referred to the ARV rollout programme at KEH or screened by the Paediatric AIDS Clinical Trial Group (PACTG) for potential participation in a clinical drug trial taking place at KEH.

Viral characteristics of infants as measured by DNA and RNA PCR testing are shown in Table X. The overall MTCT rate in this non-breastfeeding population was 11.9% (CI: 6.70-19.00), of which seven (50.0%) were intrapartum and seven (50.0%) were *in utero* HIV-1 transmissions. *In utero* and intrapartum MTCT rates were 5.9% each [95% CI: 1.68-10.28].

Table IX: Paediatric clinical characteristics and outcomes

Infant characteristics			Results
Births:			(n) %
• Total deliveries			110 (91.7)
• Total live births			117
• Singletons			103 (93.6)
• Twin pairs			7 (6.4)
Gender: (n = 117)			n (%)
• Male			57 (48.7)
• Female			60 (51.3)
Anthropometry: Weight at:	Median weight (kg)	Weight for age z-scores*	
		Male	Female
Birth	3.1 (1.2-4.1)	-1	-0.7
Four weeks	3.8 (2.0-5.5)	-0.6	-1.5
Six weeks	4.6 (2.6-8.0)	-0.2	-0.2
Median length at birth (cm)			47 (35.0-60.0)
Median head circumference at birth (cm)			34 (28.0-38.0)
Infant outcome by study completion (6 week-post delivery) (n = 117)			n (%)
Alive			104 (88.9)
Demised; 2wks (n = 1); 4wks (n = 1)			2 (1.7)
Lost to follow up; 4 weeks (n = 3); 6 weeks (n = 6)			9 (7.7)
Withdrawn due to a change of infant feeding mode of formula to breast at four weeks (n = 4)			2 (1.7)
Infection status as at 6 week study completion (n = 117)			n (%)
HIV-1 infected			14 (11.9)
Exposed uninfected			90 (76.9)
Unknown due to; infant demise; lost to follow-up or withdrawal			13 (11.1)

Footnotes:

**Weight for age z-scores calculated using WHO reference tables (WHO, 2009)*

Table X: Viral characteristics of infants

	In utero (n = 7)	Intrapartum (n = 7)	Exposed-uninfected (n = 90)
DNA PCR			
Detection at birth	HIV-1 positive (n = 7)	HIV-1 negative (n = 7)	HIV-1 negative (n = 90)
Confirmation at 6 weeks	HIV-1 positive (n = 7)	HIV-1 positive (n = 7)	HIV-1 negative (n = 90)
RNA quantitation	Median (range)	Median (range)	
Birth	3.2 (2.5-5.6)	n/a	n/a
≥ 4 weeks	5.7 (4.6-6.5)	5.2 (4.6-5.8)	n/a

Footnote:
Analysis was not possible in 13 infants with unknown HIV-1 status due to; withdrawal due to breastfeeding (n=2), demise (n=2) or lost to follow-up (n=9)

For the purpose of addressing the primary objectives of this study, all further investigations and evaluations include only the intrapartum infected infants and their mothers compared to the exposed-uninfected infants and their mothers.

3.3.5 Maternal and infant characteristics as risk factors for intrapartum MTCT

Table XI highlights various maternal clinical and obstetric characteristics as potential risk factors for intrapartum HIV-1 transmission. No statistically significant relationships were observed between maternal age (p = 0.24), gestational age at labour (p = 0.7) or parity (p = 0.16) and the risk of intrapartum MTCT (Unpaired t test). Similarly no statistically significant association [p = 0.7, OR: 0.63, 95% CI: 0.10-3.30] was found between the mode of delivery and intrapartum MTCT. Preterm labour did not appear to increase the risk of intrapartum MTCT (p = 0.31, OR: 1.70, 95% CI: 0.70-3.70). Data was too sparse to ascertain statistical relationships between intrapartum MTCT and the premature membrane rupture of ≥ 4 hours

duration before labour (Table XI). Antenatal care as defined by the commencement of antenatal care and the number of antenatal visits did not differ between intrapartum HIV-1 transmitting and non-transmitting women. The first antenatal consultation for HIV-1 transmitting (75.0%) vs. non-transmitting (80.0%) women occurred during the second trimester of pregnancy (13-26 weeks gestation).

Table XI also highlights the clinical characteristics of study infants in relation to intrapartum MTCT. The median birth apgar scores of nine and ten were comparable between intrapartum HIV-1 infected and exposed-uninfected infants at one and five minutes respectively. A statistically significant association was observed between intrapartum HIV-1 transmission and infants who weighed less than 2500 grams at birth ($p = 0.04$, OR: 6.80, 95% CI: 1.30-35.10). In contrast, infant gender was not associated with the risk of acquiring intrapartum HIV-1 ($p = 0.13$, OR: 3.20, 95% CI: 0.80-12.20). The Fishers exact test was used to test all associations.

Table XI: Maternal and infant characteristics as risk factors for intrapartum MTCT

Characteristics	Intrapartum Transmitting women	Non-transmitting women	*p value*	OR	95% CI
Maternal Clinical and Obstetric: (n = 113)	(n = 6)	(n = 95)			
Mode of delivery:	n (%)	n (%)			
NVD (n = 55)	3 (5.5)	51 (92.7)	*0.7***	0.63	0.10-3.30
Elective C/S (n = 37)	3 (5.5)	32 (86.5)			
Emergency C/S (n = 18)	0 (0)	15 (83.3)	*n/a*		
Preterm labour < 37 weeks (n = 13)	1(7.7)	11 (84.6)	*0.31***	1.70	0.70-3.70
PROM (n = 73)	n (%)	n (%)	0.6**	0.40	0.02-7.60
≥ 4 hours (n = 13)	0 (0)	9 (69.2)			
< 4 hours (n = 45)	5 (11.1)	40 (88.9)			
Paediatric: (n = 110)	Intrapartum infected (n = 7)	Exposed-uninfected (n = 90)	*p value*	OR	95% CI
Apgar scores at 1 minute	9	9	*0.8**	-	-0.60 to 0.40
Apgar scores at 5 minute	10	10	*0.9**	-	-0.40 to 0.50
Infant birth weight:	n (%)	n (%)	*0.04***	6.80	1.30-35.10
• < 2500 grams	3 (21.4)	9 (75.0)			
• ≥ 2500 grams	4 (4.2)	81 (95.3)			
Infant gender:	n (%)	n (%)	*0.13***	3.20	0.80-12.20
• Male	5 (9.6)	47 (90.4)			
• Female	2 (3.4)	43 (96.0)			

**Unpaired t test, **Fisher's exact test, OR =odds ratio, CI: Confidence interval; PROM: Premature rupture of membranes*

Footnote:
Among the 120 women enrolled; in utero transmitters (n=6), lost to follow-up (n=3 and on ARV's (n=4) were excluded from analysis

3.4 Laboratory findings:

3.4.1 Pharmacological dynamics of NVP

All data analysis pertaining to this laboratory component has been summarized in Table XII.

Table XII: Summary of data analysis for NVP pharmacodynamics and intrapartum MTCT

RESEARCH QUESTIONS	WOMEN	WOMEN	INFANTS	ENDPOINTS	ILLUSTRATION
PHARMACOKINETICS	**BASELINE**	**PERIPARTUM**			
1. NVP levels in systemic and genital compartments prior to NVP exposure	Plasma: 120 CVL: 120			Baseline Plasma vs. CVL NVP level	Text
2. NVP levels in systemic and genital compartments after NVP administration		Plasma: 113 CVL: 72		Peripartum plasma vs CVL NVP level	Text
3. CVL NVP levels in relation to maternal sdNVP administration		CVL: 72		Maternal NVP level vs. time of sdNVP administration	Text Fig 28
4. NVP levels in infants in relation to their mother's NVP level		Plasma: 100	Plasma: 100	Mum plasma NVP level vs Infant NVP level	Fig 29
5. NVP levels in maternal systemic and genital compartments in relation to intrapartum MTCT		Plasma: 51 CVL: 39 *(active labour only)*		a) Mum Plasma NVP vs. Intrapartum MTCT (45 NTM + 6 TM) b) Maternal CVL NVP vs. Intrapartum MTCT (35 NTM+ 4 TM)	Table XIV
5. NVP levels in infants in relation to intrapartum MTCT			Plasma: 97	Infant plasma NVP vs Intrapartum MTCT (90 EU + 7 IP)	Table XV

Footnotes: TM: Transmitting mother; NTM: non-transmitting mother

IP: intrapartum infected infant; EU: exposed uninfected infant

3.4.1.1 Maternal pharmacokinetic analysis

One hundred and thirteen of the 120 women enrolled, delivered at the health facility. Among the 113 women, 107 (94.7%) women self-administered their sdNVP during labour. The infants of three women, who did not take NVP, received two doses of NVP (0.6 ml) within 72 hours of delivery as the standard of care during the study. Other than the standard of care sdNVP regimen (200 mg) used for the prevention of mother-to-child HIV-1 transmission, no other ARV's were administered to study participants. Most women did however receive a wide array of medication used routinely during labour; including anesthetics, analgesics, pitocin, antacids and antibiotics.

Actual times of NVP administration were documented for each study participant and her respective infant or infants. Using these data, individual time intervals were calculated for NVP dosing in relation to maternal delivery and actual sampling. The median time interval between maternal dosing and delivery was 16 hours (range 0.5-504 hours). Two (1.8%) of women took their NVP dose less than two hours before delivery. All 117 infants (103 singletons and seven twin pairs) received their single dose of NVP shortly after birth. Median time interval between infant birth and NVP dosing was three hours (range: 0.5-48 hours).

Due to logistical constraints, the time of sample collection varied during the peripartum period. Among the women who delivered, plasma and CVL samples were collected during latent labour 10 (9.0%) or active labour 51 (46.0%) or post-delivery 49 (46.0%). The median time interval between maternal NVP administration and pharmacokinetic sampling was 15 hours (range: 2-717). Of the infants born to these women, a blood sample was drawn within 72 hours of birth (Table XIII).

Table XIII: NVP drug administration and sample collection

	Time interval between:	Sample Size	Median (hours)	Range (hours)
Maternal n = 110	NVP intake and latent labour sampling	10	16.5	5-132
	NVP intake and active-labour sampling	51	14.5	0.5-20
	NVP intake and post-delivery sampling	49	113.5	10-717
Infant n = 117*	NVP intake and birth sampling	117*	16.5	2-72

Footnote: insufficient sample volume did not permit NVP drug analysis in four infants*

Pre-NVP dosing: (n = 120)

An analysis of 120 plasma and 120 CVL samples taken during pregnancy (28-38 weeks) revealed an absence of NVP. These baseline data suggests that none of the study participants were currently on any NNRTI drug regimen at study entry.

Post-NVP dosing:

Analysis of 113 maternal plasma and 72 CVL samples obtained during labour or post-delivery confirmed the absence of NVP among the three women who did not administer NVP. Among the remaining 110 women, NVP was detectable in 82 (74.5%) of plasma samples and in 40 of the 72 (55.6%) of CVL samples. The median NVP concentration in the 110 plasma and 72 CVL samples collected during labour or within 72 hours post-delivery and after sdNVP (200 mg) administration were 869 ng/ml, (range: 0-3820) and 2.1 ng/ml (range: 0-99.7) respectively. This difference in NVP levels between the two compartments was statistically significant ($p < 0.0001$) [unpaired t test] and the ratio of CVL NVP: Plasma NVP

concentration was 0.002. NVP drug levels were below the 100 ng/ml therapeutic target in 39 (34.5%) of maternal plasma samples and in all 72 (100%) of CVL samples. To further assess the level of NVP absorption into the genital compartment, the NVP concentration in CVL were correlated with the time interval between NVP administration and specimen collection. The Pearson correlation coefficient revealed a negative correlation that was statistically significant (Fig 28).

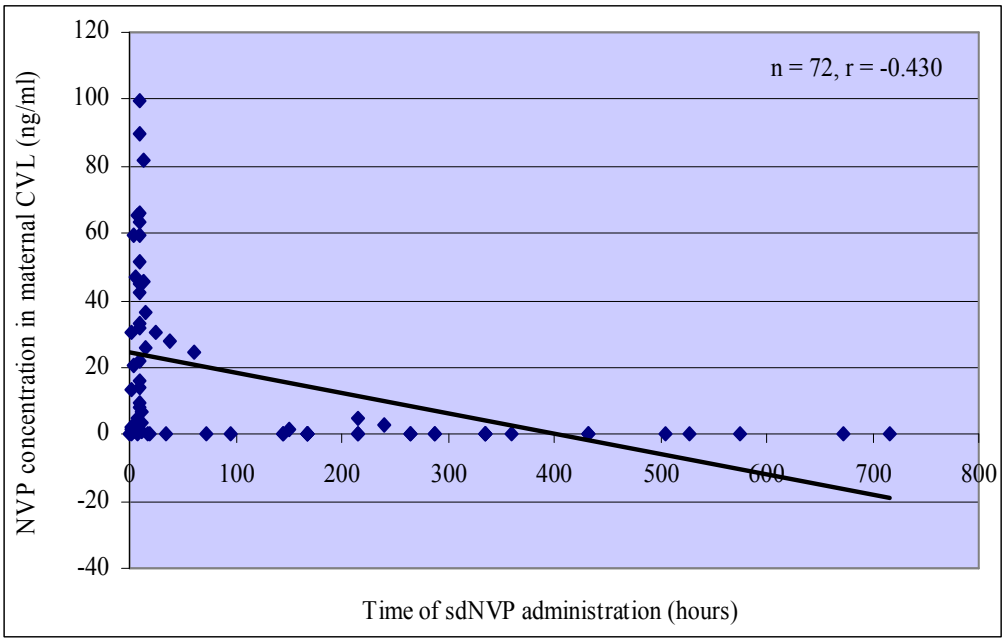


Fig 28: Association between CVL NVP concentration and sdNVP administration [n = 72, r = -0.43, $p < 0.001$]

NVP at active labour and intrapartum MTCT

Due to logistical constraints, the time of sample collection varied during the peripartum period. The samples obtained during active labour and within 20 hours of NVP dosing were best representative of the intrapartum phase. Hence these data (Table XIV) were selectively analysed to determine maternal pharmacokinetics in the systemic and genital compartments

when investigated in relation to intrapartum MTCT. Median NVP levels of 1070 ng/ml (range: 0-3080) and 24.5 ng/ml (range: 0-99.7) were found in the systemic and genital compartments respectively. Systemic NVP levels were 44 times more than genital NVP levels. This difference between compartments was statistically significant ($p < 0.0001$) [Unpaired t test]. The ratio of CVL NVP was 0.02. NVP levels were below the 100 ng/ml therapeutic target in seven (13.7%) of the 51 plasma and in all 39 maternal CVL samples collected at active labour.

Stratification according to transmission indicated that the median time interval between NVP intake and delivery was 12.5 hours (range: 3-48) among intrapartum HIV-1 transmitting and 13 hours (range: 0.5-50) among non-transmitting women ($p = 0.1$) [Mann-Whitney U test]. The median time interval between NVP self-administration and maternal pharmacokinetic sampling was 11 hours among intrapartum transmitting (range: 3-20) and 10 hours among non-transmitting women (range: 3-20 hours) respectively.

The median plasma NVP concentration among intrapartum HIV-1 transmitting and non-transmitting were 886 ng/ml (range: 112-1860) and 1060 ng/ml (range: 0-3080) respectively and not statistically significant ($p = 0.4$). The median CVL NVP concentration among intrapartum transmitting and non-transmitting women were 2.24 ng/ml (range: 0-15.8) and 25.7 (0-99.7) respectively and statistically significant ($p = 0.02$) [Table XIV].

NVP dosing at 2-48 hours revealed median plasma NVP levels of 886 ng/ml ($n = 6$) and 1023 ng/ml ($n = 31$) among intrapartum and non-transmitting women respectively. CVL samples showed median NVP levels of 2.24 ng/ml ($n = 4$) and 20.4 ng/ml ($n = 28$) in intrapartum transmitting and non-transmitting women dosed at 2-48 hours.

Table XIV: Systemic and genital NVP in association with intrapartum MTCT (active labour)

ACTIVE LABOUR (0.5-20 hours)				
	Maternal plasma (n = 51)		Maternal CVL (n = 39)	
	HIV-1 transmitting[#]	Non-transmitting	HIV-1 transmitting[#]	Non-transmitting
Sample size	6	45	4*	35
Median NVP (ng/ml) [range]	886 [112-1860]	1060 [0-3080]	2.24 [0-15.8]	25.7 [0-99.7]
*p value***	*0.4*		*0.02*	
NVP therapeutic target:	n (%)			
< 100 ng/ml	0	7 (15.6%)	4 (100%)	35 (100%)
≥ 100 ng/ml	6 (100%)	38 (84.4%)	0	0

Footnotes:

[#]*All in utero transmitters (n=6) excluded from analysis*
**Missing CVL (n=2)*
*** Unpaired t test*
< 100 ng/ml includes samples with undetectable levels of NVP

3.4.1.2 Infant pharmacokinetic analysis after sdNVP

Among the 117 infant born to 110 women, 114 (97.4%) of the infants received the standard of care sdNVP regimen (0.6 ml) within 72 hours of birth. The three infants, whose mothers did not receive NVP in labour, received the two dose regimen according to the standard of care at the time of this study. Ninety seven (85.0%) of this infant cohort received their NVP dose within six hours of birth. Blood samples were drawn from all 117 infants after NVP dosing. The median time interval between birth and NVP dosing was three hours (range: 0-48 hours). Of the 117 samples collected, samples from 113 infants were suitable and adequate for LC-MS-MS analysis. NVP was detectable in 89 (78.8%) of the infant plasma samples collected at a median time interval of 16.5 hours (range: 2-72) after dosing. Eighty (71.0%) of the infants achieved NVP drug levels above the 100 ng/ml therapeutic target, while 33 (29.2%) fell below this target. The median NVP concentration of 828 ng/ml [range: 0-3730] was eight times

higher than the therapeutic target and almost 83 times the *in vitro* inhibitory concentration (10 ng/ml) against wild-type HIV-1.

The Pearson correlation coefficient was used to determine the relationship between infant and maternal plasma NVP levels. A statistically significant association was observed between the NVP concentration in the plasma of 100 maternal-infant pairs [r = 0.36, 95% CI: 0.18-0.52, p = 0.0002] (Pearson's correlation coefficient) [Fig 29].

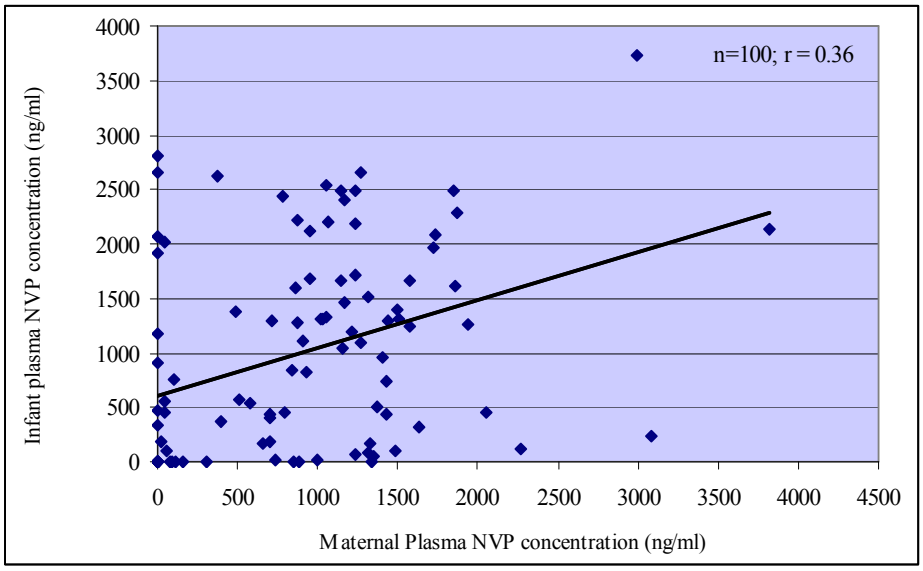


Fig 29: Association between plasma NVP levels in 100 maternal-infant pairs after sdNVP [r = 0.36, 95% CI: 0.18-0.52, p = 0.0002]

The median time interval between infant birth and NVP dosing was 2.5 hours (range: 2-28) among intrapartum HIV-1 infected infants and three hours (range: 0.5-48) among exposed-uninfected infants (p = 0.4) [unpaired t test]. NVP was detectable in four (57.1%) of intrapartum HIV-infected infants and 78 (78.8%) of exposed-uninfected infants. Table XV highlights infant pharmacological dynamics following sdNVP within 2-72 hours of birth. The

median NVP level among the intrapartum infected infants [20.3 ng/ml; range: 0-2380] was lower than the exposed-uninfected infants [933 ng/ml; range: 0-3730] but not statistically significant (p = 0.16) [Mann-Whitney U test].

Table XV: Infant pharmacological dynamics following sdNVP within 2-72 hours of birth

	Sample Size	Median (ng/ml)	Range (ng/ml)	Therapeutic target	
				< 100 ng/ml	≥ 100 ng/ml
Exposed-uninfected	90	933	(0-3730)	23 (23.7 %)	67 (70.1%)
Intrapartum-infected	7	20.3	(0-2380)	4 (4.1%)	3 (3.1%)

Footnotes:

In utero infected infants excluded from analysis (n=6)

< 100 ng/ml also includes infant samples with undetectable NVP levels

A greater proportion of exposed-uninfected infants (70.1%) had NVP drug levels above the therapeutic target, compared to those infected via the intrapartum route of transmission (3.1%) (Table XV). This observation was not statistically significant (p = 0.1; RR: 0.45; 95% CI: 0.22-0.93) [Fishers exact test].

3.4.2 Virological dynamics and intrapartum MTCT

A summary of data analysis for this study component is shown in Table XVI.

Table XVI: Summary of data analysis for virological dynamics and intrapartum MTCT

RESEARCH QUESTIONS VIROLOGICAL	WOMEN BASELINE	WOMEN PERIPARTUM	ENDPOINTS	ILLUSTRATION
1. Viral load in systemic and genital compartments prior to NVP exposure	Plasma: 120 CVL: 120	n/a	a) Baseline Plasma HIV-1 RNA vs CVL HIV-1 RNA	Table XVIIa Fig 32a
2. Viral load in systemic and genital compartments after NVP exposure	Plasma: 113 CVL: 72	Active / Post-delivery / All Plasma: 51 / 49 / 113 CVL: 39 / 20 / 72	a) Baseline Plasma HIV-1 RNA vs Peripartum HIV-1 RNA b) Baseline CVL HIV-1 RNA vs Peripartum HIV-1 RNA	Table XVIIa Fig 33a
3. Effect of NVP on viral load in systemic vs. genital compartments	Plasma:120 CVL:120	Plasma: 72 CVL: 72	a) Peripartum Plasma HIV-1 RNA vs CVL HIV-1 RNA	Table XVIIb
4 (i). Effect of NVP on viral load in the genital compartment in the absence of blood contamination in specimens	CVL:50	CVL: 19	a) Peripartum CVL HIV-1 RNA in samples with blood Contamination vs. without blood Contamination prior to NVP Exposure b) Peripartum CVL HIV-1 RNA in samples with blood contamination vs. without blood contamination after NVP Exposure	Fig 32b Fig 33b
4 (ii) Effect of NVP on viral load in systemic and genital compartments in the absence of STI's	Plasma:43 CVL: 28	Plasma: 70 CVL: 44	a) Peripartum Plasma HIV-1 RNA in women with STI vs. without STI b) Peripartum CVL HIV-1 RNA in women with STI vs. without STI	Fig 30a and Fig 30b Fig 31a and Fig 30b
5. Viral load in systemic and genital compartments prior to NVP exposure in relation to intrapartum MTCT	Plasma: 101 CVL: 101		a) Baseline Plasma HIV-1 RNA vs. Intrapartum MTCT (95 NTM + 6 IP TM) b) Baseline CVL HIV-1 RNA vs. Intrapartum MTCT (62 NTM + 4 IP TM)	Table XVIII
6. Viral load in systemic and genital compartments after NVP exposure in relation to intrapartum MTCT		Plasma: 101 CVL: 66	a) Peripartum Plasma HIV-1 RNA vs Intrapartum MTCT (95 NTM + 6 IP TM) b) Peripartum CVL HIV-1 RNA vs. Intrapartum MTCT (62 NTM + 4 IP TM)	Table XVIII
7. Change in viral load in systemic and genital compartments in relation to intrapartum MTCT	Plasma: 101 CVL: 66	Plasma: 101 CVL: 66	Viral Load changes: (Increase/Decrease/Unchanged) a) Plasma viral load change vs. Intrapartum MTCT (95 NTM + 6 IP TM) b) CVL Viral Load Change vs. Intrapartum MTCT (62 NTM + 4 IP TM)	Table XX

Footnotes: NTM: non-transmitting mother, IP TM: intrapartum transmitting mother

Table XVI continued…				
RESEARCH QUESTIONS	**WOMEN**	**WOMEN**	**ENDPOINTS**	**ILLUSTRATION**
VIROLOGICAL	**BASELINE**	**PERIPARTUM**		
8. Viral load in systemic and genital compartments prior to NVP exposure and classified according to threshold for MTCT in relation to intrapartum transmission	Plasma: 101 CVL: 66		a) Plasma Viral Load [< 1000] or [> 1000] copies/ml vs. Intrapartum MTCT b) CVL Viral Load [< 1000] or [> 1000] copies vs. Intrapartum MTCT	Table XIX
9. Viral load in systemic and genital compartments after NVP exposure and classified according to threshold for MTCT in relation to intrapartum MTCT		Plasma: 101 CVL: 66	a) Plasma Viral Load [< 1000] or [> 1000] copies vs. Intrapartum Transmission b) CVL Viral Load [< 1000] or [> 1000] copies vs. Intrapartum MTCT	Table XIX
10. STI's and viral load in relation to intrapartum transmission	Plasma: 42 CVL: 42		a) Plasma Viral Load [+ STI's] vs. Intrapartum Transmission (4 TM + 38 NTM) b) CVL Viral Load [+ STI's] vs. Intrapartum Transmission (4 TM + 38 NTM]	Table XXI
11. Ancillary viral investigations: (a) Describing the presence of NVP drug resistance mutations among intrapartum transmitting women (b) Sub typing of virus		Plasma: 6	a) Unique characteristics for the presence of NVP drug resistance mutations in IP transmitters b) Clade sequencing	Table XXI Fig 34

Footnotes: NTM: non-transmitting mother, IP TM: intrapartum transmitting mother

3.4.2.1 Maternal HIV-1 RNA (viral load): Pre vs. post NVP dosing

Of the 120 patients enrolled, HIV-1 RNA was detectable in 120 (100%) of maternal plasma and in 94 (78.3%) of whole unfractionated cervico-vaginal lavage (CVL) samples collected from women during pregnancy prior to NVP dosing. Post-NVP dose sampling of 113 women indicated that HIV-1 RNA was detectable in 106 (93.8%) of all maternal plasma and in 47 (65.3%) of the 72 unfractionated CVL samples collected after dosing.

3.4.2.2. Blood contamination of CVL: Pre vs. post NVP dosing

The presence of HIV-1 as a result of blood contamination was estimated by the use of commercially available urine dipsticks (Uricheck, RapiMED Diagnostics, SA) and visual estimation of haemoglobin concentration as per previously described method (Hart *et al*, 1999). Of the samples assayed before sdNVP, 70 (58.3%) of the maternal CVL samples had detectable blood contamination. All (100%) had quantifiable plasma HIV-1 RNA levels.

Every effort was made to avoid blood contamination during CVL sampling at the time of labour/post-delivery while also trying to collect samples that were closely representative of true intrapartum dynamics. Minimal blood contamination was detectable in 53 (73.6%) of the 72 maternal CVL samples assayed after NVP dosing. Almost 34 (47.0%) of all assayed CVL samples had quantifiable plasma virus loads.

*Footnote: *HIV-1 RNA was measured in all 72 CVL samples collected during labour/delivery.*

3.4.2.3 HIV-1 RNA in Plasma and CVL: Pre-NVP vs. Post NVP dose

Pre and post-NVP maternal plasma samples were available for 113 women (110 deliveries and three IUD's). Pre and post-NVP CVL samples were available for 72 women.

In the presence of maternal STI's (n = 43), a strong and statistically significant correlation in plasma HIV-1 RNA levels [r = 0.70, CI: 0.53-0.86, $p < 0.0001$] was observed between pre and post NVP dosing (Pearson's correlation coefficient) [Fig 30a]. In the absence of maternal STI's (n = 70), a strong [r = 0.70, 95% CI: 0.56-0.80] and statistically significant correlation ($p < 0.0001$) was found between plasma HIV-1 RNA levels at pre and post-NVP dosing [Fig 30b].

Similarly [Fig 31a] shows the strong and statistically significant correlation (r = 0.61, 95% CI: 0.32-0.80, $p < 0.001$) that was observed when comparing CVL HIV-1 RNA in pre and post NVP dose samples (n = 28) in the presence of maternal STI's. In the absence of maternal STI's, [Fig 31b] pre and post NVP sampling of the cervicovaginal compartment (n = 44) also revealed a strong and statistically significant CVL HIV-1 RNA correlation [r = 0.60, 95% CI: 0.42-0.80, $p < 0.0001$].

In the presence of maternal STI's

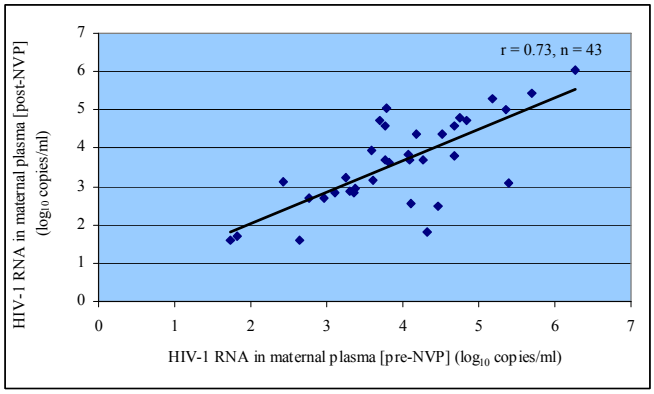


Fig 30a: Scatter-plot showing an association between levels of HIV-1 RNA (viral loads) in paired maternal plasma samples (n = 43) collected at pre and post-NVP dosing includes women with STI's [r = 0.70; 95% CI: 0.53-0.86, p < 0.0001]

In the absence of maternal STI's

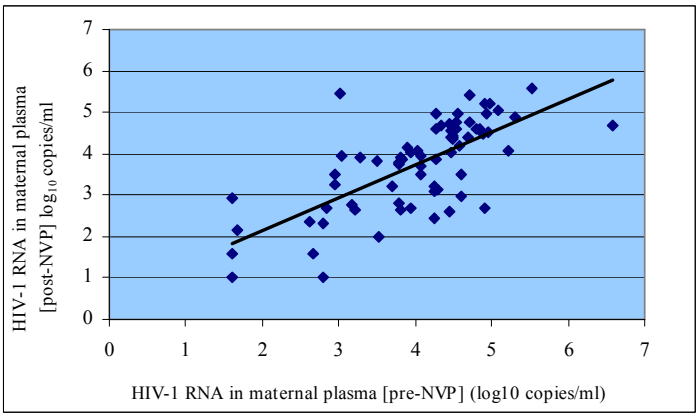


Fig 30b: Association between levels of HIV-1 RNA (viral loads) in paired maternal plasma samples (n = 70) collected pre and post-NVP dosing in the absence of maternal STI's [r = 0.70, 95% CI: 0.56-0.80, p < 0.0001]

In the presence of maternal STI's

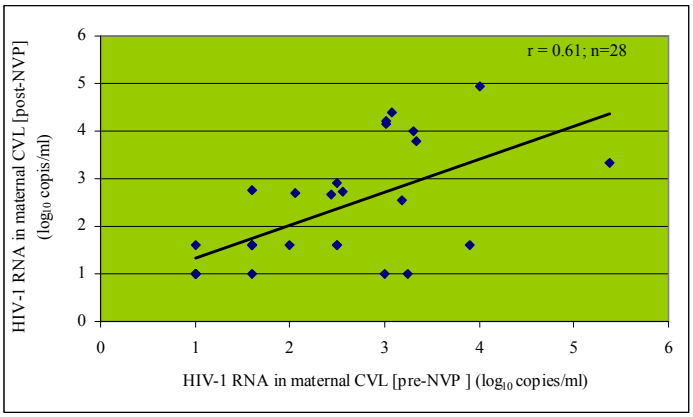


Fig 31a: Association between levels of HIV-1 RNA (viral loads) in paired maternal CVL samples (n = 28) collected pre and post-NVP dosing includes women with STI's [r = 0.61, 95% CI: 0.32-0.80, p < 0.001] '

In the absence of maternal STI's

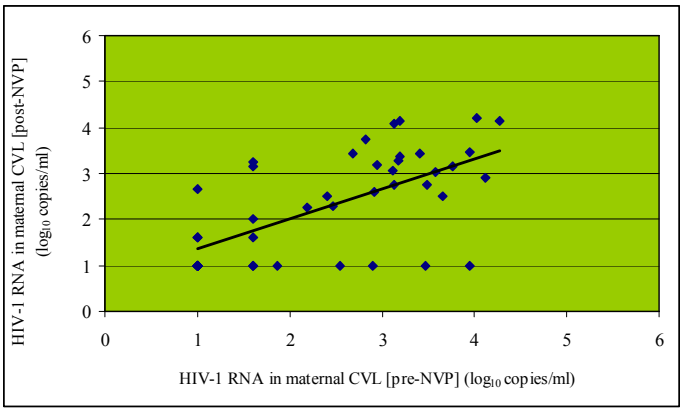


Fig 31b: Association between levels of HIV-1 RNA (viral loads) in paired maternal CVL samples (n = 44) collected pre and post-NVP dosing (in the absence of maternal STI's) [r = 0.60, 95% CI: 0.42-0.80, p < 0.0001]

Table XVIIa highlights a general trend of higher levels of HIV-1 RNA in maternal plasma when compared to CVL, irrespective of when sampling occurred. While a reduction in HIV-1 RNA levels did occur after intrapartum NVP dosing, more than 80.0% of the women in this cohort experienced no change (Table XVIIb) to their HIV-1 RNA levels in both plasma and CVL samples during active labour. These findings are further supported by the strong correlation observed when comparing pre and post-NVP HIV-1 RNA levels in both maternal systemic [$r = 0.81$, $p < 0.0001$] and genital compartments [$r = 0.80$, $p < 0.0001$] during active labour. This correlation remained strong and statistically significant in both plasma [$r = 0.75$, $p = 0.001$] and CVL [$r = 0.65$, $p < 0.0001$], independent of whether the samples were collected during latent labour, active labour or post-delivery [Table XVIIb].

Table XVIIa: Effect of NVP on HIV-1 RNA in maternal systemic and genital compartments

	n = 120		n = 113					
Maternal Sampling	Pregnancy (Pre-NVP)		Active labour (Post-NVP)		Post-delivery (Post-NVP)		ALL* (Post-NVP)	
Sample type	Plasma	CVL	Plasma	CVL	Plasma	CVL	Plasma	CVL
Sample size	120	120	51	39	49	20	113*	72*
Median HIV-1 RNA (log_{10} copies/ml)	4.1	2.8	3.9	2.36	3.7	1.9	3.9	2.1

Footnotes:
**ALL includes women sampled during the latent phase of labour (n=10) and women with IUD's (n=3)*

Table XVIIb: Overall effect of NVP on HIV-1 RNA after single-dosing

	Active labour		Post-delivery		ALL post-NVP sampling	
HIV-1 RNA changes	Plasma n = 51	CVL n = 39	Plasma n = 49	CVL n = 20	Plasma n = 113	CVL n = 72
Decreased	4 (7.8%)	2 (5.1%)	1 (2.0%)	4 (20.0%)	9 (8.0%)	7 (9.7%)
Increased	3 (5.9%)	4 (10.3%)	15 (30.6%)	4 (20%)	19 (16.8%)	12 (16.7%)
No change	44 (86.3%)	33 (84.6%)	33 (67.4%)	12 (60.0%)	85 (75.2%)	53 (73.6%)
Correlation** with pre-NVP HIV-1 RNA	r = 0.81	r = 0.80	r = 0.69	r = 0.57	r = 0.75	r = 0.65
95% CI	0.71-0.88	0.65-0.89	0.51-0.82	0.23-0.76	0.67-0.83	0.49-0.77
p value	*< 0.0001*	*< 0.0001*	*< 0.0001*	*0.0008*	*0.001*	*< 0.0001*

***Pearson's correlation coefficient*

**ALL includes women sampled during the latent phase of labour (n=10) and women with IUD's (n=3)*

Pre-NVP dose evaluation (Fig 32a) of maternal plasma-CVL pairs (n = 120) demonstrated a moderate and statistically significant correlation between the two variables [r = 0.58; p < 0.001]. This finding suggests that HIV-1 RNA in CVL was significantly influenced by HIV-1 RNA in plasma.

Post-NVP evaluation (Fig 33a) of maternal plasma-CVL pairs (n = 72) continued to suggest a similar significant association between systemic and genital compartments (p < 0.0001), despite a moderate correlation (r = 0.45) shown with Pearson's correlation coefficient.

Even after controlling for the presence of blood contamination, the dependence of CVL HIV-1 RNA on plasma HIV-1 RNA was still evident and statistically significant at both pre (Fig 32b) [p = 0.001] and post-NVP dosing (Fig 33b) [p = 0.03].

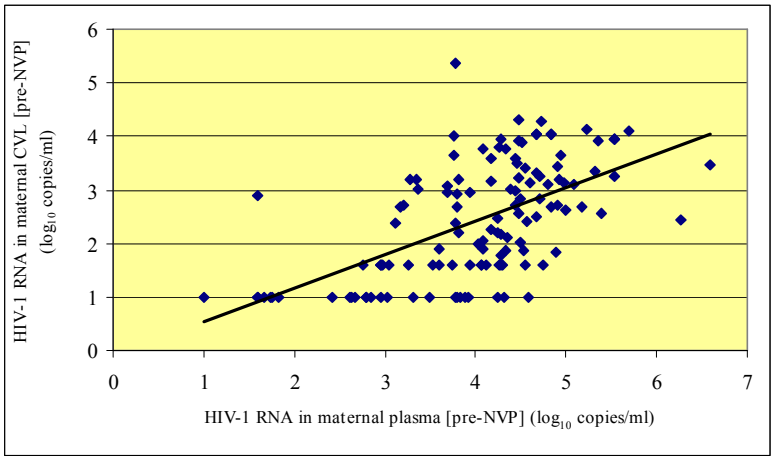


Fig 32a: Association between levels of HIV-1 RNA (viral loads) in paired maternal plasma and cervicovaginal lavage (CVL) samples collected pre-NVP dosing (n = 120). [r = 0.58, 95% CI: 0.45-0.69, p < 0.001]

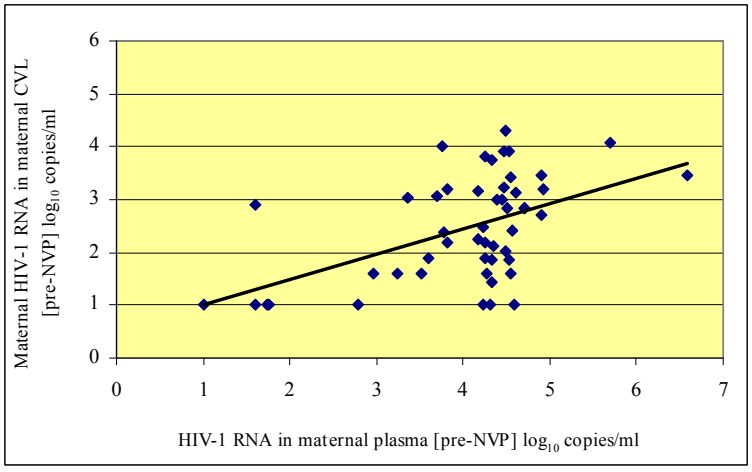


Fig 32b: Association between levels of HIV-1 RNA (viral loads) in paired maternal plasma and cervicovaginal lavage (CVL) samples collected pre-NVP dosing (n = 50) excluding samples with blood contamination. [r = 0.51, 95% CI: 0.27-0.65, p = 0.001]

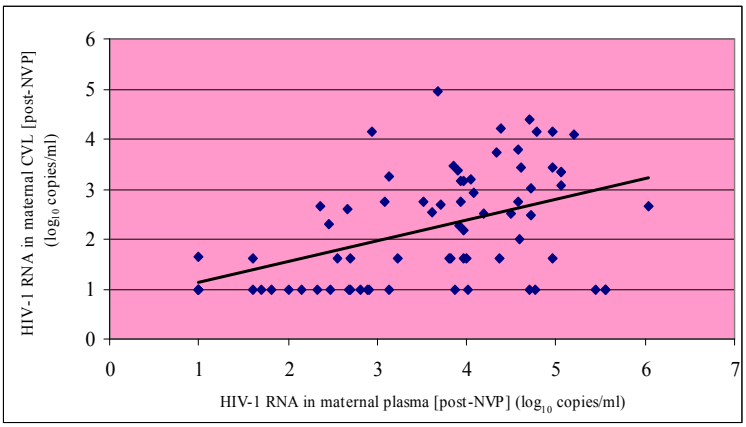


Fig 33a: Association between levels of HIV-1 RNA (viral loads) in paired maternal plasma and cervicovaginal lavage (CVL) samples collected post-NVP dosing (n = 72). [r = 0.45, 95% CI: 0.25-0.62, p < 0.0001]

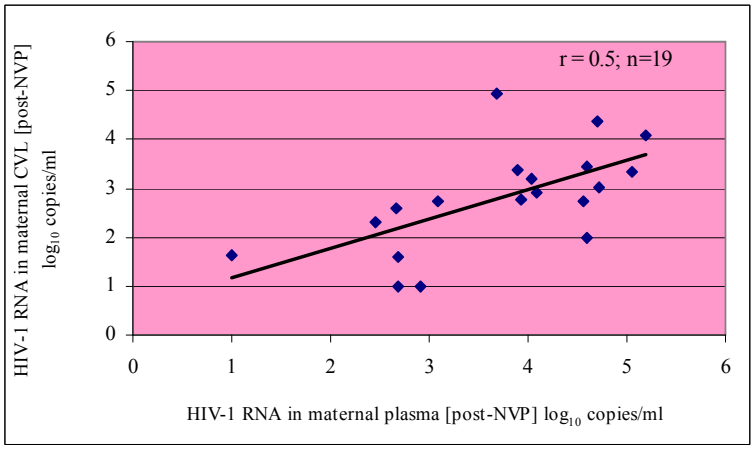


Fig 33b: Association between levels of HIV-1 RNA (viral loads) in paired maternal plasma and cervicovaginal lavage (CVL) samples collected post-NVP dosing (n = 19) excluding samples with blood contamination [r = 0.50, 95% CI: 0.04-0.77, p = 0.03]

Table XVIII compares HIV-1 RNA levels in plasma and CVL between HIV-1 transmitting (intrapartum only) and non-transmitting women at pre and post-NVP dose sampling. Firstly, an overall comparison of HIV-1 RNA levels between compartments revealed that median HIV-1 RNA levels in plasma remained consistently higher than CVL HIV-1 RNA at both pre and post-NVP dosing. This finding was statistically significant in both

intrapartum HIV-1 transmitting and non-transmitting women (Table XVII). Secondly, compared to non-transmitters, plasma HIV-1 RNA was higher in intrapartum HIV-1 transmitters at both pre [4.35; range: 3.84-6.27 $\log_{10}$ copies/ml] and post [3.86; range: 2.61-6.03 $\log_{10}$ copies/ml] NVP dosing. In contrast to plasma, the median CVL viral load was comparable between intrapartum HIV-1 transmitting and non transmitting women at both pre and post-NVP dosing (Table XVII. The association of viral load and intrapartum HIV-1 transmission was statistically significant for maternal plasma ($p = 0.02$) and not CVL ($p = 0.7$) [unpaired t test].

HIV-1 RNA classification according to the viral load threshold for the risk of intrapartum MTCT is shown in Table XIX. Women with HIV-1 RNA levels above 1000 copies/ml demonstrated an increased risk for intrapartum HIV-1 transmission. This finding was statistically significant for maternal plasma ($p = 0.02$, OR: 0.10, 95% CI: 0.01-0.70) and not CVL ($p = 0.9$, OR: 1.23, 95% CI: 0.12-12.60) [Fisher's exact test].

An overall 0.33 $\log_{10}$ viral load reduction in plasma (Table XX) following NVP dosing was not significant ($p = 0.4$) among HIV-1 transmitting women. In contrast a 0.45 $\log_{10}$ viral load reduction in the plasma of non-transmitting women was statistically significant ($p < 0.005$).

Table XVIII: Comparison of HIV-1 RNA in systemic and genital compartments of HIV-1 transmitting and non-transmitting women (n = 110)

	Intrapartum transmitting women (n = 6)		Non-transmitting women (n = 95)	
Pre-NVP dosing [1] (during pregnancy)	Plasma	CVL	Plasma	CVL
Sample size	6	6	95	95
Median HIV-1 RNA [log_{10} copies/ml]	4.35	2.71	4.15	2.72
Range	3.84-6.27	1-3.95	1.6-6.58	1.6-4.3
**p value*	*0.01*		*< 0.0001*	
Post-NVP dosing [2] (during active labour or delivery)	Plasma	CVL	Plasma	CVL
Sample size	4	4	62	62
Median HIV-1 RNA [log_{10} copies/ml]	3.86	1.83	3.73	2.17
Range	2.61-6.03	1.0-3.47	1.5-5.56	1-4.95
**p value*	*0.02*		*< 0.0001*	

Footnotes:

Among the 120 women enrolled; those with unknown transmission status (n=3), IUD's (n=3), in utero transmitters (n=6), on ARV's (n=4) or lost to follow-up (n=3) were excluded from analysis.

[1] Viral load in systemic (Plasma) and genital (CVL) compartments PRIOR to NVP exposure in relation to intrapartum MTCT

[2] Viral load in systemic (Plasma) and genital (CVL) compartments AFTER NVP exposure in relation to intrapartum MTCT

[*]Unpaired t test

Table XIX: Categorical classification of HIV-1 RNA in the systemic and genital compartments of HIV-1 transmitting and non-transmitting women after sdNVP

	Systemic compartment Plasma (n = 110)		Genital compartment CVL (n = 72)	
HIV-1 RNA copies/ml	Intrapartum transmitting N = 6	Non-transmitting n = 95	Intrapartum transmitting n = 4 #	Non-transmitting n = 62 #
	% (n)	% (n)	% (n)	% (n)
< 1000 *	1 (16.7)	68 (69.4)	3 (75)	44 (71)
≥ 1000 *	5 (83.3)	30 (30.6)	1 (25)	18 (29)
p value **	*0.02*		*0.9*	
OR	0.10		1.23	
95% CI	0.01-0.70		0.12-12.60	

Footnotes:

Among the 120 women enrolled; the following were excluded from analysis, unknown transmission status (n=3), IUD's (n=3), in utero transmitters (n=6), on ARV's (n=4) or lost to follow-up (n=3)

* *Viral load threshold of 1000 copies/ml (Garcia et al, 1999)*

** *Fisher's exact test*

\# *CVL not collected*

Table XX: HIV-1 RNA changes in the systemic and genital compartments of HIV-1 transmitting and non-transmitting women (n = 110)

		Intrapartum transmitting women (n = 6)		Non-transmitting women (n = 95)	
HIV-1 RNA (Viral load)	Maternal Sample	n (%)	Change [median] in viral load in log_{10} copies/ml after sdNVP (range)	n (%)	Change [median] in viral load in log_{10} copies/ml after sdNVP (range)
Decreased	Plasma	4 (66.7)	0.33 (0.24-1.83)	65 (68.4)	0.45 (0.02-4.2)
	CVL #	2 (50.0)	1.72 (0.48-2.95)	24 (38.7)	0.6 (0.06-2.95)
Increased	Plasma	2 (33.3)	0.03 (0.01-1.2)	30 (31.6)	0.28 (0.01-2.42)
	CVL #	1 (25.0)	0.23 (0-0.6)	20 (32.3)	1.6 (0.01-1.66)
No change	CVL #	1 (25.0)	n/a	18 (29.0)	n/a

Footnotes: Among the 120 women enrolled; the following were excluded from analysis, unknown transmission status (n=3), IUD's (n=3), in utero transmitters (n=6), on ARV's (n=4) or lost to follow-up (n=3)

3.4.2.4 Maternal STI's in relation to viral dynamics in systemic and genital compartments as risk factors for intrapartum MTCT

Maternal STI's were detected in four (66.6%) intrapartum transmitting women and in 38 (38.8%) of non-transmitting women. No significant association was observed between the presence of maternal STI's and the risk for intrapartum MTCT (p = 0.2, RR: 2.90, 95% CI: 0.60-15.40). The presence of maternal STI's was associated with higher median viral loads in both systemic and genital compartments of all women, independent of intrapartum HIV-1 transmission [Table XXI].

Table XXI: Influence of maternal STI's on viral loads in systemic and genital compartments

	STI present				STI absent			
Median HIV-1 RNA (log_{10} copies/ml)	Intrapartum transmitting n = 4		Non-transmitting n = 38		Intrapartum transmitting n = 2		Non-transmitting n = 60	
	Pre-NVP	Post-NVP	Pre-NVP	Post-NVP	Pre-NVP	Post-NVP	Pre-NVP	Post-NVP
Plasma	5.17	4.9	3.8	3.2	4.1	3.9	4.2	3.9
CVL	4.5	2.7	2.4	1.6	2.0	2.2	2.4	2.5

Footnotes:

$p \geq 0.2$ for all statistical comparisons

Analysis excludes in utero transmitting women

3.4.2.5 Ancillary viral investigations: Describing the presence of NVP drug resistance mutations among intrapartum transmitting women (n = 6)

Maternal plasma samples from the six intrapartum transmitting mothers were evaluated for the presence of NVP drug resistance mutations. The median age and gestational age among these women was 30 (27-38) years and 40 (33-40) weeks respectively. Median CD4 cell counts and median plasma viral loads were 279 (range: 76-957) cells/μl and 3.9 (range: 2.6-6.0) log_{10} (copies/ml) respectively.

Drug resistance mutations (NVP^R) to NNRTI's were detected in one of the six intrapartum transmitting women while five women lacked detectable NVP^R. Clinical, viral and immune characteristics of women in this cohort are summarized in Table XXII. Two NNRTI drug resistance mutations; Y181CY and Y188CY were identified in this mother (Appendix 8b). This patient transmitted HIV-1 to her second-born twin via the intrapartum route, while her first-born twin remained exposed-uninfected. Compared to women without NVP^R, the patient had a lower baseline CD4 cell count and higher viral loads in both systemic and genital compartments. This mother delivered twin male infants by an elective C/S within three hours of her sdNVP intake. NVP^R was detected in a plasma sample obtained seven days post-delivery after sdNVP exposure.

Quality control of all sequence data by phylogenetic tree construction (Fig 34) confirmed the absence of contamination. A sequence corresponding to protease amino acids 1-99 and reverse transcriptase amino acids 1-312 was obtained for all samples assayed. Drug resistance reports (Appendix 8a, 8b) were generated by uploading all sequence data onto the Stanford University

drug resistance database. Analysis of patient sequences confirmed a clade C viral subtype HIV infection among all of the intrapartum HIV-1 transmitting women that were assayed.

Table XXII: Characteristics of the maternal NVP drug resistance cohort (n = 6)

Intrapartum transmitting women (n = 6)	NVP^R present (n = 1)	NVP^R absent (n = 5)
Maternal CD4 cell count (cells/ul)	223	336 (76-957)
Plasma HIV-1 RNA (log_{10} copies/ml)	5.56	3.86 (2.6-6.0)
CVL HIV-1 RNA (log_{10} copies/ml)	3.95	3.1 (2.7-3.5)
Plasma NVP level (ng/ml)	0	886 (112-1860)
Interval between sdNVP and delivery (hours)	3	47 (16-132)
Mode of delivery (n = 6)		
Elective C/S (n = 3)	1 (33.3%)	2 (66.7%)
NVD (n = 3)	0	3 (100%)
Gestational age at delivery (weeks)	40	40 (39-43)
Viral subtype in mum	Clade C	Clade C
Maternal CCL3-L1 gene copy (p.d.g)	2	5 (4-7)
Intrapartum infected infants (n = 7)*		
Infant birth weight (kg)	2.2	3.3 (1.8-3.8)
Infant gender		
Male (n = 4)	1 (25.0%)	3 (75.0%)
Female (n = 3)	0	3 (100%)
Infant CCL3-L1 gene copy (p.d.g)	4	4 (4-7)

*Footnotes: * includes twins*

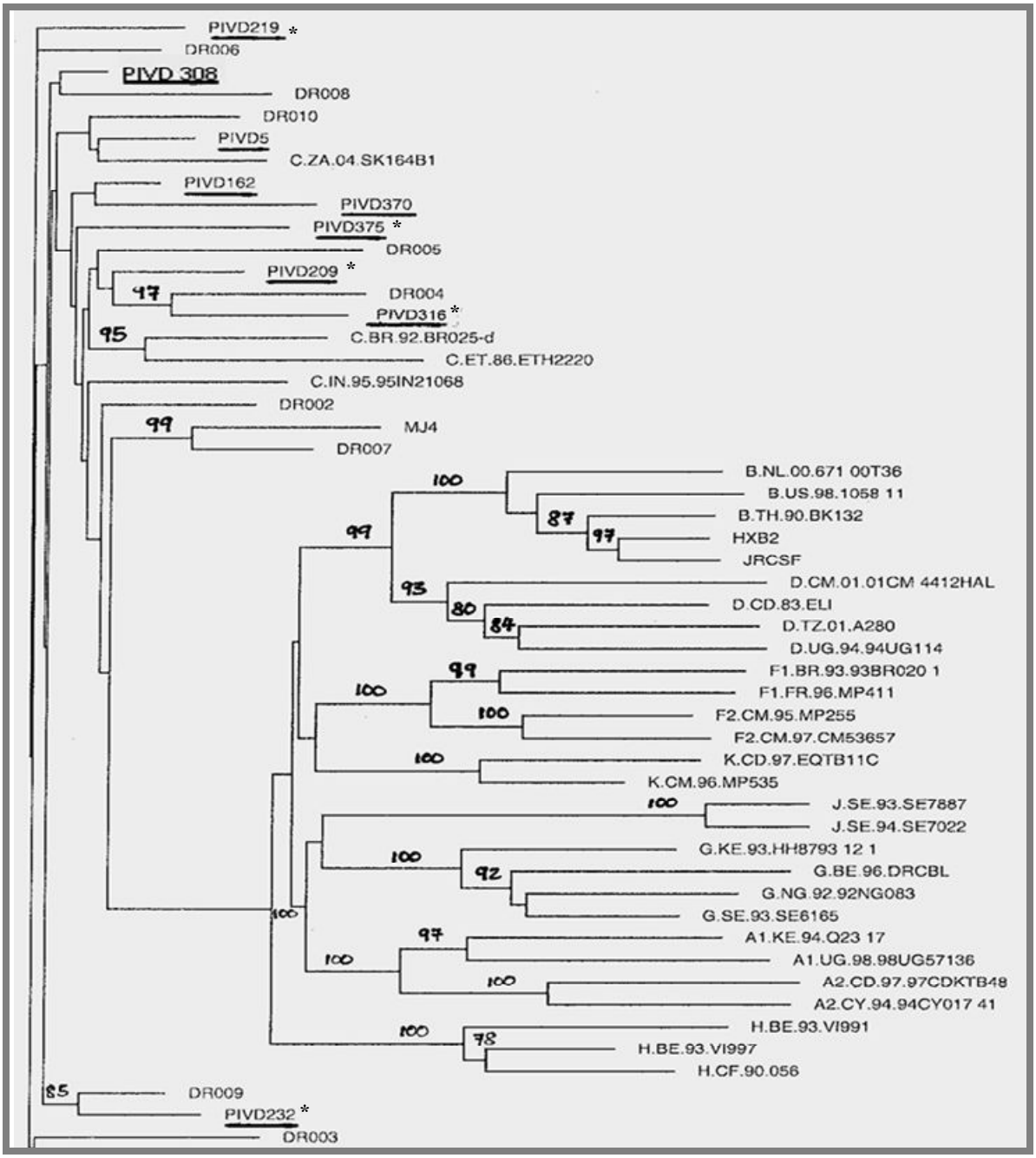


Fig 34: Phylogenetic analysis of the PIVD drug resistance subset. (*) denotes all intrapartum transmitting women (n = 6). Reference sequences were obtained from the Los Alamos database and can be accessed at (http://www.hiv.lanl.gov). Sequences were aligned using Clustal X software and corrected manually. Phylogeny construction and evaluation were performed by the neighbour-joining method (100 bootstrap replicates).

3.4.3 Immunological dynamics and intrapartum MTCT

Data analysis for this study component has been summarized in Table XXIII.

Table XXIII: Summary of data analysis for immunological dynamics and intrapartum MTCT

RESEARCH QUESTIONS	WOMEN BASELINE	WOMEN PERIPARTUM	ENDPOINTS	ILLUSTRATION
1. Immunological staging in all HIV positive participants	120		a) CD4 < 200 cells/µl b) CD4 200-350 cells/µl c) CD4 > 350 cells/µl	Table VIII
2. Immunological staging in association with HIV viral load in systemic and genital compartments prior to NVP exposure	Plasma : 120 CVL : 120		a) Baseline Plasma HIV-1 RNA vs. CD4 cell count b) Baseline CVL HIV-1 RNA vs. CD4 cell count	Fig 35a & c
3. Immunological staging in association with HIV viral load in systemic and genital compartments after NVP exposure.		Plasma: 113 CVL: 69	a) Peripartum Plasma HIV-1 RNA vs CD4 cell count b) Peripartum CVL HIV-1 RNA vs. CD4 cell count	Fig 35b & d
4. Immunological staging associated with HIV viral burden in systemic and genital compartments in relation to intrapartum MTCT		Plasma: 101 CVL: 101	a) Peripartum Plasma HIV-1 RNA stratified by CD4 stage vs. Intrapartum MTCT (6 IP TM + 95 NTM) b) Peripartum CVL HIV-1 RNA stratified by CD4 stage vs. Intrapartum MTCT (6 IP TM + 95 NTM)	Table XXIV

Footnotes: NTM: non-transmitting mother, IP TM: intrapartum transmitting mother

3.4.3.1 Maternal CD4 cell counts

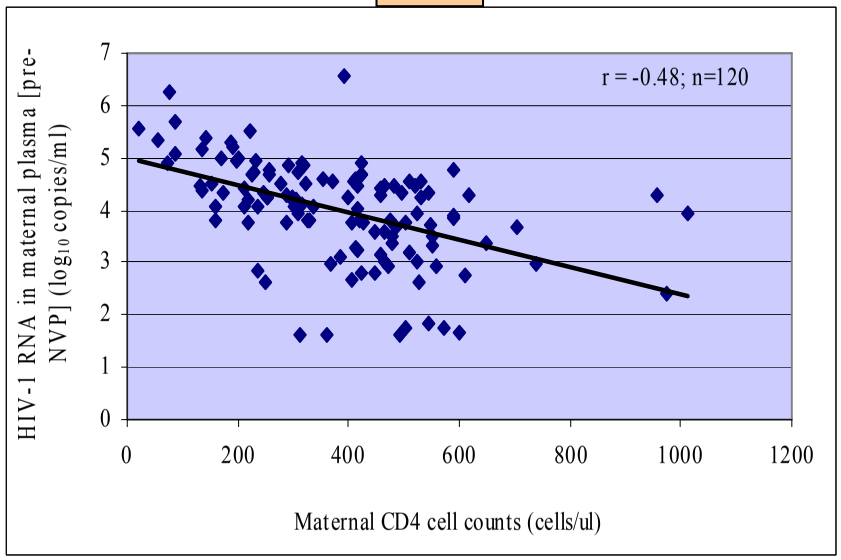
CD4 cell counts were available for 120 women during pregnancy (GA: 28-39 weeks). The median and interquartile range (IQR) of CD4 cell counts for these women was 530 cells/µl (IQR: 246-497) cells/µl. Of the 120 women, 19 (15.8%), 35 (29.2%), 37 (30.8%) and 29 (24.2%) presented with CD4 cell counts of ≤ 200 cells/µl, 200-350 cells/µl, 350-500 cells/µl and ≥ 500 cells/µl respectively.

3.4.3.2 CD4 cell counts in relation to viral dynamics in systemic and genital compartments as risk factors for intrapartum MTCT

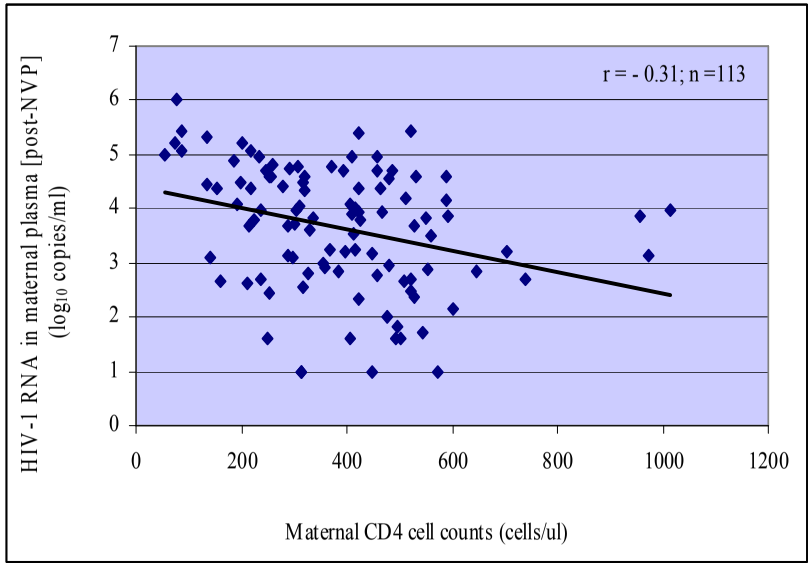
The relationship between maternal CD4 cell counts and HIV-1 RNA (viral loads) in the systemic and genital compartments of women was determined by using the Pearson correlation coefficient [Figs 35a, 35b, 35c, and 35d]. A higher maternal viral load during pregnancy and labour/delivery was associated with lower CD4 cell counts during pregnancy. This finding was statistically significant in maternal plasma ($p < 0.001$; $p < 0.001$) and CVL ($p < 0.001$; $p = 0.04$).

Footnote:
Paired t testing revealed similar statistically significant relationships between plasma HIV-1 RNA and maternal CD4 cell counts at pre ($p < 0.0001$) and post-NVP dosing ($p < 0.0001$). Similarly associations between CVL HIV-1 RNA and maternal CD4 cell counts were also statistically significant at pre ($p < 0.0001$) and post-NVP dosing ($p < 0.0001$).

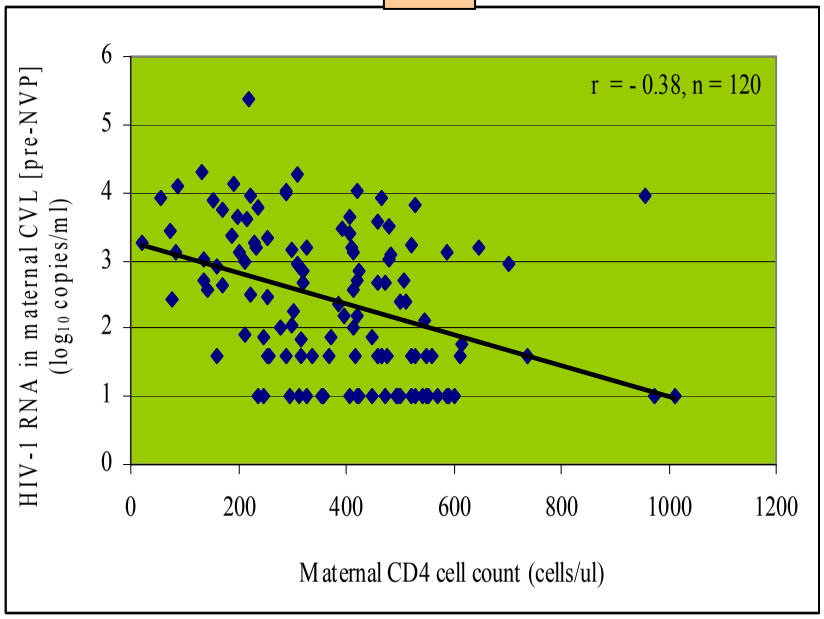
35a



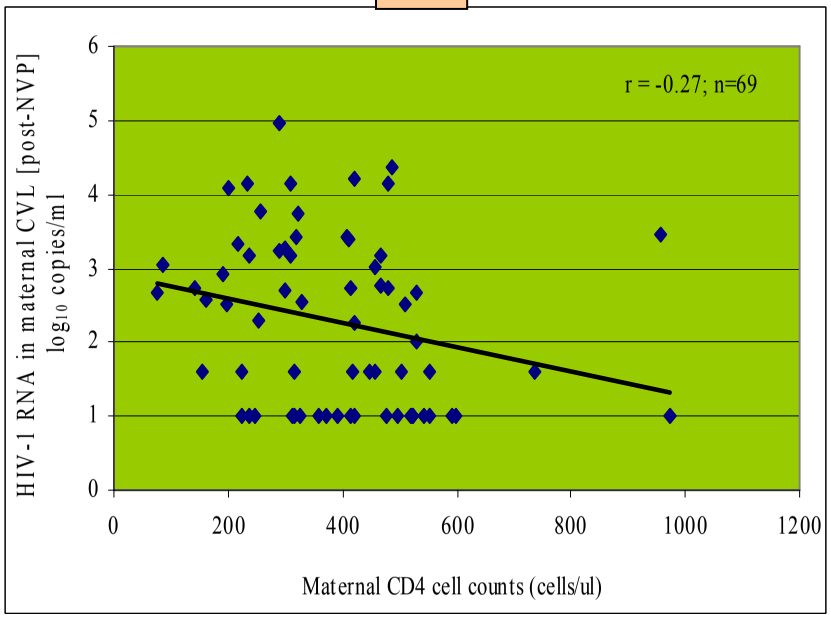
35b



35c



35d



Figs 35 (a-d): Association between CD4 cell counts and maternal viral load (HIV-1 RNA) in the systemic and genital compartments of women at pre and post-sdNVP dosing

Although not statistically significant, intrapartum transmitting women had a lower median CD4 cell count than their non-transmitting counterparts (p = 0.7) [Mann Whitney U test]. Further stratification of maternal CD4 cell counts according to the WHO CD4 classification criteria (Table XXIV) did not reveal any statistically significant associations between both groups (p = 0.4) [Fisher's exact test].

Table XXIV: CD4 cell counts among HIV-1 transmitting and non-transmitting women

Maternal CD4 cell count (cells/µl)	Intrapartum transmitting n = 6	Non-transmitting n = 95	*p value*	Total
Median (range)	279 (76-957)	402 (55-974)	*0.7***	n = 101
< 200-350*	4 (9.1%)	40 (90.9%)		n = 44
≥ 350*	2 (3.5%)	57 (96.5%)	*0.4*[#]	n = 57

Footnotes: All in utero transmitting women (n=6) were excluded from the above analysis

**WHO classification criteria (WHO, 2006c)*

***Mann-Whitney U test; [#]Fisher's exact test*

Stratification according to intrapartum HIV-1 transmission revealed no significant association between pre-NVP CD4 cell counts and pre-NVP viral loads in the systemic [r = 0.20; 95% CI: 0.25-4.69, p = 0.7] and genital compartments [r = - 0.51, 95% CI: -0.94 to 0.51; p = 0.3] of women. A similar correlation analysis of pre-NVP CD4 cell counts and post-NVP viral loads was not significant in the systemic compartment [r = -0.05; 95% CI: -0.26-4.69, p = 0.6] but significant for the genital compartment [r = - 0.36, 95% CI: -0.57 to 0.12; p = 0.001], following stratification according to intrapartum HIV-1 transmission.

3.4.3.3 Ancillary immunological investigations: Effect of host genetics on viral dynamics and intrapartum MTCT

A summary of data analysis for this study component is shown in Table XXV.

Table XXV: Summary of data analysis for ancillary study investigations and findings

RESEARCH AREAS	WOMEN	INFANTS	ENDPOINTS	ILLUSTRATION
	BASELINE			
1. Immunological: Describing host genetics (CCL3 and CCL3-L1) in relation to intrapartum MTCT	TM: 6 NTM: 28	IP: 7 EU: 28	Clinical characteristics CCL3 and CCL3-L1 gene copy expression in maternal-infant subset	Table XXVI Table XXVII
2: Immunological: Describing haplotype characteristics in relation to intrapartum MTCT	TM: 6 NTM: 28	IP: 7 EU: 28	Maternal and infant immune and virological dynamics in relation to CCL3-L1 gene expression as a marker for intrapartum MTCT	Table XXVIII
3. Birth Outcomes: Describing multiple pregnancies (twins) in relation to MTCT (Intrapartum and *In utero* transmission)	7	IP: 2 IU: 3 EU: 9	Clinical and laboratory characteristics	Table XXX

Footnotes: NTM: non-transmitting mother; TM: transmitting mother;
EU: exposed-uninfected, IU: in utero and IP: intrapartum infants

Case-controlled subsets of maternal and infant pairs, based on demographic and clinical characteristics were selected for genotypic investigation. This group of maternal-infant pairs included seven intrapartum infected infants and their corresponding "transmitting mothers" and 28 exposed-uninfected infants and their corresponding "non-transmitting" mothers. As reported in the previous section, HIV-1 transmitting women had a higher viral load in both the systemic [median: 4.36 $\log_{10}$ copies/ml] and genital [median: 3.47 $\log_{10}$ copies/ml] compartments and a lower CD4 cell count [median: 280 cells/µl] [Table XXVI].

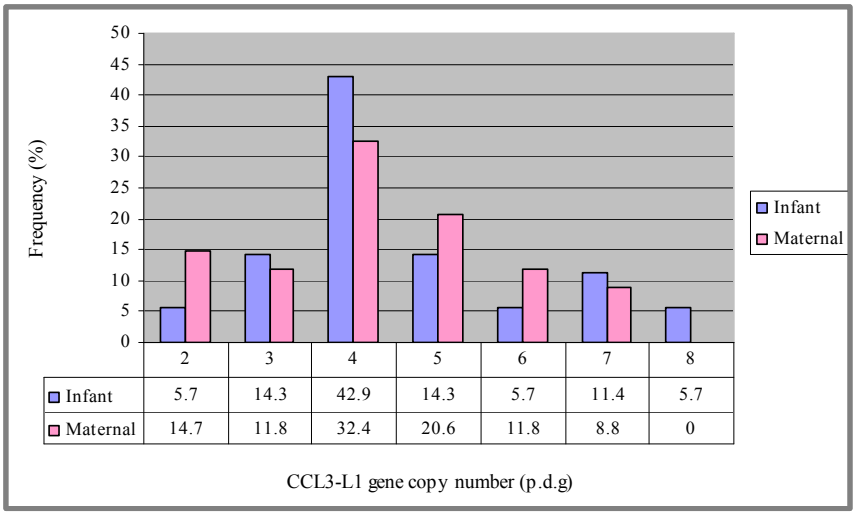
Table XXVI: Clinical characteristics of genotyping subset

Maternal cohort	HIV-1 transmitting women (n = 6)	Non-transmitting women (n = 28)	*p value**
Characteristics	Median (range)	Median (range)	
Maternal age (years)	30 (27-33)	26 (19-39)	*0.09*
Gestational age (weeks)	36 (28-37)	33 (28-39)	*0.4*
Parity	2 (0-3)	1 (0-5)	*0.2*
CD4 cell count (cells/µl)	280 (76-957)	420 (85-1013)	*0.47*
Plasma HIV-1 RNA $\log_{10}$ (copies/ml)	4.36 (3.8-6.3)	3.93 (1.74-5.2)	*0.03*
CVL HIV-1 RNA ($\log_{10}$ (copies/ml)	3.47 (1.0-5.4)	1.72 (1.0-4.12)	*0.04*
Infant cohort	Intrapartum infected (n = 7)	Exposed-uninfected (n = 28)	
Birth weight (kg)	3.25 (1.8-3.8)	3.1 (1.6-3.6)	*0.83*

*Footnotes: * Mann-Whitney U test*
All in utero infants (n=7) and their corresponding mothers (n=6) were excluded from analysis

CCL3 and CCL3-L1 gene copies

Genotypic analysis confirmed the presence of the CCL3 gene at two gene copies per diploid genome (p.d.g) in all 34 (100%) mothers and 35 (100%) infants selected for this sub-study. The detection frequency of CCL3-L1 gene copies per diploid genome ranged from 2-8 in the infants and 2-7 in the mothers of these infants (Fig 36). The overall population median in the maternal cohort was determined to be four gene copies (p.d.g) among intrapartum HIV-1 transmitting (median 4, range 2-6) and non-transmitting (median 4, range 2-7) women [Table XXVII] (p = 0.9). The overall population median in the infant cohort was similar among the intrapartum infected (4, range: 4-7) and exposed-uninfected infants (4, range: 2-8) [p = 0.4]. The Mann Whitney U test was used to test all associations.



	2	3	4	5	6	7	8
Infant	5.7	14.3	42.9	14.3	5.7	11.4	5.7
Maternal	14.7	11.8	32.4	20.6	11.8	8.8	0

Fig 36: Distribution of CCL3-L1 gene copy numbers among the maternal-infant subset

Table XXVII: CCL3-L1 gene copy expression in the selected maternal-infant subset

Maternal CCL3-L1 gene copies (p.d.g)	Intrapartum transmitting (n = 6)	Non-transmitting (n = 28)
	n (%)	n (%)
2	1 (16.7)	4 (14.3)
3	0	4 (14.3)
4*	2 (33.3)	9 (32.1)
5	2 (33.3)	5 (17.9)
6	1 (16.7)	3 (10.7)
7	0	3 (10.7)
Median (range)	4 (2-6)	4 (2-7)
Infant CCL3-L1 gene copies (p.d.g)	HIV-1 Infected (intrapartum) (n = 7)	HIV-1 exposed-uninfected (n = 28)
	n (%)	n (%)
2	0	2 (7.1)
3	0	5 (17.9)
4*	4 (57.1)	11 (39.3)
5	1 (14.3)	4 (14.3)
6	1 (14.3)	1 (3.6)
7	1 (14.3)	3 (10.7)
8	0	2 (7.1)
Median (range)	4 (4-7)	4 (2-8)

Footnote:

(*) indicates CCL3-L1 population medians in maternal and infant subsets

The median CD4 cell count among intrapartum transmitting and non-transmitting women were 279 cells/µl [range 76-957] and 421 cells/µl [range 85-1013] respectively. A correlation analysis of CCL3-L1 gene copy expression in the maternal subset did not reveal any significant relationship with maternal CD4 cell counts (n = 40, r = -0.20, p = 0.3) or maternal viral load (n = 40, r = 0.10, p = 0.4). Likewise no significant associations were observed between infant CCL3-L1 gene copy expression and maternal CD4 cell counts (n = 41; r = 0.10, p = 0.5) or maternal viral loads (n = 41, r = 0.10, p = 0.7) [Table XXVIII].

Table XXVIII: Maternal and infant immune and virological dynamics in relation to CCL3-L1 gene expression as a marker for intrapartum MTCT

Comparisons	*n*	*r*	*p values*
Maternal CD4 vs. Maternal CCL3-LI			
Maternal CD4 vs. Maternal CCL3-LI (IP)	6	0.20	*0.6*
Maternal CD4 vs. Maternal CCL3-LI (EU)	28	0.10	*0.7*
Maternal viral load vs. maternal CCL3-L1			
Maternal viral load vs. maternal CCL3-L1 (IP)	6	-0.70	*0.1*
Maternal viral load vs. maternal CCL3-L1	28	0.02	*0.9*
Maternal CD4 vs infant CCL3-L1			
Maternal CD4 vs infant CCL3-LI (IP)	7	-0.20	*0.7*
Maternal CD4 vs infant CCL3-LI (EU)	28	0.10	*0.8*
Maternal viral load and infant CCL3-L1			
Maternal viral load vs. infant CCL3-L1 (IP)	7	-0.20	*0.7*
Maternal viral load vs infant CCL3-L1 (EU)	28	0.20	*0.3*

Footnotes : r (Pearson correlation coefficient) ; IP (intrapartum) ; EU (exposed-uninfected)

Haplotype (Hap-1) characterization and intrapartum MTCT

CCL3-Hap-1 allelic representation was observed in two (33.3%) of the intrapartum transmitting women and four (14.3%) of the 28 non-transmitting women (Table XXIX). This finding was not statistically significant [p = 0.23; OR: 3.60; 95% CI: 0.62-11.40] (Fishers exact test). No significant relationships were observed between allelic Hap-1 representation and maternal plasma HIV-1 RNA (p = 0.5) or maternal CD4 cell count (p = 0.9).

CCL3-L1 Hap-1 allelic representation was detected in six (21.4%) of the exposed-uninfected infants. The absence of allelic representation among the intrapartum-infected infants did not permit statistical comparisons of CCL3-Hap-1 expression and susceptibility to intrapartum MTCT.

Table XXIX: Maternal and infant CCL3-Hap-1 allelic proportions in relation to intrapartum MTCT

Group	CCL3-Hap 1 proportions	*p value**
Intrapartum (n = 6) vs. non-transmitting women (n = 28)	2 (33.3%)	*0.23*
	4 (14.3%)	
Intrapartum infected (n = 7) vs. Exposed- uninfected infants (n = 28)	0 (0%)	*n/a*
	6 (21.4%)	

* *Fishers exact test*

3.5. Ancillary study findings:

3.5.1 Twin cohort observations

Seven pairs of twins were born during the course of this study. A summary of their clinical and laboratory characteristics are shown in Table XXX. Following two separate DNA PCR tests, nine (64.3%) of the fourteen infants were classified as exposed-uninfected, while five (35.7%) were established as HIV-1 infected. Intrapartum transmission accounted for two (40.0%) of these HIV-1 infections, while three (60.0%) were *in utero* infections. One case of discordant HIV-1 transmission was observed, whereby the first-born twin was HIV-1 negative and the second-born twin became HIV-1 infected through the intrapartum route of transmission.

The median interval between maternal NVP administration and delivery was 5 hours (range: 2-16). Median time interval between infant birth and NVP dosing was 2 hours [range: 1.5-3]. No statistically significant association was found between MTCT and maternal NVP dosing ($p = 0.1$) or infant NVP dosing ($p = 0.4$). Median age among the multiparous women was 27 years [range: 20-38] and median gestational age was 38 weeks [range: 33-38]. No statistically significant association was observed between MTCT and maternal age ($p = 0.1$) or gestational age ($p = 0.13$). Four (57.1%) twin deliveries occurred at full-term (37-41 weeks) while three (42.9%) were pre-term (< 37 weeks). While one women (16.7%) delivered by an emergency C/S, the remaining six twin deliveries (85.7%) occurred through elective C/S prior to the onset of labour.

The median maternal CD4 cell count among this multiparous cohort was 290 cell/ul [range: 160-458]. No statistical significance (p = 0.7) was found when comparing CD4 cell counts between HIV-1 transmitting [n = 3, median: 223 cell/ul, range: 218-290] and non-transmitting women [n = 4, median: 253 cell/ul, range: 160-458]. Similarly no association was found between perinatal transmission and maternal plasma HIV-1 RNA at pre-NVP (p = 0.1) and post-NVP dosing (p = 0.43). At birth sampling, the median plasma NVP concentration among HIV-1 infected infants was 1930 ng/ml [range: 0-2380], while exposed-uninfected infants had a median plasma NVP concentration of 674 ng/ml [range: 0-1640] (p = 0.3). No statistically significant associations were found when comparing median plasma NVP concentration of exposed-uninfected vs. intrapartum infected (p = 0.8) or exposed-uninfected vs. *in utero* infected (p = 0.19) infants. The Mann Whitney U test was used to test all associations.

At least one type of STI was detected in 67.0% of the HIV-1 transmitting women during antenatal screening. Approximately four (80.0%) of HIV-1 infected twins were also exposed to at least one type of maternal STI.

Infant birth weight ranged from 1.7-3.2 kg [median = 2.4 kg]. Birth weight among HIV-1 infected twins [median = 2.1 kg, range: 1.7-2.5] was significantly lower than exposed-uninfected infants [median = 2.8 kg, range: 2.1-3.2] (p = 0.01, 95% CI: 0.20-1.10) (unpaired t test). Although eleven (78.6%) infants of the PIVD twin cohort were of male gender, stratification according to HIV-1 transmission did not reveal any statistical significance (p = 0.2). Oral thrush (Candidias) was the most commonly diagnosed infection among the *in utero* HIV-1 infected infants when examined at their four and/or six week follow-up assessment.

Table XXXa: Summary of clinical and laboratory characteristics of PIVD twin cohort

PIVD STUDY ID	MATERNAL						INFANT					
	Labour HIV-1 RNA		Labour [NVP] (ng/ml)		STI	CD4 (cells/µl)	Birth Weight (kg)	Gender	HIV-1 RNA 4 week	Birth [NVP] (ng/ml)	Infections detected at 4-6 weeks	HIV-1 status 6 weeks
	Plasma	CVL	plasma	CVL								
122A	2.78	No sample	875	0	None	458	2.8	male	Undetectable	1280	oral thrush	negative
122B							3.2	male	Undetectable	888	None	Negative
162A*	4.73	No sample	1480	no sample	*Trichomonas*	290	1.7	female	4.6	99.5	oral thrush	*in utero*
162B*							1.8	male	Undetectable	2380	conjunctivitis	Intrapartum
175A	4.78	4.14	491	0	None	308	3.1	male	Undetectable	1380	None	Negative
175B							3.1	male	Undetectable	insufficient sample	None	Negative
242A	4.5	2.5	799	0	None	198	2.4	female	Undetectable	460	None	Negative
242B							2.5	male	Undetectable	1640	None	Negative
316A	5.56	1	0	0	None	223	2.2	male	Undetectable	0	None	Negative
316B*							2.1	male	6.49	0	Pneumonia	Intrapartum
370A*	5.06	3.3	1730	65.1	*Candida*	218	2.16	male	5.16	2080	oral thrush	*in utero*
370B*							2.46	male	5.84	1930	oral thrush	*in utero*
430A	2.66	2.59	80.5	0	None	160	3	male	Undetectable	0	None	Negative
430B							2.1	female	Undetectable	0	None	Negative

[NVP] = NVP concentration; (*) denotes HIV-infected infant; HIV-1 RNA (viral loads) shown as log_{10} copies/ml

One pair of HIV-1 infected twins demised as a result of respiratory-related illnesses. In addition to a low CD4 cell count, the mother of these twins had a *Trichomonas vaginalis* infection during pregnancy. Both infants had extremely low birth weights (Table XXX). This *dichotomous* HIV-1 transmitting mother infected one twin during pregnancy (*in utero)* and the other via the intrapartum route of transmission. To minimize any confusion during data analysis, this mother was classified as an "*in utero"* HIV-1 transmitting mother.

Compared to singletons, twin pregancies were significantly associated (p=0.01) with HIV-1 transmission (Table XXXb).

Table XXXb: Effect of twin pregnancy on HIV-1 transmission

	HIV-1 infected	HIV-1 uninfected
Twins (n=14)	5	9
Singletons (n=103)	9	94
p value	0.01	
OR	5.8 (95% CI: 1.34-25.1)	

*Fishers exact test

CHAPTER FOUR: DISCUSSION

During the period of the PIVD study (April-December 2006), a regimen of intrapartum maternal sdNVP and postpartum neonatal sdNVP was provided to all HIV-1 infected pregnant women and their HIV-exposed infants, as the standard of care for PMTCT at KEH, Durban. The overall HIV-1 MTCT rate at six weeks in this sdNVP-exposed and exclusively formula-fed cohort of maternal-infant pairs was 11.9% [95% CI: 6.70-19.00] with similar rates of in utero and intrapartum transmission (5.9% and 5.9% respectively). During active labour, the median NVP concentration in the maternal systemic compartment [1070 ng/ml] was almost 44 times higher than the NVP levels detected in the genital compartment [24.5 ng/ml] ($p < 0.001$). NVP drug levels were below the 100 ng/ml therapeutic target in seven (13.7%) of 51 plasma and in all 39 CVL samples. The median plasma NVP level detected among infants at birth was 83 times above the IC_{50} WT (10 ng/ml) and eight times higher than the 100 ng/ml therapeutic target for NVP. More than 71.0% of the infants achieved NVP drug levels above the therapeutic target. These findings confirm that the role of sdNVP in PMTCT was primarily one of infant prophylaxis. This was further supported by relatively unchanged maternal HIV-1 RNA (viral load) at the time of active labour, in both systemic and genital compartments. HIV-1 transmitting women had significantly higher viral loads than their non-transmitting counterparts in systemic and genital compartments, before and after intrapartum sdNVP administration. In terms of perinatal transmission this observation was only statistically significant for plasma ($p = 0.02$) and not CVL ($p = 0.7$). For clarity and ease of comparison with other studies, the present study will be referred to as the PIVD study in this chapter.

Pharmacokinetic assessment using the LC-MS-MS quantitation method in this PIVD study of women, who received the single dose NVP (200 mg) regimen as standard of care to prevent mother-to-child transmission of HIV-1 in the South African PMTCT program, demonstrated that the median NVP concentration of 1070 ng/ml, [range 0-3080] in maternal plasma collected from 51 women during active labour and within 20 hours of NVP dosing. NVP fell within the range detected in controlled phase II clinical trials that employed either HPLC or LC-MS-MS quantitation methods. Mirochnick and others first reported a median maternal plasma NVP concentration of 1663 ng/ml [range: 447-2639] in the first pharmacokinetic study investigating the potential of single dose NVP (200 mg) in prevention of MTCT among 18 HIV-1 infected pregnant women and their neonates (Mirochnick *et al*, 1998). Similar median plasma NVP concentration were reported in subsequent phase II clinical trials; 1644 ng/ml (Musoke *et al*, 1999), 1742 ng/ml (Harms *et al*, 2005) and 1695 ng/ml (Creesey *et al* 2005).

Known to be readily absorbed after oral administration in adults, NVP readily crosses the placenta and has been an excellent candidate for single-dose (200 mg) ARV intervention during labour because of its potent antiviral effect (an IC_{50} or *in vitro* inhibitory concentration of 10 ng/ml) (Musoke *et al*, 1999; Mirochnick *et al*, 1998). In the PIVD study, NVP drug levels failed to reach the 100 ng/ml therapeutic target in just 13.7% of women at active labour (0.5-20 hours). In contrast, the median NVP concentration found in maternal CVL (24.5 ng/ml, range: 0-99.7) was significantly lower than plasma NVP levels during active labour [$p < 0.0001$]. This finding was consistent with the observations by several researchers who found that the NNRTI group of compounds did not penetrate well into cervicovaginal fluids (Ellerbrock *et al*, 2001; Tirado *et al*, 2004 and Kwara *et al*, 2008). A group of researchers from the University of North Carolina used novel methods to profile various ARV drugs based

on intracellular and intracellular genital tract exposure relative to plasma. They found that the penetration of ARV agents into the genital tract was strongly dictated by the degree of protein binding and the drug's affinity for α1-acid glycoprotein. In comparison to plasma, more than 80.0% of high-protein bound drugs were recovered at a much lower concentration within the genital tract. The relative extent of drug penetration into the genital tract was similar between males and females. In the PIVD study, NVP in CVL failed to meet the 100 ng/ml therapeutic target in all 39 women studied at active labour (0.5-20 hours). Despite high plasma NVP levels, the corresponding female genital tract concentration of high protein bound NNRTI drugs like NVP remained remarkably low (Kashuba, 2006; Cohen *et al*, 2007). It was therefore not surprising that median NVP levels in active labour were almost 44 times higher in maternal plasma (1070 ng/ml) compared to CVL (24.5 ng/ml) in the PIVD study. These findings were consistent with previous reports of poor NNRTI drug penetration into the genital compartment (Ellerbrock *et al*, 2001; Tirado *et al*, 2004; Kashuba, 2006; Cohen *et al*, 2007 and Kwara *et al*, 2008).

The CVL NVP: Plasma NVP ratio [0.02] observed during active labour in this PIVD study was within the range observed in a recent study (Kwara *et al*, 2008). While no statistically significant association was attained between perinatal transmission and NVP concentration in the systemic compartment ($p = 0.4$), this relationship was significant in the genital compartment ($p = 0.02$). The observation of sub-therapeutic drug levels during active labour in the female genital tract despite optimal NVP dosing remains clinically relevant. Since sdNVP for the indication of PMTCT does not reduce maternal viral load, other ARV's are therefore required for optimal absorption and viral reduction in genital fluids and hence also reducing infant exposure to HIV-1 during labour and delivery. Studies by Kashuba and others have

demonstrated that excellent ARV drug penetration (within the female genital tract) was achievable through the use of the nucleoside reverse transcriptase inhibitors or NRTI class of compounds. NRTI's like ZDV, 3TC and Tenofovir were found to accumulate very successfully in genital secretions [Kashuba, 2006; Dumond *et al*, 2007; Kwara *et al* 2008]. The efficacy of NRTI drugs like ZDV (in PMTCT) have already been demonstrated by the successful outcomes of clinical drug trials like the PACTG 076 (Connor *et al*, 1994) and PHPT (Lallemant *et al*, 2000), in addition to field efficacy studies (Dabis *et al*, 1999; 2005). Unlike other drugs, ZDV becomes fully active within the placenta, enters the fetal circulation and has the ability to rapidly achieve drug levels comparable to those in maternal blood (Kumar *et al*, 1994).

A dual ARV regimen of (ZDV and sdNVP) is currently a WHO recommended option for PMTCT. This dual regimen will not only be beneficial for prevention of drug resistance (associated with sdNVP) but several studies have demonstrated that short-course ZDV regimens are also more efficacious in achieving optimal HIV-1 RNA (viral load) suppression in maternal genital secretions in addition to reducing MTCT. Chuachoowong and others reported a substantial and rapid reduction of HIV-1 RNA levels in maternal CVL with as little as one to two weeks of ZDV administered near the end of pregnancy (Chuachoowong *et al*, 2000). Similarly following short-course ZDV treatment, both plasma and genital secretions showed a reduction in HIV-1 RNA. Compared to plasma, a more rapid virus clearance was observed in genital secretions (Mbori-Ngacha *et al*, 2003). These reports are encouraging and have tremendous bearing on reducing vertical and horizontal transmission in resource-poor settings.

Almost 71.0% of the infants in PIVD study were able to achieve plasma NVP drug levels (birth) above the 100 ng/ml therapeutic target within 72 hours of birth. The median NVP concentration of 828 ng/ml in infant plasma was eight times higher than the therapeutic target (100 ng/ml) and almost 83 times above the *in vitro* [IC_{50} WT] (10 ng/ml). In a phase I/II safety and toxicity trial performed in Uganda, a single dose of intrapartum maternal and postpartum infant NVP, resulted in infant serum NVP levels of more than 150 times the *in vitro* [IC_{50} WT] (Musoke *et al*, 1999). This trial also reported a median cord blood NVP concentration of 1175 ng/ml which exceeded the 100 ng/ml therapeutic target in 100% of their infant cohort. Median infant serum or plasma NVP levels from other phase II clinical trials were 925 ng/ml [64-2030] (Mirochnick *et al*, 1998) and 1291 ng/ml [114-2910] respectively (Harms *et al*, 2005). While the median infant plasma NVP level (828 ng/ml) observed in the present PIVD study was slightly lower than previously described, this level was within the range described in previous studies (Mirochnick *et al*, 1998, Harms *et al*, 2005).

A statistically significant relationship was observed between the NVP concentration in the plasma of a 100 maternal-infant pairs [r = 0.36, 95% CI: 0.18-0.52, p = 0.0002] (Pearson's correlation coefficient). These findings concurred with similar observations by (Harms *et al*, 2005) and confirm the transplacental passage of NVP (Mirochnick *et al*, 1998). In the PIVD study, median plasma NVP levels of exposed-uninfected infants of 933 ng/ml fell within the drug concentration range (875-1471)] of exposed-uninfected infants at 6-8 weeks reported by Jackson and others (Jackson *et al*, 2006) in a phase III (HIVNET 012) clinical trial. The median NVP concentration of 20.3 ng/ml (range: 0-2380) found among intrapartum infected infants in the PIVD study was far below the expected therapeutic target and below the NVP concentration range described by (Jackson *et al*, 2006). Phase I studies of safety and

pharmacokinetics of NVP in Ugandan and American women demonstrated that a 200 mg/ml dose of NVP administered to HIV-1 infected women in labour was rapidly absorbed and achieved NVP drug levels of more than 10 times the concentration required to inhibit antiviral activity by 50% (IC_{50}) in the newborn at delivery. When the infant received an additional dose of 2 mg/kg (48-72 hours of birth), infant NVP concentration was maintained at greater than the 100 ng/ml target for up to seven days postnatally (Mirochnick *et al*, 1998; Musoke *et al*, 1999). Recommended dosing of sdNVP has been 2-48 hours for women and 6-72 hours for infants (Guay *et al*, 1999). While sdNVP is easily self-administered, there has been wide variability regarding the timing of the drug dose in relation to delivery. In the current study, more than 70% of women self-administered their NVP dose within 2-48 hours and more than 80.0% of this infant cohort received their NVP dose less than six hours of birth. No association was found between sdNVP dosing and intrapartum HIV-1 transmission ($p = 0.4$). While some studies reported a decreased cord blood concentration when maternal dosing took place at less than two hours of delivery (Mirochnick *et al*, 2003), Chi and others found that MTCT was unaffected by the timing of the maternal or infant NVP dose, provided dosing occurred within reasonable proximity to the time delivery (Chi *et al*, 2005).

The HIVNET 012 (Guay *et al*, 1999; Jackson *et al*, 2003) and SAINT (Moodley *et al*, 2003) trials evaluated the provision of intrapartum sdNVP and postpartum maternal NVP and postpartum neonatal sdNVP. In the NVAZ trial, a post-exposure prophylactic regimen of sdNVP and one week of Zidovudine or ZDV was administered to the infants of untreated mothers (Taha *et al*, 2003). In the South African PEP study, investigators compared two post-exposure prophylaxis regimens (sdNVP vs. 6 weeks of ZDV) administered to the infants of untreated mothers (Gray *et al*, 2005). Authors of the NVAZ study strongly recommended that

when it is not possible to administer intrapartum NVP to the mother, it might still be beneficial to provide this intervention to the infant. The infant could also be afforded additional protection by a second ARV such as ZDV. This study indicated that dual therapy of sdNVP coupled with one week of ZDV given to the infant was far superior to a single dose of NVP alone (Taha *et al*, 2003). In slight contrast, the PEP study investigators reported that an infant only sdNVP regimen was as efficacious as six weeks of ZDV therapy (Gray *et al*, 2005). Both the South African PEP (Gray *et al*, 2005) and the Malawian NVAZ (Taha *et al*, 2003) studies provided compelling evidence for the safe and efficacious implementation of an infant only PEP strategy as an alternate intervention to reduce MTCT.

Since April 2008 a dual regimen of ZDV starting from 28 weeks gestational age and sdNVP in labour has now been prescribed as the standard of care for HIV-1 infected pregnant women (PMTCT programme). Their subsequently HIV-exposed infants also receive sdNVP at birth, followed by a seven day course of ZDV (WHO, 2006b). This new development is extremely positive as findings from this and several other studies underscore the need for dual ARV regimens.

Using an ultra-sensitive HIV-1 RNA quantitation method [COBAS AmpliPrep/Cobas TaqMan system, 2006], HIV-1 RNA detection rates of 78.3% and 65.3% were observed in the analysis of maternal CVL samples collected at pre and post-NVP dosing respectively. CVL HIV-1 RNA detection rates reported in this study concur with several other studies (Kovacs *et al*, 1999; Hart *et al*, 1999; Goulston *et al*, 1998; Iversen *et al*, 1998; Fiore *et al*, 2003). The variable detection rates reported across these studies have been attributed to CVL collection procedures and HIV-1 RNA quantitation methods. The COBAS system (used in this study)

has only been validated (FDA approved) in human plasma samples. Detection rates from this study demonstrate the potential application of this assay in the quantitation of CVL HIV-1 RNA. Besides a high throughput, this commercial COBAS assay offers increased detection sensitivity with a lower limit of detection of 40 vs. 400 copies/ml typically achieved when using conventional Roche Amplicor assays (Liegler, 2006). Despite the presence of blood contamination in some CVL samples collected during labour sampling, all 72 CVL samples that were collected at post-NVP dosing were assayed for HIV-1 RNA. This was undertaken as several published studies have shown that minimal blood contamination of genital samples does not significantly affect genital viral load levels (Hart *et al*, 1999; Reichelderfer *et al*, 2000 and Cu-Uvin *et al*, 2006).

Median maternal plasma HIV-1 RNA (viral loads) did not vary significantly between pregnancy (4.1 $\log_{10}$ copies/ml), active-labour (3.9 $\log_{10}$ copies/ml) or post-delivery (3.7 $\log_{10}$ copies/ml) sampling. Likewise median CVL HIV-1 RNA during pregnancy, active labour and post-delivery were 2.8; 2.4 and 1.9 ($\log_{10}$ copies/ml) respectively.HIV-1 transmitting women had significantly higher viral loads than their non-transmitting counterparts in systemic and genital compartments, before and after intrapartum sdNVP administration. A general trend of higher HIV-1 RNA levels in maternal plasma as compared to CVL was consistently observed independent of sampling. This finding concurs with similar observations by Cu-Uvin *et al*, 2006. Overall, women who transmitted HIV-1 had significantly higher HIV-1 RNA levels than their non-transmitting counterparts at pre and post-NVP dosing in their systemic compartments. Maternal genital viral load (CVL HIV-1 RNA) was comparable between both groups at pre and post-NVP dosing. In terms of intrapartum MTCT, these findings were only significant for maternal plasma ($p = 0.02$) and not for CVL ($p = 0.7$). This observation

supports previous reports wherein plasma was seen as a much stronger predictor for MTCT (Mofenson, 1997; Quinn *et al*, 2000; Kovacs *et al*, 2001). Women with viral loads above the threshold for mother-to-child transmission (> 1000 copies/ml) only demonstrated an increased risk of intrapartum HIV-1 transmission in their plasma samples ($p = 0.02$) and not CVL ($p = 0.9$).

In their study of patterns and predictors of genital tract HIV-1 RNA levels over a 36-month period, Cu-Uvin and others reported that plasma HIV-1 RNA correlated strongly with genital tract HIV-1 RNA (Cu-Uvin *et al*, 2006). They also found plasma HIV-1 RNA to be the strongest predictor of CVL fluid HIV-1 RNA detection. Even though cases of discordance are known to occur (Rasheed, 1998), genital tract HIV-1 RNA is unlikely to be found when plasma HIV-1 RNA is undetectable.

In the PIVD study, both intra [e.g. pre-NVP dose vs. post-NVP dose] and inter-compartmental [plasma vs. CVL] HIV-1 RNA comparisons have revealed strong and statistically significant relationships. These positive associations remained constant even after controlling for confounding variables such as CVL blood contamination and detection of sexually transmitted infections. The PIVD study findings of moderate to strong correlation between systemic and genital HIV-1 RNA were consistent with other studies of HIV-1 infected women who either received or didn't receive ARV drug therapy (Andréoletti *et al*, 2003; Si-Mohamed *et al,* 2000; Iversen *et al*, 1998; Kovacs *et al*, 1999; Hart *et al*, 1999; Cu-Uvin and Caliendo, 1997; Cu-Uvin *et al*, 2006; Goulston *et al*, 1998) and in studies of pregnant and non-pregnant HIV-1 infected women (Shaheen *et al*, 1999). This finding suggests that HIV-1 RNA in CVL was significantly influenced by HIV-1 RNA in plasma (Kovacs *et al*, 2001; Cu-Uvin and Caliendo,

1997; Cu-Uvin *et al*, 2006; and Fiore *et al*, 2003). In contrast, some studies have demonstrated a lack or poor viral load correlation (Rasheed, 1998; Shepard *et al*, 2000 and Garcia-Bujalance *et al*, 2004) between both compartments. Similar to the reports of variable HIV RNA detection rates, discrepancies between published studies could be due to patient selection bias, methodological differences in respect of CVL sample collection or even choice of HIV-1 RNA quantitation assay.

Findings of strong correlation between pre and post-NVP dose HIV-1 RNA levels in both systemic [$r = 0.81$, $p < 0.0001$] and genital compartments [$r = 0.80$, $p < 0.0001$] further supports another crucial observation wherein *no viral load reduction* took place in more than 80.0% of study women (at the time of active labour). Interestingly, this relationship remained strong and statistically significant in both compartments, even during a combination analysis that included active labour, post-delivery and latent labour maternal CVL samples. While unchanged viral loads were indicative of the lack of maternal effect, these findings serve to confirm the role of sdNVP as being primarily a function of prophylaxis for infants. These findings are important in elucidating the role of sdNVP (for prevention of mother-to-child HIV-1 transmission), particularly due to current concerns over NVP-related drug resistance selection. This observation supports studies of "infant only" post-exposure prophylaxis. Researchers from the South African PEP study (Gray *et al*, 2005) concluded that sdNVP acted purely as infant prophylaxis. Initial reports from the benchmark HIVNET 012 trial almost a decade ago, found no significant difference between maternal plasma HIV-1 RNA levels at baseline and delivery. Even then, HIVNET 012 investigators postulated that the efficacy of sdNVP in prevention of vertical HIV-1 transmission was unlikely to be caused by a reduction of maternal viral load (Musoke *et al*, 1999).

In the PIVD study, CD4 cell counts were available for 120 women during pregnancy (GA: 28-39 weeks). The median and interquartile range (IQR) of CD4 cell count of these women was 530 cells/µl (IQR: 246-497) cells/µl. Of the 120 women, 19 (15.8%), 35 (29.2%), 37 (30.8%) and 29 (24.2%) presented with CD4 cell counts of ≤ 200 cells/µl, 200-350 cells/µl, 350-500 cells/µl and ≥ 500 cells/µl respectively. Nineteen (15.8%) of these women had a CD4 count of < 200 cells/µl and were eligible for antiretroviral therapy in admission into the ARV rollout programme at KEH. The median CD4 cell count among these women was 142 cells/µl [range: 21-198]. All nineteen women were referred to the ARV rollout clinic (Philani Family Clinic, KEH) and commenced on a comprehensive eight-week ARV drug adherence training programme prior to labour and delivery. As at the final postnatal study visit at six weeks, ARV treatment (3TC, d4T and NVP) was reported to have been initiated in four (21.0%) of the women prior to delivery, while the remaining 15 (78.9%) women had not commenced ARV treatment. The WHO currently recommends that all patients with a CD4 cell count of ≤ 350 cells/µl be given access to HAART (WHO, 2006c). Early identification of pregnant women in need of ARV drugs and initiation of therapy as early as possible in pregnancy would greatly reduce MTCT rates in developing regions. ARV treatment of these women not only benefits their own health but more significantly remains the best prophylaxis against MTCT (McIntyre, 2007).

In the present study, a higher maternal viral load during pregnancy and labour/delivery was associated with lower CD4 cell counts during pregnancy. This finding was statistically significant in maternal plasma ($p < 0.001$; $p < 0.001$) and CVL ($p < 0.001$; $p = 0.04$). Despite the absence of statistical significance, intrapartum transmitting women had a lower median CD4 cell count than their non-transmitting counterparts ($p = 0.7$). Maternal immune depletion

has been shown to correlate with vertical HIV-1 transmission. An increased risk of vertical transmission was noted with lowered CD4 T cell counts (European Collaborative study, 1994; Mofenson *et al*, 1999b; Luzuriaga, 2007). In the South African PEP study, a CD4 cell count of ≤ 500 cells/µl was associated with a two-fold increase in transmission (Gray *et al*, 2005). Increased maternal HIV-1 RNA (viral load) was associated with decreased or lower CD4 cell counts. This finding was statistically significant in maternal plasma and CVL. Women with high plasma HIV-1 RNA and low CD4 cell counts were more likely to also have high CVL HIV-1 RNA. Similar relationships were described by Kovacs and others (Kovacs *et al*, 1999). In concordance with a report from an Italian study (Fiore *et al*, 2003); maternal CD4 cell counts in this current study also correlated negatively with plasma and CVL HIV-1 RNA, even after stratification according to intrapartum transmission.

The overall mother-to-child transmission rate observed in this study at six weeks was 11.9% [95% CI: 6.70-19.00]. While direct comparison to other studies is difficult due to variable study designs and settings, the MTCT rate observed here was comparable to transmission rates reported by several clinical trials and studies performed on the African continent (that investigated treatment arms/regimens containing sdNVP): HIVNET 012 (*Uganda*) [11.8%; 95% CI: 8.20-15.50] (Guay *et al*, 1999; Jackson *et al*, 2003); SAINT (*South Africa*) [12.3%; 95% CI: 9.70-15.00] (Moodley *et al*, 2003); NVAZ (*Malawi*) [15.3%; 95% CI: 12.10-18.50] (Taha *et al*, 2003) and PEP (*South Africa*) [11.9%, 95% CI: 8.80-15.00] (Gray *et al*, 2005).

In the PIVD study, the MTCT *in utero* rate was 5.9% [95% CI: 1.68-10.28]. Likewise the intrapartum MTCT rate was 5.9% [95% CI: 1.68-10.28]. Without any ARV intervention or breastfeeding exposure, perinatal HIV-1 transmission rates have been documented to range

from 5-10% *in utero* and 10-20% intrapartum (De Cock *et al*, 2000). MTCT rates in this study were comparable with rates described in several studies/clinical trials investigating sdNVP-containing treatment arms/regimens. The *in utero* MTCT rates reported from such studies included; 8.2% (HIVNET 012, Guay *et al*, 1999); 8.5% (SAINT, Moodley *et al*, 2003); 10.2% (NVAZ, Taha *et al*, 2003) and 7.0% (PEP, Gray *et al*, 2005). The intrapartum MTCT rates included; 11.8% (HIVNET 012, Guay *et al*, 1999); 8.9% (SAINT, Moodley *et al*, 2003); 12.1% (NVAZ, Taha *et al*, 2003) and 11.9% (PEP, Gray *et al*, 2005).

Advanced maternal age is a well-known risk factor for poor pregnancy outcome and increased MTCT (Mayaux *et al*, 1995). To control for this confounding factor, all antenatal attendees aged $\geq$ 39 years were excluded from participation in the present study. As a result, no statistically significant association was observed between maternal age and intrapartum MTCT ($p = 0.24$).

In contrast to other MTCT studies (Fang *et al*, 1995; Stratton *et al*, 1999), no association was observed between maternal gestational age at labour and intrapartum MTCT in this study ($p = 0.7$). An infant birth weight of less than 2500 grams was significantly associated with intrapartum MTCT ($p = 0.04$). This finding was consistent with findings in numerous MTCT studies (Landesman *et al*, 1996; Stratton *et al*, 1999, Jourdain *et al*, 2007). The observation of infant gender not being a significant risk factor for intrapartum MTCT ($p = 0.13$, OR: 3.20) was in agreement with the findings from the meta-analysis of nine African studies where no association between infant gender and early perinatal transmission (Breastfeeding and HIV International Transmission Study Group, 2004).

Mode of delivery (NVD vs. C/S) was equally distributed in the current study and was not associated with an increased risk of intrapartum MTCT [p = 0.7]. Comparisons for deliveries by emergency C/S were not possible due to a lack of statistical power. While the use of an elective C/S prior to the onset of labour and rupture of membranes has been widely advocated as an effective intervention for protection against intrapartum HIV-1 transmission (European mode of delivery collaboration, 1999), there have also been conflicting reports regarding its clinical utility among HIV-1 infected women. In the meta-analysis review undertaken by Read and colleagues, the use of an elective C/S over a NVD translated to a 50.0% reduction of MTCT risk among women with high plasma viral load or those who were not on HAART (Read and Newell, 2006). However, data from the WITS study highlighted an association between maternal morbidity and use of an elective C/S (Read *et al*, 1999) among HIV-1 infected women. Similarly the ACTG 185 trial reported an increased rate of infectious maternal complications among HIV-1 infected women undergoing surgical deliveries (Mofenson *et al*, 1999a). Despite its potential risks, an elective C/S negates any likelihood of the premature rupture of membranes and also minimizes an infant's contact with contaminated maternal cervicovaginal fluids during a normal delivery. Taking this into consideration, the CDC issued guidelines stating that the risk of infectious maternal complications was not of significant frequency or severity to outweigh the potential benefits of reduced transmission with the use of elective caesarean section. The CDC also recommended that the elective C/S be offered as an option to all HIV-1 infected women provided they were aware of the potential risks and benefits (CDC 2002; Luzuriaga, 2007).

Findings from the Mandelbrot, Landesman and Magder research groups have found statistically significant associations between a rupture of membranes of greater than four hours

and the risk of intrapartum MTCT (Mandelbrot *et al*, 1996; Landesman *et al*, 1996 and Magder *et al*, 2005). Due to a lack of statistical power, this comparison was not possible in the present study. While some large cohort studies have reported an association between intrapartum MTCT and preterm labour (Landesman *et al*, 1996; Mandelbrot *et al*, 1996; European collaborative study, 1992 and Kuhn *et al*, 1999), this was not the occurrence in the present study ($p = 0.31$, $OR = 1.70$).

In spite of two decades of research on MTCT and HIV-1, there has been very little study of the risks of multiple pregnancies among women with HIV-1 infection. The twinning rate observed in the PIVD study was 6.4%. Nine (64.3%) of the twin infants were exposed-uninfected, while five (35.7%) were confirmed to be HIV-1 infected. Among those infected, intrapartum MTCT accounted two (40.0%), while the remaining three (60.0%) were infected *in utero*. Intriguingly, more than 80.0% of the HIV-1 infected twins were also exposed to at least one type of maternal STI. While 78.6% of the twin infants were of male gender, this finding was not associated with perinatal HIV-1 transmission ($p = 0.2$). Likewise findings from a large French perinatal cohort (Scavalli *et al*, 2007) also reported no relationship between gender and twin HIV-1 transmission. Similar to findings from the singleton cohort, low birth weight among the PIVD twin cohort was also significantly associated with MTCT ($p = 0.01$). All twin deliveries occurred by elective C/S, with the exception of one emergency C/S. The birth order of twins has long been associated with a variable risk of HIV-1 infection. Studies by Goedert and associates as well as Duliège and colleagues have shown that the majority of twin pairs were concordant in terms of HIV-1 infection (Goedert *et al*, 1991; Duliège *et al*, 1995). Those two groups also found first-born twins to be at an increased risk for MTCT, compared to second-born twins. In the PIVD cohort seven women delivered twins.

Of these, three (42.9%) also transmitted HIV-1 to their infants. One mother transmitted HIV-1 via the *in utero* mode to both her babies. The occurrence of a *dichotomous* transmitting mother with one *in utero*-infected twin and one intrapartum-infected twin in this study was also quite unusual. One case of discordant HIV-1 transmission was also observed. This mother transmitted HIV-1 to her second-born twin via the intrapartum mode, while her first-born twin remained inexplicably HIV-1 exposed-uninfected until the end of this study at six weeks. The precise mechanism for the increased risk of HIV-1 acquisition among first-born twins remains an unsolved mystery. Compared to singletons, twin pregnancies were highly associated with HIV-1 transmisson ($p = 0.01$) in this study.

A high prevalence rate of multiparous pregnancies is not uncommon within the African setting. This together with the high MTCT rates of this region, merits further investigation into the pregnancy-related outcomes of multiparous HIV-1 infected women.

Tremendous research efforts and progress have resulted in the WHO's current support and recommendation of more efficacious prophylactic regimens for PMTCT (in resource-constrained settings) such as the dual regimen of sdNVP and ZDV, in the absence of maternal HAART (WHO, 2006b). However due consideration must be given to the challenges associated with large scale implementation of such programmes in these resource-limited settings. Studies have shown that ZDV and sdNVP are not easy to apply in Africa (Msellati *et al*, 2001; Temmerman *et al*, 2003; Stringer *et al*, 2003a). While combined use may not be more complex, universal ARV coverage still remains a huge challenge. A recent UNAIDS report revealed that in 2007, ~49.0% of women received single-dose NVP, ~26.0% received a combination of two ARV drugs and ~8.0% received a combination of three ARV drugs (UNAIDS/WHO/UNICEF, 2008b).

Despite more efficacious ARV combinations (as demonstrated from various clinical drug trials), few low-resourced country programmes have yet been able to go beyond single dose NVP or even achieve good coverage with this regimen. In 2007, reasonable ARV coverage (for PMTCT) was observed in some African countries including SA (57.0%), Kenya (69.0%), Mozambique (46.0%), however ARV coverage in countries like India (14.0%) and Democratic Republic of Congo, Ethiopia and Nigeria (<10.0%) was extremely inadequate (UNAIDS/WHO/UNICEF, 2008). More concerning was the observation that many of the countries with poor ARV coverage were also geographically located within well-known HIV-1 epicenters (Africa and Asia) of the world. ARV coverage among HIV-exposed infants was less than 20.0% in 2007 (UNAIDS/WHO/UNICEF, 2008b).

Deficiencies in various MTCT components such as VCT uptake, receipt of results, uptake of ARV drugs and post-delivery follow up were cited as reasons for the sub-optimal implementation of PMTCT programmes in some African countries (Colvin *et al*, 2007). When the present PIVD study was undertaken, ARV drug adherence training was conducted for a period of eight weeks. While this was the standard of care, this unusually long training process for antenatal HAART did not prove beneficial to some women. Despite their eligibility for antenatal HAART, some women were either still in the process of training or were too far along in their pregnancy (> 34 weeks) to attain the optimal benefit of antenatal HAART. While there is a substantial body of literature from clinical trials regarding the efficacy of short-course ARV prophylaxis in reducing transmission from mother to child, data pertaining to the operational effectiveness (most especially sub-Saharan Africa) of national programmes remains sparse. Proportions of women who were somehow missed by their PMTCT programmes have been reported as Kenya (17.0%); Ethiopia (57.0%) and Zimbabwe

(59.0%) (Bolu *et al*, 2007). Data regarding operational efficacy has also been conflicting. While a Kenyan study found that the sdNVP regimen to have low efficacy (Quaghebeur *et al*, 2004), a South African study demonstrated the feasibility and effectiveness of implementing large scale PMTCT programmes in an urban public sector setting (Coetzee *et al*, 2005). Colvin and others also found PMTCT programmes (using sdNVP) to be efficacious in reducing early transmission of HIV-1 in operational settings (Colvin *et al*, 2007). Fear, discrimination and stigma remain some of the main impediments to participation of women in the PMTCT programme. A study carried out in Botswana highlighted the negative attitudes of healthcare workers, lack of partner support and stigma associated with infant feeding choice as some of the barriers which led to poor PMTCT uptake (Kebaabetswe, 2007).

Use of sdNVP and postpartum prophylaxis (PEP) in infants still has a significant role to play in PMTCT and will continue to be of benefit in settings where women: a) do not access routine antenatal care services, b) are not offered antenatal counseling and testing, c) arrive at hospital in advanced labour neither counseled nor tested, d) refuse to take their intrapartum NVP or miss their dose or e) where babies are born at home/or en-route to hospital and f) where healthcare workers wish to avoid maternal NVP dosing (Gray *et al*, 2005). PEP serves as an alternative post-delivery strategy to improve the uptake of VCT, attendance of infant feeding counseling and access to ARV therapy. Apart from the sdNVP PEP regimen being simpler to implement, it also abates concerns of NVP drug resistance in the mother. NVP-associated drug resistance does however remain a significant challenge among HIV-1 infected infants (Martinson et al, 2004). Infant only PEP is an effective means of reaching, counseling and treating women who missed opportunities for the commonly practiced voluntary counseling and HIV-1 testing during pregnancy or antenatal VCT. Postpartum VCT or PP-

VCT has been reported as being a valuable option (Chersich *et al*, 2002) among women who missed antenatal counseling and testing opportunities. A recent study (n = 493) offering HIV testing to women who accessed immunization or acute infection services at child health clinics in Kenya was viewed as an acceptable and valuable option by many of the clinic attendees. Approximately 472 (95.7%) of women cited a keen interest in the benefits regular HIV testing, point of care testing, infant feeding counseling and access to treatment to prevent MTCT. A large proportion of women 98 (79.0%) who did not access antenatal care services were tested in the study by Chersich and others (Chersich *et al*, 2008). The best strategy for attaining the MTCT rates of (< 2.0%) often only seen in developed countries remains "*universal access to ARV drugs*". For PMTCT, this means urgent early identification of HIV-1 infected pregnant women in need of ARV therapy and initiation of therapy in these high-risk women as early as possible in pregnancy. "ARV treatment of these women not only benefits their own health but remains the best prophylaxis against MTCT" (McIntyre, 2007).

While the sdNVP regimen is a successful and cost-effective treatment option for prevention of mother-to-child HIV transmission in many resource-constrained countries, there has however been much controversy surrounding its use for this purpose due to the rapid emergence of NVP-associated drug resistance mutations (NVP^R). While these resistance mutations may disappear over time (Eshleman *et al*, 2001), there has been concern that administration of NVP to HIV-1 infected pregnant women may permit selection of NNRTI drug resistance mutations which could compromise the success of a patient's future therapeutic options with ARV drugs like NNRTI's (Sullivan, 2002; Kantor *et al*, 2003; Eshleman *et al*, 2004; Jourdain *et al*, 2004). Eshleman and others have also shown that avoidance of maternal intrapartum sdNVP and provision of (sdNVP and ZDV) to infants only was effective in eliminating the development

of maternal NNRTI drug resistance and reducing the occurrence of NVP^R among infants (Eshleman *et al*, 2006). Compared to a regimen of maternal and infant sdNVP, development of NVP^R among infants occurred less frequently in infants exposed to "*infant only PEP*" (Gray *et al*, 2005). Alternatively the development of NVP^R could also be reduced in infants when both mother and infant received a short course of combivir (ZDV and 3TC) in addition to sdNVP (McIntyre *et al*, 2005).

NVP drug resistance testing was only undertaken among the intrapartum HIV-1 transmitting women to determine plausible reasons for the intrapartum HIV-1 transmission occurring in this group, independent of sdNVP dosing at labour. While five HIV-1 transmitting women lacked detectable resistance to NNRTI, NVP^R was observed in one HIV-1 transmitting woman. Compared to women who lacked NVP^R, the patient with drug resistant mutations had a lower baseline CD4 cell count and higher viral load in both systemic and genital compartments. In keeping with findings from the analysis of two local PMTCT cohorts in Soweto and KZN (Martinson *et al*, 2004), two commonly occurring NNRTI resistance mutations (Y181CY and Y188CY) were also detected in this HIV-1 transmitting mother. Furthermore, sub-typing of the virus confirmed a *clade C* infection among all intrapartum transmitting women. This finding was consistent with the viral subtype, known to dominate in the Sub-Saharan region (Spira *et al*, 2003).

In addition to ARV therapy, prevention and elimination of biological risk factors like STI's are simple and effective PMTCT interventions. In a study by Wilkinson and others in the rural Hlabisa district (KZN), investigators observed that on any given day, 29.4% of the resident female population had one or more detectable STI and that more than 48.0% of the women

were asymptomatic (Wilkinson *et al*, 1999). While screening for STI's in the PIVD cohort was carried out mainly to rule out confounding risk factors, it was rather concerning to note that 47 (39.2%) of the maternal cohort had at least one or more STI detected at antenatal screening. This finding underscores the need for ongoing STI-related counseling and education throughout the duration of pregnancy. In addition, two (2.5%) of women had two types of STI's. Independent of the type of STI, detection of these preventable and curable infections during pregnancy remains clinically relevant due to the resulting adverse maternal (e.g. infertility, puerperal sepsis, chorioamniotis) and neonatal complications (e.g. pneumonia, conjunctivitis) (Moodley and Sturm, 2000). The deadly synergy between HIV-1 and STI's presents a dual dilemma and further complicates this equation. Researchers have shown strong epidemiological evidence of an increased risk of HIV-1 acquisition and transmission in the presence of STI's (Pham-Kanter *et al*, 1996; Kharsany *et al*, 2006). HIV-1 infected pregnant women with untreated STI's continue to act as viral reservoirs of infection for both horizontal and vertical HIV-1 transmission (Wilkinson *et al*, 1997; Sturm *et al*, 1998).

Although the number of *Bacterial vaginosis* infection cases reported in the present study was lower compared to global estimates by the World Health Organization (WHO, 2001), the prevalence rate of other STI's such as; *Treponema pallidum* (0.83%), *Trichomonas vaginalis* (15%) and *Candida albicans* (20.8%) concurred with reports from local and international studies (WHO, 2001; Pham-Kanter *et al*, 1996; Kharsany *et al*, 1997 and Johnson *et al*, 2005b). No statistically significant association was found between STI detection and intrapartum MTCT in the present study [p = 0.2, RR: 2.90, 95% CI: 0.60-15.40]. The presence of any STI was however found to increase HIV-1 RNA levels in both systemic and genital compartments of HIV-1 transmitting women at the time of labour/delivery. These findings

were in accordance with numerous other reports of increased viral shedding in STI presence and a strong association with increased HIV-1 transmission risk (Ghys *et al*, 1997; Pham-Kanter *et al*, 1996; Moodley and Sturm, 2000; Moodley *et al*, 2002; Taha *et al*, 1998; Coombs *et al*, 2003; Mbopi-Kéou *et al*, 2000; Cu-Uvin *et al*, 2001).

In South Africa, STI's are still managed syndromically (Department of Heath, 1999), however studies on MTCT risk factors have found the treatment of such infections at the onset of prenatal care to be quite ineffective for prevention for perinatal HIV-1 transmission (Fawzi *et al*, 2001). The investigators cited treatment failure, re-infection of treated individuals or incident infections after onset of antenatal care, as important predictors of HIV-1 transmission due to ascending infection (Fawzi *et al*, 2001). In the PIVD study all 47 women with detectable STI's were contacted to return for appropriate clinical management. Only 19 (40.6%) of these women sought treatment before delivery. Likewise Wilkinson and others found that 98.0% of symptomatic women in their study also failed to seek treatment. A substantial proportion of STI's, therefore, remain untreated as health-seeking behaviors are influenced by recognition of symptoms, access to health services, attitudes of service providers and gender (Wilkinson *et al*, 1997, 1999; Sturm *et al*, 1998 and Frohlich *et al*, 2007).

Ongoing STI counseling, intensive screening and testing (including partners), promotion of condom usage, safe sex practices and aggressive STI treatment are simple interventions with tremendous impact. Along with enhancing antenatal care and improving pregnancy and neonatal outcomes (Moodley and Sturm, 2000), this simple and cost-effective strategy has great potential in also reducing and preventing both horizontal and vertical HIV-1

transmission. Given that STI treatment is provided at no cost to all attendees in most public health settings, this presents healthcare workers with an invaluable opportunity for strengthening HIV-1 prevention and STI management. In addition, the integration of STI services with family planning and antenatal services could potentially have a huge impact on both epidemics (Frohlich *et al*, 2007).

Novel findings of increased HIV-1 susceptibility with reduced CCL3-L1 gene copy numbers prompted the investigation of this unique immune marker in the PIVD study. A case-control subset of maternal-infant pairs was selected for CCL3-L1 gene copy determination, identification of single nucleotide polymorphisms and haplotype-1 characterization. The observed population median of four in HIV-1 transmitting and non-transmitting women of the PIVD study fell within the range determined by a large international study (Gonzalez *et al*, 2005) and was consistent with reports from local studies (Meddows-Taylor *et al*, 2006; Kuhn *et al*, 2007). In their landmark study, Gonzalez and others observed wide variability in the median number of CCL3-L1 gene copies across various human populations that extended across various continents. Adults and children bearing a higher number of CCL3-L1 gene copies *relative to their population median* were at a lower risk of acquiring HIV-1 infection, while those individuals possessing a lower gene copy number than the population median displayed marked enhanced HIV/AIDS susceptibility (Gonzalez *et al*, 2005). Similar to findings by Meddows-Taylor and others, no relationship was found between maternal CD4 cell counts and infant CCL3-L1 gene copy number (Meddows-Taylor *et al*, 2006). In contrast to the same study, a statistically significant association between infant CCL3-L1 gene copy numbers and maternal viral loads was observed in the PIVD study. There are two plausible reasons as to why the PIVD study was unable to detect any significant association between

increased CCL3-L1 gene copy numbers and reduced HIV-1 susceptibility in both mothers ($p = 0.9$) and infants ($p = 0.4$). The small sample size of the genotyping subset may have not been statistically powered to adequately address this relationship. Another possible explanation could be attributable to the use of NVP itself. In keeping with the observations by Gonzalez *et al*, 2005, local researchers at the NICD (Kuhn *et al*, 2007) successfully confirmed a similar association between high infant CCL3-L1 gene copy numbers and reduced HIV-1 susceptibility within the context of the South African black population. Their finding was however limited to detection "*only in the absence of maternal NVP"* or high maternal viral load (Kuhn *et al*, 2007). Coincidentally the cohort genotyped by Tiemessen and others included mother-infant sample pairs from the previously described South African PEP study wherein mothers were untreated (Gray *et al*, 2005). The investigators postulated that NVP could possess antiviral or immunomodulatory effects that may partially alter the role of CCR5 and its ligands during HIV-1 transmission (Kuhn *et al*, 2007). In the PIVD study, 94.7% of women and 100% of infants received sdNVP as PMTCT standard of care. Kuhn and others found that sdNVP influenced CCL3 production and have suggested that ARV drugs used for PMTCT could possibly obscure genotype-transmission relationships (Kuhn *et al*, 2007). The CCL3-L1 gene dose may be an important genetic correlate of vaccine responsiveness (Gonzalez *et al*, 2005). ARV drugs may also affect the immunogenicity of vaccines that rely on appropriate support from this component of innate immunity. In light of current evidence, sdNVP still remains the cornerstone of therapy for PMTCT in many resource-poor settings and further investigation into the indirect consequences of ARV drugs used for this purpose would be extremely valuable.

Paximadis and others investigated the role of a unique CCL3 haplotype (CCL3-Hap-1) in respect of HIV-1 susceptibility and MTCT (Paximadis *et al*, 2008). They found a lower Hap-1 representation among *in utero* infected infants as opposed to intrapartum infected infants. In the PIVD study, CCL3-L1 Hap-1 allelic representation was detected in six (21.4%) of the exposed-uninfected infants. The absence of allelic representation among the intrapartum-infected infants (n = 7) did not permit a statistical comparison between CCL3-Hap-1 expression and susceptibility to intrapartum MTCT. Consistent with observations by Paximadis and others, no significant relationship was observed between CCL3-Hap-1 presence and maternal CD4 cell counts or maternal viral loads (Paximadis *et al*, 2008). The role played by CCL3-Hap-1 in MTCT is very novel and further investigation in larger maternal-infant cohorts could shed more light into these very intriguing immune dynamics.

CONCLUSION

Detectable CVL HIV-1 RNA (viral load) that correlated well with plasma HIV-1 RNA, in conjunction with sub-optimal NVP drug concentration in maternal CVL during active labour suggests that HIV-1 infected women continue to act as reservoirs for both vertical and horizontal HIV-1 transmission throughout the duration of pregnancy. Early identification of women who need HAART and initiation of such therapy as early as possible during pregnancy not only benefits maternal health but remains the best prophylaxis against mother-to-child HIV-1 transmission. Universal access to HAART and improving strategies to optimize coverage of the current dual ARV regimen of sdNVP and ZDV for PMTCT remain urgent research priorities for several resource-limited settings. Early diagnosis and aggressive treatment of maternal STI's are simple, cost-effective interventions that not only enhance antenatal care, improve pregnancy and neonatal outcomes but also reduce and prevent PMTCT.

STUDY LIMITATIONS

1) This study was limited by the use of cervicovaginal lavage (CVL) fluid. This method results in the dilution of mixed cervicovaginal secretions. All CVL samples were analyzed as *whole unfractionated* samples. While all HIV-1 RNA (viral loads) [copies/ml] were $\log_{10}$ transformed before statistical analysis, the *dilution factor* known to result during the CVL sample collection procedure was not taken into consideration during the analysis of HIV-1 RNA in this study. This widely-accepted practice has been noted in several publications and was also corroborated through personal communication with several research experts (Williamson, 2007; Puren, 2007; Hart, 2007; Kovacs, 2007; Cu-Uvin, 2007 and Reichelderfer, 2007).

2) While all maternal (labour) samples were only collected after maternal sdNVP dosing, data reported in this study represents sampling during active labour and post-delivery.

3) CVL labour sampling was not undertaken among women who had: 1) already progressed well into their labour upon arrival at labour ward; 2) bulging or ruptured membranes or 3) signs of excessive blood contamination.

4) Wet mount microscopy was the screening method employed to detect STI's, as opposed to gram staining, culture or molecular PCR techniques. Wet mount microscopy is also a valid method for STI screening recommended by the World

Health Organisation (WHO, 2005). While the prevalence rates reported in this study could have been under-estimated, the main purpose of undertaking antenatal STI screening was to rule out possible confounding factors which may have influenced detection of HIV-1 RNA (viral load) in maternal CVL.

5) The small number of HIV-1 infected infants (n = 14) in this study, resulted in a smaller subset for CCL3-L1 genotyping analysis. This may have reduced the power of statistical analysis to adequately address relationships observed elsewhere.

6) The Tandem Mass spectrophotometry method used to quantify NVP in maternal CVL has only been validated for use in plasma samples at the UCT host laboratory.

7) The drug reference standard (NVP) used at the UCT host laboratory was a generic drug obtained from Aspen Pharmacare.

8) The Cobas AmpliPrep/Cobas TaqMan system utilized for HIV-1 RNA (viral load) quantitation in CVL is currently only FDA-approved for use in human plasma samples.

RECOMMENDATIONS

- Further investigation into ARV pharmacology and the effects of drug interaction, drug penetration and drug affinity in various bodily fluids and viral compartments.
- Earlier identification of HIV-1 infected, pregnant women and commencement of HAART in these women as soon as possible to improve overall maternal health and prevent MTCT. This strategy prevents the unnecessary exposure of infants to ARV drugs.
- Improve access and optimize coverage of the current dual regimen of ZDV and sdNVP for PMTCT [where HAART is unavailable]. This regimen reduces the likelihood of the development of NNRTI drug-resistant mutations typically associated with NVP monotherapy.
- Promotion of the infant PEP strategy among healthcare workers as a valuable alternate strategy for reaching those women who: a) were missed by PMTCT, b) booked late for antenatal visits, c) arrived in advanced labour with unknown HIV status, d) refused or missed their intrapartum sdNVP dosing and also in instances where infants are born at home or on route to hospital.
- Due consideration should be given to CCL3-L1 and other immune markers, in light of their implications in vaccine development.
- Further exploration into the antiviral and immunomodulatory effects of NVP as well as other ARV drugs.
- Continued STI screening by wet mount microscopy and aggressive treatment of STI's during the course of pregnancy, with partner involvement where possible.

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APPENDICES

Appendix 1: MTCT rates and ARV prophylactic options for PMTCT in breastfeeding populations (Moodley and Coovadia, 2008)

	ANTEPARTUM				INTRAPARTUM	POSTPARTUM					MTCT rate (%)	
	< 14w	14-28w	28-34w	≥34-36w	LABOUR	1w	4w	6w	2m	6m	8w-24m	15m - 24m
Malawi (NVAZ)						NVP					20.9	
HIVNET 012					ZDV	ZDV					20	25.8
South Africa (PEP)						ZDV					18.1	
Ivory Coast ANRS				ZDV							18	22.5
Zimbabwe					NVP	NVP					17.8	
Ivory Coast CDC				ZDV							16.5	22.5
Malawi (NVAZ)					NVP	ZDV+NVP					16.3	
Zimbabwe					ZDV+NVP	ZDV+NVP					15.5	
Malawi (NVAZ)						ZDV+NVP					15.3	
South Africa (PEP)						NVP					14.3	
PETRA					ZDV+3TC						14.2	20
Malawi (NVAZ)					NVP	NVP					14.1	
SAINT					NVP	NVP					12.3	
HIVNET 012					NVP	NVP					11.8	15.7

Footnotes- (w): weeks; (m): months, (ZDV): Zidovudine, (3TC): Lamivudine, (NVP): Nevirapine, (ddI): Didanosine

Appendix 1 continued

	ANTEPARTUM				INTRAPARTUM	POSTPARTUM (ARV's and TRANSMISSION %)						
	< 14w	14-28w	28-34w	≥34-36w	LABOUR	1w	4w	6w	2m	6m	8w-24m	15m - 24m
SAINT					ZDV	ZDV+					9.3	
Botswana (HARVARD)				ZDV	ZDV+NVP	ZDV+	ZDV				9.1	
PETRA					ZDV+3TC	ZDV+					8.9	18.1
Ivory Coast ANRS				ZDV	ZDV+NVP	ZDV+					6.5	
PETRA				ZDV+3TC		ZDV+					5.7	14.9
Tanzania (MITRA)				ZDV+3T	ZDV+3TC	ZDV+	3TC				5.1	
Ivory Coast ANRS				ZDV+3T	ZDV+3TC+NVP	ZDV+					4.7	
Botswana (HARVARD)				ZDV	ZDV+NVP	ZDV+	ZDV				4.3	
Botswana (HARVARD)				ZDV	ZDV	ZDV+	ZDV				3.7	
Uganda, Rwanda				ZDV+dd	ZDV+ddI	ZDV+	NVP				2.4	
						NVP						
Uganda, Rwanda				ZDV+dd	ZDV+ddI	ZDV+	3TC					
						3TC						

Footnotes- (w): weeks; (m): months, (ZDV): Zidovudine, (3TC): Lamivudine, (NVP): Nevirapine, (ddI): Didanosine

Appendix 2: MTCT rates and ARV prophylactic options for PMTCT in non-breastfeeding populations

(Moodley and Coovadia, 2008)

	ANTEPARTUM				INTRAPARTUM	POSTPARTUM (ARV's and TRANSMISSION %)					
	<14w	14-28w	28-34w	≥34-36w	LABOUR	1w	4w	6w	2m	6m	8w-24m
Thailand PHPT				ZDV		ZDV					10.5
Thailand CDC-1999				ZDV		ZDV					9.4
Thailand PHPT				ZDV		ZDV					8.6
PACTG076		ZDV				ZDV					8.3
Thailand PHPT			ZDV			ZDV					6.5
Thailand PHPT-2			ZDV			ZDV					6.3
Botswana (HARVARD)				ZDV	ZDV+NVP	ZDV+NVP	ZDV				5.6
Thailand PHPT			ZDV			ZDV					4.7
Thailand CDC-2004				ZDV	ZDV+NVP	ZDV+NVP					4.6
Thailand Ministry				ZDV+3TC		ZDV					2.8
Thailand PHPT-2			ZDV		ZDV+NVP	ZDV					2.8
Thailand PHPT-2			ZDV		ZDV+NVP	ZDV+NVP					2
France ANRS		ZDV+3TC				ZDV+3TC					1.6
PACTG316	MONO/DUAL/DUAL+PI	ZDV+NVP				ZDV+NVP					1.4

Footnotes- (w): weeks; (m): months, (ZDV): Zidovudine, (3TC): Lamivudine, (NVP): Nevirapine, (ddI): Didanosine

Appendix 3a: Standard of care – ARV's for the prevention of mother-to-child HIV-1 transmission (WHO, 2006b)

	HAART available			HAART not available	
	Maternal HAART indicated	Maternal HAART considered[a]	Maternal HAART not yet indicated	Capacity to deliver full range of ARVs for PMTCT exists	Capacity to only deliver minimal range of ARVs for PMTCT exists (e.g. AZT not available)
	A	B	C		D
Mother					
Antepartum	HAART	HAART	AZT starting at 28 weeks or as soon as feasible thereafter		-
Intrapartum	HAART	HAART	AZT + single dose NVP[b] Consider[c]: 3TC		Single dose NVP
Postpartum	HAART	HAART	Consider[c]: AZT + 3TC for 7 days		-
Infant	AZT for 7 days[d]	AZT for 7 days[d]	Single dose NVP + AZT for 7 days[d]		Single dose NVP

PMTCT = Prevention of Mother-to-Child Transmission of HIV HAART = Highly Active Antiretroviral Therapy ARV = Antiretroviral
AZT = Azidothymidine or Zidovudine 3TC = Lamivudine NVP = Nevirapine

[a] **Maternal HAART considered:** the revised WHO adult guidelines recommend HAART be considered for patients with clinical stage I and II with CD4 cell count below 350 10^6 cells/L, particularly if closer to 200-250 10^6 cells/L. Toxicity to the initiation of long-term NVP-containing HAART may be a concern in pregnant women with CD4 count between 250 and 350 10^6 cells/L. However recent data from resource-limited countries among pregnant and post partum women in Africa and Thailand suggest a low toxicity associated with the use of NVP in this context. The expert consultation concluded that NVP-containing HAART can be considered in this subgroup, or alternatively a triple-NRTI regimen.

[b] **If the woman receives at least 4 weeks of AZT during pregnancy**, omission of maternal NVP dose may be considered.

[c] **If the woman is symptomatic and conditions to deliver the following interventions exist**, a seven-day tail of AZT + 3TC given to the mother after delivery can be considered to reduce the emergence of NVP resistance, and is advised if HAART is foreseeable expected to be started soon after delivery..

[d] **If the mother receives less than 4 weeks of AZT or HAART during pregnancy**, infant AZT dosing should be extended to 4 weeks.

Appendix 3b: Women presenting around delivery and having received no ARV's for PMTCT (WHO, 2006b)

	Woman in labour, known to be HIV positive with no prior antiretroviral			No maternal ARV PMTCT prophylaxis
	Capacity to deliver full range of ARVs for PMTCT exists		Capacity to deliver only minimal range of ARVs for PMTCT exists (e.g. AZT not available)	
	Option 1	Option 2		
Mother				
Intrapartum	Single dose NVP + AZT	AZT + 3TC	Single dose NVP	-
Postpartum	-	AZT + 3TC for 7 days	-	-
Infant	Single dose NVP + AZT for 4 weeks	AZT + 3TC for 7 days	Single dose NVP	Single dose NVP + AZT for 4 weeks

PMTCT = Prevention of Mother-to-Child Transmission of HIV ARV = Antiretroviral
AZT = Azidothymidine or Zidovudine 3TC = Lamivudine NVP = Nevirapine

In all cases, mothers need to be assessed postpartum for need for therapy.

Appendix 4a: NVP resistance (NVP^R) data from clinical studies - sdNVP only

Name of study/ trial sdNVP Only	Genotyping system	Sampling time point	NVP^R prevalence among mothers	NVP^R prevalence among infants	Dominant clade at trial or study location
NVAZ	Viroseq™ (Applied Biosystems)	6-8 weeks	69.2% Eshleman *et al*, 2005a	87.0% Eshleman *et al*, 2005b	Subtype C (Malawi)
PMTCT PROGRAM	Allele-specific RT PCR for K103N	4 weeks	40% Lehman *et al*, 2005	No data	no data
HIVNET 012	Viroseq™	6 weeks	25.1% Eshleman *et al*, 2004	45.8% Eshleman *et al*, 2001	Subtype A/D (Uganda)
HIVNET 006	Viroseq™	6 weeks	20% Jackson *et al*, 2000	No data	Subtype A/D (Uganda)
Resistance study: PMTCT cohort	Viroseq™	6 weeks	44% Martinson *et al*, 2004	48% Martinson *et al*, 2004	Subtype C South Africa
HPTN 023	TruGen HIV-1 kit	8 weeks	34.4% Lee *et al*, 2005	No data	Subtype E/B Zimbabwe

Appendix 4b: NVP resistance (NVP^R) data from clinical studies - sdNVP as part of combination therapy

Name of trial or a study with sdNVP as part of combination therapy	Genotyping system	Sample time point	NVP^R prevalence among mothers	NVP^R prevalence among infants	Dominant clade at trial or study location
PACTG 316	TruGen HIV-1	6 weeks	14.7% Cunningham *et al*, 2002	No data	USA (B), Europe (B) Brazil (F)
PHPT-2	Viroseq™	6 weeks	No data	10.3% Ngo-Giang *et al*, 2005	Subtype E/B (Thailand)
TOPS	Sequencing analysis	6 weeks	60.3% McIntyre *et al*, 2005	77.8% McIntyre *et al*, 2005	Subtype C South Africa
ANRS 1201 DITRAME	Sequence RT & Sequence navigator	4 weeks	33.3% Chaix *et al*, 2004; 2006	23.1% Chaix *et al*, 2004;2006	Circulating recombinant forms (CRF) Cote'd Ivoire
Thailand CDC	TruGen HIV-1 kit	4 weeks	17.4% Chalermchokcharoenkit *et al*, 2004	20% Chalermchok-charoenkit *et al*, 2004	Subtype A/E Thailand
DITRAME PLUS	No data	4 weeks	1.14% Chaix *et al*, 2005	6% Chaix *et al*, 2005	Circulating recombinant forms (CRF) Abidjan, Cote d'Ivoire
SAINT	No data	4-6 weeks	No data	52.5% Sullivan, 2002	Subtype C South Africa
MASHI	Viroseq™	One month postpartum	45% Shapiro *et al*, 2006	No data	Subtype C Botswana
DREAM	No data	8-12 weeks	12% Palombi *et al*, 2005	No data	Mozambique

Appendix 5: Patient case report form (CRF)

PHARMACO-IMMUNOLOGICAL-VIROLOGICAL DYNAMICS IN INTRAPARTUM HIV-1 TRANSMISSION (PIVD STUDY)

PRE-NVP DOSING (BASELINE ANTENATAL VISIT)

Study ID: PIVD

Patient Initials:

ANC No:

Hospital No:

Informed consent obtained: Yes/no Enrolment date: /

Age: (yrs) Gestational age at visit:

Parity: Gravida: WR: Rh:

HIV status:

Was NVP tab dispensed: Yes/no Date of NVP issue: / /

Date of NVP expiry: / /

NOTES:

_____

_____

_____

_____

_____

LABORATORY EVALUATIONS	DONE Y/N	DATE	RESULT
CD4 cell count			cells/ml
HIV-1 ELISA			
FBC			Hb WCC Platelets
SAMPLE COLLECTION			
CVL (10 ml)			
Blood (x2 EDTA)			
CVL volume collected			
CVL pH @ collection			
CVL blood level			
SAMPLE STORAGE			
Whole blood aliquoted			No of 1ml (WB) aliquots stored:
Plasma spun & aliquoted			No of 1ml (P) aliquots stored:
CVL aliquoted			No of 1ml (CVL) aliquots stored:
LABORATORY ASSAYS	**DONE Y/N**	**DATE OF ASSAY**	**RESULTS**
Wet mount microscopy			
CVL HIV RNA PCR			copies/ml
Plasma HIV RNA PCR			copies/ml
Plasma NVP concentration			ng/ml
CVL NVP concentration			ng/ml
CCL3 gene copy no			copy/pdg
CCL3L1 gene copy no			copy/pdg
OTHER			

POST-NVP DOSING (LABOUR/DELIVERY)

Date of onset of labour: / / Time am/pm

NVP administered? Yes/no Date: / / Time am/pm

Was more than one dose given? yes/no Time am/pm

Date of rupture of membranes: / / Time am/pm

Mode of delivery: NVD Emergency C/S Other

Episiotomy: Yes/No Instrumental delivery: Forceps/vacuum extraction

No of live infants born: Gestational age at delivery:

Apgar score: (1min) (5min)

NOTES :

_____

_____

Baby Study ID:

Date of birth: / / Time of birth: / / Sex: Male/ Female

Birth weight: kg OFC: cm Length: cm

Baby NVP given: yes/no Date: / / Time: am/pm

Was a 2nd dose given? Yes/No Date: / / Time: am/pm

Condition of baby: Well /unwell

NOTES :

_____

_____

LABORATORY EVALUATIONS	DONE Y/N	DATE	RESULT
FBC			
SAMPLE COLLECTION			
(MUM) CVL (10 ml)			
Blood (X2 EDTA)			
Total volume CVL collected			
pH of CVL			
Blood level of CVL			
(INFANT) Blood (X1 EDTA)			
SAMPLE STORAGE			
Mum whole blood aliquoted			No of 1ml (WB) aliquots stored:
Mum blood spun & plasma aliquoted			No of 1ml (P) aliquots stored:
CVL vortexed & aliquoted			No of 1ml (CVL) aliquots stored:
Infant blood spun & plasma aliquoted			No of 200ul aliquots stored:
LABORATORY ASSAYS	**DONE Y/N**	**DATE OF ASSAY**	**RESULTS**
Mum CVL HIV-1 RNA PCR			copies/ml
Mum Plasma HIV-1 RNA PCR			copies/ml
Mum Plasma NVP concentration			ng/ml
Mum CVL NVP concentration			ng/ml
Mum CCL3 gene copy no			copy/p.d.g
Mum CCL3-L1 gene copy			copy/p.d.g
Infant plasma HIV-1 RNA PCR			copies/ml
Infant plasma NVP concentration			ng/ml
Infant HIV-1 DNA PCR			

INFANT FOLLOW-UP ASSESSMENTS

4-WEEK POST DELIVERY:

Date of visit: / / Baby weight: kg

Is baby still being formula-fed? Yes/No

Condition of baby: Well /unwell

NOTES:

LABORATORY EVALUATIONS	DONE Y/N	DATE	RESULT
INFANT SAMPLE COLLECTION			
Blood (X1 EDTA)			
SAMPLE STORAGE			
Blood spun and plasma aliquoted			No of 200ul (P) aliquots stored:
LABORATORY ASSAYS	**DONE Y/N**	**DATE OF ASSAY**	**RESULTS**
HIV DNA PCR			
HIV RNA PCR			copies/ml
NVP concentration			ng/ml

INFANT FOLLOW-UP ASSESSMENTS

6-WEEKS POST DELIVERY:

Date of visit: / / Baby weight: kg

Is baby still being bottle-fed? Yes/No

Condition of baby: Well /unwell

NOTES:

LABORATORY EVALUATIONS	DONE Y/N	DATE	RESULT
INFANT SAMPLE COLLECTION			
Blood (X1 EDTA)			
SAMPLE STORAGE			
Whole blood aliquoted for host genetics			No of 200ul (P) aliquots stored:
Blood spun & plasma aliquoted			No of 200ul (P) aliquots stored:
LABORATORY ASSAYS	**DONE Y/N**	**DATE OF ASSAY**	**RESULTS**
HIV DNA PCR			
NVP concentration			ng/ml
CCL3 gene copy no			copy/p.d.g
CCL3-L1 gene copy no			copy/p.d.g

End of study

Appendix 6: Reagents and solutions – NVP quantitation assay

A) Nevirapine stock solution:

A Nevirapine stock solution was first prepared by weighing out 0.001 grams of Nevirapine powder (drug reference standard) obtained from Aspen Pharmacare, South Africa). A calibrated analytical balance (Satorius) was used for accuracy. The Nevirapine powder was dissolved in one milliliter (1000 µl) of HPLC grade Acetonitrile (MERCK, Germany) to obtain a final concentration of 1 mg/ml. All stocks were stored at -80^{o}C after use.

B) Reserpine stock solution:

0.001 grams of Reserpine powder (Sigma, St Louis, MO) was weighed out using a calibrated analytical balance (Satorius). Reserpine powder was reconstituted with one milliliter (1000 µl) of HPLC grade Acetonitrile (MERCK, Germany) and served as the internal standard in the assay.

C) 100 µg/ml Nevirapine working standard:

1 ml of Acetonitrile (MERCK, Germany) was added to a clean eppendorf tube. A 100 µl volume of Acetonitrile was removed and discarded. This was replaced with 100 µl of Nevirapine stock (1 mg/ml) [from **A** above]. The eppendorf tube was vortexed thoroughly and labeled appropriately.

D) 10 µg/ml Nevirapine working standard:

1 ml of Acetonitrile (MERCK, Germany) was added to an eppendorf. A 100 µl volume of Acetonitrile was removed and discarded. This was replaced with 100 µl of Nevirapine Working standard (100 µg/ml) [from **C** above]. The eppendorf tube was vortexed thoroughly and labeled appropriately.

E) Nevirapine calibration standards [S1- S10] *for plasma samples:*

Calibration standards contained ten non-zero calibrators. A calibration curve was run with every batch of samples run throughout the analysis period to evaluate the

calibration status of the assay and to also calculate the concentration of each unknown study sample within the batch. Calibration standards were made up in drug-free plasma obtained from the blood bank at Groote Schuur Hospital, Cape Town. Calibration standards were spiked to the final concentrations of 100, 200, 500, 800, 1000, 1300, 1500, 2000, 5000, and 10 000 ng/ml respectively.

F) Quality Control Samples for plasma samples

Three quality control samples, [C1 (low), C2 (medium), and C3 (high)] with concentrations of 300 ng/ml (C1), 1800 ng/ml (C2) and 9000 ng/ml (C3) respectively were used to monitor the performance of the Nevirapine quantitation assay. These quality control samples were equally interspersed among unknown patient samples during each sample run.

G) Protein Precipitation Solution:

Protein Precipitation contained 80% Methanol: 20% 0.2 M Zinc Sulphate (v: v)

For a 250 ml volume of protein precipitation solution:
Measure 200 ml of HPLC grade Methanol (MERCK, Germany). Add 50 ml of 0.2 M Zinc Sulphate (Sigma, St Louis, MO), followed by 150 µl of Reserpine (1 mg/ml) [from B]. Mix thoroughly and store in cold room when not in use.

H) 0.2 M Zinc Sulphate

Weigh out 28.75 grams of Zinc Sulphate (Sigma, St Louis, MO). Add 450 ml of deionised water (MERCK, Germany). Place onto a mixer until dissolved. Pour mixture into a measuring cylinder and top up to the 500 ml mark.

I) Mobile Phase:

Consisted of two bottles of reagents:
Bottle A: 10% Methanol
Bottle B: 97% Methanol + 3% 10 mM Ammonium Acetate in 0.1% Glacial Acetic Acid

Bottle A: 10% Methanol

Pour 100 ml of Methanol (MERCK, Germany) into a measuring cylinder. Add 900 ml of deionised water (MERCK, Germany). Cover with parafilm. Invert gently. Filter using vacuum filter apparatus. Sonnicate solution for ten minutes. Label appropriately and store at room temperature.

Bottle B: (preparations)

0.1% Glacial Acetic Acid

Pour 1000 ml of deionised water (MERCK, Germany) into a clean bottle. Remove 1 ml and discard. Add 1 ml of concentrated analytical grade Glacial Acetic Acid (Pal Chemicals). Re-cap the bottle, invert to mix and label appropriately.

10 mM Ammonium Acetate in 0.1% Glacial Acetic Acid

Weigh out 0.38544 grams of Ammonium Acetate (MERCK, Germany). Measure 450 ml of 0.1% of Glacial Acetic Acid from above. Add Ammonium Acetate (MERCK, Germany) to it. Mix until dissolved. Top up to 500 ml with 0.1% Glacial Acetic Acid from above and label appropriately.

Bottle B: (final)

Measure 970 ml of Methanol (MERCK, Germany). Add 30 ml of 10 mM Ammonium Acetate in 0.1% Glacial Acetic Acid from above. Mix and filter using vacuum filter apparatus. Sonnicate solution for 10 minutes, label appropriately and store at room temperature.

Appendix 7: Wet mount microscopy for STI screening (WHO, 2005)

WET MOUNT MICROSCOPY

Direct microscopic examination of vaginal discharge can aid in diagnosis of yeast infection (*Candida albicans*), bacterial vaginosis and trichomoniasis.	
Collect specimen	Take a sample of discharge with a swab from the side walls or deep in the vagina where discharge accumulates.
Prepare slide	Mix specimen with 1 or 2 drops of saline on a glass slide and cover with a coverslip.
What to look for	Examine at 100X magnification and look for typical jerky movement of motile trichomonads. Examine at 400X magnification to look for yeast cells and trichomonads. To make identification of yeast cells easier in wet mount slides, mix the vaginal swab in another drop of saline and add a drop of 10% potassium hydroxide to dissolve other cells.
Important	Look for evidence of other vaginal or cervical infections—multiple infections are common.

Appendix 8a: Drug resistance report for study patient without NNRTI drug resistant mutations

STANFORD UNIVERSITY
HIV DRUG RESISTANCE DATABASE
A curated public database designed to represent, store, and analyze the divergent forms of data underlying HIV drug resistance

HOME GENOTYPE-RX GENOTYPE-PHENO GENOTYPE-CLINICAL HIVdb PROGRAM

HIVdb: Genotypic Resistance Interpretation Algorithm

SeqID: PIVD162.TXT_1 PIVD162 Date: 14-Feb-2008

Summary Data

Sequence includes PR: codons: 1 - 99
Sequence includes RT: codons: 1 - 312

There are no insertions or deletions

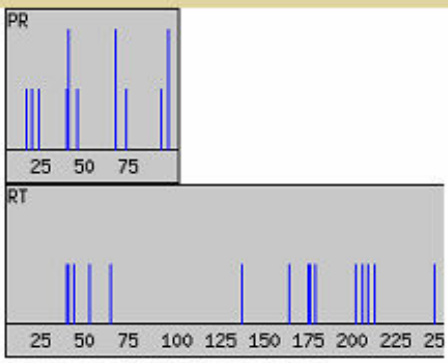
Subtype and % similarity to closest reference isolate:

1. *PR: C (90.9%)*
2. *RT: C (93.8%)*

Sequence Quality Assessment

Gene	QA Problem	Codons
PR	Stop Codons, Frame Shifts:	*None*
PR	B,D,H,V,N:	*None*
PR	Unusual Residues:	*None*

Gene	QA Problem	Codons
RT	Stop Codons, Frame Shifts:	*None*
RT	B,D,H,V,N:	*None*
RT	Unusual Residues:	*None*



Blue lines indicate differences from consensus B; tall blue lines indicate sites associated with drug resistance.
Red lines indicate QA problems.

Drug Resistance Interpretation

PI Major Resistance Mutations: None
PI Minor Resistance Mutations: None
Other Mutations: T12S, I15V, L19I, E35D, M36I, R41K, L63LAPV, H69K, L89M, I93L

	Protease Inhibitors
atazanavir (ATV)	Susceptible
darunavir (DRV)	Susceptible
fosamprenavir (FPV)	Susceptible
indinavir (IDV)	Susceptible
lopinavir (LPV)	Susceptible
nelfinavir (NFV)	Susceptible
saquinavir (SQV)	Susceptible
tipranavir (TPV)	Susceptible

PR Comments

The following 2 of the 21 tipranavir RESIST study mutations were present: M36I, H69K (Baxter J et al J Virology 2006).
M36I/V are weakly associated with PI resistance when present with other mutations. M36I occurs commonly in certain non-subtype B viruses. M36L/T are rare variants of uncertain significance.
L63P is a common polymorphism that becomes even more common in persons receiving PIs.
H69K is a highly polymorphic residue that was weakly associated with a decreased virologic response to TPV in the RESIST trials.
I93L is a common polymorphism that becomes even more common in persons receiving PIs.

Drug Resistance Interpretation

NRTI Resistance Mutations:	None
NNRTI Resistance Mutations:	None
Other Mutations:	V35T, E36A, T39E, S48T, V60IV, I135V, S162C, K173A, Q174KR, D177E, T200A, E204DE, Q207A, R211K, V245Q, A272P, T286V, E291D, V292I, I293V

Nucleoside RTI		Non-Nucleoside RTI	
lamivudine (3TC)	Susceptible	**delavirdine (DLV)**	Susceptible
abacavir (ABC)	Susceptible	**efavirenz (EFV)**	Susceptible
zidovudine (AZT)	Susceptible	**etravirine (ETR)**	Susceptible
stavudine (D4T)	Susceptible	**nevirapine (NVP)**	Susceptible
didanosine (DDI)	Susceptible		
emtricitabine (FTC)	Susceptible		
tenofovir (TDF)	Susceptible		

RT Comments

None

Mutation Scoring

	ATV	DRV	FPV	IDV	LPV	NFV	SQV	TPV
Total:	0	0	0	0	0	0	0	0

	3TC	ABC	AZT	D4T	DDI	FTC	TDF	DLV	EFV	ETR	NVP
Total:	0	0	0	0	0	0	0	0	0	0	0

STANFORD UNIVERSITY
HIV DRUG RESISTANCE DATABASE
A curated public database designed to represent, store, and analyze the divergent forms of data underlying HIV drug resistance

HOME GENOTYPE-RX GENOTYPE-PHENO GENOTYPE-CLINICAL HIVdb PROGRAM

HIVdb: Genotypic Resistance Interpretation Algorithm

SeqID: PIVD316.TXT_1 PIVD316 Date: 14-Feb-2008

Summary Data

Sequence includes PR: codons: 1 - 99
Sequence includes RT: codons: 1 - 312

There are no insertions or deletions

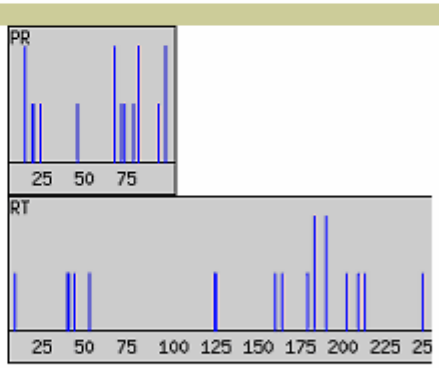
Subtype and % similarity to closest reference isolate:

1. *PR: C (90.9%)*
2. *RT: C (93.7%)*

Sequence Quality Assessment

Gene	QA Problem	Codons
PR	Stop Codons, Frame Shifts:	*None*
PR	B,D,H,V,N:	*None*
PR	Unusual Residues:	*None*

Gene	QA Problem	Codons
RT	Stop Codons, Frame Shifts:	*None*
RT	B,D,H,V,N:	*None*
RT	Unusual Residues:	*None*



Blue lines indicate differences from consensus B; tall blue lines indicate sites associated with drug resistance.
Red lines indicate QA problems.

Drug Resistance Interpretation

PI Major Resistance Mutations: None
PI Minor Resistance Mutations: **L10LIM**
Other Mutations: K14KR, I15V, L19I, R41K, L63T, C67S, H69K, T74S, V77I, L89M, I93IL

	Protease Inhibitors
atazanavir (ATV)	Susceptible
darunavir (DRV)	Susceptible
fosamprenavir (FPV)	Susceptible
indinavir (IDV)	Susceptible
lopinavir (LPV)	Susceptible
nelfinavir (NFV)	Potential low-level resistance
saquinavir (SQV)	Susceptible
tipranavir (TPV)	Susceptible

PR Comments

M is an atypical mutation at codon 10.

The following 1 of the 21 tipranavir RESIST study mutations were present: H69K (Baxter J et al J Virology 2006).
L10I/V/F/R are associated with resistance to each of the PIs when present with other mutations. L10I/V occur commonly in 5-10% of untreated persons. L10FRY are nonpolymorphic.
H69K is a highly polymorphic residue that was weakly associated with a decreased virologic response to TPV in the RESIST trials.
T74S is associated with reduced NFV susceptibility. It occasionally occurs in untreated persons. T74P is a nonpolymorphic mutation that occurs more commonly in heavily treated patients. It has been associated with decreased virological response to TPV but probably is associated with resistance to multiple PIs.
V77I is a common polymorphism that is associated with NFV therapy.
I93L is a common polymorphism that becomes even more common in persons receiving PIs.

Drug Resistance Interpretation

NRTI Resistance Mutations:	None
NNRTI Resistance Mutations:	**Y181CY, Y188CY** ←
Other Mutations:	P4S, V35T, E36A, T39E, S48T, K122E, D123GS, A158AS, S162A, D177N, T200A, Q207E, R211KR, V245Q, A272Q, T286A, E291D, V292I, I293V

Nucleoside RTI	
lamivudine (3TC)	Susceptible
abacavir (ABC)	Susceptible
zidovudine (AZT)	Susceptible
stavudine (D4T)	Susceptible
didanosine (DDI)	Susceptible
emtricitabine (FTC)	Susceptible
tenofovir (TDF)	Susceptible

Non-Nucleoside RTI	
delavirdine (DLV)	High-level resistance
efavirenz (EFV)	Intermediate resistance
etravirine (ETR)	Intermediate resistance
nevirapine (NVP)	High-level resistance

RT Comments

The following 1 of the 13 etravirine DUET study mutations were present: Y181C (Katlama C et al, IAS 2007).
Y181C/I/V cause high-level resistance to NVP and DLV and low-level resistance to EFV. Y181C/I/V reduces ETR susceptibility by 5-10 fold and lays the mutational foundation for the development of higher levels of ETR resistance. Y181C increases susceptibility to AZT and TDF.
Y188C causes high-level resistance to NVP and low-level resistance to EFV and DLV; its effect on ETR is not known.

Y181CY partially reverses AZT and possibly d4T resistance caused by other mutations. AZT mutations in this isolate include: none.

Mutation Scoring

	ATV	DRV	FPV	IDV	LPV	NFV	SQV	TPV
L10ILM	2	2	2	2	2	2	2	2
T74S	0	0	0	0	0	10	0	0
Total:	2	2	2	2	2	12	2	2

	3TC	ABC	AZT	D4T	DDI	FTC	TDF	DLV	EFV	ETR	NVP
Y181CY	0	0	0	0	0	0	0	60	20	30	60
Y188CY	-	-	-	-	-	-	-	25	25	10	60
Total:	0	0	0	0	0	0	0	85	45	40	120

Appendix 9: Preparation of a mini agarose gel (1%)

A gel casting tray was rinsed and dried. The ends of the casting tray were sealed with tape before the tray was placed onto a level surface. The level of the comb was adjusted so that it rested with a few millimeters of space between the teeth and the tray. This allowed for the formation of wells in the agarose gel.

An agarose gel (1%) was prepared by combining 50 ml of 1x Tris-Borate-EDTA or TBE buffer [Bulk 10x TBE buffer, Invitrogen, UK] and 1 tablet (0.5 grams) Agarose (Bioline, USA) in a glass bottle. The bottle was capped loosely and the agarose solution was placed into a microwave for two minutes on high power (or until agarose solution was clear). The agarose solution was gently swirled to mix, and then allowed to stand on the work bench for five minutes. Once the solution had cooled, 0.5 μg/ml Ethidium Bromide (Sigma, St Louis, MO), was added into the agarose solution and the entire solution was carefully poured into the prepared casting tray. The gel was allowed to solidify (5-10 minutes) and the comb was carefully removed.

Maternal & infant samples

Appendix 10: Representative CCL3 sequence data

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C327-CCL3-end…  #1  TCATGAGTTG AGAGCTGAGA GTTAGAGAAT AGCTCAAAGA
C289-CCL3       #1  TCATGAGTTG AGAGCTGAGA GTTAGAGAAT AGCTCAAAGA
C194-CCL3       #1  TCATGAGTTG AGAGCTGAGA GTTAGAGAAT AGCTCAAAGA
M400-CCL3       #1  TCATGAGTTG AGAGCTGAGA GTTAGAGAAT AGCTCAAAGA
C232-CCL3       #1  TCATGAGTTG AGAGCTGAGA GTTAGAGAAT AGCTCAAAGA
C113-CCL3       #1  TCATGAGTTG AGAGCTGAGA GTTAGAGAAT AGCTCAAAGA
C05-CCL3        #1  TCATGAGTTG AGAGCTGAGA GTTAGAGAAT AGCTCAAAGA
C351-CCL3       #1  TCATGAGTTG AGAGCTGAGA GTTAGAGAAT AGCTCAAAGA
C45-CCL3        #1  TCATGAGTTG AGAGCTGAGA GTTAGAGAAT AGCTCAAAGA

C327-CCL3-end… #41  TGCTATTCTT GGATATCCTG AGCCCCTGTG GTCACCAGGG
C289-CCL3      #41  TGCTATTCTT GGATATCCTG AGCCCCTGTG GTCACCAGGG
C194-CCL3      #41  TGCTATTCTT GGATATCCTG AGCCCCTGTG GTCACCAGGG
M400-CCL3      #41  TGCTATTCTT GGATATCCTG AGCCCCTGTG GTCACCAGGG
C232-CCL3      #41  TGCTATTCTT GGATATCCTG AGCCCCTGTG GTCACCAGGG
C113-CCL3      #41  TGCTATTCTT GGATATCCTG AGCCCCTGTG GTCACCAGGG
C05-CCL3       #41  TGCTATTCTT GGATATCCTG AGCCCCTGTG GTCACCAGGG
C351-CCL3      #41  TGCTATTCTT GGATATCCTG AGCCCCTGTG GTCACCAGGG
C45-CCL3       #41  TGCTATTCTT GGATATCCTG AGCCCCTGTG GTCACCAGGG

C327-CCL3-end… #81  ACCCTGAGTT GTGCAACTTA GCATGACAGC ATCACTACGC
C289-CCL3      #81  ACCCTGAGTT GTGCAACTTA GCATGACAGC ATCACTACGC
C194-CCL3      #81  ACCCTGAGTT GTGCAACTTA GCATGACAGC ATCACTAYGC
M400-CCL3      #81  ACCCTGAGTT GTGCAACTTA GCATGACAGC ATCACTACGC
C232-CCL3      #81  ACCCTGAGTT GTGCAACTTA GCATGACAGC ATCACTACGC
C113-CCL3      #81  ACCCTGAGTT GTGCAACTTA GCATGACAGC ATCACTAYGC
C05-CCL3       #81  ACCCTGAGTT GTGCAACTTA GCATGACAGC ATCACTAYGC
C351-CCL3      #81  ACCCTGAGTT GTGCAACTTA GCATGACAGC ATCACTAYGC
C45-CCL3       #81  ACCCTGAGTT GTGCAACTTA GCATGACAGC ATCACTAYGC
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13 September 2007

C327-CCL3-en…	#121	TTAAAAATTT CCCTCCTCAC CCCCAGATTC CATTTCCCCA
C289-CCL3	#121	TTAAAAATTT CCCTCCTCAC CCCCAGATTC CATTTCCCCA
C194-CCL3	#121	TTAAAAATTT CCCTCCTCAC CCCCAGATTC CATTTCCCCA
M400-CCL3	#121	TTAAAAATTT CCCTCCTCAC CCCCAGATTC CATTTCCCCA
C232-CCL3	#121	TTAAAAATTT CCCTCCTCAC CCCCAGATTC CATTTCCCCA
C113-CCL3	#121	TTAAAAATTT CCCTCCTCAC CCCCAGATTC CATTTCCCCA
C05-CCL3	#121	TTAAAAATTT CCCTCCTCAC CCCCAGATTC CATTTCCCCA
C351-CCL3	#121	TTAAAAATTT CCCTCCTCAC CCCCAGATTC CATTTCCCCA
C45-CCL3	#121	TTAAAAATTT CCCTCCTCAC CCCCAGATTC CATTTCCCCA
C327-CCL3-en…	#161	TCCGCCAGGG CTGCCTATAA AGAGGAGAGC TGGTTTCAGA
C289-CCL3	#161	TCCGCCAGGG CTGCCTATAA AGAGGAGAGC TGGTTTCAGA
C194-CCL3	#161	TCCGCCAGGG CTGCCTATAA AGAGGAGAGC TGGTTTCAGA
M400-CCL3	#161	TCCGCCAGGG CTGCCTATAA AGAGGAGAGC TGGTTTCAGA
C232-CCL3	#161	TCCGCCAGGG CTGCCTATAA AGAGGAGAGC TGGTTTCAGA
C113-CCL3	#161	TCCGCCAGGG CTGCCTATAA AGAGGAGAGC TGGTTTCAGA
C05-CCL3	#161	TCCGCCAGGG CTGCCTATAA AGAGGAGAGC TGGTTTCAGA
C351-CCL3	#161	TCCGCCAGGG CTGCCTATAA AGAGGAGAGC TGGTTTCAGA
C45-CCL3	#161	TCCGCCAGGG CTGCCTATAA AGAGGAGAGC TGGTTTCAGA
C327-CCL3-en…	#201	CTTCAGAAGG ACACGGGCAG CAGACAGTGG TCAGTCCTTT
C289-CCL3	#201	CTTCAGAAGG ACACGGGCAG CAGACAGTGG TCAGTCCTTT
C194-CCL3	#201	CTTCAGAAGG ACACGGGCAG CAGACAGTGG TCAGTCCTTT
M400-CCL3	#201	CTTCAGAAGG ACACGGGCAG CAGACAGTGG TCAGTCCTTT
C232-CCL3	#201	CTTCAGAAGG ACACGGGCAG CAGACAGTGG TCAGTCCTTT
C113-CCL3	#201	CTTCAGAAGG ACACGGGCAG CAGACAGTGG TCAGTCCTTT
C05-CCL3	#201	CTTCAGAAGG ACACGGGCAG CAGACAGTGG TCAGTCCTTT
C351-CCL3	#201	CTTCAGAAGG ACACGGGCAG CAGACAGTGG TCAGTCCTTT
C45-CCL3	#201	CTTCAGAAGG ACACGGGCAG CAGACAGTGG TCAGTCCTTT

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C289-CCL3       #241  CTTGGCTCTG CTGACACTCG AGCCCACATT CCGTCACCTG
C194-CCL3       #241  CTTGGCTCTG CTGACACTCG AGCCCACATT CCGTCACCTG
M400-CCL3       #241  CTTGGCTCTG CTGACACTCG AGCCCACATT CCGTCACCTG
C232-CCL3       #241  CTTGGCTCTG CTGACACTCG AGCCCACATT CCGTCACCTG
C113-CCL3       #241  CTTGGCTCTG CTGACACTCG AGCCCACATT CCGTCACCTG
C05-CCL3        #241  CTTGGCTCTG CTGACACTCG AGCCCACATT CCGTCACCTG
C351-CCL3       #241  CTTGGCTCTG CTGACACTCG AGCCCACATT CCGTCACCTG
C45-CCL3        #241  CTTGGCTCTG CTGACACTCG AGCCCACATT CCGTCACCTG

C327-CCL3-en... #281  CTCAGAATCA TGCAGGTCTC CACTGCTGCC CTTGCTGTCC
C289-CCL3       #281  CTCAGAATCA TGCAGGTCTC CACTGCTGCC CTTGCTGTCC
C194-CCL3       #281  CTCAGAATCA TGCAGGTCTC CACTGCTGCC CTTGCTGTCC
M400-CCL3       #281  CTCAGAATCA TGCAGGTCTC CACTGCTGCC CTTGCTGTCC
C232-CCL3       #281  CTCAGAATCA TGCAGGTCTC CACTGCTGCC CTTGCTGTCC
C113-CCL3       #281  CTCAGAATCA TGCAGGTCTC CACTGCTGCC CTTGCTGTCC
C05-CCL3        #281  CTCAGAATCA TGCAGGTCTC CACTGCTGCC CTTGCTGTCC
C351-CCL3       #281  CTCAGAATCA TGCAGGTCTC CACTGCTGCC CTTGCTGTCC
C45-CCL3        #281  CTCAGAATCA TGCAGGTCTC CACTGCTGCC CTTGCTGTCC

C327-CCL3-en... #321  TCCTCTGCAC CATGGCTCTC TGCAACCAGT TCTCTGCATC
C289-CCL3       #321  TCCTCTGCAC CATGGCTCTC TGCAACCAGT TCTCTGCATC
C194-CCL3       #321  TCCTCTGCAC CATGGCTCTC TGCAACCAGT TCTCTGCATC
M400-CCL3       #321  TCCTCTGCAC CATGGCTCTC TGCAACCAGT TCTCTGCATC
C232-CCL3       #321  TCCTCTGCAC CATGGCTCTC TGCAACCAGT TCTCTGCATC
C113-CCL3       #321  TCCTCTGCAC CATGGCTCTC TGCAACCAGT TCTCTGCATC
C05-CCL3        #321  TCCTCTGCAC CATGGCTCTC TGCAACCAGT TCTCTGCATC
C351-CCL3       #321  TCCTCTGCAC CATGGCTCTC TGCAACCAGT TCTCTGCATC
C45-CCL3        #321  TCCTCTGCAC CATGGCTCTC TGCAACCAGT TCTCTGCATC
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C327-CCL3-en…	#361	ACGTGAGTCT GAGTTTCGTT GTGGGTATCA CCACTCTCTG
C289-CCL3	#361	ACGTGAGTCT GAGTTTCGTT GTGGGTATCA CCACTCTCTG
C194-CCL3	#361	ACGTGAGTCT GAGTTTCGTT GTGGGTATCA CCACTCTCTG
M400-CCL3	#361	ACGTGAGTCT GAGTTTCGTT GTGGGTATCA CCACTCTCTG
C232-CCL3	#361	ACGTGAGTCT GAGTTTCGTT GTGGGTATCA CCACTCTCTG
C113-CCL3	#361	ACGTGAGTCT GAGTTTCGTT GTGGGTATCA CCACTCTCTG
C05-CCL3	#361	ACGTGAGTCT GAGTTTCGTT GTGGGTATCA CCACTCTCTG
C351-CCL3	#361	ACGTGAGTCT GAGTTTCGTT GTGGGTATCA CCACTCTCTG
C45-CCL3	#361	ACGTGAGTCT GAGTTTCGTT GTGGGTATCA CCACTCTCTG
C327-CCL3-en…	#401	GCCATGGTTA GACCACATCA ATCTTTTCTT GTGGCCTAAA
C289-CCL3	#401	GCCATGGTTA GACCACATCA ATCTTTTCTT GTGGCCTAAA
C194-CCL3	#401	GYCATGGTTA GACCACATCA ATCTTTTCTT GTGGCCTAAA
M400-CCL3	#401	GCCATGGTTA GACCACATCA ATCTTTTCTT GTGGCCTAAA
C232-CCL3	#401	GCCATGGTTA GACCACATCA ATCTTTTCTT GTGGCCTAAA
C113-CCL3	#401	GYCATGGTTA GACCACATCA ATCTTTTCTT GTGGCCTAAA
C05-CCL3	#401	GYCATGGTTA GACCACATCA ATCTTTTCTT GTGGCCTAAA
C351-CCL3	#401	GYCATGGTTA GACCACATCA ATCTTTTCTT GTGGCCTAAA
C45-CCL3	#401	GYCATGGTTA GACCACATCA ATCTTTTCTT GTGGCCTAAA
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C289-CCL3	#441	AGCCCCCAAG AGAAAAGAGA ACTTCTTAAA GGGCTGCCAA
C194-CCL3	#441	AGCCCCCAAG AGAAAAGAGA ACTTCTTAAA GGGCTGCCAA
M400-CCL3	#441	AGCCCCCAAG AGAAAAGAGA ACTTCTTAAA GGGCTGCCAA
C232-CCL3	#441	AGCCCCCAAG AGAAAAGAGA ACTTCTTAAA GGGCTGCCAA
C113-CCL3	#441	AGCCCCCAAG AGAAAAGAGA ACTTCTTAAA GGGCTGCCAA
C05-CCL3	#441	AGCCCCCAAG AGAAAAGAGA ACTTCTTAAA GGGCTGCCAA
C351-CCL3	#441	AGCCCCCAAG AGAAAAGAGA ACTTCTTAAA GGGCTGCCAA
C45-CCL3	#441	AGCCCCCAAG AGAAAAGAGA ACTTCTTAAA GGGCTGCCAA

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C289-CCL3     #481  ACATCTTGGT CTTTCTCTTT AAGACTTTTA TTTTTATCTC
C194-CCL3     #481  ACATCTTGGT CTTTCTCTTT AAGACTTTTA TTTTTATCTC
M400-CCL3     #481  ACATCTTGGT CTTTCTCTTT AAGACTTTTA TTTTTATCTC
C232-CCL3     #481  ACATCTTGGT CTTTCTCTTT AAGACTTTTA TTTTTATCTC
C113-CCL3     #481  ACATCTTGGT CTTTCTCTTT AAGACTTTTA TTTTTATCTC
C05-CCL3      #481  ACATCTTGGT CTTTCTCTTT AAGACTTTTA TTTTTATCTC
C351-CCL3     #481  ACATCTTGGT CTTTCTCTTT AAGACTTTTA TTTTTATCTC
C45-CCL3      #481  ACATCTTGGT CTTTCTCTTT AAGACTTTTA TTTTTATCTC

C327-CCL3-en… #521  TAGAAGGGGT CTTAGCCCCC TAGTCTCCAG GTATGAGAAT
C289-CCL3     #521  TAGAAGGGGT CTTAGCCCCC TAGTCTCCAG GTATGAGAAT
C194-CCL3     #521  TAGAAGGGGT CTTAGCCCCC TAGTCTCCAG GTATGAGAAT
M400-CCL3     #521  TAGAAGGGGT CTTAGCCCCC TAGTCTCCAG GTATGAGAAT
C232-CCL3     #521  TAGAAGGGGT CTTAGCCCCC TAGTCTCCAG GTATGAGAAT
C113-CCL3     #521  TAGAAGGGGT CTTAGCCCCC TAGTCTCCAG GTATGAGAAT
C05-CCL3      #521  TAGAAGGGGT CTTAGCCCCC TAGTCTCCAG GTATGAGAAT
C351-CCL3     #521  TAGAAGGGGT CTTAGCCCCC TAGTCTCCAG GTATGAGAAT
C45-CCL3      #521  TAGAAGGGGT CTTAGCCCCC TAGTCTCCAG GTATGAGAAT

C327-CCL3-en… #561  CTAGGCAGGG GCAGGGGAGT TACAGTCCCT TTTACAGATA
C289-CCL3     #561  CTAGGCAGGG GCAGGGGAGT TACAGTCCCT TTTACAGATA
C194-CCL3     #561  CTAGGCAGGG GCAGGGGAGT TACAGTCCCT TTTACAGATA
M400-CCL3     #561  CTAGGCAGGG GCAGGGGAGT TACAGTCCCT TTTACAGATA
C232-CCL3     #561  CTAGGCAGGG GCAGGGGAGT TACAGTCCCT TTTACAGATA
C113-CCL3     #561  CTAGGCAGGG GCAGGGGAGT TACAGTCCCT TTTACAGATA
C05-CCL3      #561  CTAGGCAGGG GCAGGGGAGT TACAGTCCCT TTTACAGATA
C351-CCL3     #561  CTAGGCAGGG GCAGGGGAGT TACAGTCCCT TTTACAGATA
C45-CCL3      #561  CTAGGCAGGG GCAGGGGAGT TACAGTCCCT TTTACAGATA
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C289-CCL3	#601	GAAAAACAGG GTTCGAAACG AATCAGTTAG CAAGAGGCAG
C194-CCL3	#601	GAAAAACAGG GTTCGAAACG AATCAGTTAG CAAGAGGCAG
M400-CCL3	#601	GAAAAACAGG GTTCGAAACG AATCAGTTAG CAAGAGGCAG
C232-CCL3	#601	GAAAAACAGG GTTCGAAACG AATCAGTTAG CAAGAGGCAG
C113-CCL3	#601	GAAAAACAGG GTTCGAAACG AATCAGTTAG CAAGAGGCAG
C05-CCL3	#601	GAAAAACAGG GTTCGAAACG AATCAGTTAG CAAGAGGCAG
C351-CCL3	#601	GAAAAACAGG GTTCGAAACG AATCAGTTAG CAAGAGGCAG
C45-CCL3	#601	GAAAAACAGG GTTCGAAACG AATCAGTTAG CAAGAGGCAG
C327-CCL3-en…	#641	AATCCAGGGC TGCTTACTTC CCAGTGGGGT ATGTTGTTCA
C289-CCL3	#641	AATCCAGGGC TGCTTACTTC CCAGTGGGGT ATGTTGTTCA
C194-CCL3	#641	AATCCAGGGC TGCTTACTTC CCAGTGGGGT ATGTTGTTCA
M400-CCL3	#641	AATCCAGGGC TGCTTACTTC CCAGTGGGGT ATGTTGTTCA
C232-CCL3	#641	AATCCAGGGC TGCTTACTTC CCAGTGGGGT ATGTTGTTCA
C113-CCL3	#641	AATCCAGGGC TGCTTACTTC CCAGTGGGGT ATGTTGTTCA
C05-CCL3	#641	AATCCAGGGC TGCTTACTTC CCAGTGGGGT ATGTTGTTCA
C351-CCL3	#641	AATCCAGGGC TGCTTACTTC CCAGTGGGGT ATGTTGTTCA
C45-CCL3	#641	AATCCAGGGC TGCTTACTTC CCAGTGGGGT ATGTTGTTCA
C327-CCL3-en…	#681	CTCTCCAGCT CACTCTAGGT CTCCCAGGAG CTCTGTCCCT
C289-CCL3	#681	CTCTCCAGCT CACTCTAGGT CTCCCAGGAG CTCTGTCCCT
C194-CCL3	#681	CTCTCCAGCT CACTCTAGGT CTCCCAGGAG CTCTGTCCCT
M400-CCL3	#681	CTCTCCAGCT CACTCTAGGT CTCCCAGGAG CTCTGTCCCT
C232-CCL3	#681	CTCTCCAGCT CACTCTAGGT CTCCCAGGAG CTCTGTCCCT
C113-CCL3	#681	CTCTCCAGCT CACTCTAGGT CTCCCAGGAG CTCTGTCCCT
C05-CCL3	#681	CTCTCCAGCT CACTCTAGGT CTCCCAGGAG CTCTGTCCCT
C351-CCL3	#681	CTCTCCAGCT CACTCTAGGT CTCCCAGGAG CTCTGTCCCT
C45-CCL3	#681	CTCTCCAGCT CACTCTAGGT CTCCCAGGAG CTCTGTCCCT

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C289-CCL3     #721  TGGATGTCTT ATGAGAGATG TCCAAGGCTT CTCTTGGGTT
C194-CCL3     #721  TGGATGTCTT ATGAGAGATG TCCAAGGYTT CTCTTGGGTT
M400-CCL3     #721  TGGATGTCTT ATGAGAGATG TCCAAGGCTT CTCTTGGGTT
C232-CCL3     #721  TGGATGTCTT ATGAGAGATG TCCAAGGCTT CTCTTGGGTT
C113-CCL3     #721  TGGATGTCTT ATGAGAGATG TCCAAGGYTT CTCTTGGGTT
C05-CCL3      #721  TGGATGTCTT ATGAGAGATG TCCAAGGYTT CTCTTGGGTT
C351-CCL3     #721  TGGATGTCTT ATGAGAGATG TCCAAGGYTT CTCTTGGGTT
C45-CCL3      #721  TGGATGTCTT ATGAGAGATG TCCAAGGYTT CTCTTGGGTT
C327-CCL3-en… #761  GGGGTATGAC TTCTTGAACC AGACAAAATT CCCTGAAGAG
C289-CCL3     #761  GGGGTATGAC TTCTTGAACC AGACAAAATT CCCTGAAGAG
C194-CCL3     #761  GGGGTATGAC TTCTTGAACC AGACAAAATT CCCTGAAGAG
M400-CCL3     #761  GGGGTATGAC TTCTTGAACC AGACAAAATT CCCTGAAGAG
C232-CCL3     #761  GGGGTATGAC TTCTTGAACC AGACAAAATT CCCTGAAGAG
C113-CCL3     #761  GGGGTATGAC TTCTTGAACC AGACAAAATT CCCTGAAGAG
C05-CCL3      #761  GGGGTATGAC TTCTTGAACC AGACAAAATT CCCTGAAGAG
C351-CCL3     #761  GGGGTATGAC TTCTTGAACC AGACAAAATT CCCTGAAGAG
C45-CCL3      #761  GGGGTATGAC TTCTTGAACC AGACAAAATT CCCTGAAGAG
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C289-CCL3     #801  AACTGAGATA AGAGAACAGT CCGTTCAGGT ATCTGGATCA
C194-CCL3     #801  AACTGAGATA AGAGAACAGT CCGTTCAGGT ATCTGGATCA
M400-CCL3     #801  AACTGAGATA AGAGAACAGT CCGTTCAGGT ATCTGGATCA
C232-CCL3     #801  AACTGAGATA AGAGAACAGT CCGTTCAGGT ATCTGGATCA
C113-CCL3     #801  AACTGAGATA AGAGAACAGT CCGTTCAGGT ATCTGGATCA
C05-CCL3      #801  AACTGAGATA AGAGAACAGT CCGTTCAGGT ATCTGGATCA
C351-CCL3     #801  AACTGAGATA AGAGAACAGT CCGTTCAGGT ATCTGGATCA
C45-CCL3      #801  AACTGAGATA AGAGAACAGT CCGTTCAGGT ATCTGGATCA
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C327-CCL3-en…	#841	CACAGAGAAA CAGAGAACCC ACTATGAAGA GTCAAGGAGA
C289-CCL3	#841	CACAGAGAAA CAGAGAACCC ACTATGAAGA GTCAAGGAGA
C194-CCL3	#841	CACAGAGAAA CAGAGAACCC ACTATGAAGA GTCAAGGAGA
M400-CCL3	#841	CACAGAGAAA CAGAGAACCC ACTATGAAGA GTCAAGGAGA
C232-CCL3	#841	CACAGAGAAA CAGAGAACCC ACTATGAAGA GTCAAGGAGA
C113-CCL3	#841	CACAGAGAAA CAGAGAACCC ACTATGAAGA GTCAAGGAGA
C05-CCL3	#841	CACAGAGAAA CAGAGAACCC ACTATGAAGA GTCAAGGAGA
C351-CCL3	#841	CACAGAGAAA CAGAGAACCC ACTATGAAGA GTCAAGGAGA
C45-CCL3	#841	CACAGAGAAA CAGAGAACCC ACTATGAAGA GTCAAGGAGA

C327-CCL3-en…	#881	AAGAAGGATA CAGACAGAAA CAAAGAGACA TTTCTCAGCA
C289-CCL3	#881	AAGAAGGATA CAGACAGAAA CAAAGAGACA TTTCTCAGCA
C194-CCL3	#881	AAGAAGGATA CAGACAGAAA CAAAGAGACA TTTCTCAGCA
M400-CCL3	#881	AAGAAGGATA CAGACAGAAA CAAAGAGACA TTTCTCAGCA
C232-CCL3	#881	AAGAAGGATA CAGACAGAAA CAAAGAGACA TTTCTCAGCA
C113-CCL3	#881	AAGAAGGATA CAGACAGAAA CAAAGAGACA TTTCTCAGCA
C05-CCL3	#881	AAGAAGGATA CAGACAGAAA CAAAGAGACA TTTCTCAGCA
C351-CCL3	#881	AAGAAGGATA CAGACAGAAA CAAAGAGACA TTTCTCAGCA
C45-CCL3	#881	AAGAAGGATA CAGACAGAAA CAAAGAGACA TTTCTCAGCA

C327-CCL3-en…	#921	AAAATGCCCA AATGCCTTCC AGTCACTTGG TCTGAGCAAG
C289-CCL3	#921	AAAATGCCCA AATGCCTTCC AGTCACTTGG TCTGAGCAAG
C194-CCL3	#921	AAAATGCCCA AATGCCTTCC AGTCACTTGG TCTGAGCAAG
M400-CCL3	#921	AAAATGCCCA AATGCCTTCC AGTCACTTGG TCTGAGCAAG
C232-CCL3	#921	AAAATGCCCA AATGCCTTCC AGTCACTTGG TCTGAGCAAG
C113-CCL3	#921	AAAATGCCCA AATGCCTTCC AGTCACTTGG TCTGAGCAAG
C05-CCL3	#921	AAAATGCCCA AATGCCTTCC AGTCACTTGG TCTGAGCAAG
C351-CCL3	#921	AAAATGCCCA AATGCCTTCC AGTCACTTGG TCTGAGCAAG
C45-CCL3	#921	AAAATGCCCA AATGCCTTCC AGTCACTTGG TCTGAGCAAG

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C289-CCL3     #961   CYTGCCTTCC TCAACTGCTC GGGGATCAGA AGCTGCCTGG
C194-CCL3     #961   CCTGCCTTCC TCAACTGCTC GGGGATCAGA AGCTGCCTGG
M400-CCL3     #961   CCTGCCTTCC TCAACTGCTC GGGGATCAGA AGCTGCCTGG
C232-CCL3     #961   CCTGCCTTCC TCAACTGCTC GGGGATCAGA AGCTGCCTGG
C113-CCL3     #961   CCTGCCTTCC TCAACTGCTC GGGGATCAGA AGCTGCCTGG
C05-CCL3      #961   CCTGCCTTCC TCAACTGCTC GGGGATCAGA AGCTGCCTGG
C351-CCL3     #961   CCTGCCTTCC TCAACTGCTC GGGGATCAGA AGCTGCCTGG
C45-CCL3      #961   CCTGCCTTCC TCAACTGCTC GGGGATCAGA AGCTGCCTGG

C327-CCL3-e… #1001   CCTTTTCTTC TGAGCTGTGA CTCGGGCTCA TTCTCTTCCT
C289-CCL3    #1001   CCTTTTCTTC TGAGCTGTGA CTCGGGCTCA TTCTCTTCCT
C194-CCL3    #1001   CCTTTTCTTC TGAGCTGTGA CTCGGGCTCA TTCTCTTCCT
M400-CCL3    #1001   CCTTTTCTTC TGAGCTGTGA CTCGGGCTCA TTCTCTTCCT
C232-CCL3    #1001   CCTTTTCTTC TGAGCTGTGA CTCGGGCTCA TTCTCTTCCT
C113-CCL3    #1001   CCTTTTCTTC TGAGCTGTGA CTCGGGCTCA TTCTCTTCCT
C05-CCL3     #1001   CCTTTTCTTC TGAGCTGTGA CTCGGGCTCA TTCTCTTCCT
C351-CCL3    #1001   CCTTTTCTTC TGAGCTGTGA CTCGGGCTCA TTCTCTTCCT
C45-CCL3     #1001   CCTTTTCTTC TGAGCTGTGA CTCGGGCTCA TTCTCTTCCT

C327-CCL3-e… #1041   TTCTCCACAG TTGCTGCTGA CACGCCGACC GCCTGCTGCT
C289-CCL3    #1041   TTCTCCACAG TTGCTGCTGA CACGCCGACC GCCTGCTGCT
C194-CCL3    #1041   TTCTCCACAG TTGCTGCTGA CACGCCGACC GCCTGCTGCT
M400-CCL3    #1041   TTCTCCACAG TTGCTGCTGA CACGCCGACC GCCTGCTGCT
C232-CCL3    #1041   TTCTCCACAG TTGCTGCTGA CACGCCGACC GCCTGCTGCT
C113-CCL3    #1041   TTCTCCACAG TTGCTGCTGA CACGCCGACC GCCTGCTGCT
C05-CCL3     #1041   TTCTCCACAG TTGCTGCTGA CACGCCGACC GCCTGCTGCT
C351-CCL3    #1041   TTCTCCACAG TTGCTGCTGA CACGCCGACC GCCTGCTGCT
C45-CCL3     #1041   TTCTCCACAG TTGCTGCTGA CACGCCGACC GCCTGCTGCT
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C327-CCL3-e… #1081  TCAGCTACAC CTCCCGGCAG ATTCCACAGA ATTTCATAGC
C289-CCL3    #1081  TCAGCTACAC CTCCCGGCAG ATTCCACAGA ATTTCATAGC
C194-CCL3    #1081  TCAGCTACAC CTCCCGGCAG ATTCCACAGA ATTTCATAGC
M400-CCL3    #1081  TCAGCTACAC CTCCCGGCAG ATTCCACAGA ATTTCATAGC
C232-CCL3    #1081  TCAGCTACAC CTCCCGGCAG ATTCCACAGA ATTTCATAGC
C113-CCL3    #1081  TCAGCTACAC CTCCCGGCAG ATTCCACAGA ATTTCATAGC
C05-CCL3     #1081  TCAGCTACAC CTCCCGGCAG ATTCCACAGA ATTTCATAGC
C351-CCL3    #1081  TCAGCTACAC CTCCCGGCAG ATTCCACAGA ATTTCATAGC
C45-CCL3     #1081  TCAGCTACAC CTCCCGGCAG ATTCCACAGA ATTTCATAGC
C327-CCL3-e… #1121  TGACTACTTT GAGACGAGCA GCCAGTGCTC CAAGCCCRGT
C289-CCL3    #1121  TGACTACTTT GAGACGAGCA GCCAGTGCTC CAAGCCCGGT
C194-CCL3    #1121  TGACTACTTT GAGACGAGCA GCCAGTGCTC CAAGCCYGGT
M400-CCL3    #1121  TGACTACTTT GAGACGAGCA GCCAGTGCTC CAAGCCCGGT
C232-CCL3    #1121  TGACTACTTT GAGACGAGCA GCCAGTGCTC CAAGCCCGGT
C113-CCL3    #1121  TGACTACTTT GAGACGAGCA GCCAGTGCTC CAAGCCYGGT
C05-CCL3     #1121  TGACTACTTT GAGACGAGCA GCCAGTGCTC CAAGCCYGGT
C351-CCL3    #1121  TGACTACTTT GAGACGAGCA GCCAGTGCTC CAAGCCYGGT
C45-CCL3     #1121  TGACTACTTT GAGACGAGCA GCCAGTGCTC CAAGCCYGGT
C327-CCL3-e… #1161  GTCATGTAAG TGCCAGTCTT CCTGCTCACC TCTATGGAGG
C289-CCL3    #1161  GTCATGTAAG TGCCAGTCTT CCTGCTCACC TCTATGGAGG
C194-CCL3    #1161  GTCATGTAAG TGCCAGTCTT CCTGCTCACC TCTATGGAGG
M400-CCL3    #1161  GTCATGTAAG TGCCAGTCTT CCTGCTCACC TCTATGGAGG
C232-CCL3    #1161  GTCATGTAAG TGCCAGTCTT CCTGCTCACC TCTATGGAGG
C113-CCL3    #1161  GTCATGTAAG TGCCAGTCTT CCTGCTCACC TCTATGGAGG
C05-CCL3     #1161  GTCATGTAAG TGCCAGTCTT CCTGCTCACC TCTATGGAGG
C351-CCL3    #1161  GTCATGTAAG TGCCAGTCTT CCTGCTCACC TCTATGGAGG
C45-CCL3     #1161  GTCATGTAAG TGCCAGTCTT CCTGCTCACC TCTATGGAGG
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C327-CCL3-e… #1201  TAGGGAGGGT CAGGGTTGGG GCAGAGACAG GCCAGAAGGC
C289-CCL3    #1201  TAGGGAGGGT CAGGGTTGGG GCAGAGACAG GCCAGAAGGC
C194-CCL3    #1201  TAGGGAGGGT CAGGGTTGGG GCAGAGACAG GCCAGAAGGC
M400-CCL3    #1201  TAGGGAGGGT CAGGGTTGGG GCAGAGACAG GCCAGAAGGC
C232-CCL3    #1201  TAGGGAGGGT CAGGGTTGGG GCAGAGACAG GCCAGAAGGC
C113-CCL3    #1201  TAGGGAGGGT CAGGGTTGGG GCAGAGACAG GCCAGAAGGC
C05-CCL3     #1201  TAGGGAGGGT CAGGGTTGGG GCAGAGACAG GCCAGAAGGC
C351-CCL3    #1201  TAGGGAGGGT CAGGGTTGGG GCAGAGACAG GCCAGAAGGC
C45-CCL3     #1201  TAGGGAGGGT CAGGGTTGGG GCAGAGACAG GCCAGAAGGC

C327-CCL3-e… #1241  TATCCTGGAA AGGCCCAGCC TTCAGGAGCC TATCGGGGAT
C289-CCL3    #1241  YATCCTGGAA AGGCCCAGCC TTCAGGAGCC TATCGGGGAT
C194-CCL3    #1241  TATCCTGGAA AGGCCCAGCC TTCAGGAGCC TATCGGGGAT
M400-CCL3    #1241  YATCCTGGAA AGGCCCAGCC TTCAGGAGCC TATCGGGGAT
C232-CCL3    #1241  TATCCTGGAA AGGCCCAGCC TTCAGGAGCC TATCGGGGAT
C113-CCL3    #1241  TATCCTGGAA AGGCCCAGCC TTCAGGAGCC TATCGGGGAT
C05-CCL3     #1241  TATCCTGGAA AGGCCCAGCC TTCAGGAGCC TATCGGGGAT
C351-CCL3    #1241  TATCCTGGAA AGGCCCAGCC TTCAGGAGCC TATCGGGGAT
C45-CCL3     #1241  TATCCTGGAA AGGCCCAGCC TTCAGGAGCC TATCGGGGAT

C327-CCL3-e… #1281  ACAGGACGCA GGGCTCCGAG GTGTGACCTG ACTTGGAGCT
C289-CCL3    #1281  ACAGGACGCA GGGCWCYGAG GTGTGACCTG ACTTGGAGCT
C194-CCL3    #1281  ACAGGACGCA GGGCTCCGAG GTGTGACCTG ACTTGGAGCT
M400-CCL3    #1281  ACAGGACGCA GGGCTCCGAG GTGTGACCTG ACTTGGAGCT
C232-CCL3    #1281  ACAGGACGCA GGGCTCCGAG GTGTGACCTG ACTTGGAGCT
C113-CCL3    #1281  ACAGGACGCA GGGCTCCGAG GTGTGACCTG ACTTGGAGCT
C05-CCL3     #1281  ACAGGACGCA GGGCTCCGAG GTGTGACCTG ACTTGGAGCT
C351-CCL3    #1281  ACAGGACGCA GGGCTCCGAG GTGTGACCTG ACTTGGAGCT
C45-CCL3     #1281  ACAGGACGCA GGGCTCCGAG GTGTGACCTG ACTTGGAGCT
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C327-CCL3-e… #1321  GGAGTGAGGC ATGTGTTACA GAGTCAGGAA GGGCTGCCCC
C289-CCL3    #1321  GGAGTGAGGC ATGTGTTACA GAGTCAGGAA GGGCTGCCCC
C194-CCL3    #1321  GGAGTGAGGC ATGTGTTACA GAGTCAGGAA GGGCTGCCCC
M400-CCL3    #1321  GGAGTGAGGC ATGTGTTACA GAGTCAGGAA GGGCTGCCCC
C232-CCL3    #1321  GGAGTGAGGC ATGTGTTACA GAGTCAGGAA GGGCTGCCCC
C113-CCL3    #1321  GGAGTGAGGC ATGTGTTACA GAGTCAGGAA GGGCTGCCCC
C05-CCL3     #1321  GGAGTGAGGC ATGTGTTACA GAGTCAGGAA GGGCTGCCCC
C351-CCL3    #1321  GGAGTGAGGC ATGTGTTACA GAGTCAGGAA GGGCTGCCCC
C45-CCL3     #1321  GGAGTGAGGC ATGTGTTACA GAGTCAGGAA GGGCTGCCCC
C327-CCL3-e… #1361  AGCCCAGAGG AAAGGGACAG GAAGAAGGAG GCAGCGGGAC
C289-CCL3    #1361  AGCCCAGAGG AAAGGGACAG GAAGAAGGAG GCAGCGGGAC
C194-CCL3    #1361  AGCCCAGAGG AAAGGGACAG GAAGAAGGAG GCAGCGGGAC
M400-CCL3    #1361  AGCCCAGAGG AAAGGGACAG GAAGAAGGAG GCAGCGGGAC
C232-CCL3    #1361  AGCCCAGAGG AAAGGGACAG GAAGAAGGAG GCAGCGGGAC
C113-CCL3    #1361  AGCCCAGAGG AAAGGGACAG GAAGAAGGAG GCAGCGGGAC
C05-CCL3     #1361  AGCCCAGAGG AAAGGGACAG GAAGAAGGAG GCAGCGGGAC
C351-CCL3    #1361  AGCCCAGAGG AAAGGGACAG GAAGAAGGAG GCAGCGGGAC
C45-CCL3     #1361  AGCCCAGAGG AAAGGGACAG GAAGAAGGAG GCAGCGGGAC
C327-CCL3-e… #1401  ACTCTGAGGG CCACCCCTAC TGAGTCACTG AGAGAAGCTC
C289-CCL3    #1401  ACTCTGAGGG CCACCCCTAC TGAGTCACTG AGAGAAGCTC
C194-CCL3    #1401  ACTCTGAGGG CCACCCCTAC TGAGTCACTG AGAGAAGCTC
M400-CCL3    #1401  ACTCTGAGGG CCACCCCTAC TGAGTCACTG AGAGAAGCTC
C232-CCL3    #1401  ACTCTGAGGG CCACCCCTAC TGAGTCACTG AGAGAAGCTC
C113-CCL3    #1401  ACTCTGAGGG CCACCCCTAC TGAGTCACTG AGAGAAGCTC
C05-CCL3     #1401  ACTCTGAGGG CCACCCCTAC TGAGTCACTG AGAGAAGCTC
C351-CCL3    #1401  ACTCTGAGGG CCACCCCTAC TGAGTCACTG AGAGAAGCTC
C45-CCL3     #1401  ACTCTGAGGG CCACCCCTAC TGAGTCACTG AGAGAAGCTC
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C327-CCL3-e… #1441  TCTAGACAGA GATAGGCAGG GGGCCCCTGA AAGAGGAGCA
C289-CCL3    #1441  TCTAGACRGA GATAGGCAGG GGGCCCCTGA AAGAGGAGCA
C194-CCL3    #1441  TCTAGACRGA GATAGGCAGG GGGCCCCTGA AAGAGGAGCA
M400-CCL3    #1441  TCTAGACAGA GATAGGCAGG GGGCCCCTGA AAGAGGAGCA
C232-CCL3    #1441  TCTAGACAGA GATAGGCAGG GGGCCCCTGA AAGAGGAGCA
C113-CCL3    #1441  TCTAGACRGA GATAGGCAGG GGGCCCCTGA AAGAGGAGCA
C05-CCL3     #1441  TCTAGACRGA GATAGGCAGG GGGCCCCTGA AAGAGGAGCA
C351-CCL3    #1441  TCTAGACRGA GATAGGCAGG GGGCCCCTGA AAGAGGAGCA
C45-CCL3     #1441  TCTAGACRGA GATAGGCAGG GGGCCCCTGA AAGAGGAGCA

C327-CCL3-e… #1481  AGCCCTGAGC TGCCCAGGAC AGAGAGCAGA ATGGTGGGGC
C289-CCL3    #1481  AGCCCTGAGC TGCCCAGGAC AGAGAGCAGA ATGGTGGGGC
C194-CCL3    #1481  AGCCCTGAGC TGCCCAGGAC AGAGAGCAGA ATGGTGGGGC
M400-CCL3    #1481  AGCCCTGAGC TGCCCAGGAC AGAGAGCAGA ATGGTGGGGC
C232-CCL3    #1481  AGCCCTGAGC TGCCCAGGAC AGAGAGCAGA ATGGTGGGGC
C113-CCL3    #1481  AGCCCTGAGC TGCCCAGGAC AGAGAGCAGA ATGGTGGGGC
C05-CCL3     #1481  AGCCCTGAGC TGCCCAGGAC AGAGAGCAGA ATGGTGGGGC
C351-CCL3    #1481  AGCCCTGAGC TGCCCAGGAC AGAGAGCAGA ATGGTGGGGC
C45-CCL3     #1481  AGCCCTGAGC TGCCCAGGAC AGAGAGCAGA ATGGTGGGGC

C327-CCL3-e… #1521  CATGGTGGGC CCAGGATTCC CCTGCTGGAT TCCCCAGTGC
C289-CCL3    #1521  CATGGTGGGC CCAGGATTCC CCTGCTGGAT TCCCCAGTGC
C194-CCL3    #1521  CATGGTGGGC CCAGGATTCC CCTGCTGGAT TCCCCAGTGC
M400-CCL3    #1521  CATGGTGGGC CCAGGATTCC CCTGCTGGAT TCCCCAGTGC
C232-CCL3    #1521  CATGGTGGGC CCAGGATTCC CCTGCTGGAT TCCCCAGTGC
C113-CCL3    #1521  CATGGTGGGC CCAGGATTCC CCTGCTGGAT TCCCCAGTGC
C05-CCL3     #1521  CATGGTGGGC CCAGGATTCC CCTGCTGGAT TCCCCAGTGC
C351-CCL3    #1521  CATGGTGGGC CCAGGATTCC CCTGCTGGAT TCCCCAGTGC
C45-CCL3     #1521  CATGGTGGGC CCAGGATTCC CCTGCTGGAT TCCCCAGTGC
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C327-CCL3-e… #1561  TTAACTCTTC CTCCCTTCTC CACAGCTT
C289-CCL3    #1561  TTAACTCTTC CTCCCTTCTC CACAGCTTCC TAACCAAGCG
C194-CCL3    #1561  TTAACTCTTC CTCCCTTCTC CACAGCTTCC TAACCAAGCG
M400-CCL3    #1561  TTAACTCTTC CTCCCTTCTC CACAGCTTCC TAACCAAGCG
C232-CCL3    #1561  TTAACTCTTC CTCCCTTCTC CACAGCTTCC TAACCAAGCG
C113-CCL3    #1561  TTAACTCTTC CTCCCTTCTC CACAGCTTCC TAACCAAGCG
C05-CCL3     #1561  TTAACTCTTC CTCCCTTCTC CACAGCTTCC TAACCAAGCG
C351-CCL3    #1561  TTAACTCTTC CTCCCTTCTC CACAGCTTCC TAACCAAGCG
C45-CCL3     #1561  TTAACTCTTC CTCCCTTCTC CACAGCTTCC TAACCAAGCG

C289-CCL3    #1601  AAGCCGGCAG GTCTGTGCTG ACCCCAGTGA GGAGTGGGTC
C194-CCL3    #1601  AAGCCGGCAG GTCTGTGCTG ACCCCAGTGA KGAGTGGGTC
M400-CCL3    #1601  AAGCCGGCAG GTCTGTGCTG ACCCCAGTGA GGAGTGGGTC
C232-CCL3    #1601  AAGCCGGCAG GTCTGTGCTG ACCCCAGTGA GGAGTGGGTC
C113-CCL3    #1601  AAGCCGGCAG GTCTGTGCTG ACCCCAGTGA KGAGTGGGTC
C05-CCL3     #1601  AAGCCGGCAG GTCTGTGCTG ACCCCAGTGA KGAGTGGGTC
C351-CCL3    #1601  AAGCCGGCAG GTCTGTGCTG ACCCCAGTGA KGAGTGGGTC
C45-CCL3     #1601  AAGCCGGCAG GTCTGTGCTG ACCCCAGTGA KGAGTGGGTC

C289-CCL3    #1641  CAGAAATATG TCAGCGACCT GGAGCTGAGT GCCTGAGGGG
C194-CCL3    #1641  CAGAAATATG TCAGCGACCT GGAGCTGAGT GCCTGAGGGG
M400-CCL3    #1641  CAGAAATATG TCAGCGACCT GGAGCTGAGT GCCTGAGGGG
C232-CCL3    #1641  CAGAAATATG TCAGCGACCT GGAGCTGAGT GCCTGAGGGG
C113-CCL3    #1641  CAGAAATATG TCAGCGACCT GGAGCTGAGT GCCTGAGGGG
C05-CCL3     #1641  CAGAAATATG TCAGCGACCT GGAGCTGAGT GCCTGAGGGG
C351-CCL3    #1641  CAGAAATATG TCAGCGACCT GGAGCTGAGT GCCTGAGGGG
C45-CCL3     #1641  CAGAAATATG TCAGCGACCT GGAGCTGAGT GCCTGAGGGG
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C289-CCL3    #1681  TCCAGAAGCT TCGAGGCCCA GCGACCTCGG TGGGCCCAGT
C194-CCL3    #1681  TCCAGAAGCT TCGAGGCCCA GCGACCTCGG TGGGCCCAGT
M400-CCL3    #1681  TCCAGAAGCT TCGAGGCCCA GCGACCTCGG TGGGCCCAGT
C232-CCL3    #1681  TCCAGAAGCT TCGAGGCCCA GCGACCTCGG TGGGCCCAGT
C113-CCL3    #1681  TCCAGAAGCT TCGAGGCCCA GCGACCTCGG TGGGCCCAGT
C05-CCL3     #1681  TCCAGAAGCT TCGAGGCCCA GCGACCTCGG TGGGCCCAGT
C351-CCL3    #1681  TCCAGAAGCT TCGAGGCCCA GCGACCTCGG TGGGCCCAGT
C45-CCL3     #1681  TCCAGAAGCT TCGAGGCCCA GCGACCTCGG TGGGCCCAGT
C289-CCL3    #1721  GGGGAGGAGC AGGAGCCTGA GCCTTGGGAA CATGCGTGTG
C194-CCL3    #1721  GGGGAGGAGC AGGAGCCTGA GCCTTGGGAA CATGCGTGTG
M400-CCL3    #1721  GGGGAGGAGC AGGAGCCTGA GCCTTGGGAA CATGCGTGTG
C232-CCL3    #1721  GGGGAGGAGC AGGAGCCTGA GCCTTGGGAA CATGCGTGTG
C113-CCL3    #1721  GGGGAGGAGC AGGAGCCTGA GCCTTGGGAA CATGCGTGTG
C05-CCL3     #1721  GGGGAGGAGC AGGAGCCTGA GCCTTGGGAA CATGCGTGTG
C351-CCL3    #1721  GGGGAGGAGC AGGAGCCTGA GCCTTGGGAA CATGCGTGTG
C45-CCL3     #1721  GGGGAGGAGC AGGAGCCTGA GCCTTGGGAA CATGCGTGTG
C289-CCL3    #1761  ACCTCCACAG CTACCTCTTC TATGGACTGG TTGTTGCCAA
C194-CCL3    #1761  ACCTCCACAG CTACCTCTTC TATGGACTGG TTGTTGCCAA
M400-CCL3    #1761  ACCTCCACAG CTACCTCTTC TATGGACTGG TTGTTGCCAA
C232-CCL3    #1761  ACCTCCACAG CTACCTCTTC TATGGACTGG TTGTTGCCAA
C113-CCL3    #1761  ACCTCCACAG CTACCTCTTC TATGGACTGG TTGTTGCCAA
C05-CCL3     #1761  ACCTCCACAG CTACCTCTTC TATGGACTGG TTGTTGCCAA
C351-CCL3    #1761  ACCTCCACAG CTACCTCTTC TATGGACTGG TTGTTGCCAA
C45-CCL3     #1761  ACCTCCACAG CTACCTCTTC TATGGACTGG TTGTTGCCAA
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C289-CCL3    #1801  ACAGCCACAC TGTGGGACTC TTCTTAACTT AAATTTTAAT
C194-CCL3    #1801  ACAGCCACAC TGTGGGACTC TTCTTAACTT AAATTTTAAT
M400-CCL3    #1801  ACAGCCACAC TGTGGGACTC TTCTTAACTT AAATTTTAAT
C232-CCL3    #1801  ACAGCCACAC TGTGGGACTC TTCTTAACTT AAATTTTAAT
C113-CCL3    #1801  ACAGCCACAC TGTGGGACTC TTCTTAACTT AAATTTTAAT
C05-CCL3     #1801  ACAGCCACAC TGTGGGACTC TTCTTAACTT AAATTTTWAT
C351-CCL3    #1801  ACAGCCACAC TGTGGGACTC TTCTTAACTT AAATTTTAAT
C45-CCL3     #1801  ACAGCCACAC TGTGGGACTC TTCTTAACTT AAATTTTAAT

C289-CCL3    #1841  TTATTTATAC TATTTAGTTT TTGTAATTTA TTTTCGATTT
C194-CCL3    #1841  TTATTTATAC TATTTAGTTT TTGTAATTTA TTTTCGATTT
M400-CCL3    #1841  TTATTTATAC TATTTAGTTT TTGTAATTTA TTTTCGATTT
C232-CCL3    #1841  TTATTTATAC TATTTAGTTT TTGTAATTTA TTTTCGATTT
C113-CCL3    #1841  TTATTTATAC TATTTAGTTT TTGTAATTTA TTTTCGATTT
C05-CCL3     #1841  TTATTTATAC TATTTAGTTT TTGTAATTTA TTTTCGATTT
C351-CCL3    #1841  TTATTTATAC TATTTAGTTT TTGTAATTTA TTTTCGATTT
C45-CCL3     #1841  TTATTTATAC TATTTAGTTT TTGTAATTTA TTTTCGATTT

C289-CCL3    #1881  CACAGTGTGT TTGTGATTGT TTGCTCTGAG AGTTCCCCTG
C194-CCL3    #1881  CACAGTGTGT TTGTGATTGT TTGCTCTGAG AGTTCCCCTG
M400-CCL3    #1881  CACAGTGTGT TTGTGATTGT TTGCTCTGAG AGTTCCCCTG
C232-CCL3    #1881  CACAGTGTGT TTGTGATTGT TTGCTCTGAG AGTTCCCCTG
C113-CCL3    #1881  CACAGTGTGT TTGTGATTGT TTGCTCTGAG AGTTCCCCTG
C05-CCL3     #1881  CACAGTGTGT TTGTGATTGT TTGCTCTGAG AGTTCCCCTG
C351-CCL3    #1881  CACAGTGTGT TTGTGATTGT TTGCTCTGAG AGTTCCCCTG
C45-CCL3     #1881  CACAGTGTGT TTGTGATTGT TTGCTCTGAG AGTTCCCCTG
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C289-CCL3    #1921  TCCCCTCCCC STTCCCTCAC ACCGCGTCTG GTGACAACCG
C194-CCL3    #1921  TCCCCTCCCC STTCCCTCAC ACCGCGTCTG GTGACAACCG
M400-CCL3    #1921  TCCCCTCCCC CTTCCCTCAC ACCGCGTCTG GTGACAACCG
C232-CCL3    #1921  TCCCCTCCCC CTTCCCTCAC ACCGCGTCTG GTGACAACCG
C113-CCL3    #1921  TCCCCTCCCC STTCCCTCAC ACCGCGTCTG GTGACAACCG
C05-CCL3     #1921  TCCCCTCCCC STTCCCTCAC ACCGCGTCTG GTGACAACCG
C351-CCL3    #1921  TCCCCTCCCC STTCCCTCAC ACCGCGTCTG GTGACAACCG
C45-CCL3     #1921  TCCCCTCCCC STTCCCTCAC ACCGCGTCTG GTGACAACCG

C289-CCL3    #1961  AGTGGCTGTC ATCRGCCTGT GTAGGCAGTC ATGGCACCAA
C194-CCL3    #1961  AGTGGCTGTC ATCAGCCTGT GTAGGCAGTC ATGGCACCAA
M400-CCL3    #1961  AGTGGCTGTC ATCAGCCTGT GTAGGCAGTC ATGGCACCAA
C232-CCL3    #1961  AGTGGCTGTC ATCAGCCTGT GTAGGCAGTC ATGGCACCAA
C113-CCL3    #1961  AGTGGCTGTC ATCAGCCTGT GTAGGCAGTC ATGGCACCAA
C05-CCL3     #1961  AGTGGCTGTC ATCAGCCTGT GTAGGCAGTC ATGGCACCAA
C351-CCL3    #1961  AGTGGCTGTC ATCAGCCTGT GTAGGCAGTC ATGGCACCAA
C45-CCL3     #1961  AGTGGCTGTC ATCAGCCTGT GTAGGCAGTC ATGGCACCAA

C289-CCL3    #2001  AGCCACCAGA CTGACAAATG TGTATCGGAT GCTTTTSTTC
C194-CCL3    #2001  AGCCACCAGA CTGACAAATG TGTATCGGAT GCTTTTGTTC
M400-CCL3    #2001  AGCCACCAGA CTGACAAATG TGTATCGGAT GCTTTTGTTC
C232-CCL3    #2001  AGCCACCAGA CTGACAAATG TGTATCGGAT GCTTTTGTTC
C113-CCL3    #2001  AGCCACCAGA CTGACAAATG TGTATCGGAT GCTTTTGTTC
C05-CCL3     #2001  AGCCACCAGA CTGACAAATG TGTATCGGAT GCTTTTGTTC
C351-CCL3    #2001  AGCCACCAGA CTGACAAATG TGTATCGGAT GCTTTTGTTC
C45-CCL3     #2001  AGCCACCAGA CTGACAAATG TGTATCGGAT GCTTTTGTTC
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C289-CCL3    #2041  AGGGCTGTGA TCGGCCTGGG GAAATAATAA AGATGCTCTT
C194-CCL3    #2041  AGGGCTGTGA TCGGCCTGGG GAAATAATAA AGATGCTCTT
M400-CCL3    #2041  AGGGCTGTGA TCGGCCTGGG GAAATAATAA AGATGCTCTT
C232-CCL3    #2041  AGGGCTGTGA TCGGCCTGGG GAAATAATAA AGATGCTCTT
C113-CCL3    #2041  AGGGCTGTGA TCGGCCTGGG GAAATAATAA AGATGCTCTT
C05-CCL3     #2041  AGGGCTGTGA TCGGCCTGGG GAAATAATAA AGATGCTCTT
C351-CCL3    #2041  AGGGCTGTGA TCGGCCTGGG GAAATAATAA AGATGCTCTT
C45-CCL3     #2041  AGGGCTGTGA TCGGCCTGGG GAAATAATAA AGATGCTCTT
C289-CCL3    #2081  TTAAAAGGTA AACCAGTATT GAGTTTG
C194-CCL3    #2081  TTAAAAGGTA AACCAGTATT GAGTTTG
M400-CCL3    #2081  TTAAAAGGTA AACCAGTATT GAGTTTG
C232-CCL3    #2081  TTAAAAGGTA AACCAGTATT GAGTTTG
C113-CCL3    #2081  TTAAAAGGTA AACCAGTATT GAGTTTG
C05-CCL3     #2081  TTAAAAGGTA AACCAGTATT GAGTTTG
C351-CCL3    #2081  TTAAAAGGTA AACCAGTATT GAGTTTG
C45-CCL3     #2081  TTAAAAGGTA AACCAGTATT GAGTTTG
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