In utero HIV infection in South Africa: the potential for cure



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Komama, umuntu ngumuntu ngabantu

For the mothers, you are not alone, together we are all people

Abstract

It is well established that for HIV-infected infants, early combination antiretroviral therapy initiation reduces morbidity, mortality, and latent viral reservoir size. The latter, in addition to the uniquely tolerant neonatal immune response, raises the question of whether there is potential for HIV cure or remission by treating HIV-infected infants in the first hours of life.

I coordinated the recruitment of nearly 200 *in utero* HIV-infected infants in KwaZulu-Natal, South Africa, half of which were identified using point-of-care testing within 48 hours of birth, which enabled significantly earlier treatment initiation. Due to exposure to transplacental maternal antiretroviral therapy, and prophylaxis postnatally, these HIV-infected infants had low to undetectable HIV RNA and even DNA levels pre-treatment, creating diagnostic uncertainty. Despite this promising start to life, the mortality and study attrition rates were high. For those infants who remained on the study, treatment failure was common, and not associated with HIV drug resistance, but instead non-adherence to treatment. Prophylaxis exposure, along with this sub-optimal treatment adherence meant that there was no discernible difference in reservoir size or decline for those infants who started treatment very early, compared to those starting at the standard age of one to two weeks. Females were found to be at higher risk of *in utero* HIV-infection, which may also have implications for HIV cure potential.

Where mother-to-child HIV prevention measures are available, *in utero* HIV-infection occurs in a vulnerable subset of mothers for whom antiretroviral therapy self-administration, and administration to their infected child, is difficult to achieve, no matter how early it is started. There is an urgent need to address the particular challenges facing mothers of HIV-infected infants, and at the same time to explore long-acting therapeutic options or cure interventions.

Contributions to this Thesis

First and foremost, I would like to express my deepest gratitude for the families enrolled in the studies this thesis is based upon. Without their participation; their blood, sweat and tears, this research would have been impossible. Also, to the Ucwaningo Lwabantwana study team, whose dedication and compassion enabled recruitment, and data collection.

I am very grateful to the following individuals who have contributed their expertise to the work presented in this thesis.

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- **Andreas Groll** from TU Dortmund University, Germany, advised on data modelling, and wrote the model code.
- **Maria C. Puertas** from IrsiCaixa, Spain, ran the HIV DNA ddPCR.
- **David Bonsall** and colleagues from the University of Oxford performed most of the HIV deep sequencing.
- **Matthew Bull and Thomas Connor** from Cardiff University, UK performed some of the HIV deep sequencing.
- **Nick Grayson** from the University of Oxford analysed the HIV deep sequencing data.
- **Katya Govender** from Africa Health Research Institute, South Africa, performed plasma ART quantification.
- **Emily Adland** from the University of Oxford undertook some of the *gag* sequencing and all of the VRC and interferon assays.
- **Timothy Rostron** from the University of Oxford performed the HLA typing.
- **Pieter Jooste** and team from Kimberley Hospital, South Africa provided the cord blood samples.

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When you set out for Ithaka
ask that your way be long,
full of adventure, full of instruction
- Constantine P. Cavafy

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Glossary of terms

Units of measurement

°C	degrees Celcius
Ct	cycle threshold value
g	gram
kb	kilobase
kg	kilogram
km	kilometre
min	minute
mL	millilitre
mm	millimetre
mM	millimolar
MOI	multiplicity of infection
ng	nanogram
nM	nanomolar
RFI	relative fluorescence index
rpm	revolutions per minute
s	second
μF	microfarad
μg	microgram
μL	microlitre
μm	micrometre
V	volt
xg	times gravity

Acronyms and abbreviations

ABC	abacavir
AIDS	acquired immunodeficiency syndrome
ART	antiretroviral therapy
ARV	antiretroviral drug
ATZ	atazanavir
AZT	zidovudine
BCG	Bacillus Calmette–Guérin

bNAbs	broadly neutralising antibodies
CAP/CTM	COBAS AmpliPrep/COBAS TaqMan HIV-1 Test
cART	combination antiretroviral therapy
CCR	chemokine receptor
CD	cluster of differentiation
CDC	Centre for Disease Control and Prevention, United States
CI	confidence interval
CMV	cytomegalovirus
CO_2	carbon dioxide
Covid-19	coronavirus disease 2019
CTL	cytotoxic T cell
CXCR	chemokine receptor
CYP	cytochrome P450
d4T	stavudine
DBS	dried blood spot
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DC	dendritic cell
ddPCR	droplet digital PCR
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DRM	drug resistance mutation
DTG	dolutegravir
EDTA	ethylenediaminetetraacetic acid
EFV	efavirenz
EID	early infant diagnosis
ELISA	enzyme-linked immunosorbent assay
FCS	foetal calf serum
FDA	Food and Drug Administration, United States
FRESH	Females Rising through Education, Support and Health
FTC	emtricitibine
GAMM	generalised additive mixed model
GFP	green fluorescent protein
gp	glycoprotein
GXP	GeneXpert HIV-1 qualitative test
H_2O	water
HIV	human immunodeficiency virus
HIVDR	HIV drug resistance
HLA	human leukocyte antigen
HPLC	high performance liquid chromatography
HR	hazard ratio
IC_{80}	80% inhibitory concentration
IFN	interferon
IL	interleukin
INSTI	integrase strand transfer inhibitor

IP	intrapartum
IQR	interquartile range
IU	*in utero*
IUGR	intra-uterine growth restriction
KZN	KwaZulu-Natal
LASSO	least absolute shrinkage and selection operator
LC-MS/MS	liquid chromatography–mass spectrometry
LMIC	low and middle income countries
ln	Natural log (e)
LPV	lopinavir
LPVr	ritonavir-boosted lopinavir
LTR	long terminal repeat
MIT	Massachusetts Institute of Technology
MGH	Massachusetts General Hospital
MTCT	mother-to-child transmission
NGS	next generation sequencing
NHLS	National Health Laboratory Service, South Africa
NIH	National Institutes of Health, United States
NNRTI	non-nucleoside reverse transcriptase inhibitor
NRTI	nucleoside/nucleotide reverse transcriptase inhibitor
NVP	nevirapine
OR	odds ratio
PBMC	peripheral blood mononuclear cell
PCP	*Pneumocystis (jirovecii)* pneumonia
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cell
PEHSS	Paediatric Early HAART and Strategic Treatment Interuption study
PENTA	Paediatric European Network for Treatment of AIDS
PI	protease inhibitor
PK	pharmacokinetic
PMTCT	prevention of mother-to-child transmission
PoC	point-of-care
PPV	positive predictive value
PR	prevalence ratio
qVOA	quantitative viral outgrowth assay
RAL	raltegravir
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RT	reverse transcriptase
RTV	ritonavir
SARS-Cov-2	severe acute respiratory syndrome coronavirus 2
SIV	simmian immunodeficiency virus
SoC	standard-of-care
TAM	thymidine analog mutation
TB	*Mycobacterium tuberculosis*

TDF	tenofovir disproxil
TFG	transforming growth factor
Th	T helper cell
TLR	toll-like receptor
TNA	total nucleic acid
TNF	tumor necrosis factor
UL	Ucwaningo Lwabantwana study
UNAIDS	Joint United Nations Programme on HIV and AIDS
USD	United States dollar
VRC	viral replicative capacity
VS	viral suppression
WHO	World Health Organisation
XCI	X chromosome inactivation

Amino acid abbreviations

A	alanine
C	cysteine
D	aspartic acid
E	glutamic acid
F	phenylalanine
G	glycine
H	histidine
I	isoleucine
K	lysine
L	leucine
M	methionine
N	asparagine
P	proline
Q	glutamine
R	arginine
S	serine
T	threonine
V	valine
W	tryptophan
Y	tyrosine

Publications relevant to this thesis

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Chapter 1

Introduction

1.1 Epidemiology

1.1.1 Origin and emergence of HIV

18 months after first descriptions of Acquired Immunodeficiency Syndrome (AIDS) in adults (Gottlieb et al. 1981), in 1982 the United States Center for Disease Control and Prevention (CDC) reported four infants with unexplained cellular immunodeficiency and opportunistic infection, whose mothers had suspected or confirmed AIDS (O'Reilly et al. 1982). Although it was not until the following year that the aetiological agent for AIDS was isolated; the virus later named human immunodeficiency virus (HIV) (Barré-Sinoussi et al. 1983; Coffin et al. 1986), it was obvious that AIDS could be transmitted from mother-to-child.

HIV originated via multiple zoonotic transmissions of various strains of Simian Immunodeficiency Virus (SIV) from non-human primates in Western Africa in the early 20th century (Sharp and Hahn 2011), resulting in several HIV lineages in humans; HIV type 1 (HIV-1) groups M-P and HIV type 2 (HIV-2) groups A–H (Hemelaar 2012). In the 1970's group M HIV-1 started rapidly spreading around the world and evolved into subtypes and circulating recombinant forms (Hemelaar et al. 2018). By 1987 an explosive epidemic of sub-type C HIV-1 had begun in Southern Africa (UNAIDS 2003). Today an estimated 75 million people have been infected with HIV worldwide (UNAIDS 2019a).

1.1.2 HIV worldwide: current situation and global targets

After a peak of 1.7 million in 2004, the advent of life-saving combination antiretroviral therapy (cART) and public health prevention campaigns led to global AIDS-related deaths declining to 770 000 in 2018 (UNAIDS 2019b). In 2011 the landmark HPTN 052 trial demonstrated that treatment of HIV-infected individuals prevents onward spread (Cohen et al. 2004). In 2014 the Joint United Nations Programme on HIV/AIDS (UNAIDS) recognised this Treatment as Prevention strategy in their release of an ambitious three-part goal, for 90% of people living with HIV to be aware of their status, 90% of people diagnosed with HIV to be initiated on cART, and for 90% of those on treatment to be taking it successfully, evident by suppressed plasma viraemia. Modelling suggested that the achievement of this goal by 2020 would have led to the AIDS epidemic ending by 2030 (UNAIDS 2014). Although there has been immense global success heading towards these 90-90-90 targets, numbers are not on track. In 2018 nearly four out of five people living with HIV knew their status, and nearly two-thirds were on cART, but only half of people living with HIV were virally suppressed, fuelling the HIV epidemic (UNAIDS 2019b, Figure 1.1).

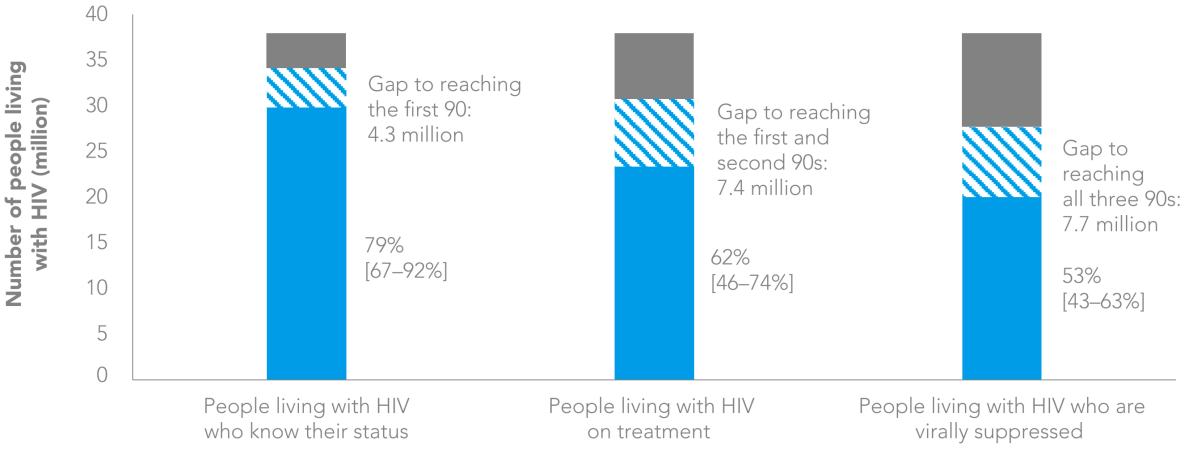


Figure 1.1: UNAIDS 90-90-90 HIV testing and treatment cascade 2018. Worldwide figures. Figure reproduced with permission from UNAIDS Global AIDS Update 2019.

1.1.3 Paediatric HIV worldwide

HIV from mother-to-child-transmission (MTCT) vastly outnumbers the rarer routes of paediatric infection, which include unsafe healthcare practices (Mir et al. 2019), or from adults via surrogate breastfeeding (Goedhals et al. 2012; Tsai et al. 2016) or sexual abuse (Lindegren et al. 1998). The scale-up of cART for pregnant women living with HIV has led to a 41% reduction in paediatric HIV incidence since 2010 (Figure 1.2). Although promising, this still translated to 160 000 new paediatric infections in 2018 (UNAIDS 2019b). There are approximately 1.7 million children living with HIV worldwide, with two-thirds living in sub-Saharan Africa (UNAIDS 2019c, Figure 1.3). Only half of the world's HIV-infected children are receiving cART, less than for adults, with the gap widening (UNAIDS 2019b). As a result, children are disproportionately represented in AIDS-related mortality (UNAIDS 2019c).

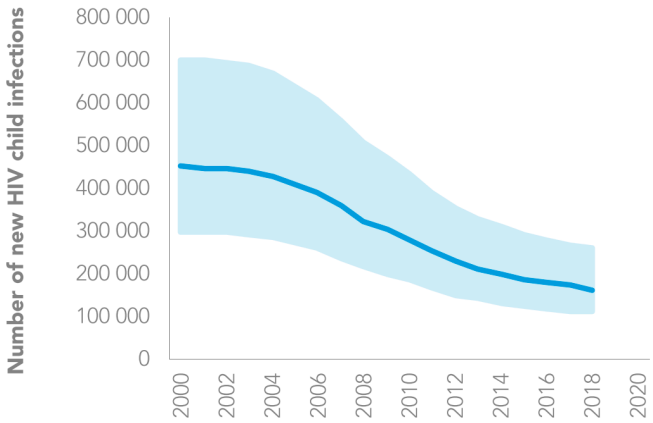


Figure 1.2: Paediatric HIV incidence (0-14 years) 2000-2018. Worldwide there has been a 41% reduction since 2010. Reproduced with permission from UNAIDS Global AIDS Update 2019.

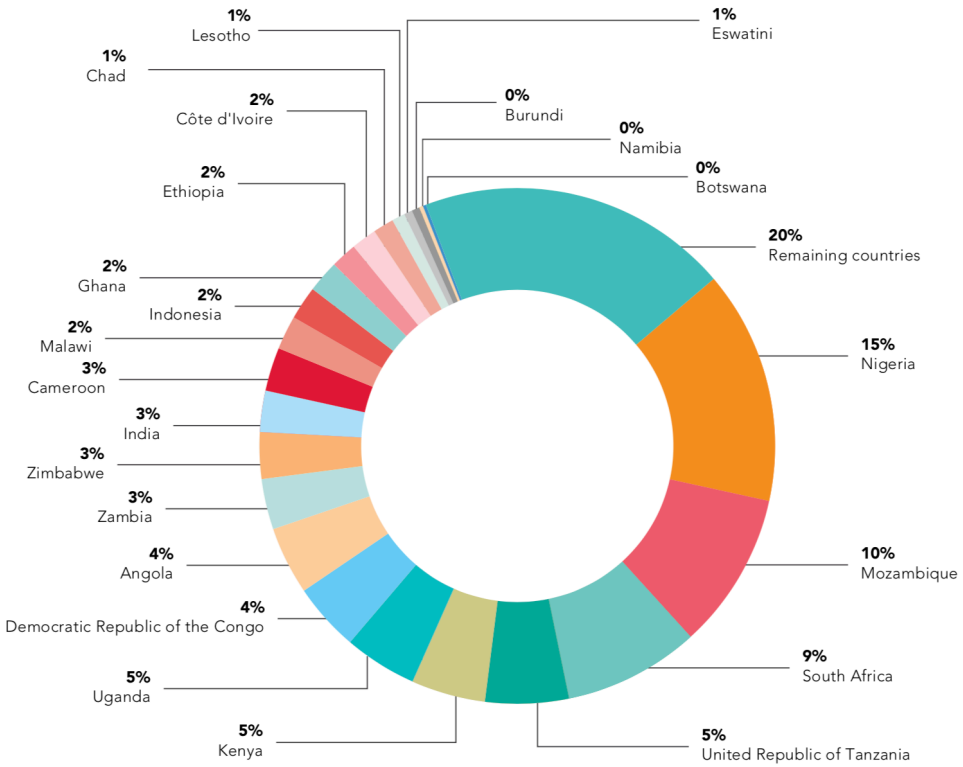


Figure 1.3: Geographic distribution of new paediatric HIV infections 2018. Figure reproduced with permission from UNAIDS Start Free Stay Free AIDS Free 2019 Report.

1.1.4 HIV in South Africa: an historical perspective

South Africa's infamous HIV hyperepidemic "the cutting edge of a continental apocalypse" (Hawthorne 2000) was primed by a unique historical and political setting at the end of apartheid in 1990. At this time HIV seroprevalence in South Africa was 0.7%, but continuing inequality, migrant workers, and violence propelled HIV spread. By 2000 HIV seroprevalence had escalated to 25% in South Africa, and 36% in the province of KwaZulu-Natal (KZN) (Fassin and Schneider 2003). Initial HIV/AIDS response plans by the President Nelson Mandela were promising, but were hampered by controversies and inefficiencies (Simelela et al. 2015). Mandela's successor Thabo Mbeki, allied with other "HIV dissidents" from around the world, publicly stating that AIDS was not caused by HIV and refusing to provide "poisonous" cART to the people of South Africa. In 2002 the Treatment Action Campaign activist group came to force resulting in Mbeki being overruled by the South African Constitutional Court and ordered to provide the antiretroviral drug (ARV), nevirapine (NVP) to pregnant women, later followed by a slow roll-out of cART to AIDS patients (Fassin and Schneider 2003). It has been estimated that Mbeki's delay in providing ART to South Africans resulted in an excess of 330 000 deaths (Chigwedere et al. 2008). On face-value this was an inexcusable atrocity, but to understand Mbeki's actions, one has to recognise his paradigm. In the shadow of apartheid Mbeki feared white people conspiring against black Africans, and this mistrust and inequality still contributes to issues seen with HIV in South Africa today.

1.1.5 HIV in South Africa today

In 2018, 20% of the South African population (equating to 7.7 million people) were living with HIV, and another 240 000 people were newly infected. Although this HIV incidence in absolute numbers sounds formidably high, a declining incidence-to-prevalence ratio shows promise for curbing the epidemic (UNAIDS 2019b). Furthermore, a recent household-based testing study in KZN, found a 43% reduction in HIV incidence since 2012 (Vandormael et al. 2019).

cART coverage for pregnant women in South Africa is 87%, resulting in a low MTCT rate of 4.9% or 14 000 children per annum, despite a high antenatal HIV seroprevalence (UNAIDS 2019b). Similar to the rest of the world, in South Africa the HIV treatment cascade is more difficult in children, which resulted in 4400 paediatric AIDS-related deaths in 2018 (ibid.).

1.2 Pathogenesis

1.2.1 The virus and its replication cycle

HIV is a member of the *Lentivirus* genus: a retrovirus distinguished by long disease latency and use of the CD4 surface receptor for cell entry (Kirchhoff 2013). The most abundant cell type expressing the CD4 receptor are the T lymphocyte helper cells (Th cells), which mediate both humoral and cellular immune responses. Other cells expressing the CD4 receptor include monocytes, macrophages and dendritic cells (DCs) (Maartens et al. 2014).

The HIV-1 genome is 9.7kb long and consists of nine genes flanked by the 3' and 5' long terminal repeat sequences (LTR). The three classical retroviral genes, *gag*, *pol* and *env* encode structural proteins (capsid [p24], matrix [p17] and nucleocapsid [p7]), the enzymes (reverse transcriptase [RT], protease, and integrase), and envelope glycoproteins (gp41 and gp120) respectively. Six smaller genes have regulatory and accessory functions (Abfalterer et al. 2018).

HIV's genetic material exists as RNA that can be retrotranscribed into DNA for integration into the host genome as a means of replication. The HIV replication cycle takes one to two days and usually ends with cell death (Kirchhoff 2013). Replication begins when gp120 binds to the host CD4 receptor, triggering conformational change, exposing the chemokine binding domains, which interact with the co-receptor, usually chemokine receptor 5 (CCR5), but alternatively the CXCR4 receptor. gp41 can then mediate fusion of the viral and host cell membranes (Chen 2019), releasing the viral core into the cytoplasm. RT then retrotranscribes viral RNA forming linear

double-stranded DNA which is actively transported into the nucleus to be irreversibly integrated into the host genome (Symons et al. 2018). Once integrated, the HIV provirus is transcribed by host RNA Polymerase II along with the host genome. After translation, viral proteins and genomic RNA move to the cell membrane where they are assembled, budded off and released as immature virions. Protease then cleaves the precursor proteins, forming the mature HIV structure (Freed 2015, Figure 1.4).

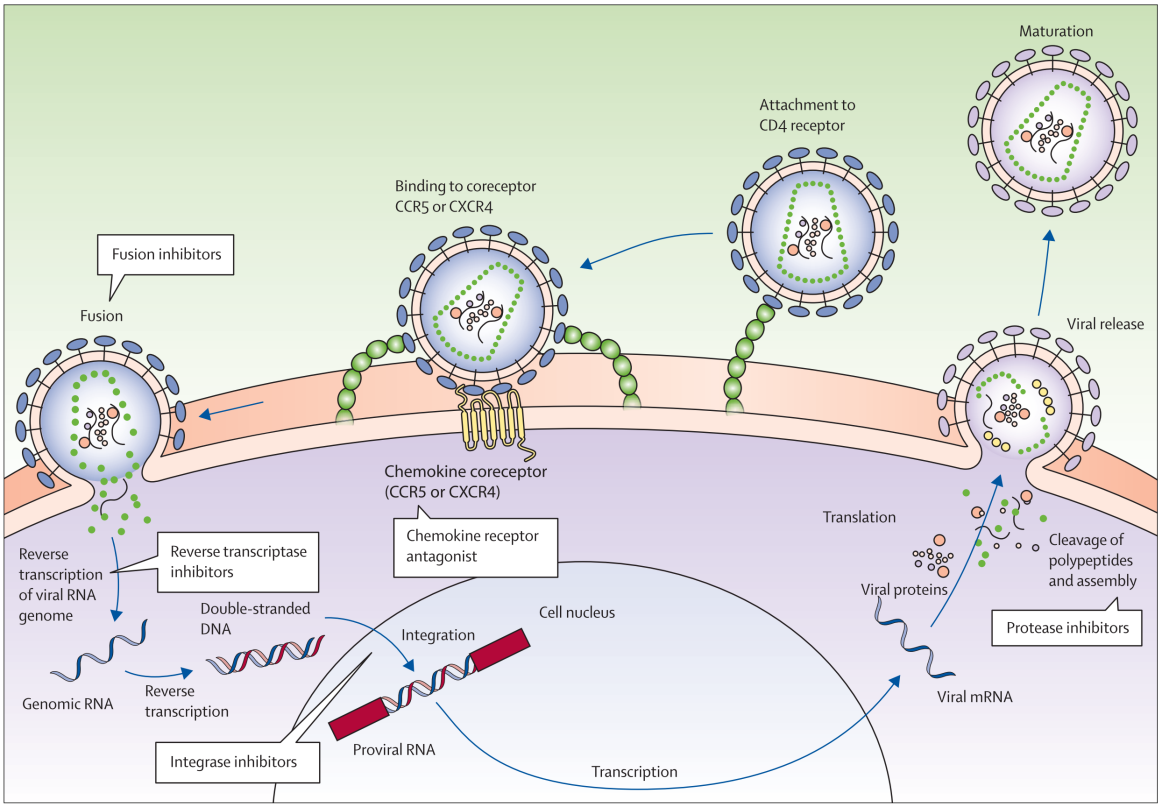


Figure 1.4: HIV replication cycle and antiretroviral drug sites of action. Reproduced with permission from Maartens et al. Lancet 2014

1.2.2 Viral latency

Due to the high surface expression of CCR5, and available dNTPs for reverse transcription, HIV infection preferentially occurs in activated Th cells. Usually infected cells producing viral antigen are targeted and cleared by the immune system, whereas in an "accident of timing", HIV can infect activated CD4+ T cells transitioning into a resting state, thus evading immune surveillance (Shan et al. 2017). This small population of cells retains the potential to reactivate thus is the latent HIV reservoir.

HIV DNA exists inside the cell nucleus as three forms; integrated provirus, 2-LTR circles, and linear unintegrated DNA, with the first being the most stable and responsible for virion production (Zack et al. 1990; Bukrisky et al. 1991). HIV RT is error prone, resulting in approximately 90% of integrated HIV DNA being replication incompetent (Ho et al. 2013; Bruner et al. 2016; Hiener et al. 2017; Bui et al. 2017).

The latent HIV reservoir is already establishing when plasma viraemia is first detectable (Ananworanich et al. 2016; Leyre et al. 2020), and evidence from a non-human primate study suggests that reservoir seeding occurs from as early as 3 days post-infection (Whitney et al. 2014). In untreated adult HIV infection, the latent HIV reservoir peaks at about 4 weeks after infection, then lowers slightly to a set point of 3-3.3 $\log_{10}$ HIV DNA copies per million peripheral blood mononuclear cells (PBMC) (Ananworanich et al. 2016; Goujard et al. 2006; Minga et al. 2008). The reservoir size in adults is similar to that seen in ART naive HIV-infected infants at birth (Scott-Algara et al. 2010). HIV DNA can be found in PBMC, gut-associated lymphoid tissue (Chun et al. 2008), lymph nodes (Fletcher et al. 2014), the central nervous system (Sturdevant et al. 2015) and tissue macrophages throughout the body (Saksena et al. 2010). However, HIV DNA levels in PBMC are the most readily accessible and have been shown to be an independent risk factor for disease progression in untreated HIV-infected children and adults (Rouzioux et al. 2005; Goujard et al. 2006), therefore are an accepted surrogate measure for the body-wide viral burden.

Measuring the latent HIV reservoir

Measuring the peripheral blood latent HIV reservoir is challenging. With no known biomarker for latently HIV-infected cells (Sengupta and R. F. Siliciano 2018), HIV reservoir measurement must directly measure the virus, most commonly by some variation of polymerase chain reaction (PCR). However, quantifying HIV provirus when the frequency is as low as one copy per million PBMC in early treated adults (Ananworanich et al. 2016) is technically difficult. The different forms of HIV and the high proportion of defective virus means that a simple quantification of total HIV DNA via PCR over-estimates the amount of HIV DNA with potential to reactivate. The

gold standard for HIV DNA quantification is the quantitative Viral Outgrowth Assay (qVOA), which measures the frequency of resting CD4+ T cells that produce infectious HIV after a single round of *in vitro* T cell activation, but this is a cumbersome method and underestimates the amount of replication competent virus by 60-fold (Ho et al. 2013). In development are multiplex PCR methods to quantify replication competent HIV DNA more accurately (Bruner et al. 2019).

Latent HIV reservoir decay on treatment

cART can only block active HIV replication, thus has no direct effect on the latent HIV reservoir. Instead, cART prevents new cells from being infected allowing HIV-infected cells to die or be cleared. HIV DNA decay kinetics in children and adults are best modelled as biphasic, with a rapid decline in the first month, followed by a slower decline due to the various infected cell populations and their heterogenous turn-over rates (Blankson et al. 2000; De Rossi et al. 2002). The residual HIV-infected cell population is enriched with long-lived CD4+ T memory cells and stem-like cells (Strain et al. 2003; Jaafoura et al. 2014).

HIV decay eventually plateaus due to the self-propagation of latently infected cells via homeostatic mechanisms and antigen stimulation (Chomont et al. 2009). This clonal proliferation is demonstrated by discrete CD4+ T cells with identical proviral sequences and integration sites, and the absence of viral evolution for patients on long-term cART, even in lymphoid tissue, which were once thought to be ART "sanctuary sites" (W. R. McManus et al. 2019).

When cART is interrupted, viral replication is no longer inhibited allowing the stochastic reactivation of latently HIV-infected cells. This results in virion production, infection of other CD4+ T cells, and subsequent exponential plasma viraemia, usually within days to weeks of treatment interruption in children (Zanchetta et al. 2008; Martínez-Bonet et al. 2015; Pankau et al. 2018). Thus HIV infection necessitates life-long cART treatment.

1.2.3 *In utero* mother-to-child transmission and prevention

Without prevention measures, 30-40% of children born to mothers with chronic HIV infection are infected with HIV *in utero* (IU), intrapartum (IP) or during breastfeeding, with approximate rates of transmission for the separate routes being 5-10%, 10-20% and 10-20% respectively (De Cock et al. 2000; Marinda et al. 2007). Risk of IU HIV transmission is inverse to the duration of cART and proportional to maternal plasma viral load at delivery (Mandelbrot et al. 2015). Furthermore, treating HIV-infected mothers with cART prior to pregnancy and ensuring suppressed maternal plasma viral load throughout the antenatal period, eliminates the risk of perinatal (IU and IP) HIV transmission (ibid.).

The risk of IU HIV transmission is more than two-fold higher when the mother is newly infected with HIV during pregnancy, probably because of the peaking plasma viral load, but possibly also due to immune dysregulation during acute infection or transmitted founder virus traits (L. F. Johnson et al. 2012; Taha et al. 2011). Compounding this risk of transmission in acute HIV infection is the increased risk of HIV acquisition for women ante- and post-partum. Women late in pregnancy are estimated to have three-fold, and postpartum four-fold increased risk of HIV infection per unprotected sex act compared to non-pregnant women (Thomson et al. 2018). Now that WHO recommend all HIV-infected women to be on lifelong cART, chronic HIV infection has less contribution towards overall MTCT rate, resulting in acute maternal HIV infection accounting for one-third of new paediatric HIV infections in Africa (L. F. Johnson et al. 2012), and possibly more since last estimated.

Aside from maternal plasma viral load and acute HIV infection, other factors known to increase IU HIV transmission risk include maternal co-infections, such as malaria, *Mycobacterium tuberculosis* (TB), cytomegalovirus (CMV), syphilis and bacterial vaginosis (Blanche 2020; Farquhar et al. 2010; Mwapasa et al. 2006; Frank et al. 2012; Taha and Gray 2000). Related, is the increased risk of IU HIV transmission in the

presence of chronic chorioamnionitis; inflammation of the placental membranes (Chi et al. 2006).

HIV transmission, whether adult-to-adult or mother-to-child, is inefficient. Of all the potential diverse quasispecies, 80% of the time, a single genetic variant transmits transplacentally (Russell et al. 2011); the transmitted-founder virus. Transmission of HIV is in part a stochastic process, whereby most HIV is unable to pass the mucosal or placental barrier. Additionally, it has been found that in perinatal HIV transmission, a minor, lower fitness variant is transmitted, that has a consensus-like sequence, suggesting selection pressure (Naidoo et al. 2017). However, other than CCR5 tropism, no single phenotypic trait has been consistently found in transmitted-founder viral strains (Shaw and Hunter 2012).

The mechanism for IU HIV transmission is not completely understood. The placenta consists of millions of foetal chorionic villi, invading into the maternal decidua where they are surrounded by maternal blood. Each villus consists of foetal blood vessels, stromal cells and placental macrophages-like (Hofbauer) cells enclosed by synctiotrophoblasts (Figure 1.5). This materno-foetal barrier, only a few cells thick, is highly effective at defending against HIV. How HIV crosses this barrier is controversial. Some studies have shown that synctiotrophoblasts can be infected directly with free virus (David et al. 1992; Douglas et al. 1991), but this has been disputed (Kilani et al. 1997). Possibly, the virus is endocytosed and transported, or instead structural disruptions occur (Behbahani et al. 2000). Hofbauer cells can be infected with HIV and play a key role in limiting HIV replication and transmission in their release of regulatory cytokines (IL-10 and TFG-β) (E. L. Johnson and Chakraborty 2012). Other immune functions of the placenta include the release of anti-viral Type I interferons (IFN-1) by trophoblasts (E. L. Johnson and Chakraborty 2016). IU HIV transmission potentially also involves the transplacental transfer of maternal HIV-infected cells. This theory is supported by studies which found reduced risk of HIV transmission for infants with discordance to maternal human leukocyte antigen (HLA) class I alleles (Mackelprang et al. 2008; Macdonald et al. 1998; Polycarpou et al. 2002), suggesting that maternal

HIV-infected cells were more likely to be recognised and cleared by the infant immune system thus reducing infection risk.

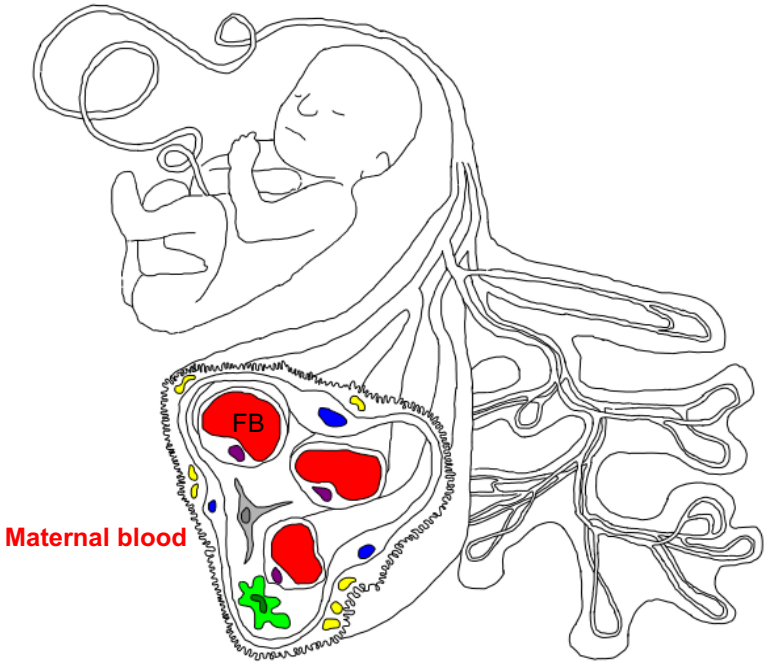


Figure 1.5: Schematic cross-sectional diagram of a foetal villus. Maternal blood and foetal blood (FB) are separated by an endothelial cell (purple nuclei), synctiotrophoblast (yellow nuclei), and cytotrophoblast (blue nuclei) monolayers, with fibroblasts (grey) and Hofbauer cells (green) in between. Adapted from Buerki-Thurnherr et al. Swiss Medical Weekly 2012.

A small modelling study undertaken prior to the advent of ART used postnatal infant HIV viral kinetics to calculate that 95% of IU transmission occurs in the final 8 weeks prior to delivery, with a median time of 14 days (Rouzioux et al. 1995). In addition, in a study of antenatal zidovudine (AZT) prophylaxis for prevention of MTCT (PMTCT), the rate of IU HIV transmission was 5% when starting AZT at 35 weeks gestation compared with 1-2% when starting AZT at 28 weeks gestation, suggesting that a significant proportion of transmissions occur between 28 and 35 weeks gestation (Lallemant et al. 2000). The accuracy of these data now that there is a greater contribution of acute maternal infection is unknown. However, it is still true that the suppression of maternal plasma viral load has increasing urgency with advancing gestation. If a mother has a high plasma viral load late in pregnancy, ways to reduce the risk of all modes of transmission include; switching to a more potent cART regimen,

performing an elective caesarean section prior to labour, intrapartum AZT, avoidance of breastfeeding and intensifying postpartum infant prophylaxis (Blanche 2020). In South Africa, switching the maternal cART regimen, oral cART during labour and dual infant prophylaxis, consisting of AZT and NVP, for 12 weeks, as opposed to 6 weeks of NVP only, are the current recommendations (Republic of South Africa National Department of Health 2019b).

1.2.4 Disease progression

After entering the blood or breaching the mucosa, HIV is bound by the DC-SIGN receptor on immature conventional DCs, triggering DC maturation and migration to draining lymph nodes. There, the DCs antigen present, activating naive T cells, thus stimulating a primary adaptive immune response. This process also facilitates body-wide HIV spread, as in the lymph nodes the DCs also transfer HIV to CD4+ T cells via the immunological synapse, an efficient method of cell infection (Martín-Moreno and Muñoz-Fernández 2019). Subsequent pathogenesis of HIV infection can be broadly thought of as having two interrelated components; CD4+ T cell depletion and chronic immune activation.

CD4+ T cell depletion

Within days of infection, HIV spreads to local and distal mucosal sites, particularly the gastrointestinal tract, the primary site of HIV pathogenesis (George and Asmuth 2014). The majority of CD4+ T cells in the gut-associated lymphoid tissue are targeted and die in the first weeks of infection, with preferential infection of T helper 17 subset (Th17) cells, impairing both the structural and immune function of the gut (Kewenig et al. 1999; Brenchley et al. 2008). Although infants have lower levels of activated CD4+ T lymphocytes peripherally, they have high levels in the gut (Bunders et al. 2012). Even though cART is effective at restoring circulating levels of CD4+ T cells, it has little effect on the immune reconstitution in the gut (George and Asmuth 2014).

After this initial immunological insult, the progression of untreated HIV disease is a slower decline in CD4+ T cells, which initially can be compensated for with increased

production. Most CD4+ T cells die by pyroptosis, a highly inflammatory form of programmed cell death, causing bystander cell death, which then recruits more CD4+ T cells, causing a vicious cycle of inflammation and cell death (Doitsh et al. 2014, Figure 1.6). Eventually proliferation can no longer keep up with depletion and immunodeficiency ensues.

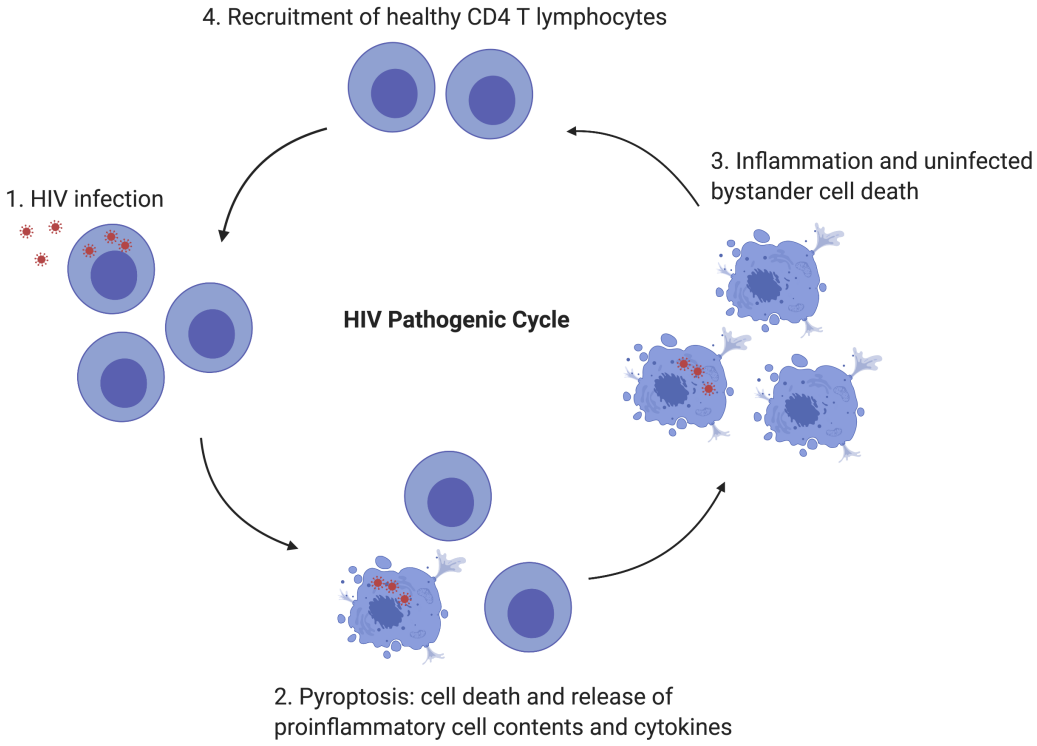


Figure 1.6: HIV pathogenic cycle; fueled by pyroptosis, a highly inflammatory form of programmed cell death. Adapted from Doitsh et al. Nature 2014. Created on Biorender.com

Immune activation

The degree of an HIV-infected child's immune activation, as measured by expression of cytotoxic T cell (CD8+, cytotoxic lymphocyte, CTL) and Th cell activation surface markers (HLA-DR and/or CD38+) early in infection can predict disease progression independently from plasma viral load (Paul et al. 2005; Resino et al. 2006; Ssewanyana et al. 2007; Prendergast et al. 2012). This immune activation is reduced by cART, but does not return to normal levels, even in IU HIV-infected infants who start cART

within hours of life (Garcia-Broncano et al. 2019). Immune activation contributes to the higher than background rate of non-AIDS mortality in HIV-infected adults, including cardiovascular disease, metabolic disease and non-AIDS related malignancies (Farahani et al. 2017). The concern for HIV-infected children is that the cumulative risk secondary to immune activation puts them at the greatest long-term risk for non-AIDS related morbidity and mortality.

The origin of this immune activation is dependent on the interplay between host and viral factors, including microbial translocation, viral fitness and co-infections.

Aside from HIV's direct stimulation of the innate and adaptive immune responses, another key driver of HIV-related immune activation is microbial translocation from the gut lumen into the circulation due to HIV enteropathy. Plasma markers of microbial translocation such as bacterial cell wall lipopolysaccharide, and microbial DNA are increased in HIV-infected individuals and are correlated with lymphocyte activation, exhaustion and proliferation, higher levels of proinflammatory cytokines including IFN-α, IL-6, TNF-α, and increased numbers of HIV-target cells (Claiborne et al. 2019; Brenchley et al. 2006; Ericsen et al. 2016; Zevin et al. 2016) further amplifying infection. Microbial translocation has been shown to predict disease progression in children (Papasavvas et al. 2011; Muenchhoff et al. 2018; Pilakka-Kanthikeel et al. 2014) and to persist even after years of cART (Pilakka-Kanthikeel et al. 2012). Due to the relative immaturity of the newborn gut, particularly for premature infants (Chernikova et al. 2018), it is possible that microbial translocation has a more detrimental effect in infant HIV infection.

Viral factors can also drive immune activation, in particular, higher levels of immune activation are seen with transmitted founder viruses with higher replication capacity (Claiborne et al. 2015).

Coinfections, particularly CMV increase immune activation in HIV infection (Boulougoura and Sereti 2016). CMV is common in sub-Saharan Africa, with most children infected

in the first year of life (Kaye et al. 2008). CMV co-infection in HIV-infected infants has been shown to increase activated (HLA-DR+ CD38+) and apoptosis vulnerable (CD95+ Bcl-2-) CD8+ T cells (Slyker et al. 2012). This activation contributes to faster HIV disease progression and higher rates of neurological sequelae seen in HIV/CMV-coinfected untreated children (Doyle et al. 1996; Kovacs et al. 1999; Nigro et al. 1996). However, more recently, effective HIV-treatment has been shown to reduce this accelerated disease progression in African CMV/HIV co-infected infants (Kfutwah et al. 2017).

1.2.5 Natural history in children

Untreated HIV disease progresses more rapidly in infants compared to adults. For untreated adults, average survival is 90% at 5 years and 60% at 10 years (Collaborative Group 2000), while survival of African infants following perinatal HIV infection is 50% at 1 year, even after adjusting for background infant mortality (Marston et al. 2011, Figure 1.7). In paediatric HIV infection, plasma HIV RNA levels rise rapidly to reach a set point of about 6 $\log_{10}$ HIV RNA copies per mL, before slowly declining to a set point $\sim$5 $\log_{10}$ HIV RNA copies per mL 3-6 years later (PENTA 1998; Rouet et al. 2003). This contrasts to adult HIV infection where peak plasma viraemia of $\sim$7 $\log_{10}$ HIV RNA copies per mL is reached in one to two weeks, before quickly falling to a set point of $\sim$4-5 $\log_{10}$ HIV RNA copies per mL six weeks later (Dong et al. 2017, Figure 1.8). The persistently higher plasma viral loads seen in infants are attributed to a less effective infant anti-HIV adaptive immune response (Adkins et al. 2004).

The natural history of untreated paediatric HIV infection is essentially bimodal, with 50% being rapid progressors who become symptomatic and die in the first 1-2 years of life, and the rest progressing more slowly (Duliege et al. 1992; Blanche et al. 1997). Paediatric non-progressors are a rarer phenotype (5-10%), and are characterised by high plasma viral loads, normal for age CD4+ T cell counts and low immune activation, similar to the non-pathogenic phenotype seen in natural non-human primate SIV hosts (Muenchhoff et al. 2016). Much more rarely, paediatric non-progressors can

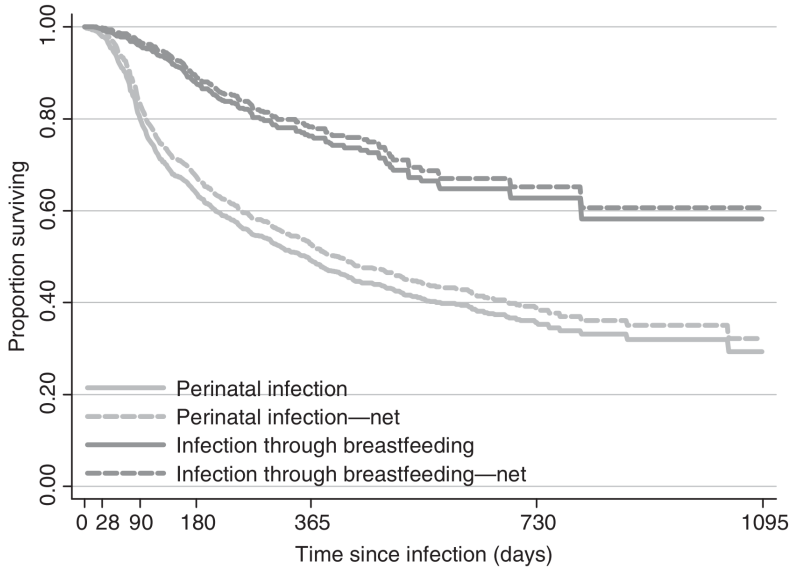


Figure 1.7: Perinatal HIV mortality in the first years of life. Net rates exclude additional non-HIV related mortality. Reproduced with permission from Marston et al. Int J Epi 2011

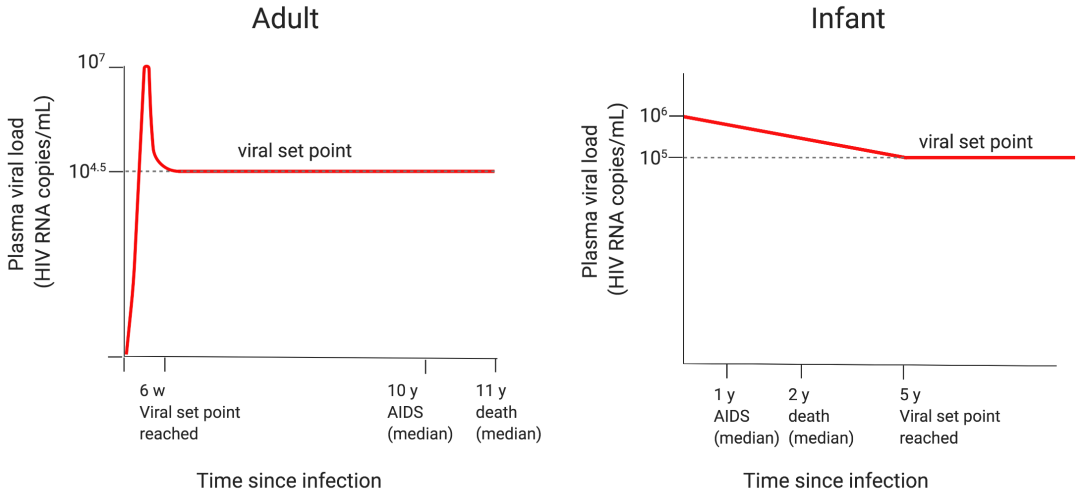


Figure 1.8: Adult versus paediatric HIV viral dynamics in untreated infection. Adapted from Goulder et al. Nat Rev Imm 2016. Created on Biorender.com

spontaneously control HIV viraemia. Although, this paediatric elite controller phenotype occurs more rarely than in adults (0.01 vs 0.1%), almost exclusively in females, and is infrequently long-lived (Vieira et al. 2019).

1.2.6 Clinical manifestations in children

HIV presentation in infancy is commonly a severe lower respiratory tract infection; often the fungal opportunistic *Pneumocystis jiroveci* (PCP) in combination with pathogens such as CMV. Additional presenting features include; failure to thrive, chronic diarrhoea, hepatosplenomegaly, lymphadenopathy, persistent oral candidiasis and HIV encephalopathy (Chintu et al. 2002; WHO 2007b). PCP is the leading cause of death with a peak incidence at 3-6 months of age (Simonds et al. 1993). AIDS-defining illnesses and death in infancy cannot be predicted from CD4+ T cell count, which is why treatment at the earliest opportunity is prioritised for infants (Violari et al. 2008).

1.3 HIV diagnosis

1.3.1 Tests

The standard clinical diagnostic HIV test is an enzyme-linked immunosorbent antibody (ELISA) test, a sensitive laboratory-based test that detects HIV-specific antibodies in serum. In South Africa, because of high demand for testing, the first line HIV test is a point-of-care (PoC) fourth generation rapid HIV test, which detects p24 antigen and HIV-specific antibodies. This test is performed on finger-prick blood and results are available within minutes.

Infants born to HIV-infected mothers have transplacentally acquired maternal anti-HIV antibodies in circulation. The median age for seroreversion of HIV-exposed uninfected infants is 14 months and 14-20% of infants still have detectable maternal anti-HIV antibodies in circulation at 18 months of age (Gutierrez et al. 2012; Chatpornvorarux et al. 2019). For this reason, infants under the age of 18 months require direct viral detection both as an initial and confirmatory test, and between 18 and 24

months as a confirmatory test following a positive antibody test (Republic of South Africa National Department of Health 2019a).

Nucleic acid amplification by PCR is the standard HIV diagnostic test in infants, which, depending on the test, can detect HIV RNA, DNA or both (total nucleic acid, TNA), is either qualitative or quantitative, and can be performed on whole blood, dried blood spot (DBS) or plasma. Quantitative plasma RNA PCR is also known as a plasma viral load. Since 2014 the standard of care infant HIV test in South Africa has been the COBAS AmpliPrep/COBAS TaqMan (CAP/CTM) version two (Roche Molecular Diagnostics), which detects HIV TNA by PCR test on plasma, whole blood or DBS and is performed at nine National Health Laboratory Service (NHLS) centres nationwide (Haeri Mazanderani et al. 2018a).

The CAP/CTM test is a quantitative PCR used in order to achieve a qualitative result. The reaction uses an HIV specific probe, which fluoresces when it binds to the HIV target. The more HIV present, the greater the intensity of fluorescence. The number of thermal cycles required to produce a fluorescent signal is called the cycle threshold (Ct) value and is inversely proportional to the amount of HIV in an infant blood sample (Haeri Mazanderani et al. 2019). The test cycles a set number of times and if no fluorescence occurs, the result is negative. Whereas, if any fluorescence is produced, the test result is positive, but high Ct values, or weak fluorescent signal (relative fluorescence index; RFI) could either be a result of non-specific binding or low HIV concentration (Figure 1.9). In South Africa, the NHLS in-house guidelines interpret an instrument positive result with a Ct value >32 and RFI <5, as an indeterminate result (Maritz et al. 2017). Thus, an indeterminate test result is not a function of the test itself, instead is an interpretation of the test output, and is strongly recommended by WHO to avoid overdiagnosis (WHO 2018).

WHO have approved two HIV TNA PCR test kits that can be used as a PoC, or near-PoC test operated by non-laboratory staff. One of these tests, the Gene Xpert HIV-1 Qualitative TNA PCR (GXP, Cepheid) has particular relevance in South Africa, as

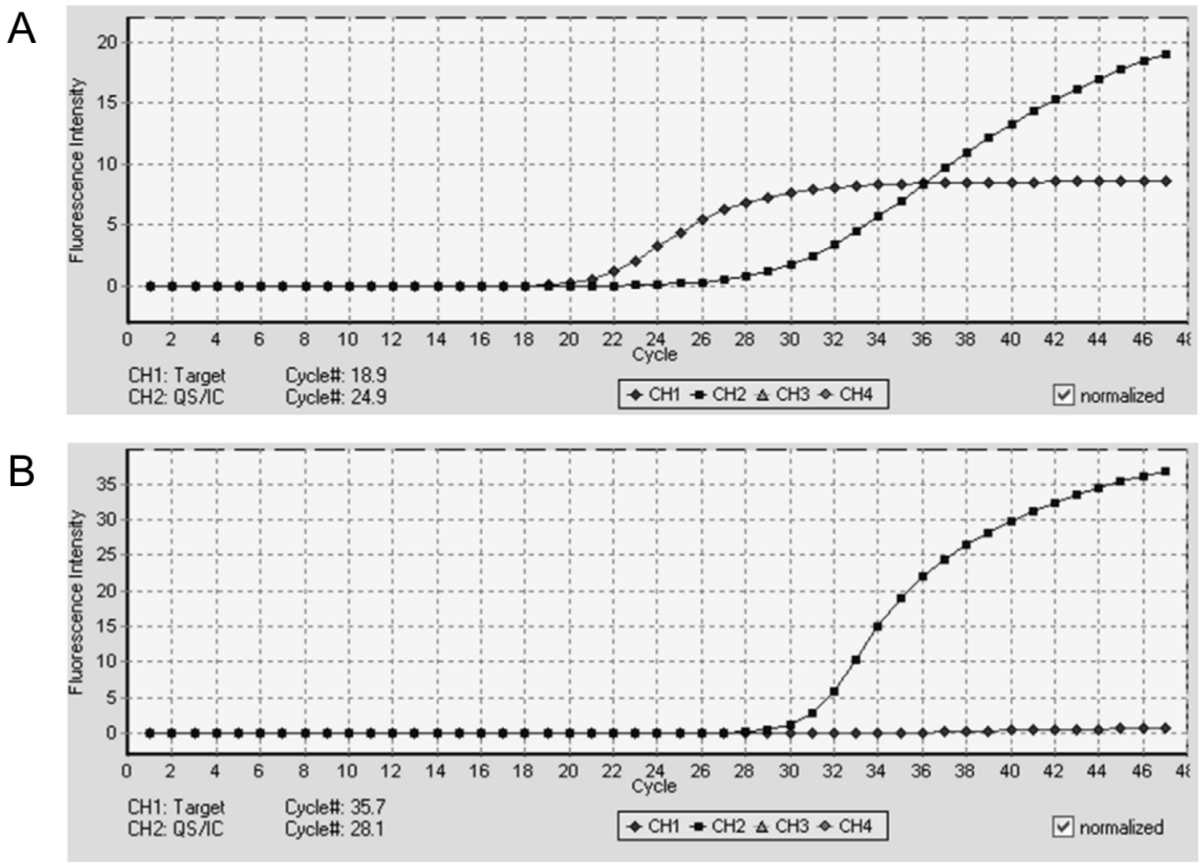


Figure 1.9: HIV total nucleic acid PCR curves. Amplification curves using COBAS AmpliPrep/COBAS TaqMan (CAP/CTM) V2, extracted from AMPLILINK software. HIV curves are indicated by diamonds and internal control curves by squares. (A) Positive PCR example, demonstrated by the low cycle threshold (Ct) and high relative fluorescence intensity (RFI). (B) indeterminate PCR example, demonstrated by the high Ct and low RFI. Adapted from Maritz et al. JCV 2017

the Xpert machine is already used in most clinical laboratories in South Africa for TB testing (Vassall et al. 2017).

1.3.2 Immunological markers

Quantification of patient T lymphocyte subsets is important for monitoring immune function. In the South African clinical setting basic flow cytometry is used to routinely monitor CD4+ T cell count in peripheral blood. In the research setting, CD8+ T cells are also quantified, as HIV disease progression is associated with increasing activation and expansion of HIV-specific and bystander CTLs (Lu et al. 2015). Both CD4+ T cell and CD8+ T cell counts can be given as an absolute count, or as a percentage of total lymphocytes.

Young children have higher numbers of CD4+ T cells compared to older children and adults. This relative lymphocytosis slowly declines to reach adult values by 6-8 years of age (WHO 2007b; European Collaborative Study 2003), therefore interpretation of a CD4 count must take into account age. CD4 percentage has less intra- and inter-person variability, so is the preferred parameter for monitoring immunological status in children (Wongsawat et al. 2010; WHO 2007b).

The WHO HIV disease staging system is used in South Africa and has four age-stratified immunological stages (WHO 2007b, Table 1.1). The immunological category gives an estimated risk of disease progression for a given age, but does not necessarily correlate with clinical stage (Shah 2006; WHO 2007b; European Collaborative Study 2001; Blanche et al. 1997). More informative than a one-off CD4 percentage is the overall trend, as the rate of CD4 percentage decline is more predictive of future disease progression (Cozzi Lepri et al. 1998). With early cART, the peripheral blood CD4+ T cell count can normalise, masking persisting immune dysfunction. Therefore, the CD4:CD8 ratio can better describe immunological status for children on cART (Sainz et al. 2013).

Table 1.1: WHO immunological classification for HIV infection 2007

HIV-associated immunodeficiency	**<11 months (%CD4+)**	**12-35 months (%CD4+)**	**36-59 months (%CD4+)**	**>5 years (cells/μL or %CD4+)**
None or not significant	>35	>30	>25	>500
Mild	30-35	25-30	20-25	350-499
Advanced	25-29	20-24	15-19	200-349
Severe	<25	<20	<15	<200 or <15%

1.3.3 Early infant diagnosis

HIV diagnosis in children occurs either as targeted testing of a child when there is high clinical suspicion for HIV disease or as part of a screening programme for all infants born to HIV-infected mothers, with the latter approach being the best way to prevent morbidity and mortality.

Beginning in 2002, the South African Early Infant Diagnosis (EID) programme tested all HIV-exposed infants at 6 weeks of age to efficiently diagnose IP infection and the less common IU infection, also coinciding with a scheduled immunisation clinic visit (Moyo et al. 2018). But as the use of antenatal cART increased, rates of IP infection declined, until the ratio of IU:IP was 3:1. Furthermore, in 2012 it was shown that by 6 weeks of age 20% of IU HIV-infected infants had already died or were lost to follow-up (Lilian et al. 2012). Therefore, birth testing was introduced, first only for high-risk infants, then in 2015 for all HIV-exposed infants (Republic of South Africa National Department of Health 2019b). A year after the introduction of routine birth testing in South Africa, the national coverage was estimated at 93% (Moyo et al. 2018). The birth test is followed by another test at 10 weeks of age, 4 weeks after most infants have completed ART prophylaxis, then again at 6 months, both at scheduled immunisation clinic visits.

1.4 HIV treatment

1.4.1 Antiretroviral drug classes and modes of action

ARV classes target different stages of the HIV replication cycle (Figure 1.4, on page 7). The five ARV classes available in South Africa are summarised in Table 1.2 and are all once or twice daily oral medication.

Table 1.2: Classes of antiretroviral drugs commonly used in South Africa.

Mode of action	Class of ARV	Drugs
Reverse transcriptase inhibitors	Non-nucleoside reverse transcriptase inhibitors (NNRTIs)	efavirenz (EFV)
		nevirapine (NVP)
	Nucleoside reverse transcriptase inhibitors (NRTIs)	abacavir (ABC)
		emtricitibine (FTC)
		stavudine (d4T)
		zidovudine (AZT)
	Nucleotide reverse transcriptase inhibitor (NRTI)	tenofovir disoproxil (TDF)
Protease inhibitors	Protease inhibitors (PIs)	lopinavir (LPV)
		ritonavir (RTV)
		atazanavir (ATZ)
Integrase strand inhibitors	Integrase strand inhibitors (INSTIs)	raltegravir (RAL)
		dolutegravir (DTG)

Only those commonly used as first or second line therapy in pregnant women and infants are listed.

1.4.2 Adult combination antiretroviral therapy

Three ARVs are the minimum required to effectively and durably suppress plasma viraemia to prevent disease progression and resistance development in adults, and this finding has been extrapolated to children (Jordan et al. 2002; Feng et al. 2019). First line cART usually consists of two nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) with different drug resistance mutation profiles (the NRTI backbone) with an additional third agent from a different drug class.

The adult NRTI backbone

AZT is less preferable as it requires twice daily dosing and commonly causes anaemia (FDA 2020). Additionally, abacavir (ABC) is avoided because it can trigger a potentially fatal hypersensitivity reaction (Hetherington et al. 2001). This reaction is

associated with HLA-B*57:01, a rare (<1%) allele in the South African population (Saag et al. 2008; G. Q. Lee et al. 2017). Nevertheless, the first line NRTI backbone recommended for adults, including pregnant women in South Africa is tenofovir disoproxil and emtricitabine (TDF+FTC) or TDF and lamivudine (3TC) (Republic of South Africa National Department of Health 2019a).

The adult third agent

In 2018 WHO updated their recommendation for the cART third agent to be the new integrase strand inhibitor (INSTI) dolutegravir (DTG) (WHO 2018). This has superseded the previous recommendations for a non-nucleoside reverse transcriptase inhibitor (NNRTI) efavirenz (EFV) (WHO 2016), because of increasing rates of pre-treatment NNRTI resistance (Hamers et al. 2018), and more reassuring data on the small increased risk of neural tube defects when used in early pregnancy (Zash et al. 2019). However, DTG only began a slow introduction to South Africa in recent months. Therefore, most South African pregnant women living with HIV on first line cART are taking the once daily fixed-dose combination pill containing TDF, FTC and EFV.

Second line therapy in adults

Switching to second-line therapy from an NNRTI-based regimen for adults in South Africa is based on a plasma viral load >1000 HIV RNA copies per mL for a duration of 6 months, despite sufficient measures to improve ART adherence. Standard second line therapy was PI-based (AZT, 3TC/FTC and ritonavir-boosted-lopinavir (LPVr)) up until 2019 (Republic of South Africa National Department of Health 2015b). Now DTG is the recommended second-line third agent (Republic of South Africa National Department of Health 2019a).

1.4.3 Paediatric combination antiretroviral therapy

Safety, formulation and pharmacokinetic (PK) issues significantly limit ARV options in children, particularly for neonates.

The paediatric NRTI backbone

TDF is avoided in children given the risk of renal toxicity and reduction in bone mineral density (Aurpibul and Puthanakit 2015). The WHO recommended first-line NRTI backbone in children is ABC and 3TC, both of which come in suspensions and crushable pills. However, there is limited PK data for the use ABC in infants <3 months of age, and none for infants in the first two weeks of life. This requires the use of AZT in neonates, which can be substituted with stavudine (D4T) in the occurrence of anaemia (Republic of South Africa National Department of Health 2019a).

The paediatric third agent

Because of limited PK data, EFV is not recommended for use in children <3 years of age or <10kg (ibid.). The next NNRTI of choice is NVP, which, as the first ARV to be used as infant prophylaxis, has extensive safety and PK data from birth and is available as a suspension. NVP is inferior compared with the PI LPVr at maintaining plasma viral suppression in in children <3 years old (Palumbo et al. 2010; Violari et al. 2012). Therefore LPVr is the third agent of choice in young children.

LPVr is the only infant formulated PI available in South Africa. LPVr is a suspension of a 4:1 ratio of lopinavir (LPV) and ritonavir (RTV). LPV has low oral bioavailability due to rapid metabolism by cytochrome P450 3A4 (CYP3A4) but co-administering LPV with low-dose RTV, a CYP3A4 inhibitor, increases the LPV area under the plasma drug concentration-time curve (Moyle and Back 2001). Despite this, LPVr still requires twice daily dosing in children. LPVr is insoluble thus is suspended in a mixture of ethanol and propylene glycol (42% and 15% respectively) which are foul-tasting. Furthermore, these excipients have caused cardiotoxicity in preterm infants, triggering an FDA black box warning against the use of LPVr suspension in infants <42 weeks corrected gestational age (NIH 2020). Once dispensed and outside a refrigerator, LPVr suspension is only stable for 6 weeks, necessitating frequent pharmacy visits (IATT 2015).

Given the aforementioned concerns with LPVr use in children, in 2018 WHO changed its recommendation from LPVr to DTG (WHO 2018). However, PK data for DTG in children <20kg has not been established, requiring the use of either LPVr or another INSTI raltegravir (RAL). RAL has been used as prophylaxis and treatment in children down to 2 kg (IMPAACT P1066 and IMPAACT P1110, Clarke et al. 2020; Nachman et al. 2018), but this drug has a much lower genetic barrier to resistance, and comes as oral granules for suspension, requiring complex preparation (Clarke et al. 2020; Archary et al. 2020). DTG dispersible tablets have been evaluated in infants from four weeks of age as part of the IMPAACT P1093 and Odyssey trials with registration of the formulation expected in 2020 (`https://impaactnetwork.org/studies/P1093.asp`, `http://odysseytrial.org`). The only paediatric formulation INSTI available in the South African public health sector are non-crushable 50 mg tablets, hence why LPVr suspension is still the mainstay of infant therapy.

Second line therapy in paediatrics

If a child on a PI-based regimen has a plasma viral load remain over 1000 HIV RNA copies per mL over a 2 year period, then resistance testing is recommended. If no PI resistance is detected, then the treatment remains unchanged. If PI resistance is present, then the resistance genotype is sent, along with the clinical information, to the National Third-line Committee in Johannesburg who decide on appropriate therapy (Republic of South Africa National Department of Health 2019a).

1.4.4 Adjuvant therapies

Prophylactic dosing of trimethoprim/sulfamethoxazole (co-trimoxazole) significantly reduces PCP and serious bacterial infection-related morbidity and mortality in HIV-infected infants (Thea et al. 1996; Chintu et al. 2004; Zar et al. 2010). The South African recommendations are that HIV-infected infants continue once daily co-trimoxazole from 6 weeks until 1 year, then until 5 years only if the CD4 is <25% or the child is symptomatic (Republic of South Africa National Department of Health 2019a). Additionally, given the high rate of malnutrition and food insecurity in HIV-infected

children in South Africa it is recommended that all HIV-children and infants are prescribed a daily multivitamin dose (Kimani-Murage et al. 2011; Republic of South Africa National Department of Health 2019a).

1.5 Treatment failure

1.5.1 Definitions

Monitoring HIV plasma viral load is superior to using clinical or immunological criteria to assess treatment response in children and adults (Joram et al. 2017; Costenaro et al. 2014; Davies et al. 2011; Westley et al. 2012). Viral suppression refers to a plasma viral load below the detection threshold of standard viral assays and is the goal of cART (WHO 2016). Virological failure refers to the inability to achieve or maintain plasma HIV viral load below a setting-dependent threshold. WHO define virological failure as a plasma viral load >1000 HIV RNA copies per mL, on two separate occasions at least 3 months apart, after at least 6 months of cART (ibid.), and this is the definition used for children and adults in South Africa. WHO's more relaxed definition of virological failure aims for what is achievable in resource-limited settings. However, low-level viraemia should not be ignored, as it indicates an increased risk of future virological failure (Hermans et al. 2017).

Rates of virological failure in children are higher than in adults. A meta-analysis estimated that only two-thirds of children initiated on cART in Low and Middle Income Countries (LMIC) remain in care with a suppressed viral load (Boerma et al. 2016). The overall estimate for paediatric HIV treatment failure in South Africa is unknown, but studies from across the provinces suggest a prevalent problem, particularly at the extremes of the paediatric age range (Meyers et al. 2011; Teasdale et al. 2018; Kuhn et al. 2020). Kuhn and colleagues followed 73 HIV-infected infants from birth in Johannesburg, South Africa. Of the 84% remaining in care at 2 years, only half had achieved and maintained viral suppression (Kuhn et al. 2020).

Two prevailing and interdependent causes for treatment failure in children are HIV drug resistance (HIVDR) and suboptimal cART adherence.

1.5.2 HIV drug resistance

Due to a large viral population, high turnover rate, and RT's propensity for introducing mutations, HIV has exceptional potential for generating genetic diversity, including the development of drug resistance mutations (DRMs) (Wei et al. 1995; Mansky and Temin 1995; O'Neil et al. 2002; Abram et al. 2010).

Clinically significant levels of HIVDR can either be present before starting or re-starting cART: pre-treatment drug resistance, or acquired on cART: acquired drug resistance. Pre-treatment drug resistance occurs either from transmitted drug resistance or prior exposure to ART either as treatment or prophylaxis. Acquired drug resistance occurs when serum ART levels drop below the therapeutic index, allowing viral replication and selection for HIV with DRMs (Clutter et al. 2016).

With the increasing use of cART worldwide, the rate of resistance is growing (Gupta et al. 2017). The concern with maternal HIVDR is two-fold. First the increased risk of transmission due to viraemia, and second, the risk of transmitting HIV resistant to both prophylactic and therapeutic ART to the infant. A study of 78 South African HIV-infected pregnant women with treatment failure found that 73% of women had DRMs, of which three-quarters had NNRTI DRMs and half had NRTI DRMs (Adeniyi et al. 2019).

A South African national survey in 2014-16 found NNRTI pre-treatment HIVDR in children <18 months of age was 62.7% and for NRTIs 13.9% (WHO 2017b). There is a paucity of data worldwide on acquired HIVDR in HIV-infected children (ibid.). A meta-analysis in 2017 found a high level of acquired HIVDR observed in children with treatment failure (ibid.). However, this study is outdated, with a focus on children on NNRTI-based therapy. In a recent study of 236 South African children with treatment failure, 9.3% had PI mutations (95% major mutations), although 27% had received

Table 1.3: Major drug resistance mutations of commonly used first and second line ART in South African mothers and children.

Non-nucleoside reverse transcriptase inhibitor resistance mutations													
Position	**100**	**101**	**103**	**106**	**181**	**188**	**190**	**230**					
Consensus	**L**	**K**	**K**	**V**	**Y**	**Y**	**G**	**M**					
Efavirenz	I	EP	NS	AM	CIV	LCH	ASE	L					
Nevirapine	I	EP	NS	AM	CIV	LCH	ASE	L					
Nucleoside/nucleotide reverse transcriptase inhibitor resistance mutations													
	Non-thymidine analogue mutations					**Thymidine analogue mutations**						**Multi-drug resistance mutations**	
Position	**184**	**65**	**70**	**74**	**115**	**41**	**67**	**70**	**210**	**215**	**219**	**61**	**151**
Consensus	**M**	**K**	**K**	**L**	**Y**	**M**	**D**	**K**	**L**	**T**	**K**	**T**	**Q**
Lamivudine	VI	R										Insertion	M
Emtricitabine	VI	R										Insertion	M
Abacavir	VI	R	E	VI	F	L			W	FY		Insertion	M
Tenofovir	***	R	E			L		R	W	FY		Insertion	M
Stavudine	***	R	E			L	N	R	W	FY	QE	Insertion	M
Zidovudine	***	***	*	*		L	N	R	W	FY	QE	Insertion	M
Protease inhibitor resistance mutations													
Position	**32**	**33**	**46**	**47**	**48**	**50**	**54**	**76**	**82**	**84**	**88**	**90**	
Consensus	**V**	**L**	**M**	**I**	**G**	**I**	**I**	**L**	**V**	**I**	**N**	**L**	
Atazanavir	I	F	IL	V	VM	L	VTALM		ATFS	V	S	M	
Lopinavir	I	F	IL	V A	VM	V	VTALM	V	ATFS	V		M	
Integrase strand inhibitor resistance mutations													
Position	**66**	**92**	**118**	**138**	**140**	**143**	**148**	**155**	**263**	**263**			
Consensus	**T**	**E**	**G**	**E**	**G**	**Y**	**Q**	**N**	**R**	**R**			
Raltegravir	AIK	Q	R	KAT	SAC	RC	HRK	H	K	K			
Dolutegravir	K	Q	R	KAT	SAC		HRK	H	K	K			

Red text: Highest level of phenotypic resistance and the strongest clinical evidence for interfering with successful therapy. Blue text: Reduced susceptibility or virological response. Plain text: Reduced susceptibility in combination with other resistance mutations from the same antiretroviral class. *Changes at this position infer increased sensitivity, the degree to which according to the number of *. Amino acid abbreviations: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine. Adapted from https://hivdb.standford.edu/

the historical unboosted-RTV therapy, which has a high-risk for developing DRMs (Makatini et al. 2019).

1.5.3 Resistance mechanisms

Common major DRMs are summarised in Table 1.3. DRM mechanisms vary, but major mutations are an amino acid substitution or insertion that either individually or in combination result in a viral enzymatic conformational change altering drug binding to one or more ARVs from the same class. Additionally, accessory mutations can compensate for reduced viral fitness.

NNRTIs

The NNRTI binding site is small and well-defined thus a single amino acid change can result in high-level resistance. There is significant cross resistance in the common DRMs for EFV and NVP which has implications for maternal EFV use and NVP as infant prophylaxis.

NRTIs

In most cases NRTI DRMs confer high-level resistance to one or more NRTIs. M184V is the most common NRTI DRM and is virtually ubiquitous in children with virological failure while on 3TC (Miller et al. 2002). A group of Thymidine Analogue Mutations (TAMs) are associated with AZT and d4T use (Table 1.3). Once more than three or four TAMs accumulate, they also confer resistance to other NRTIs, such as ABC and TDF. Following this, specific further mutations can confer high-level resistance to all NRTIs.

PIs

PI DRMs are classed as either major or accessory. Generally, more than three major DRMs will impair PI susceptibility (`https://hivdb.stanford.edu`). Accessory DRMs restore viral fitness but many are actually naturally occurring polymorphisms, and their significance is questionable in the absence of major PI DRMs (Gordon et al. 2003; Wensing et al. 2017).

INSTIs

Resistance to RAL develops quickly, with only one or two mutations required to significantly reduce susceptibility (Han et al. 2017). There is low cross-resistance of RAL resistance to DTG, which maintains a high genetic barrier to resistance (ibid.).

1.5.4 Resistance testing

Most commonly HIVDR testing uses genotypic analysis performed using Sanger sequencing. This method can detect DRMs which are present in >20% of the viral quasispecies. Commercial Sanger sequencing kits cost $100-300 USD (Inzaule et al.

2016) therefore, resistance testing in the event of first line treatment failure in low income settings is not cost effective (Phillips et al. 2014).

Next-Generation sequencing (NGS) methods can sequence millions of DNA fragments simultaneously, producing full viral genome sequences for viral quasispecies in multiple samples per run. This dramatically reduces the running cost per sample, with scope for centralised, high-throughput laboratories. NGS readouts quantify DRMs, including those with a prevalence of <20%, however data analysis is complex, and bioinformatic pipelines are not standardised resulting in variable sensitivity and accuracy (E. R. Lee et al. 2020). Furthermore, low prevalence DRMs detected can arise from PCR error inherent to high-throughput sequencing (Jabara et al. 2011).

Once obtained, the list of patient DRMs are entered into a programme, such as the Stanford Online HIV Drug Resistance Database (`https://hivdb.stanford.edu`), which uses a meta-analysis of clinical and *in vitro* studies to calculate the level of resistance (high, intermediate, low or potential) for all the relevant ARVs.

1.5.5 Treatment adherence

Treatment adherence describes the degree to which the prescription is followed. cART adherence can be directly measured using serum drug levels; however, results only indicate cART ingestion in the previous days. More commonly, cART adherence is assessed indirectly with patient or caregiver missed dose recall. Self-reports, although the simplest method of adherence measurement, are subject to social desirability bias and questionaries are not evidence-based (Vreeman et al. 2019). Bypassing questioning are "pill counts" and pharmacy records, where used medication containers are returned for left-over medication to be quantified. However, this method relies on the containers being returned, no stockpiled medication, and no repeated doses following infant spitting. One South African study found no correlation between infant plasma viral load and returned medication measurements nor caregiver adherence reports (Teasdale et al. 2013). The gold standard for measuring adherence is electronic dose monitoring, such as the Medication Event Monitoring System (MEMS) that records each time the

medication bottle is opened, but this method is usually only available in the research setting.

Adherence can be considered a spectrum, but it is useful to have an estimate of what level of adherence is required for a specific cART regimen to achieve plasma viral suppression. Two studies of South African infants prescribed PI therapy found that >80-85% adherence to any of the three prescribed medications significantly decreased the odds of viraemia (Teasdale et al. 2013; Müller et al. 2009). Similarly, for adults on NNRTI-based cART, >80% adherence by pill counts results in <10% virological failure rate (Martin et al. 2008).

1.5.6 Risk factors for non-adherence

A meta-analysis of over 20 000 HIV-infected pregnant women from around the world found that only 74% of women had >80% cART adherence during pregnancy (Nachega et al. 2012), similar to non-pregnant women (Henegar et al. 2015). Risk factors for non-adherence in South African pregnant women include younger age, shorter duration of cART, depression and unplanned pregnancy (Woldesenbet et al. 2020; Brittain et al. 2018; Psaros et al. 2014). The postpartum period is well-described as being the riskiest time for cART non-adherence (Henegar et al. 2015; Huntington et al. 2015; Adams et al. 2015), with an estimated non-adherence rate of 50% (Nachega et al. 2012). Caregivers' own cART adherence practices often extend to their child (Humphrey et al. 2019). A study in rural South Africa, found maternal non-adherence to cART during pregnancy predicted an 80% reduction in odds of infant prophylaxis non-adherence (Cook et al. 2018).

Like any human behaviour, the reasons behind cART adherence are individual, complex and dynamic. WHO developed a framework for exploring barriers to chronic medication adherence (WHO 2003), and an example for paediatric HIV is shown in Table 1.4. HIV-related stigma and non-disclosure of HIV are significant barriers to HIV care for African women (Hodgson et al. 2014). In South Africa, non-disclosure of HIV status to male partners during pregnancy is associated with maternal cART

non-adherence, but it is not clear whether this effect is causal, as non-disclosure is also associated with intimate partner violence, alcohol use, younger age, and less financial security, all of which could increase non-adherence risk (Brittain et al. 2019; Adeniyi et al. 2017; Ramlagan et al. 2018; Makin et al. 2008). However, undoubtedly, non-disclosure to the household or partner requires the concealment of paediatric cART administration, which can negatively impact adherence.

Table 1.4: Barriers to paediatric ART adherence

Dimension	Paediatric HIV examples
Condition-related	Child looking well
	Lifelong illness
Medication-related	Medication burden i.e. multiple syrups twice daily
	ART formulation issues e.g. bad-taste
Socio-economic	Caregiver's competing demands e.g. childcare, employment, food
Healthcare system	Frequency of appointments
	Transport costs
	Long wait clinic wait times
	Negative healthcare worker attitudes
Individual	HIV-related stigma
	Depression
	Caregiver health
	Caregiver beliefs and concerns regarding ART

Adapted from: Adherence to long-term therapies, evidence for action. WHO 2003

Better socioeconomic conditions are associated with superior cART adherence for South African children (Teasdale et al. 2013), furthermore non-adherence to placebo has been shown to be strongly predictive of increased mortality risk for HIV-infected African infants (S. A. Walker et al. 2009). Thus, non-adherence is a marker for unmeasured caregiver effects; whether it be health-seeking behaviour, poverty or violence at home; non-adherence corresponds to a vulnerable infant.

In South Africa, adherence counselling sessions from nurses or lay-counsellors are designed to provide information and psychosocial support at the individual patient level. But an observational study of routine counselling clinics in rural KZN found HIV counselling sessions were brief and insufficient (Coetzee et al. 2016). Additionally, rates

of re-suppression following enhanced adherence counselling in children are lower than adults, indicating a patient group with more challenges (Ford et al. 2019).

1.6 Potential for paediatric HIV cure

1.6.1 Definition of cure

Lifelong viral suppression with cART is indeed possible, however, HIV stigma, drug resistance, side effects of long-term therapy, ongoing immune inflammation, the cost of cART, as well as high rates of non-adherence, all contribute to the rationale for paediatric HIV cure. A sterilizing cure, is defined as complete eradication of HIV from the body, whereas a "functional cure" is long-term remission: when plasma viraemia is undetectable in the absence of ART, even if complete eradication has not occurred. The latent HIV reservoir is the ultimate barrier to HIV cure which is why substantial efforts have been made to determine how to minimize it. To date there have only been two people thought to be cured of HIV (Gupta et al. 2019; Hutter et al. 2009). Both men had haematological malignancies for which they received allogenic haematopoietic stem cell transplants from donors homozygous for CCR5 deletion. Bar this extreme, and by no means scalable approach, the only intervention that has been shown to reduce the size of the latent HIV reservoir reproducibly is cART early in infection.

1.6.2 Post-treatment control

Some studies have shown that lower peripheral reservoirs on cART can delay plasma viral rebound following analytic treatment interruption in adults (J. P. Williams et al. 2014; Li et al. 2016), however other studies of adults treated very early in HIV infection, with extremely low latent HIV reservoirs rebounded quickly after treatment interruption (Chun et al. 2010; Henrich et al. 2014; Colby et al. 2018). Mathematical models estimate that peripheral blood HIV DNA levels must be decreased by 50-1000-fold in order to increase the time before rebound following treatment interruption from weeks to a year (Hill et al. 2014; Pinkevych et al. 2015). There have been fewer examples of analytical treatment interruption studies in children, but two studies have demonstrated better viraemic control in children with lower HIV DNA levels (Kuhn

et al. 2018; Tagarro et al. 2018). The most extreme example of a delay in rebound following treatment interruption is where an HIV-infected individual can maintain low to undetectable plasma viral loads despite withdrawal of therapy; post-treatment controllers. Adult post-treatment controllers are more likely to have been treated in primary infection, where the occurrence is predicted as high as 15%, and have lower latent HIV reservoirs (Sáez-Cirión et al. 2013; Assoumou et al. 2015; Steingrover et al. 2008; Namazi et al. 2018). There have been three well-described reports of paediatric post-treatment controllers (Persaud et al. 2013; Violari et al. 2019; Frange et al. 2016). The most famous example is the Mississippi Baby, who initiated cART at 30 hours of life then had 27 months of HIV remission following treatment interruption at 18 months of age (Persaud et al. 2013). This case triggered the great interest in very early infant HIV treatment, which is thought to harness the unique neonatal immune response.

1.6.3 The neonatal immune response

The newborn immune response is broadly described as tolerogenic, not from immaturity, but as a continuation of the active immunomodulatory response required *in utero*. Foetuses have adapted to avoid inflammatory responses that can cause preterm delivery (Vitoratos et al. 2006), in particular, to non-inherited maternal antigens, and in preparation for the barrage of foreign antigens on entering the extra-uterine world. The key features of a newborn immune response are an anti-inflammatory innate immune response and a weaker cell-mediated response.

With limited immunological memory, newborns have a heavy reliance on their innate immune system and on passive immunity acquired from the mother transplacentally and via breastmilk. Neonatal innate immune response results in activation of the extra-cellular Th cell responses (Th2 and Th17), rather than the intracellular Th cell response (Th1) via a unique cytokine profile released by DCs and monocytes; higher IL-6, IL-23, IL-1β and less IL-12, IFN-γ compared to adults following toll-like receptor (TLR) stimulation (Kollmann et al. 2009; De Wit et al. 2004; Nguyen et al. 2010).

This Th2/17 bias, may dampen the immunopathologic response to HIV. Furthermore, the same innate cells also produce an overall anti-inflammatory cytokine profile, with lower IFN-1 and higher IL-10 levels (Kollmann et al. 2009; De Wit et al. 2004; Nguyen et al. 2010).

With less Th1 activation, newborns mount a less frequent and lower magnitude adaptive anti-HIV CTL response (Thobakgale et al. 2007; Scott et al. 2001; Shalekoff et al. 2009; Garcia-Broncano et al. 2019). This weak response is insufficient to control HIV viraemia, as demonstrated by the high plasma viral loads and lack of selection of viral escape mutations in infants (Adland et al. 2015). Moreover, HIV-infected infants do not typically have HIV-specific CD4+ T cell responses in peripheral blood (S. Huang et al. 2008; Thobakgale et al. 2007). That said, infant gut associated lymphoid tissue is home to a large subset of clonally expanded mucosal memory CD4+ T cells (Bunders et al. 2012), which suggests that effective adaptive cellular immunity does occur in newborns, just outside of the peripheral blood compartment.

The immune system is complex, with many more unique features of the newborn immune system under investigation, in particular potentially favourable newborn antiviral innate immune responses via natural killer cells and innate lymphoid cells (Yu et al. 2018).

However, life in the outside world is full of other immune triggering exposures, such as microbial translocation through an immature gut, co-infections, in particular CMV, and immunisations, including the BCG immunisation at birth. These inflammatory proponents, along with poor adaptive immune responses, and a virus pre-adapted to shared maternal HLA-mediated CTL responses, results in the overall balance tipping towards immune dysregulation and HIV disease progression. Therefore, there may just be a short window to suppress HIV with cART in the first weeks of life in order to exploit the immunotolerant state and preserve immune function (Goulder et al. 2016; Garcia-Broncano et al. 2019).

1.7 Overview of this thesis

Paediatric HIV is still a significant problem in South Africa and treatment is difficult. There is anecdotal evidence that very early cART initiation, in the first hours of life, for *in utero* HIV-infected infants, can induce HIV remission or cure, negating the need for life-long ART. For this thesis I set out to recruit a cohort of IU HIV-infected infants in KwaZulu-Natal South Africa, in order to determine the feasibility of point-of-care testing to initiate cART within 48 hours of birth, and explore the impact this has on infant viral burden in the first years of life, as a measure for the potential for cure. The first two data chapters of this thesis deals with these questions, while the rest of the thesis examines discoveries from my work on the study in South Africa, and include important features that could influence the potential for *in utero* HIV infection cure.

- Chapter three describes the cohort's recruitment, retention, and plasma viral suppression rates, and explores HIV drug resistance and non-adherence as possible reasons for treatment failure.
- Chapter four investigates the impact timing of cART initiation has on infant latent HIV viral reservoir levels.
- Chapter five outlines new complexities in diagnosing HIV at birth for infants exposed to ART as prophylaxis.
- Chapter six delves deeper into some of the maternal barriers to ART adherence using second-generation mother-to-child HIV transmission as an example for discussion.
- Chapter seven reviews the plausibility for the seemingly higher risk for girls to be *in utero* HIV-infected, and then explores the clinical, virological and immunological features of male versus female *in utero* HIV infection.

Detailed aims and hypotheses are described in each individual chapter.

Chapter 2

Methods

Chapter-specific methods are presented in the Methods sections of the relevant chapters. Contributions are explicitly stated where relevant.

2.1 The Ucwaningo Lwabantwana Cohort

Ucwaningo Lwabantwana (UL) (meaning 'Learning from Children') is an ongoing observational prospective cohort study that began in 2015, and was designed to determine the feasibility of very early (<48 hours of life) cART initiation for *in utero* HIV-infected infants.

2.1.1 Study setting

UL is set in the province of KwaZulu-Natal (KZN), South Africa, where 85% of the population are black African, and 80% speak isiZulu as their first language. Approximately 60% of KZN's population live in the World Bank's 2015 definition of poverty (<$1.9 USD per day, Statistics South Africa 2018) and 69% of females are in long-term unemployment, along with 60% who never completed secondary school (Statistics South Africa 2016b).

The KZN Department of Health run a successful PMTCT programme, with free life-long access to cART for all pregnant women, and HIV testing at every antenatal clinic visit. This results in a very low IU HIV transmission rate of 0.6%, but given the high

antenatal seroprevalence (40%) the number of IU HIV-infected infants in KZN still equates to ∼450 per year (Figure 2.1 Goga et al. 2018).

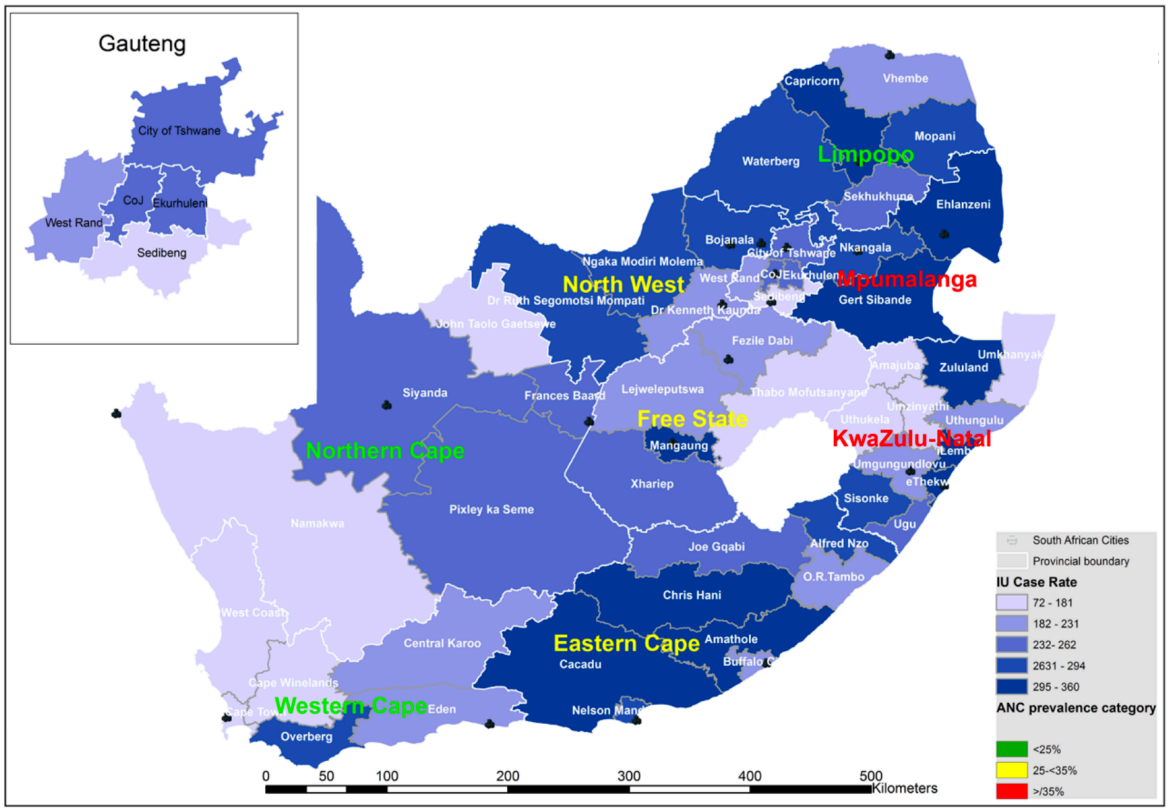


Figure 2.1: Antenatal HIV seroprevalence and *in utero* HIV transmission rate by South African province and district, 2016-2017. ANC: antenatal clinic. IU: *in utero*. Reproduced with permission from Goga et al. South African Med J. 2018

96% of births in South Africa occur in a healthcare facility (Statistics South Africa 2016b), therefore maternity units provide a concentrated inpatient population of HIV-exposed neonates, where routine HIV birth testing is also performed. The following hospitals were selected as UL study sites for their combination of large size and location within a district with a high antenatal HIV seroprevalence (Republic of South Africa National Department of Health 2015a). The hospital locations are displayed in Figure 2.2.

Edendale Hospital, Pietermaritzburg

Secondary level hospital with approximately 580 deliveries per month, located 80 km west from Durban in the uMgungundlovu district, which in 2015 had an estimated

antenatal HIV seroprevalence of 46.2% (95% CI 39.3 - 53.1).

Stanger Hospital, KwaDukuza

Secondary level hospital with approximately 520 deliveries per month, located 80 km north from Durban in the iLembe district, which in 2015 had an estimated antenatal HIV seroprevalence of 44.3% (95% CI 38.3 - 50.5).

Mahatma Gandhi Memorial Hospital, Phoenix, Durban North

Secondary level hospital with approximately 600 deliveries per month, located 30 km north from Durban in the eThekwini district, which in 2015 had an estimated antenatal HIV seroprevalence of 46.2% (95% CI 43.0 - 49.5).

Queen Nandi Regional Hospital, Empangeni

Secondary level hospital dedicated exclusively to the care of obstetrical, gynaecological and neonatal patients, with approximately 700 deliveries per month, located 170 km north from Durban. This hospital is also the neonatal referral centre for a large population in the uThungulu district, which in 2015 had an estimated antenatal HIV seroprevalence 45.9% (95% CI 37.0 - 55.1).

2.1.2 Study recruitment and HIV testing

At each study site, Monday to Friday, all HIV-infected mothers on the postnatal ward were interviewed by research staff as soon as possible after delivery to assess risk of IU HIV transmission. High-risk was defined as one or more of the following:

- Maternal seroconversion; a documented negative rapid HIV-1 antibody test in pregnancy followed by a positive result later in pregnancy or at delivery.

- <4 weeks of maternal cART prior to delivery

- Suboptimal cART adherence during pregnancy; defined as missing three or more consecutive cART doses



Figure 2.2: Ucwaningo Lwabantwana study site locations. Map reproduced from www.google.com/maps

- Most recent plasma viral load >1000 HIV RNA copies per mL

Note that maternal cART was available free of charge from South African Department of Health clinics, which in the first instance was EFV/TDF/FTC once daily fixed-combination oral pills. Deviation from this regimen occurred when clinically indicated, with indications including renal impairment or first line therapy treatment failure.

Infants of high-risk mothers were tested for HIV-1 using PoC TNA PCR on whole blood (GXP, Cepheid) or where PoC testing was not available, by overnight plasma HIV RNA viral load measurement (Nuclisens EasyQ v2.0 HIV-1 RNA PCR, bioMérieux). The results of these tests had no indeterminate range.

All HIV PCR test results in South Africa were released by NHLS weekly via email. Mothers from the same hospitals who either declined PoC testing or who were discharged before their infant could be tested, and those who delivered at the hospitals' local clinics with a positive or indeterminate result from the standard-of-care (SoC)

laboratory-based DBS TNA PCR (CAP/CTM, Roche) test on their infant at birth were traced. If contact was successful, the mothers were asked to consent for study enrolment. Turnaround time for the SoC test result was approximately 4–7 days.

Eligibility criteria for enrolment via any method of testing included an IU HIV-infected infant initiated on cART <21 days of life. An IU HIV-infected infant was defined as an infant with a positive or indeterminate TNA PCR taken <48 hours of age that was later confirmed positive via further testing from a separate blood sample i.e. all cases diagnosis required two or more positive nucleic acid tests.

For analysis purposes, the infants were sub-grouped into PoC and SoC according to whether or not they were first diagnosed using a PoC test.

2.1.3 Infant ART

All HIV-exposed infants were given prophylactic ART as soon as possible after birth by the hospital staff, consisting of at least daily NVP, and additional twice daily AZT for infants deemed high risk of HIV-1 infection, in accordance with the South African National PMTCT Guidelines. After the first positive infant HIV-1 test result was received, blood for confirmatory testing and baseline measurements was taken and cART consisting of twice daily NVP, AZT and 3TC oral suspensions was initiated. This neonatal regimen was switched to LPVr, 3TC and ABC oral suspensions at 42 weeks corrected gestational age for infants <2.5 kg at birth or at 1 month of age for all other infants. All cART was given in accordance to South African guidelines (Appendix I, page 165).

2.1.4 Participant follow-up

Maternal and infant HIV care and ART were provided by the hospital HIV clinics. In parallel, the study participants were seen in the on-site research clinic, monthly for 6 months, and 3 monthly thereafter. During follow-up, data was collected and additional support was provided for the caregivers, including regular one-on-one sessions from trained HIV counsellors in isiZulu, paid transport to clinic, appointment reminders

via phone, family support groups and home visits where required. Loss to follow-up was defined as an infant that had not returned to the HIV clinic after 6 months, despite active tracing.

2.1.5 Ethics

The UL study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal and the Oxfordshire Research Ethics Committees. Written informed consent for the infant and mother's participation in the study was obtained from the mother or infant's legal guardian.

2.2 Infant blood sampling

2.2.1 Phlebotomy schedule

Peripheral venous blood was drawn from the infants in the UL cohort at each scheduled follow up visit. Blood sample volumes were calculated per kg body weight and in according with South African Research Guidelines (Stellenbosch University Health Research Ethics Committee 2015). Blood was drawn from the mothers at baseline and during follow-up if there was clinical suspicion of virological failure, or in special circumstances, such as acutely infected mothers.

2.2.2 Clinical samples

Participant blood samples were sent to an accredited clinical laboratory (Neuberg Global, Amanzimtoti, Durban) for lymphocyte subset (CD4+ and CD8+ T cell) quantification by flow cytometry (BD) and plasma viral load (HIV-1 RNA PCR, NucliSens). The limit of detection for plasma viral load quantification was 20 HIV RNA copies per mL or 100 HIV RNA copies per mL where the sample volume was low. Lipid profiles, transaminases and haemoglobin were also monitored to assess for ART side effects, as per the South African guidelines. Where indicated, HIVDR testing was performed on plasma by Sanger sequencing using the HIV-1 Genotyping kit (Applied Biosystems) to identify DRMs, and resistance phenotype was derived from the Online Stanford Database (`https://hivdb.stanford.edu/`).

2.2.3 Separation of plasma and isolation of PBMCs

Fresh blood samples in EDTA were sent to the core laboratory of HIV Pathogenesis Programme, University of KwaZulu-Natal, for separation into plasma, and isolation and cryopreservation of PBMC within 8 hours of collection.

Whole blood was spun for 10 minutes at 500 xg to separate plasma. Plasma was spun again for 10 minutes, then aliquoted into 1.8 mL vials to be stored at -80°C. The remaining blood was diluted to the original volume with PBS, layered onto Histopaque-1077 (Sigma-Aldrich), in a ratio of 1:2.25 and spun for 30 minutes at 500 xg without brakes. The buffy coat layer containing PBMC was transferred to a new tube with a Pasteur pipette, washed twice with PBS then resuspended in R10 medium (10% FCS in RPMI with 1% penicillin-streptomycin and 1% L-glutamine, Sigma-Alrich) for cell counting. PBMC were spun down and resuspended drop-wise in FCS containing 10% DMSO (Sigma-Alrich) and aliquoted into 1 mL vials of 5-10 million cells. The vials were placed in a pre-chilled StrataCooler freezing container (Aligent) that allows a constant rate of cooling at 1°C per minute. The container was kept at -80°C overnight, then transferred to liquid nitrogen for long-term storage.

2.3 Serum ART levels

Serum ART quantification was performed by Katya Govender (Africa Health Research Institute, South Africa). Eleven ARVs; FTC, TDF, LPV, RTV, NVP, ABC, 3TC, AZT, ATZ, DTG and EFV, were simultaneously analysed and quantified in patient plasma samples using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Calibration standards and quality control samples were prepared using drug free, donor human plasma. The bioanalytical LC-MS/MS method for the quantification of ARVs in plasma was developed and validated according to FDA guidelines.

The ARV analytes were extracted from samples using protein precipitation and separated on a Zorbax Eclipse Plus C18, 2.1 x 50 mm, 3.5 μm HPLC column (Aligent) using gradient elution. Mobile phase A consisted of water with 0.1% formic acid and

B, acetonitrile with 0.1% formic acid. The column oven was set at 40°C, a sample volume of 2 μL was injected and the flow rate was set to 0.2 mL per minute. MS data acquisition was performed using polarity switching; for simultaneous analyte ion selection and detection in positive and negative modes. TDF, FTC, 3TC, LPV, RTV, NVP, ABC, AZT, ATZ and DTG were analysed in the positive mode, using 6-amino-nicotinic acid as an internal standard and EFV was analysed in the negative mode using deuterated efavirenz as an internal standard.

Data were collected and quantitated using Analyst Software, version 1.6.2 (SCIEX). The calibration standard concentration range was 150–5000 ng per mL for EFV, 90–3000 ng per mL for TDF, 20–800 ng per mL for FTC and 180–6000 ng per mL for 3TC, LPV, RTV, NVP, ABC, AZT, ATZ and DTG. The standard calibration curves for all analytes showed good linearity. All calibration standards and quality control samples were within 15% of the nominal value for both within-day and between-day runs. Plasma ART levels below the standard concentration range (limit of detection) were deemed negative, whereas any quantifiable level was assigned positive.

2.4 Deep sequencing HIV

The majority of the maternal and infant HIV was deep-sequenced by Dr David Bonsall's research group (Big Data Institute, University of Oxford, UK) according to their customized method, described elsewhere in detail (Bonsall et al. 2018) and summarised as follows. Note that a few initial samples were sequenced by Matthew Bull, Thomas Connor and colleagues from Cardiff University, UK, using similar methods.

Total RNA was extracted using the NucliSens easyMAG system (bioMérieux) and concentrated with Agencourt RNAClean XP (Beckman Coulter). Libraries retaining directionality were prepared using the SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian (Takara Bio) with specific modifications to the manufacturer protocol. The RNA was denatured at 72°C with tagged random hexamers, followed by cDNA synthesis using in-house sets of 96 i7 and 96 i5 indexed primers (Lamble

et al. 2013), and a maximum of 12 PCR cycles, in 25% of the reaction volume recommended. Ribosomal cDNA was not depleted. 5 μL of each amplified library were pooled in 96-plex. The pool was cleaned with a lower ratio than recommended of Agencourt AMPure XP, to eliminate shorter libraries. The size distribution and concentration of the 96-plex was assessed using a High Sensitivity D1000 ScreenTape assay on a TapeStation system (Agilent) and a Qubit dsDNA HS Assay (Thermo Fisher Scientific). A total of 500 ng of pooled libraries was hybridized (SeqCap EZ Reagent kit, Roche) to a mixture of custom HIV-specific biotinylated 120-mer oligonucleotides (xGen Lockdown Probes, Integrated DNA Technologies, Coralville, IA, USA), then captured by streptavidin-conjugated beads. Unbound DNA was washed off the beads (SeqCap EZ Hybridization and Wash kit, Roche), and the remaining libraries were then PCR amplified for sequencing using a MiSeq instrument (Illumina) resulting in read length up to 300 nucleotide paired-end.

2.5 Total HIV DNA quantification

PBMC were thawed in a 37°C waterbath and washed twice in R10. 2-3 million PBMC were washed in PBS, pelleted and resuspended in 1 μL per 100 000 cells with custom-prepared lysis buffer. The lysed extracts were stored then shipped at -20°C to Dr Mari Carmen Puertas (IrsiCaixa, AIDS Research Institute, Spain) for total HIV DNA quantification.

Total HIV DNA was quantified by droplet digital PCR (ddPCR; BioRad), as previously described (Morón-López et al. 2017). A total of 2–3 μL of cell lysate was mixed with ddPCR supermix (Bio-Rad) and primers/probes (FAM/HEX-ZEN-Iowa BlackFQ double-quenched probes, Integrated DNA Technologies) in a total volume of 20 μL to generate droplets, according to manufacturer's recommendations. Two sets of primer/probes specific for HIV conserved regions; 5'LTR and *gag*, were used to minimise patient primer mismatch (Table 2.1).

Table 2.1: Total HIV DNA ddPCR primer and probe sequences

Target	Primer/probe	Sequence (5'-3')	Strand
HIV-1 U5'-LTR	LTR-U5 integrated	GTTCGGGCGCCACTGCTAG	Forward
	LTR-R integrated	TTAAGCCTCAATAAAGCTTGCC	Reverse
	New integrated-2 Probe	CCAGAGTCACACAACAGACGGGCA	Probe
HIV-1 *gag*	HIV_F (SCA)	CATGTTTTCAGCATTATCAGAAGGA	Forward
	HIV_R (SCA)	TGCTTGATGTCCCCCCACT	Reverse
	HIV Probe (SCA)	CCACCCCACAAGATTTAAACACCATGCTAA	Probe
Cellular RPP30 gene	RPP30-F	GATTTGGACCTGCGAGCG	Forward
	RPP30-R	GCGGCTGTCTCCACAAGT	Reverse
	RPP30 Probe	CTGACCTGAAGGCTCT	Probe

The HIV DNA count was normalised using cellular RPP30 housekeeping gene quantification to give a value per million PBMC with a 95% Poisson confidence interval, as estimated across duplicates by the QuantaSoft^{TM} software (BioRad, Hindson et al. 2013). A limit of detection for each sample was estimated according to input cell number. Where HIV DNA was undetectable, the final value was recorded as the limit of detection. Results were excluded if HIV DNA was undetectable and the limit of detection was >25 copies per million PBMC, or where there was interference from erythrocyte contamination. All HIV DNA values were $\log_{10}$ transformed prior to analysis.

2.6 HIV *gag* Sanger sequencing

HIV *gag* from UL samples were sequenced by myself or Dr Emily Adland (University of Oxford, UK).

Up to 1 mL of patient plasma was thawed at room temperature, spun at 13 000 rpm at 4°C for 1.5 hours then supernatant was removed to give a final volume of 140 μL. Viral RNA was isolated from the concentrated plasma using the QIAamp Viral RNA Mini Kit according to the manufacturer's protocol (Qiagen). The HIV *gag-protease* region was amplified by reverse transcription (RT) using Superscript III One-Step Reverse Transcriptase kit (Invitrogen, Tables 2.2, 2.3, 2.4). Two rounds of PCR were used to amplify full-length *gag* using BioTaq Polymerase (Bioline, Tables 2.2, 2.5, 2.6).

Table 2.2: HIV *gag* sequencing primers

Region	Primer	Sequence (5'-3')	Strand	HXB2 coordinates
Gag-pro	2cRx	AAATCTCTAGCAGTGGCGCCCGAACAG	Forward	512 to 539
	623Fi	AAATCTCTAGCAGTGGCGCCCGAACAG	Reverse	2851 to 2825
Gag (outer)	5'OP	CTAGCAGTGGCGCCCGAACA	Forward	627 to 646
	3'OP	GCAGTCTTTCATTTGGTGTCCTTC	Reverse	2023 to 2045
Gag (inner)	5'IP	ACTCGGCTTGCTGAAGTGC	Forward	696 to 714
	3'IP	CAATTTCTGGCTATGTGCCC	Reverse	1984 to 2003

Table 2.3: RT PCR mastermix

Reagent	Volume (μL)
H_20	14.4
x2 reaction mix	20
623Fi primer (10 mM)	0.8
2cRx primer (10 mM)	0.8
Superscript III enzyme	0.8
RNA	4
Total	**36.8**

Table 2.4: RT PCR conditions

Step		Temperature	Duration
1	cDNA synthesis and pre-denaturation	55°C	30 min
2	Denaturation	94°C	2 min
3	Denaturation	94°C	15 s
	Annealing	55°C	30 s
	Elongation	68°C	2 min
4	Repeat step 3, 34 times		
5	Elongation	68°C	5 min
6	Storage	4°C	

Table 2.5: *Gag* PCR mastermix

Reagent	Volume (μL)
H_2O	37
Reaction Buffer	5
5' primer (10 mM)	1
3' primer (10 mM)	1
dNTP	0.5
BioTaq enzyme	0.5
Magnesium	3
DNA	2
Total	**50**

Volumes were doubled for second round

Table 2.6: *Gag* PCR conditions

Step		Temperature	Duration
1	Denaturation	94°C	2 min
2	Denaturation	94°C	2 min
	Annealing	60/57°C ∗	30 s
	Elongation	72°C	1 min
3	Repeat step 2, 19 times		
4	Denaturation	94°C	2 min
	Annealing	57/54°C ∗	30 s
	Elongation	72°C	1 min
5	Repeat step 4, 19 times		
6	Elongation	72°C	5 min
7	Storage	4°C	

∗ first temperature stated is used for first round (outer primers), the second for the second round (inner primers)

PCR products were purified using a QIAquick PCR purification kit according to manufacturer's instructions (Qiagen). Population sequences were generated using Sanger sequencing with the Big Dye ready reaction terminator mix v3 (Applied Biosystems) in a thermocycler (30 cycles of; 10 seconds at 96°C, 5 seconds at 50 °C, 4 minutes at 60°C). Reaction product was precipitated using sodium acetate, spun at 4°C at 3500 rpm for 1.5 hours, washed with ice-cold 70% ethanol and dried in the thermocycler for 1 minute. Sequences were read by the Department of Zoology, University of Oxford. Sequence data were analysed using Sequencher v4.8 (Gene Codes Corporation). Nucleotides for each gene were aligned manually in Se-Al v2.0a11 (University of Edinburgh) or Geneious v10.0.9 (Biomatters). Maximum-Likelihood phylogenetic trees were generated using PhyML v3.0 (`http://www.hiv.lanl.gov`) and visualised using Figtree v1.2.2 (`http://tree.bio.ed.ac.uk/software/figtree/`).

2.7 Statistical analysis

Statistical analyses were performed, and graphs drawn using R Software version v3.6.1 (R Core Team 2019) and Prism v8 (GraphPad Software). Clinical and laboratory results were described using absolute numbers, percentages, medians and interquartile range (IQR). Comparisons were performed using the Chi-Squared and Fisher's exact tests for categorical variables and the t-test or Mann-Whitney U test for parametric or non-parametric continuous variables respectively. Paired continuous variables were compared using the paired t-test or Wilcoxon matched-pairs signed rank test for parametric and non-parametric variables respectively. Univariate, and multivariate linear regression with a small number of covariates, were calculated using the `lm` R function. When the number of candidate covariates was large, a penalised model was used, whereby the set of relevant predicting covariates was selected by the LASSO penalty approach (Tibshirani 1997) and the optimal penalty parameter (ξ) was determined via 10-fold cross validation using the `glmnet` R package. Univariate survival analyses were calculated using Kaplan-Meier curves, and groups were compared using the Log-Rank test. Multivariate survival analyses were undertaken with the Cox-proportional hazards model using the `coxph` R function when the number of variables included was

small in comparison to the number of subjects. Again, where the number of candidate variables was large, the Cox model was penalised using the LASSO approach. Potentially non-linear models were estimated and visualised using a Generalised Additive Mixed Model (GAMM) which also accounts for covariates, including random effects and non-linear effects, using the `mgcv` R package (Wood 2017). Strength of association was calculated using Pearson's or Spearman's correlations for parametric or non-parametric correlations respectively. When unclear, data were either visualised in a histogram or the D'Agostino and Pearson omnibus normality test to determine data distribution. All p-values were two-sided with an alpha of 0.05.

Chapter 3

Very early treated *in utero* HIV-infected infants

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3.1 Introduction

The life-saving effect of early cART for perinatally HIV-infected infants is undoubted. The landmark randomised South African Children with HIV Early Antiretroviral Therapy (CHER) trial demonstrated that treating perinatally HIV-infected infants at a median age of 7 weeks, rather than deferring until CD4+ T cell count declines, at a median of 20 weeks, mortality and AIDS is reduced by 76% (Violari et al. 2008). Subsequently, the international general consensus has been to start cART as soon an infant tests HIV positive, even while awaiting diagnosis confirmation (WHO 2016). Furthermore, in 2013 a famous case report described the 18 month old Mississippi Baby's HIV remission following cART initiation at 30 hours of life. Although 27 months later the infant was required to restart cART following plasma viraemia rebound, the idea of

inducing HIV remission by treating IU HIV-infected within hours of birth had already been sparked.

There is certainly biological plausibility to this prospect. Initiation of cART for children early in HIV infection reduces the size of the viral reservoir, the most consistent correlate of cure or remission (Sharaf et al. 2018). PoC testing and cART initiation can be implemented very soon after birth, (Technau et al. 2017a) and, therefore, very soon after infection, since most IU infections arise late in pregnancy (Rouzioux et al. 1995). Furthermore, the tolerogenic immune response in early life mitigates against immune activation (Goulder et al. 2016) preserving immune function and possibly limiting reservoir seeding (Rajasuriar et al. 2015; Krebs and Ananworanich 2016).

However, additional factors other than limiting the viral reservoir via early cART initiation, including immune, host genetic, virological and environmental factors, are likely to influence the ability to achieve cure or remission for HIV-infected infants (Shiau et al. 2018). Moreover, a recent study in South Africa suggests that outcomes in very early-treated IU-infected infants are not successful (Technau et al. 2018; Kuhn et al. 2020).

3.1.1 Hypothesis and aims

The Ucwaningo Lwabantwana study provided the opportunity to investigate the clinical impact of very early (<48 hours after birth) cART initiation for IU HIV-infected infants, with the hypothesis that earlier cART initiation could lower latent HIV reservoirs enhancing plasma viral suppression rates. Specific aims addressed in this chapter include:

1. To characterise the clinical characteristics of IU-HIV infected infants in the era of an effective prevention of mother-to-child transmission programme.

2. To determine treatment outcomes, measured by viral suppression, for very early treated IU HIV-infected infants.

3. To further explore the factors, including cART non-adherence and drug resistance, underlying success or failure of early cART in this group.

3.2 Chapter specific methods

3.2.1 Study participants and comparison groups

Results from the Ucwaningo Lwabantwana cohort (as described in Chapter 2 on page 39) up until May 31 2019 were analysed.

To assess the impact of the current PMTCT programme, the Ucwaningo Lwabantwana infants' baseline results were also compared to those infants from the historical PEHSS cohort. PEHSS (Paediatric Early HAART and Strategic Treatment Interruption Study Mphatswe et al. 2007; Prendergast et al. 2008) was also set in Durban, KwaZulu-Natal but in 2002-2005 when there was no ART available to treat adults for HIV infection and perinatal ART prophylaxis was single dose NVP for both mother and infant. Raw data from the PEHSS cohort were available for all 50 mother/IU HIV-infected infant pairs at baseline.

To assess the impact of very early cART initiation, I considered outcomes for the whole Ucwaningo Lwabantwana cohort together and then compared two subgroups of IU-infected infants according to the timing of test they were first identified by: the PoC and Standard-of-care (SoC) groups. Comparisons included: time to cART initiation, maternal and infant baseline characteristics, time to viral suppression, viral rebound rates, mortality, loss to follow-up and viral suppression rates at 12 and 24 months. Plasma viral rebound was defined as one plasma viral load measurement >1000 HIV RNA copies per mL, or two consecutive measurements >100 HIV RNA copies per mL, both following a plasma viral load lower than the detectable limit. To assess the influence of HIVDR on virological outcomes, DRMs were quantified and plasma ART levels were measured to evaluate potential non-adherence to treatment.

3.2.2 ART resistance testing

Where plasma samples were available at baseline for the mothers and infants and for infants in the event of treatment failure, HIV RNA was deep sequenced using Next Generation Sequencing methods, as described in Chapter 2 on page 46. Raw sequencing reads were blasted against Los Alamos Sequence Database `http://www.hiv.lanl.gov/` HIV-1 "Web Alignments 2017" to obtain the HXB2 alignment positions for each read. Reads covering possible drug resistance locations were translated to provide the amino acid percentages. Sequences where drug resistance mutation locations had coverage of more than 20 reads were retained. Variants that were found in >10% of the sequence reads were documented. Viral amino acid mutations were uploaded to the online Stanford University Genotypic Resistance Interpretation Algorithm v8.8 (`http://hivdb.stanford.edu/`) to obtain the drug resistance phenotype. All levels of resistance to antiretroviral drugs commonly used in South Africa were included. The presence of minor PI resistance mutations in the absence of major PI mutations as defined by the Stanford database were disregarded. In some cases, infant HIVDR was undertaken for clinical reasons using Sanger sequencing and these results were also included.

3.3 Results

3.3.1 Baseline characteristics

Between July 01, 2015 and January 31, 2019, 151 IU-infected infants were enrolled onto the Ucwaningo Lwabantwana study (Figure 3.1). In all 151 cases, infant ART prophylaxis was initiated within minutes of birth prior to diagnosis of IU infection. Two-thirds of infants (103/151, 68%) received both NVP and AZT, the remaining one-third (48/151, 32%) receiving NVP or AZT only. Of these 151 IU-infected infants, 76 were identified through PoC (n=70) or overnight viral load (n=6) testing of infants, and cART was initiated at a median age of 26 hours (IQR 18–38). A further 75 infants were diagnosed through SoC testing, in whom cART was initiated at a median age of

10 days (IQR 8–13). ART prophylaxis did not differ significantly between the PoC and SoC groups (Table 3.1).

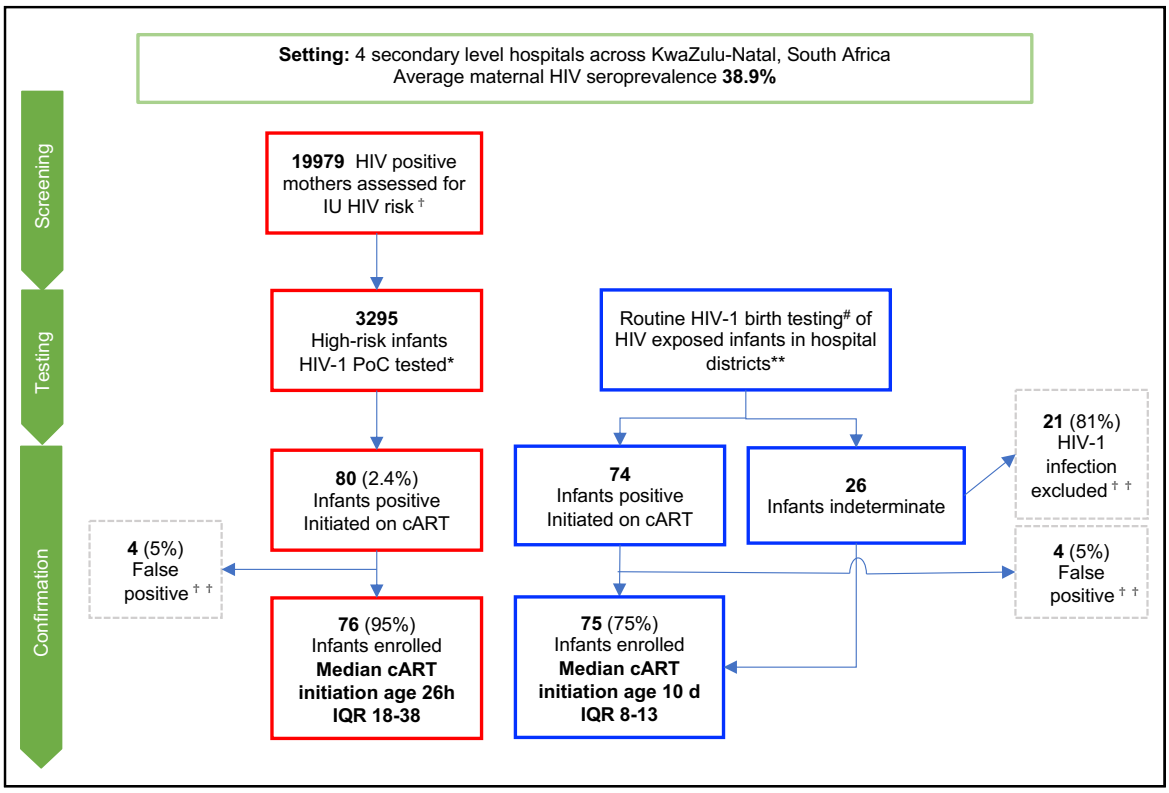


Figure 3.1: Ucwaningo Lwabantwana study profile. † High risk; during pregnancy the mother either seroconverted, had <4 weeks cART, poor cART adherence by history or a documented plasma viral load >1000 HIV RNA copies per mL ∗Tested with PoC whole blood GeneXpert (GXP) HIV-1 Qualitative TNA PCR; but when not available an overnight HIV RNA test; plasma Nuclisens EasyQ v2.0 HIV-1 RNA PCR. # Laboratory-based dried blood spot TNA PCR (CAP/CTM HIV-1 Qualitative Test V2) ∗∗iLembe, uMgungundlovu, eThekwini and uThungulu. ††Confirmatory testing included; TNA PCR via CAP/CTM or GXP, HIV RNA PCR via Nuclisens or in selected cases more sensitive methods.

With respect to timing of maternal infection, 31% (47/151) of the IU-infected infants were born to mothers designated as seroconverting during the pregnancy. This included 41 mothers who had a negative HIV test during pregnancy followed subsequently by a positive test; and six, in whom the estimated seroconversion date, midway between last negative test and first positive test, was also during pregnancy. In 28% (42/151) of cases, the infants were born to mothers known to have been HIV-infected prior to conception, including six who were themselves infected via MTCT (Table 3.1). The remaining 62/151 (41%) mothers' first HIV test during the pregnancy was HIV-positive and the timing of infection was unknown (Table 3.1). Due to lacking

Table 3.1: Baseline clinical information of mother and *in utero* HIV-infected infant pairs

	All		Point-of-care Tested		Standard-of-care Tested		
	(n=151)		(n=76)		(n=75)		
		IQR/%		IQR/%		IQR/%	P-value of PoC vs SoC
MOTHER							
Median maternal age (years)	24.7	(21.5–30.0)	24.1	(21.4–27.9)	26.3	(22.0–26.7)	0.088
Timing of maternal HIV infection							
Chronic; vertical	6	4.0%	3	4.0%	3	4.0%	0.41
Chronic; horizontal	36	23.8%	14	18.4%	22	29.3%	
Acute	47	31.1%	27	35.5%	20	26.7%	
Unknown	62	41.1%	32	42.1%	30	40.0%	
Previous MTCT	7	4.6%	4	5.3%	3	4.0%	1.0
Median number of other children	1	(0–2)	1	(0–2)	1	(0–2)	0.25
Timing of maternal cART initiation							
Pre-pregnancy	31	20.5%	13	17.1%	18	24.0%	0.33
During pregnancy >12 weeks prior to delivery	50	33.1%	26	34.2%	24	32.0%	
During pregnancy <12 weeks prior to delivery	33	21.9%	16	21.1%	17	22.7%	
During labour or at delivery	20	13.2%	14	18.4%	6	8.0%	
Postnatally	17	11.3%	7	9.2%	10	13.3%	
Maternal cART regimen at enrollment							
1st line (NNRTI)	146	96.7%	75	98.7%	71	94.7%	0.30
2nd line (PI)	3	2.0%	1	1.3%	2	2.7%	
*None**	2	1.3%	0	0%	2	2.7%	
Self-reported antenatal cART non-adherence/refusal**	63	55.3%	30	54.5%	33	55.9%	0.69
Median plasma viral load (HIV RNA copies/mL)	4400	(520–40 500)	12 500	(1400–160 000)	2200	(133–12 500)	0.0002
Median absolute CD4+ T cell count (cells/μL)	452	(318–637)	442	(298–604)	462	(348–641)	0.45
Median CD4:CD8	0.46	(0.31–0·69)	0.44	(0.30–0·62)	0.5	(0.34–0·75)	0.10
INFANT							
Sex							
Male	55	36.4%	20	26.3%	35	46.7%	0.011
Female	96	63.6%	56	73.7%	40	53.3%	
Median gestation at birth (weeks)†	38	(36–39)	37	(36–39)	38	(36–40)	0.24
Median birth weight (kg)	2.82	(2.40–3.14)	2.81	(2.36–3.09)	2.86	(2.50–3.20)	0.44
Small for gestational age††	32	21.20%	16	21.1%	16	21.3%	1.0
Exclusively breastfed	120	79.5%	69	90.8%	51	68.0%	0.0011
Neonatal admission ‡	46	30.5%	23	30.3%	23	30.7%	1.0
Infant ART prophylaxis	151	100%	76	100%	75	100%	1.0
Nevirapine only	47	31.1%	26	34.2%	21	28.0%	0.45
Zidovudine only	1	0.7%	0	0.0%	1	1.3%	
Nevirapine and Zidovudine	103	68.2%	50	65.8%	53	70.7%	
Median plasma viral load (HIV RNA copies/mL)	8350	(1500–67 500)	29 500	(3325–342 500)	3350	(300–22 500)	<0.0001
Median absolute CD4+ T cell count (cells/μL)	2035	(1263–2700)	1682	(1012–2299)	2444	(1811–3147)	<0.0001
Median CD4+ T cell percentage	43	(35–52)	39	(31.8–49.5)	46	(37–53)	0.011
Median CD4:CD8	1.86	(1.13–2.84)	1.54	(1.05–2.65)	2.08	(1.36–3.21)	0.043
Standard-of-care TNA PCR birth test result							
Positive	136	90.1%	66	86.8%	70	93.3%	0.25
Indeterminate	13	8.6%	8	10.5%	5	6.7%	
Negative	2	1.3%	2	2.6%	0	0%	
Age of cART initiation (hours or days)	4d	(26h–10d)	25.6h	(17·7–37·9h)	10d	(8–13d)	<0.0001

∗Two mothers had cART initiation delayed beyond enrolment while TB infection was excluded. ∗∗Of those who initiated cART prior to labour. †Six infants had unknown gestational age at birth. ††As classified by InterGrowth 21st charts. ‡Direct to neonatal ward or to the paediatric ward aged < 28 days of life.

antenatal care, or seroconversion late in pregnancy, cART had not been initiated in 25% (37/151) of mothers prior to the onset of labour. Among the 75% (114/151) of mothers who initiated cART prior to the onset of labour, 63 (55%) self-reported poor cART adherence, of which nine (8%) omitted all doses. Timing of maternal infection and maternal cART initiation did not differ between the PoC and SoC groups (Table 3.1).

The majority of IU-infected infants were healthy, term babies (median gestation 38 weeks [IQR 36–39], median birth weight 2.82 kg [IQR 2.4–3.14]) (Table 3.1). However, 32/151 (21%) were small-for-gestational age (birth weight <10th centile on Inter-Growth 21st charts, Villar et al. 2014) and 46/151 (31%) of infants required hospital admission in the first 28 days of life. As previously reported (Piwoz et al. 2006) the number of female IU-infected infants exceeded that of males (96 vs 55). For reasons that are unclear, the sex difference was substantially greater in the PoC group (females:males 2.8:1) compared to the SoC group (females:males 1.1:1; p=0.011) (Table 3.1).

3.3.2 Baseline viral load

The first ('baseline') measurements of plasma viral load and CD4+ T cell count in the PoC and SoC groups were taken, respectively, at a median of 1 day and 10 days of age, and thus after 1 and 10 days of prophylactic ART (NVP alone or AZT/NVP). As expected, therefore, at baseline, the SoC infant group had significantly lower plasma viral loads than the PoC group (median 3350 vs 29 500 HIV RNA copies per mL; p<0.0001), higher CD4+ T cell counts (median 2444 vs 1682 cells per μL; p<0.0001) and higher CD4+ T cell percentage, (median 46% versus 39%, p=0.01; Figure 3.2A-C). However, by 1 month of age, differences between the PoC and SoC groups in plasma viral load, absolute CD4+ T cell count and CD4+ T cell percentage had disappeared (Figure 3.2A-C).

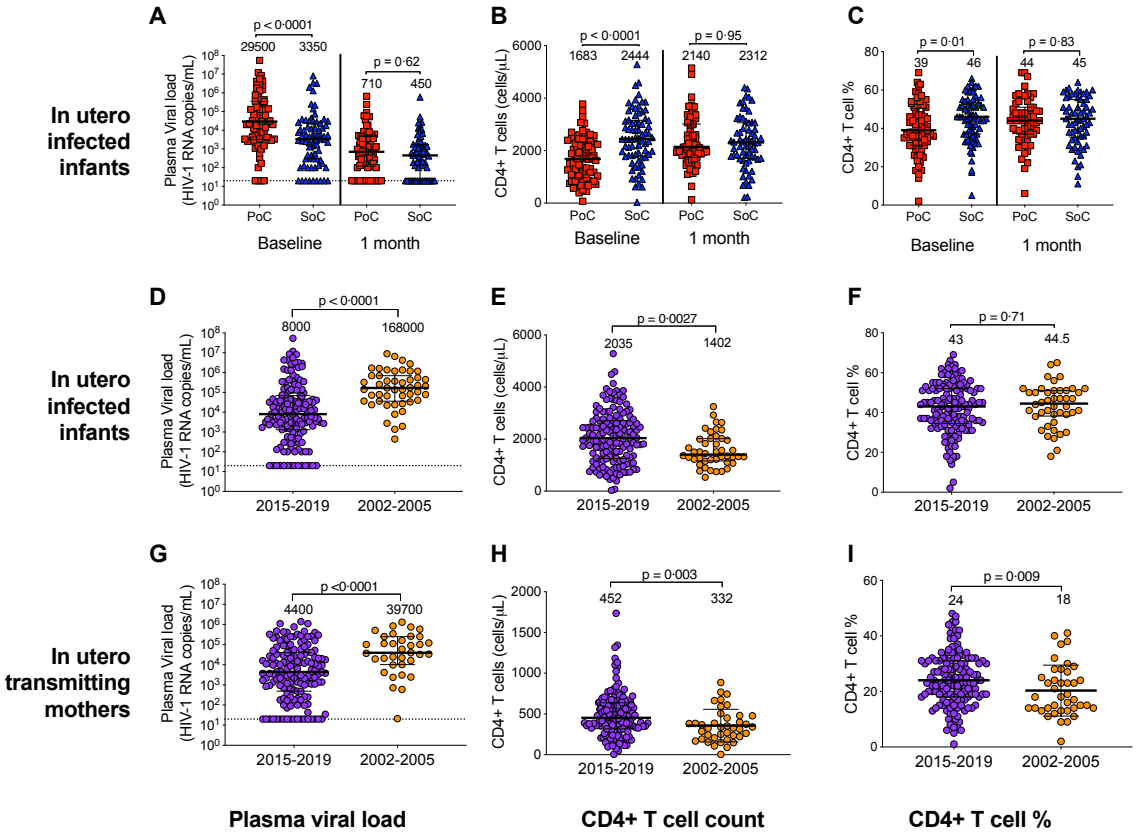


Figure 3.2: Effects of very early ART initiation and universal maternal cART on baseline plasma HIV viral load and CD4+ T cell count. (A) Plasma HIV RNA viral load, (B) absolute CD4+ T cell count and (C) CD4+ T cell percentage of the IU HIV-infected infants at baseline (<21 days of life, prior to cART initiation) (left) and at 1 month of age (right), where PoC were diagnosed using a point-of-care HIV TNA PCR or overnight RNA PCR test and treated very early (median age 26 hours) and SoC were diagnosed by the standard-of-care laboratory-based HIV PCR test and treated early (median age 10 days). (D) Plasma HIV RNA viral load, (E) absolute CD4+ T cell count and (F) CD4+ T cell percentage of the IU HIV-infected infants within 21 days of birth where 2015–2019 is the current cohort (Ucwaningo Lwabantwana) and 2002–2005 are the cohort from the same population prior to universal antenatal cART (PEHSS). (G) Plasma HIV RNA viral load, (H) absolute CD4+ T cell count and (I) CD4+ T cell percentage of the mothers of the IU HIV-infected infants shown in panel D-F, within a month of delivery. Median values displayed. All p-values calculated using the Mann-Whitney U test.

Baseline infant plasma HIV viral loads were significantly lower and absolute CD4+ T cell counts significantly higher in the current Ucwaningo Lwabantwana cohort of 151 IU-infected infants compared to the 2002–2005 PEHSS cohort of 50 IU-infected infants, also in KZN, South Africa (Figure 3.2D-F), when cART was not available to mothers during pregnancy. Similarly, in the IU transmitting mothers, plasma viral loads were lower in the current Ucwaningo Lwabantwana cohort compared to the PEHSS cohort (4400 vs 39 700 RNA copies per mL $p<0.0001$, Figure 3.2G). In the 23 mothers from the Ucwaningo Lwabantwana cohort who had no ART prior to labour and plasma viral load measurement within 1 week of delivery, plasma viral load was similar to the PEHSS cohort (16 000 vs 39 700 HIV RNA copies per mL, $p=0.09$, not shown). It is noteworthy that as many as 19/151 (12.5%) of the Ucwaningo Lwabantwana cohort of IU-infected infants had undetectable plasma viral loads (<20 HIV RNA copies per mL or <100 HIV RNA copies per mL) at baseline, consistent with the notion that, in the majority of IU-infected infants, there is significant intrauterine ART exposure transplacentally.

To investigate further whether delaying cART initiation to day 10 compared to initiation on day 1 of life might affect the size of the viral reservoir in the PoC and SoC groups of infants at the time of cART initiation, HIV DNA viral loads were measured by ddPCR in a subset of 35 infants, selected according to sample availability. Even though the plasma HIV RNA loads were 1.2–1.3 $\log_{10}$ higher in the PoC group, HIV DNA viral loads measured at the same timepoint showed minimal difference between the groups (<0.07 $\log_{10}$ copies HIV DNA per million PBMC; $p=0.43$, Figure 3.3). Together with the data showing a lack of difference in plasma viral loads between the PoC and SoC groups at 1 month of age, these findings suggest that SoC testing and cART initiation at median 10 days does not bring any significant disadvantage compared with PoC testing and cART initiation at median 26 hours in a setting where prophylactic ART is initiated in all babies born to HIV-positive mothers within minutes of birth and ART exposure commonly occurs *in utero*.

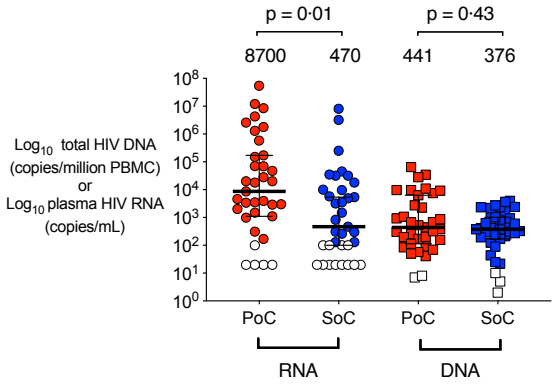


Figure 3.3: Baseline infant proviral HIV DNA levels compared to plasma RNA. Baseline infant plasma HIV RNA levels (copies per mL) (left) and proviral HIV DNA levels (copies per million PBMC) as measured by droplet digital PCR (right) for a subset of infants at baseline, a comparison for PoC, and SoC infants. Median values and interquartile range displayed. Open symbols are below the limit of detection (20 or 100 copies per mL for RNA and variable for each individual test for DNA (2-10 copies per million PBMC)). P-values calculated using the Mann-Whitney U test.

3.3.3 Mortality

To assess initial outcome in SoC and PoC-treated, IU-infected infants, next the 114/151 infants from the Ucwaningo Lwabantwana cohort who were born >12 months prior to the time of analysis were evaluated. Excluding four infants (two PoC- and two SoC-tested) who transferred out in the first week of life to receive care at external clinics, 26 of the remaining 110 (24%) had either died (n=8, 7.3%), or were lost to follow-up (n=18, 16.4%) (Figure 3.4A). Among the latter, six died subsequent to defaulting clinic appointments. Thus, in the cohort overall (n=151), 15 infants died. The probability of death by age 3 months was 6.4% (95% CI 2.3–10.3%) and was 9.3% by 1 year (95% CI 4.4-14.1%; not shown) with a median age of death of 3.5 months. A penalised Cox-proportional hazard model identified three independent predictors of mortality: low infant birthweight; neonatal hospital admission and high infant baseline plasma HIV RNA viral load, from a possible 24 covariates (Table 3.2).

3.3.4 Treatment outcomes

Of the infants retained on the study at 12 months of age, 15/110 (13.6%) had not achieved suppression of plasma viraemia on cART by 6 months and 28/110 (25.5%) had achieved only short-term suppression of viraemia, rebounding by 12 months (Figure

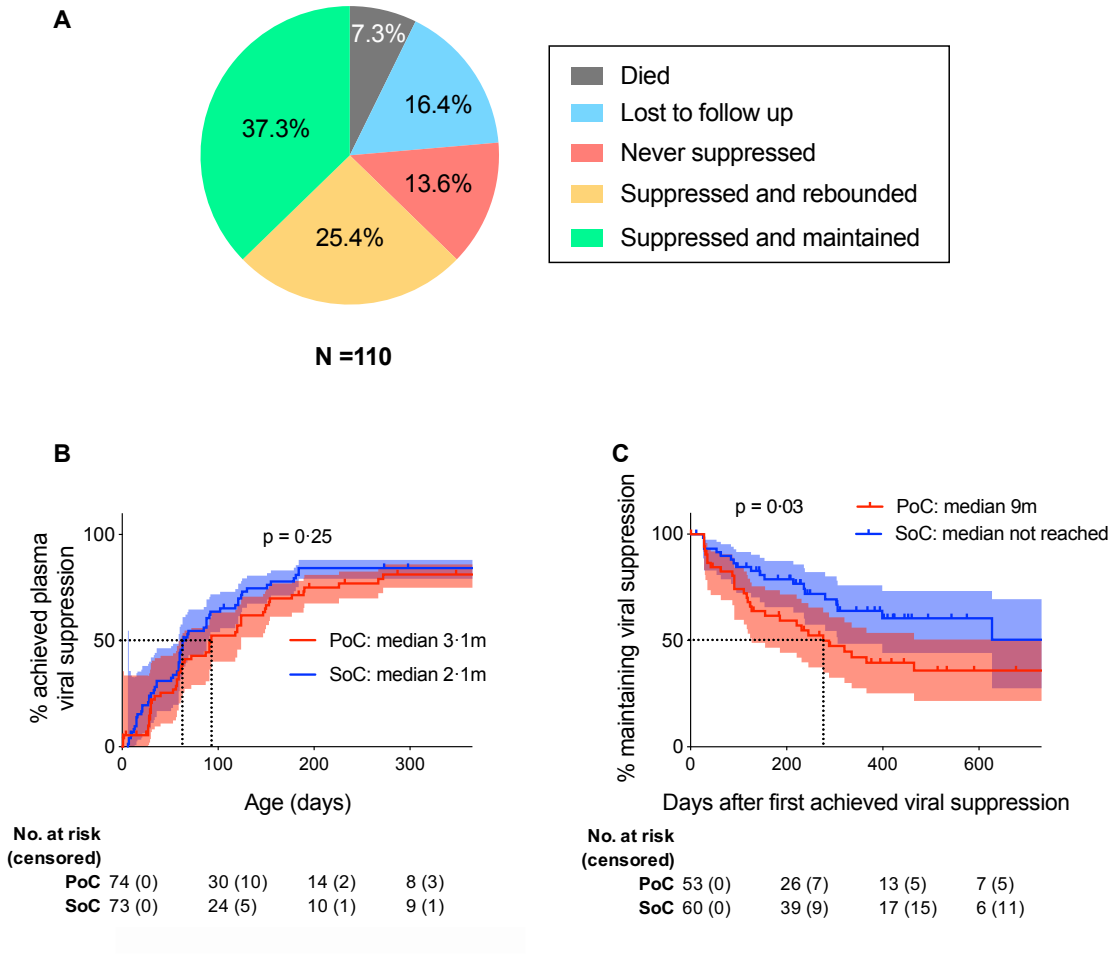


Figure 3.4: Infant outcomes: retention, mortality and plasma viral suppression. (A) Proportions of infants with the opportunity for 12 months follow-up (excluding four infants who were withdrawn by the investigators) who died, were lost to follow-up, those who did not achieve plasma viral suppression by 6 months of age, those who did achieve plasma viral suppression by 6 months of age but had rebounded by 12 months of age, and those who achieved plasma viral suppression by 6 months of age and maintained suppression to at least 12 months of age. (B) Kaplan-Meier analysis of time to plasma viral suppression; a comparison of PoC and SoC groups. (C) Kaplan-Meier analysis of time to plasma viral rebound; a comparison of PoC and SoC groups.

Table 3.2: Multivariate analysis of mortality, plasma viral suppression and rebound predictors

	Mortality		Time to Suppression		Time to Rebound	
Baseline variable	ß coefficient	Multiplicative effect on hazard rate	ß coefficient	Multiplicative effect on hazard rate	ß coefficient	Multiplicative effect on hazard rate
Group SoC					-0.443	0.642
Study site						
Infant birth weight (kg)	-0.566	0.568				
Infant sex male					-0.071	0.931
Infant gestational age at birth (weeks)					0.057	1.059
Small for gestational age						
Timing of maternal HIV infection						
Days of maternal ART in pregnancy					0.002	1.002
Self-reported maternal ART non-adherence						
Maternal age (years)					-0.027	0.973
Infant prophylaxis						
Age infant ART initiation						
Infant CD4 count (cells/μL)						
Infant CD4%			0.008	1.008		
Infant CD8 count (cells/μL)			0.007	1.007		
Infant CD8%						
Infant CD4:CD8						
Infant $\log_{10}$ HIV RNA copies/mL	0.062	1.064	-0.360	0.700		
Maternal CD4 count (cell/μL)						
Maternal CD8 count (cells/μL)						
Maternal CD4:CD8						
Maternal $\log_{10}$ HIV RNA copies/mL						
Infant feeding						
Neonatal hospital admission	0.529	1.700			-0.206	0.814

A classical Cox model was used with a LASSO penalty to determine the effect of 24 variables at baseline on the rate of the following outcomes; mortality, plasma viral suppression and plasma viral rebound. The relevant covariates selected by the model are shown, with grey cells representing the irrelevant covariates eliminated via the LASSO. The β-coefficient gives the influence of the variable in the units stated, and the "multiplicative effect on hazard rate" ($\text{Exp}(\beta)$) gives the factor by which the rate to the outcome is affected by the variable in the units stated, analogous to a hazard ratio. Variables are highlighted, green for decreasing the rate of the outcome, orange for increasing it.

3.4). Only 37.3% of the 110 IU-infected infants enrolled had achieved and maintained suppression of viraemia on cART to >12 months of age. 40 out of the 84 infants on the study at 12 months were also analysed 24 months post-enrolment. 14/17 (82.4%) of those infants who had suppressed by 6 months and maintained suppression to 12 months, continued to maintain viraemic suppression to 2 years of age. In contrast, only 2/17 (12%) of those whom only had short term suppression between 6 and 12 months, and 0/6 of those who had not suppressed by 6 months, achieved and maintained suppression to 2 years (Figure 3.5).

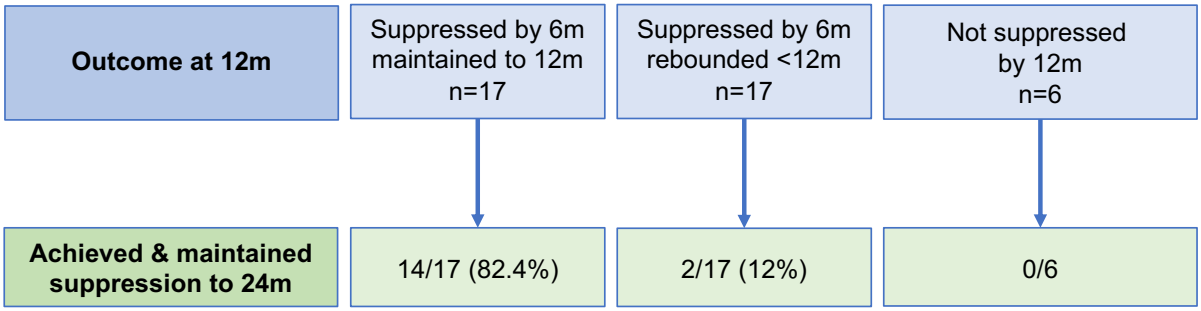


Figure 3.5: Infant outcomes at 24 months. 40 infants were 24 months beyond enrolment and were grouped in accordance to their outcome at the 12 month analysis. The number from each group who achieved and maintained plasma viral suppression until 24 months of age are shown.

3.3.5 Outcome predictors

In order to further investigate why sustained suppression of viraemia in early-treated infants on ART is so infrequent, I first compared the PoC and SoC groups. Although there was no difference in mortality or retention on the study at 12 months (p=0.75, and p=0.19, respectively), in the univariate analyses, infants in the SoC group tended to achieve suppression of viraemia earlier, and to maintain suppression of viraemia longer than those in the PoC group (Figure 3.4B-C, p=0.25 and p=0.03, respectively). These findings held true in the multivariate analyses, with a strong predictor at baseline for rebound being the PoC group compared with the SoC group, Table 3.2. The strongest predictive factor at baseline for time to viral suppression was baseline infant plasma viral load and was not influenced by the age at which the infant initiated cART. Aside from SoC vs PoC group influencing time to infant viral rebound, longer

duration of maternal cART in pregnancy and older infant gestational age at birth was also found to be associated with a faster rate of rebound, while older maternal age, male infants, and being admitted to hospital in the neonatal period was associated with a slower rate (Table 3.2). There was no significant change to the rate of viral rebound when relaxing the definition of viral rebound to a level >1000 HIV RNA copies per mL only (Figure 3.6). High rates of treatment failure suggest either inferior cART adherence or reduced cART effectiveness due to HIVDR.

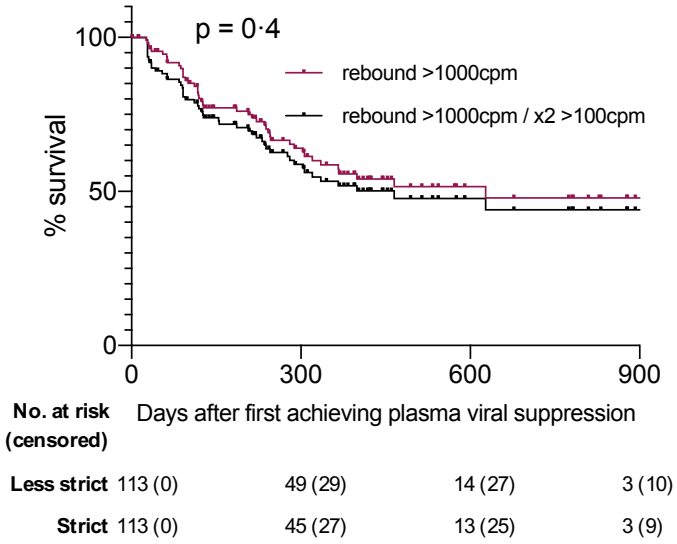


Figure 3.6: Sensitivity analysis for infant plasma viral rebound. Kaplan-Meier analysis for all infants who achieved plasma viral suppression (plasma viral load lower than the detectable limit <20 or <100 copies per mL) and the time to viral rebound. A sensitivity analysis comparing the original definition of viral rebound (plasma viral load >1000 copies per mL on one occasion, or two measurements >100 copies per mL) to a more relaxed definition of one measurement >1000 copies per mL. P-value was calculated using the Log-Rank test. cpm: HIV RNA copies per mL.

3.3.6 Transmitted HIV drug resistance

To investigate whether ART resistance might explain the high rates of virological failure on ART in the Ucwaningo Lwabantwana cohort, I first addressed the question of whether DRMs were transmitted from the mother to the child. Samples from a subset of infants (n=20, selected according to sample availability) were deep sequenced for DRMs at baseline (median age 6 days [IQR 1–26.5 days], Table 3.3). Among these 20 infants, ten achieved suppression of viraemia by 6 months of age, in seven cases without any DRMs detected, and the remainder with NNRTI DRMs detected in the

child's virus conferring high-level resistance to NVP. Similarly, among the ten infants who did not achieve suppression of viraemia by 6 months, six had no detectable DRMs and the remaining four had NNRTI DRMs at baseline also corresponding to high-level resistance to NVP. Given the short duration the infants receive NVP-based cART before switching to PI-based cART and the absence of any PI DRMs at baseline, these data are consistent with ART non-adherence rather than transmitted DRMs being the cause of virological failure in these children.

3.3.7 Acquired HIV drug resistance

I next investigated whether virological failure might have resulted from DRMs acquired in older infants. Samples were analysed from a second subset of infants (n=28, selected according to sample availability) aged >6 months (median 12 months, IQR 9–15; median viral load 30 500 HIV RNA copies per mL) with virological failure, defined as a plasma RNA viral load of >1000 HIV RNA copies per mL. Where possible, I also measured levels of ART in these plasma samples, since although ART detection reflects adherence only in the previous 24–48 hours, ART non-detection unequivocally demonstrates non-adherence during this window. For fifteen subjects, viral suppression was never subsequently achieved, of which, eight had no resistance to the infant cART regimen, while seven had high-level resistance to 3TC (Table 3.4). However, in the remaining 13 subjects, who did subsequently suppress on the same cART regimen, five had high-level 3TC resistance, and one had high-level resistance to both 3TC and ABC. This suggests that, in the absence of PI DRMs, adherence to this regimen can still achieve viral suppression.

3.3.8 ART levels

To test further the hypothesis that children with virological failure were cART non-adherent, I compared plasma ART levels in children with virological failure (n=17, Table 3.4) with children maintaining suppression of viraemia who by history were ART-adherent (n=11). All 11 of the virally suppressed infants had all three prescribed

Table 3.3: Mother and infant transmitted HIV drug resistance genotype and phenotype

					Genotype - DRMs			Resistance level		
Pair		**Days post delivery**	**Duration cART (days)**	**Plasma viral load (HIV RNA copies/mL)**	**NNRTI**	**NRTI**	**PI**	**Intermediate**	**High**	**Infant age at viral suppression (m)**
1	Mother	1	1	6300	-	-	-			1
	Child	1	0	4600	-	-	-			
2	Mother	9	150	100 000	K103N (11%)	-	-		EFV, NVP	1
	Child	9	0	830	-	-	-			
3	Mother	1	1	46 000	-	-	-			1
	Child	1	0	1100	-	-	-			
4	Mother	1	>5 years	13 000	K103N (76%), Y188L (17%)	-	-		EFV, NVP	2
	Child	1	0	150 000	Y188L (99%)	-	-		EFV, NVP	
5	Mother	1	6	4400	-	-	-			3
	Child	32	31	8700	-	-	-			
6	Mother	9	9	1 100 000	-	-	-			3
	Child	9	0	74 000	-	-	-			
7	Mother	1	22	29 000	-	-	-			4
	Child	26	25	660 000	-	-	-			
8	Mother	1	34	1400	-	-	-			5
	Child	1	0	3900	-	-	-			
9	Mother	12	17 years	2800	-	-	-			5
	Child	53	41	36 000	Y181C (99%)	-	-	EFV	NVP	
10	Mother	1	160	210 000	K103N (100%)				EFV, NVP	6
	Child	1	0	430 0000	K103N (100%)	-	-		EFV, NVP	
11	Mother	2	111	180 000	-	-	-			>6
	Child	2	0	440 000	-	-	-			
12	Mother	1	0	1 100 000	-	-	-			7
	Child	28	28	53 000	-	-	-			
13	Mother	1	23	5200	K103N (100%), V106I (100%)	-	-		EFV, NVP	>15
	Child	34	33	8200	K103N (100%), V106I (99%)	-	-		EFV, NVP	
14	Mother	18	8 years	3400	K103N (21%)	-	-		EFV, NVP	27
	Child	18	0	32 000	K103N (46%), V106A (27%), G190A (27%)	-	-		EFV, NVP	
15	Mother	11	37	28 000	K103N (99%), Y188C (16%)	-	-		EFV, NVP	>18
	Child	60	49	57 000	K103N (99%), V106A (28%), Y188C (22%)	-	-		EFV, NVP	
16	Mother	2	4	16 000	-	-	-			LTFU at 1
	Child	2	0	1 500 000	-	-	-			
17	Mother	8	161	12 000	K103N (88%)	-	-		EFV, NVP	died at 3
	Child	24	20	52 000	K103N (99%) V106M (16%)	-	-		EFV, NVP	
18	Mother	0	203	770 000	-	-	-			died at 8
	Child	0	0	2 900 000	-	-	-			
19	Mother	2	0	43000	-	-	-			LTFU at 15
	Child	2	0	350 000	-	-	-			
20	Mother	3	3	4800	V106M (97%)	-	-		EFV, NVP	LTFU at 21
	Child	3	0	40 000	-	-	-			

HIV genotype was determined using NGS methods for mother and infant pairs at baseline, subject to sample availability. DRM prevalence shown in brackets. Resistance phenotype for the ARVs commonly used in South Africa were determined from the Online Stanford Database. Median infant age at blood draw was 5.5 days (IQR 1-26 days). Participants listed in order of increasing time to infant plasma viral suppression (in months). No NRTI or PI resistance mutations were found for any of the participants.

Table 3.4: Infant HIV drug resistance genotype and phenotype at 12 months

			Genotype - DRMs			Resistance level				
Child	**Age at blood draw (m)**	**Plasma viral load (HIV RNA copies/mL)**	**NNRTI**	**NRTI**	**PI**	**Low**	**Intermediate**	**High**	**Subsequently suppressed?**	**Plasma ART detected**
1	36	17 000	-	-	-				no	none
21*	9	15 000	-	-	-				no	3TC
22	15	24 000	-	-	-				no - LTFU	none
23	6	410 000	-	-	-				no - LTFU	none
24	15	6100	-	-	-				no - LTFU	none
25	9	1 700 000	-	-	-				yes	not tested
26	8	8600	-	-	-				yes	not tested
27	12	350 000	K103N (99%)	-	-			EFV, NVP	yes	none
28	11	16 000	V106M (76%) Y188C (26%)	-	-			EFV, NVP	no	not tested
29	15	37 000	K103N (98%)	-	-			EFV, NVP	yes	not tested
30	18	8900	F227L (10%)	-	-	EFV	NVP		yes	not tested
9	15	95 000	Y181C (88%)	-	-			EFV, NVP	yes	not tested
15	6	69 000	K103N (99%) Y188C (16%)	-	-			EFV, NVP	no	none
31	6	7400	K103N (99%)	-	-			EFV, NVP	yes	none
19	12	50 000	V106M (74%) G190A (16%) F227L (12%)	-	-		EFV	NVP	no - LTFU	LPVr, 3TC, ABC
32*	9	2100	-	M184V	-	ABC		3TC	yes	LPVr, 3TC, ABC
33	6	6500	-	M184I (37%)	-	ABC		3TC	yes	none
34	15	6000	-	M184I (98%)	-	ABC		3TC	no	3TC
35*	21	480 000	-	M184V	-	ABC		3TC	no	not tested
36	12	330 000	K103N (98%)	M184V (12%)	-	ABC		3TC EFV, NVP	no - LTFU	none
37	18	15 000	V106M (92%) H221Y (99%)	M184V (99%)	-	ABC	EFV	3TC, NVP	no - LTFU	LPVr, 3TC, ABC
38	9	390 000	A98G (99%) K103N (93%)	M184V (98%)	-	ABC		3TC EFV, NVP	no	none
14*	15	88 000	Y181C G190A	M184V	-	ABC	EFV	3TC, NVP	yes	none
39	12	10 000	K103N (100%)	M184V (17%)		ABC		3TC EFV, NVP	yes	not tested
40	9	220 000	V106M (99%) Y188C (99%)	T215I (51%)	-	AZT		EFV, NVP	no	not tested
41	12	120 000	K103N (98%)	M184V (99%)		ABC		3TC EFV, NVP	yes	not tested
42*	12	24 000	K103N	M184V	-	ABC		3TC EFV, NVP	no	not tested
43	9	520 000	K103N (99%)	K65R (99%) M184V (98%)	-			ABC, 3TC EFV, NVP	yes	3TC

HIV genotype was determined using NGS methods of a selection of infants with plasma viral load >1000 RNA copies per mL, subject to sample availability. Mutation prevalence shown in brackets. ∗Indicates samples that only underwent Sanger sequencing. Resistance phenotypes for the ARVs commonly used in South African children were determined from the Online Stanford Database. Plasma ART levels were quantified using LC-MS/MS and were considered negative if at very low levels or below the level of quantification. Median infant age was 12 months, IQR 9-15 months; median viral load 30 500 HIV RNA copies per mL. Participant numbers are in continuation from Table 3.3. No PI DRMs were found for any of the participants. LTFU: loss to follow-up.

ART drugs detectable at high levels in the plasma, while 14/17 (82%) of the infants with high viral loads had either no or only one ART drug detected at therapeutic levels (p<0.0001, Figure 3.7A, Table 3.4). Together, these analyses indicate that the high frequency of failure to achieve suppression of viraemia on cART in the Ucwaningo Lwabantwana cohort of IU-infected infants is principally due to non-adherence.

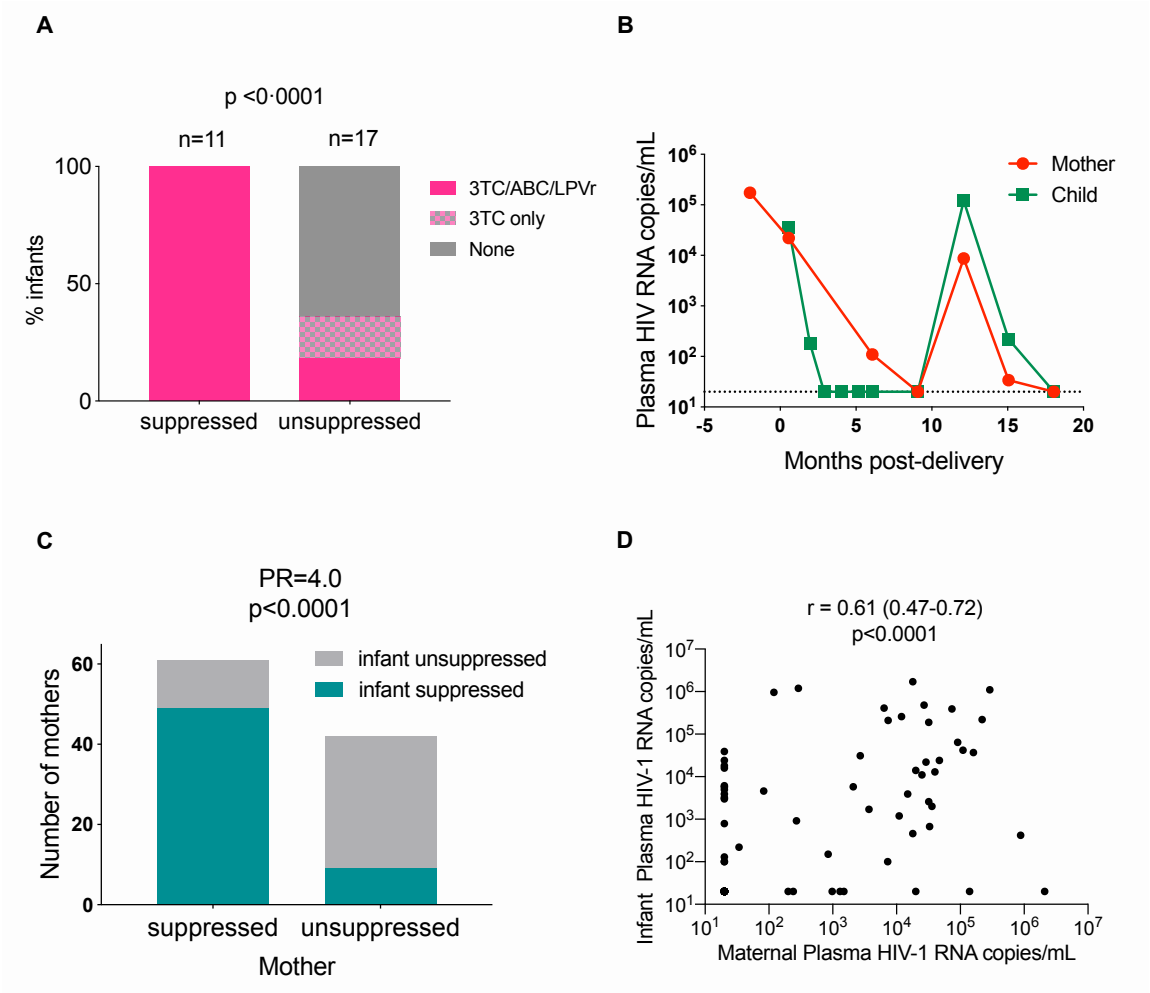


Figure 3.7: Determinants of infant plasma viraemia . (A) Results from plasma ART quantification from Table 3.4 are compared to those of 11 infants of similar age (median age 21 months) with plasma viral suppression (median duration of suppression 18 months) on cART. Negative levels includes levels below the level of quantification. P-value calculated using Fisher's exact test. (B) Plasma viral load levels over time for a mother-child pair following cART initiation, as an example of infant treatment success being determined by maternal cART adherence. Dashed line shows limit of detection (20 HIV RNA copies per mL). (C) A cross-sectional analysis of 101 mother-child pairs, >6 months on cART at the most recent timepoint of contemporaneous (within 14 days) plasma viral load measurement for the mother and child. Suppression is defined as a plasma viral load lower than the detectable limit (20 or 100 RNA copies per mL). Prevalence ratio of infant non-suppression according to maternal suppression is shown and p-value is calculated using Fisher's Exact test. (D) Spearman's non-parametric correlation of maternal and infant plasma load shown in (C).

3.3.9 Maternal treatment failure

Finally, in 101 children older than 6 months of age who had a plasma viral load measurement at the same time point as their mother, viraemia in a child of a viraemic mother was four times as common than for children with virally suppressed mothers (prevalence ratio 4.0, p<0.0001, Figure 3.7C) and there was a strong association between maternal and infant plasma viral load (r=0.61, 95% CI 0.47–0.72, p<0.0001, Figure 3.7D). Together, these data strongly support the conclusion that the same barriers to effective treatment affect both the mother and child, and if the needs of the mothers are addressed, then the challenges of administering ART to children are no longer a significant obstacle preventing ART adherence in the children.

3.4 Discussion

This study was designed to explore the impact of very early cART for IU HIV-infected children and the opportunity to facilitate cure or remission. Initial infant plasma RNA and DNA viral loads were very low, with 12% of IU HIV-infected infants having undetectable viraemia at baseline, suggesting potential to ultimately achieve cure or remission in this group. The findings, however, are striking in highlighting the high-frequency failure of cART in this group, however early in life it is delivered. Barely one-third (37%) of cART-treated infants achieved suppression of viraemia by 6 months that was maintained beyond 12 months of age. Almost one quarter (24%) of IU-infected infants either died or were lost to follow-up by 6 months. In the subset tested, high-frequency cART failure did not result from HIV drug resistance. The barriers contributing to treatment failure are the same in both mother and child. This is an important result because it means that, if the needs of the mother can be successfully addressed, the practical challenges of administering cART to children, in most cases, will also be met. If durable suppression of viraemia in HIV-infected infants is to be achieved, and the undoubted opportunities to achieve cure or remission in HIV-infected children are to be fully realised, the unmet needs of transmitting mothers urgently need to be addressed first, whilst, at the same time, exploring options for long-acting or

extended-release paediatric ART formulations. Finally, apparently superior treatment outcomes for the SoC tested group suggests that barriers to returning to clinic to collect test results selects out infants with inherently better outcomes, and that although PoC testing gives the opportunity to capture these vulnerable infants, more focus needs to be put into supporting the maintenance of cART once initiated.

In South Africa, IU HIV-infected infants usually receive cART transplacentally in addition to ART prophylaxis (AZT and NVP or NVP alone) immediately after birth (prior to HIV diagnosis). In this setting, neither this study nor another South African study by Technau and colleagues (2018) showed superiority in initiating infant cART (AZT, NVP and 3TC) within 48 hours of delivery following PoC testing and diagnosis, compared with cART initiation at a median of 10 days following SoC testing and diagnosis. Indeed, Technau reported worse retention in care for the PoC tested infants, and here it was observed that the SoC tested infants, who had returned to clinic from home for confirmatory testing, had higher rates of sustained viral suppression. Together, these findings suggest that HIV-infected infants successfully recalled from home following a positive birth test inherently may be more likely to succeed with treatment. However, the data to determine the proportion of infected infants successfully recalled were not available. It is possible that studies only analysing those infants successfully recalled may over-estimate positive treatment outcomes. The ability of PoC testing to initiate cART earlier in perinatally infected infants has been well-described in a range of African countries (Bianchi et al. 2019) and is demonstrated also here, but in countries such as South Africa where universal birth testing is already implemented, and where ART is reaching infected infants transplacentally and at birth via prophylaxis, the advantages of PoC over SoC testing may be reduced. Furthermore, these HIV-infected infants were born to mothers with a range of immune status and ART exposure, resulting in the wide heterogeneity of infant HIV DNA levels at baseline. It is possible that some of these infants were not in fact, early in infection and perhaps the relevance of initiating cART in the first hours of life has been overstated.

The high rates of treatment failure presented here, along with other studies describing the difficulties in treating paediatric HIV infection in sub-Saharan Africa, (Technau et al. 2018; Teasdale et al. 2018; Meyers et al. 2011; Abrams et al. 2017) as well as in high-resourced settings (Ruel et al. 2019; Kakkar et al. 2019) suggest a prevailing problem across the world. Perinatal HIV transmission, whether in high- or low-resourced settings, is concentrated in the most marginalised populations (Peters et al. 2018; Gnanashanmugam et al. 2019) who are often disengaged from conventional health care providers. Indeed, the Mississippi child is a case in point, (Persaud et al. 2013) whose remission only came to light following loss to follow-up and cART non-adherence. Here is strong evidence showing cART failure in the child is due to cART non-adherence and is tightly linked to cART failure in the mother. The magnitude of relationship between mother and child suppression shown here is much higher than has been recently described in older children, (Humphrey et al. 2019) showing the critical role of the mother for consistent cART administration for infants. On exploring the factors related to rate of infant viral rebound, counterintuitively, a strong predictive factor was longer duration of maternal cART in pregnancy. This could be explained by the mothers who were prescribed cART prior to pregnancy and defaulted treatment resulting in transmission *in utero*, being more likely to have difficulty administering infant cART compared to mothers who transmitted simply because they were diagnosed late in pregnancy. Interestingly, infants born at a younger gestational age and also those admitted to hospital as a neonate had lower rates of rebound, suggesting that additional support for the mother in the first weeks of life may be protective for virological control over the longer term. Older maternal age was also found to be protective against infant viral rebound. The importance of considering a child in the context of the mother is nowhere better illustrated than in the setting of perinatal HIV.

Infant mortality at 1 year was 9.3%, similar to other studies of South African IU HIV-infected infants (Technau et al. 2018) but high compared to the national estimated infant mortality rate of 27–33/1000, albeit a figure only representing reported deaths,

therefore a significant underestimate (Bamford et al. 2018). This study's figure is also likely to be an underestimate, as the outcome for some of the infants lost to follow-up was unknown. The predictors of infant mortality were low birth weight, followed by neonatal hospital admission and baseline infant plasma viral load, the latter also previously reported (Technau et al. 2018). Change of caregivers, home environment, comorbidities and other factors that were not systematically assessed by this study were likely playing pivotal roles. Moreover, the cause and circumstances of death were often not ascertained because most of the infants (9/15, 60%) died either at home or within a few hours of arrival at a hospital. This phenomenon in itself illustrates either the lack of access, poor engagement or mistrust in conventional health care providers. In-depth qualitative analysis of caregivers' experiences may illuminate areas for improvement in provision of care for HIV-infected infants.

It is important to consider the potential limitations within this study. Most crucially, in the comparisons of outcomes following PoC testing versus SoC testing, it should be emphasised that this was not a randomised controlled trial. Indeed, there were some differences between the PoC and SoC groups that were unexpected, such as the proportion of females (74% vs 53%), and the proportion exclusively breastfed (91% vs 68%), the clinical significance of which were unclear. With these differences at baseline, and the outcomes of the SoC tested infants who were not recruited onto the study unknown, a direct comparison of the PoC and SoC infants should be interpreted with caution. However, what is irrefutable, is that despite very early cART initiation, PoC tested infants' outcomes are poor. It has been suggested that INSTI drugs can achieve suppression in early treated infants faster, (Kakkar et al. 2019) which could mean that the poor viral suppression rates described here will improve with the planned move away from paediatric regimens based on the notoriously unpalatable LPVr suspension. However, the strong association between viral suppression in the mother (taking one tablet once a day) with viral suppression in the child suggests that if the mother is cART-adherent, the cART regimen being utilised here in the children is effective and that unpalatability is not the over-riding factor causing failure. Although there was

no evidence to suggest that HIVDR accounted for the high-frequency cART failure, the sample size was small, and only children with a high plasma viral load were tested, so it is possible that in some cases resistance contributed to cART failure. The direct testing of plasma showing a failure to detect cART in the majority of children with virological failure argues against this. Nevertheless, if these children continue to have viraemia and sub-optimal ART dosing, it is only a matter of time before ART resistance develops and contributes to treatment failure. Finally, this study was limited by the inability to determine the outcomes for the majority of infants who did not return to clinic and were lost to follow-up. Due to the poor coordination in HIV care in South Africa, it is possible that some of these infants relocated to another region to receive care without being formally being transferred, which may have led us to over-estimate true loss to follow-up.

ART has been widely successful in improving health, prolonging life and reducing HIV transmission events across sub-Saharan Africa. Furthermore, cART adherence in acutely infected women from a similar sociodemographic in KwaZulu-Natal with additional support can achieve excellent outcomes (Dong et al. 2017). However, the 0.6% of HIV-infected mothers in KwaZulu-Natal who are failed by the PMTCT programme and transmit HIV IU (Goga et al. 2018) are unlikely to be successful with cART treatment for their child. In the absence of long-acting therapeutic interventions that can be implemented very soon after birth, unless these unmet needs are addressed, infant HIV-related morbidity and mortality will remain high.

Chapter 4

Latent HIV reservoirs in very early treated *in utero* infected infants

4.1 Introduction

The latent viral reservoir constitutes the ultimate barrier to HIV remission or cure. The only intervention that has been shown to reduce the latent HIV reservoir in children is early initiation of cART, whereby lower reservoir levels are achieved by not only restricting the initial size (Garcia-Broncano et al. 2019; Veldsman et al. 2019) and sequence diversity (Palma et al. 2016; Van Zyl et al. 2017) of the viral reservoir, but also its cellular localisation, skewing the reservoir to exist in shorter-lived, transitional CD4+ T cell memory cells which decay faster than longer-lived central memory cells (Luzuriaga et al. 2014).

Adults treated in chronic HIV infection have a latent HIV reservoir decay half-life of 3.6 years (Finzi et al. 1999; J. D. Siliciano et al. 2003), whereas adults treated in early HIV infection have a faster HIV reservoir decay, with an initial half-life of approximately 12 weeks (Ananworanich et al. 2016). Cross-sectional and comparative analyses have shown infants starting cART <1 year (Foster et al. 2017; Uprety et al. 2017; Persaud et al. 2012), <6 months (Bitnun et al. 2019; Veldsman et al. 2019; Moragas et al. 2018), <3 months (M. McManus et al. 2016; Martínez-Bonet et al. 2015; Luzuriaga et al. 2014) and < 2 months of age (Van Zyl et al. 2015) compared with children treated at an older age, can result in a smaller latent HIV reservoir after 1-12 years on treatment. It is speculated that latent HIV reservoir decay is faster in infants

compared to adults, however HIV decay kinetic studies in infants have been small and heterogenous producing varying results, as summarised in Table 4.1. Therefore, the precise age, whether it is hours or days of life, that defines "early treatment" to realise benefit is unclear.

Table 4.1: Longitudinal HIV DNA measurements in early treated infants

	n	Median age cART initiation	cART regimen, 2NRTIs plus:	Median age first suppressed	Median age first measurement	HIV DNA Assay	Initial HIV DNA decay rate
Garcia-Broncano et al. 2019	10	1d	PI	3m	1d	ddPCR	$t_{1/2}$ 3-4w (over 6m)
Veldsman et al. 2019	7	5d	PI	3.5m	18d	qPCR	$t_{1/2}$ 2.7m (at 6m)
Uprety et al. 2017	13	2.1m	PI or NNRTI	8m	8m	ddPCR	0.5 $\log_{10}$ copies/y (over 2y)
McManus et al. 2016	13	1.9m	Not stated	4.2m	1.9m	Limiting dilution	1.04 $\log_{10}$ copies/y (over 1y)
Uprety et al. 2015	18	2m	PI	5m	2m	ddPCR	$t_{1/2}$ 7.5m (over 2y)
Luzuriaga et al. 2014	4	1.9m	PI or NNRTI	5m	1y	ddPCR	0.6 $\log_{10}$ copies/10y

HIV DNA copies are expressed per million PBMC. m: months. w: weeks. y: years. d: days. ddPCR: droplet digital PCR. qPCR: quantitative PCR.

4.2 Hypothesis and aims

The hypothesis for this study was that treatment with cART in the first hours rather than days of life could limit the size of the peripheral latent HIV reservoir for IU HIV-infected infants, and further facilitate reservoir decay, enhancing plasma virological control. The aims of the study were to use to UL cohort data to determine how timing of cART initiation for IU HIV-infected infants influences:

- Pre-treatment peripheral total HIV DNA levels in IU HIV-infected infants initiating cART on day 0-21 of life
- Rate of peripheral total HIV DNA decline in the first years on treatment

- The relationship between HIV DNA level and subsequent plasma virological control

4.3 Chapter specific methods

4.3.1 Study participants

Participants and data from the UL cohort up until December 31, 2019 were included.

4.3.2 Study definitions

- Latent HIV reservoir was estimated using total HIV DNA concentration in PBMC.

- Viral suppression was defined as one plasma viral load measurement lower than the limit of detection.

- One of two definitions of viral rebound was used; low-level rebound, one plasma viral load measurement >100 HIV RNA copies per mL, or high level rebound, one plasma viral load measurement >1000 HIV RNA copies per mL.

- A blip was defined as one plasma viral load >100 HIV RNA copies per mL preceded and followed by an undetectable plasma viral load, or a plasma viral load that increased <1 $\log_{10}$ HIV RNA copies per mL between consecutive monthly measurements during initial plasma viral load decline.

- Adherence was documented at each clinic visit using caregiver self-reports and by measuring returned medication (if available). Non-adherence was defined as evidence or self-reports of missing >2 doses since the previous blood sample.

- A viral suppression (VS) sub-group was defined by those infants who were >6 months post-enrolment with >1 HIV DNA measurement, had a plasma viral load that negatively trended i.e. was less than, the same, or <1 $\log_{10}$ higher in consecutive monthly measurements, and reached plasma viral load suppression at or before 6 months of age.

4.3.3 Statistics

HIV DNA decay

A potentially non-linear HIV DNA (on a natural log, ln, scale) decay over time in the VS infants was estimated and visualised via a Generalised Additive Mixed Model (GAMM) with a random effect (intercept only) on the participant, then again in a multivariate analysis to assess for covariates influencing HIV DNA level over time, both using the `mgcv` R package (Wood 2017). Estimates of HIV DNA decay was calculated by using the time at which the HIV DNA level (in absolute values) was half of its value at baseline, and then this was repeated from 90 days via the `predict().gam` function.

4.4 Results

4.4.1 Baseline HIV DNA

Of 191 IU HIV-infected infants, a baseline HIV DNA measurement was available for 164 infants, whose baseline characteristics are displayed in Table 4.2. Median cART initiation was 6.5 days (IQR 1-11 days, maximum 21 days). 148/164 (90.2%) infant baseline measurements were taken immediately prior to cART initiation, while the remaining 16/164 (9.8%) were after a median of 1 day of cART. The median infant baseline HIV DNA level was 2.8 $\log_{10}$ copies per million PBMC (IQR 2.3 – 3.4), and levels were variable, ranging from 0.6 – 4.8 $\log_{10}$ copies per million PBMC (Figure 4.1A). 77/164 (47%) of the infants were diagnosed by PoC testing (Table 4.2). The baseline HIV DNA levels in the PoC-tested infants did not differ from the infants diagnosed via SoC testing (p=0.7, not shown). Furthermore, there was no significant relationship between baseline HIV DNA level and infant age at the time of measurement (β coefficient = -0.01, p=0.3, Figure 4.1B), even when adjusted for infant CD4 percentage and plasma viral load (p=0.14, not shown). Similarly, there was no relationship between HIV DNA level and infant age when data were analysed separately within the PoC group and within the SoC-tested group, respectively.

Table 4.2: Baseline characteristics of all infants and the virally suppressed (VS) infants

	All infants (n=164)		Virally suppressed infants (n=98)		
	n/median	**IQR/%**	**n/median**	**IQR/%**	**p-value**
INFANT					
Sex male	60	36.6	36	36.7	1
Median birth weight (kg)	2.8	2.4-3.1	2.9	2.5-3.2	0.2
Median gestational age at birth (weeks)*	38	36-39	37.5	37-39	0.4
Dual prophylactic ART	111	67.7	72	73.5	0.2
PoC tested	77	47	41	41.8	0.1
SoC PCR result indeterminate or negative	15	9	11	11.2	0.4
Age at cART intiation (days)	6.5	1-11	7	1-11	0.6
Age at blood draw (days)**	7	1-11	7	1-11	0.6
Neonatal admission	54	32.9	29	29.6	0.4
Breastfed	130	79.3	77	78.6	0.7
CD4 count (cells/μL)	2120	1291-2702	2222	1371-2777	0.6
CD4 percentage	44.5	35.3-52	45.5	38-53	0.11
CD8 count (cells/μL)	1088	685-1587	983	616-1433	0.3
CD8 percentage	23	18-32	22	18-28	0.3
CD4:CD8	1.84	1.17-2.77	2.08	1.45-2.84	0.2
Plasma viral load copies/mL	9700	1200-56 500	3700	310-30 500	0.04
Log10 HIV DNA copies/million PBMC	2.8	2.3-3.4	2.6	2.2-3.3	0.1
MOTHER					
Age (years)	25.2	21.9-30.3	24.5	21.2-29.7	0.3
Acute maternal infection	43	26.2	29	29.6	0.6
No antenatal cART	15	9.1	8	8.2	1
Duration cART in pregnancy (days)	104	13-217	95	8-178	0.3
Self-reported maternal non-adherence†	70	54	36	48	0.5
CD4 count (cells/μL)	460	317-646	516	350-650	0.4
CD4 percentage	24	19-31	25	19-32	0.4
CD8 count (cells/μL)	952	706-1272	910	660-1332	0.6
CD8 percentage	52.5	43.3-58.8	49	41-58	0.4
CD4:CD8	0.46	0.32-0.7	0.5	0.33-0.74	0.3
Plasma viral load (HIV RNA copies/mL)	3750	488-85 957	2250	253-20 750	0.2

∗excluded from multivariate analysis because of missing data, ∗∗ replaced by "cART prior to blood draw" in multivariate analysis, †of all mothers who were initiated on cART prior to labour.

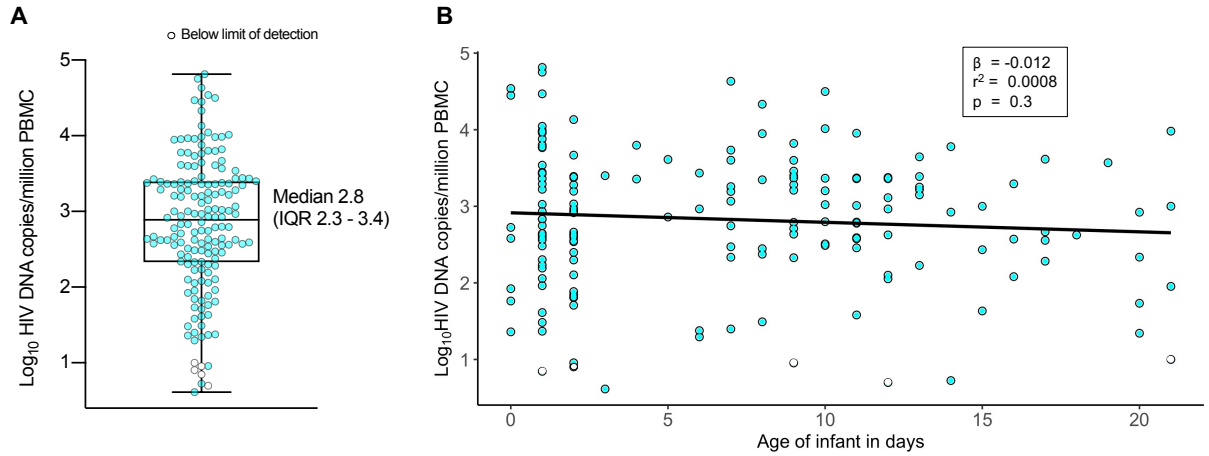


Figure 4.1: Baseline infant HIV DNA levels. (A) Baseline HIV DNA levels as determined using droplet digital PCR on PBMC from 164 infants at a median age of 7 days. Undetectable HIV DNA is represented by an open circle at the limit of detection for that measurement. (B) The same measurements displayed according to the age of the infant at the time of measurement. Unadjusted linear regression model shown by the black line.

In order to explore the potential influence of a large number of baseline maternal and infant characteristics on baseline HIV DNA, a penalised linear regression model (LASSO) was used in order to select those covariates with the strongest relationship with HIV DNA level from a possible set of 26 baseline variables, as listed in Table 4.2. In the model representing all 164 infants, relatively, the baseline covariates that were the most influential on baseline infant HIV DNA were infant plasma viral load and CD4 percentage, followed by absence of antenatal maternal cART (Figure 4.2A, Appendix III). Infant age at blood draw was not selected as influencing HIV DNA level. The selected influential covariates were then input in a (post-LASSO) multivariate linear regression to determine their absolute influence on infant HIV DNA (unstandardised β-coefficient, Table 4.3). For every $\log_{10}$ HIV RNA copies per mL increase in infant plasma viral load, HIV DNA increased by 0.2 $\log_{10}$ copies per million PBMC, and increase of 10 in CD4 percentage, HIV DNA decreased by 0.3 $\log_{10}$ copies per million PBMC. The absence of antenatal maternal cART increased infant HIV DNA by 0.6 $\log_{10}$ copies per million PBMC.

The median infant age at baseline blood draw for those infants PoC tested was 1 day (IQR 1-2). The baseline HIV DNA multivariate analyses were repeated on the PoC and SoC groups of infants separately (Figure 4.2B-C, Appendix III). In both groups,

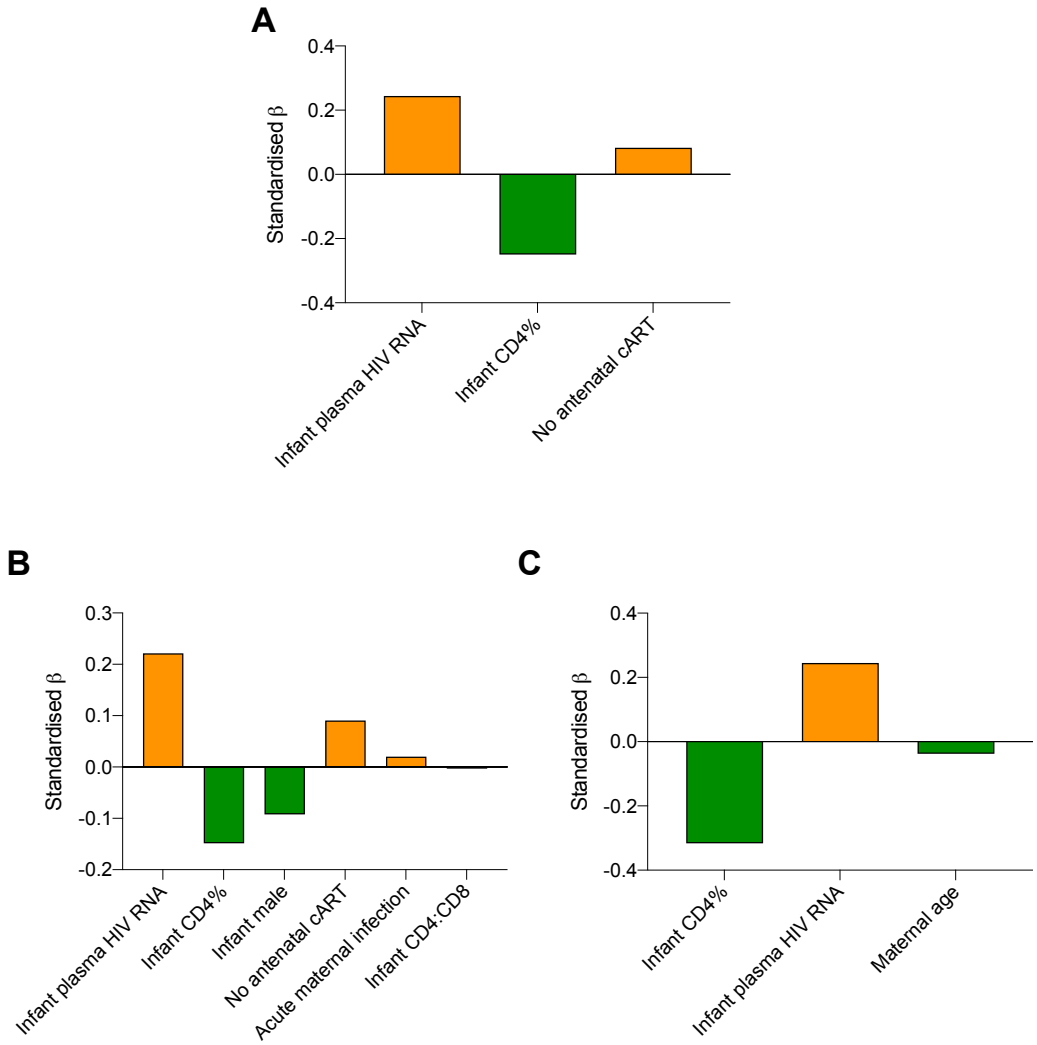


Figure 4.2: Covariates influencing infant baseline HIV DNA level. Covariates selected using a penalised linear regression model (LASSO) are shown left to right along the x-axis in order of standardized beta-coefficient (β) magnitude, which represents their relative influence on HIV DNA. Positive, orange bars indicate covariates associated with higher HIV DNA, while negative, green bars indicate covariates associated with lower HIV DNA. These selected covariates were used in the post-LASSO linear regression to calculate unpenalized, unstandardized beta-coefficients (Table 4.3). (A) shows the LASSO model for all 164 infants, while (B) and (C) show the PoC tested infant and SoC infant subgroups, respectively. VL: plasma viral load.

Table 4.3: Covariates influencing infant baseline HIV DNA level

	All infants (n=164)			PoC infants (n=77)			SoC infants (n=87)		
Variable	β	**95% CI**	**p-value***	β	**95% CI**	**p-value***	β	**95% CI**	**p-value***
Infant plasma viral load (log_{10} HIV RNA copies/mL)	0.22	0.14 to 0.3	<0.0001	0.22	0.09 to 0.35	0.001	0.26	0.17 to 0.35	<0.0001
Infant CD4 percent	-0.03	-0.04 to -0.02	<0.0001	-0.01	-0.04 to 0.01	0.2	-0.03	-0.04 to -0.02	<0.0001
No antenatal cART	0.61	0.24 to 0.98	0.002	0.76	0.13 to 1.39	0.02	–	–	–
Male infant	–	–	–	-0.49	-0.88 to -0.09	0.02	–	–	–
Acute maternal infection	–	–	–	0.29	-0.08 to 0.66	0.1	–	–	–
Infant CD4:CD8	–	–	–	-0.08	-0.28 to 0.11	0.4	–	–	–
Maternal age (years)	–	–	–	–	–	–	-0.02	-0.04 to 0	0.03
	Adjusted r^2 = 0.37			Adjusted r^2 = 0.39			Adjusted r^2 = 0.5		
	p = <0.0001			p = <0.0001			p = <0.0001		

∗p-values for post-LASSO linear regression models should be interpreted with caution as they ignore the uncertainty involved in the preceding LASSO step. β: unstandardised regression coefficient estimate.

infant plasma viral load and CD4 percent still had the strongest relationship with HIV DNA level. Male sex was additionally associated with a lower DNA level and acute maternal infection during pregnancy for the PoC-tested group (Table 4.3 and Figure 4.2B-C). It was observed that after adjusting for other influencing variables, for those infants tested at birth, males had HIV DNA levels 0.5 log_{10} HIV copies per million PBMC lower than female infants (Table 4.3).

4.4.2 HIV DNA decay

As previously described in Chapter 3, despite early treatment, in general, virological response to cART in this cohort was suboptimal. Therefore, longitudinal measurements in a sub-group of 98 infants with adequate viral suppression (VS infants, Figure 4.3) were further analysed to examine HIV DNA decay in early treated infants. To check for representativeness, the VS infants' baseline characteristics were compared to all the infants with measurements at baseline (Table 4.2). The VS infants had significantly lower baseline plasma viral load (median 3700 versus 9700 HIV RNA copies per mL, p=0.04), but baseline characteristics were otherwise similar. Median age of plasma viral load suppression was 60 days (IQR 29-90 days) and this was not significantly correlated with age of cART initiation (r^2=-0.18, p=0.08, not shown), even when adjusted for baseline CD4 percentage and plasma viral load (β=0.02, p=0.1, not shown). VS infant HIV DNA measurements were censored at a plasma viral rebound

of >1000 HIV RNA copies per mL, resulting in 274 measurements in the first 3.5 years of life, of which 40/274 (14.5%) were below the limit of detection. All measurements were put into a GAMM of HIV DNA over cART duration with random effects (intercept only) on each individual infant (Figure 4.4). The model was non-linear, well representative of the data and highly significant (adjusted R^2 0.84, p<0.0001). Rate of decay was estimated from the model, giving an initial (i.e. from baseline) half-life of 28 days. From 90 days the half-life had increased to 194 days. A multivariate GAMM was used to determine the factors influencing the change in HIV DNA over time (Table 4.4, Appendix IV). Plasma viral load over time and age at viraemia suppression significantly influenced HIV DNA decay while age of cART initiation, blips, documented non-adherence, infant sex and CD4 percentage over time did not (Table 4.4). In order to reduce the potential effect of sub-optimal adherence on reservoir decline, this analysis was re-run using the sub-group of the VS infants (n=74); those who had no blips, and whose baseline HIV DNA level was >50 copies per million PBMC, to ensure that all infants had an opportunity to halve their HIV DNA level. The decay half-life for this sub-group at baseline was shorter, at 21 days, but from 90 days the half-life in the sub-group was similar to all the VS infants at 188 days. The influence of the same covariates was similar to the whole VS group, except the HIV DNA level was approximately 0.5 $\log_{10}$ HIV DNA copies per mL lower for the PoC tested group (p=0.06), which was visualised well by instead using age of cART initiation as a continuous variable, where lower levels were seen with an age of initiation < 5 days (p=0.051, Appendix IV, page 169).

4.4.3 HIV DNA and plasma virological control

The relationship between HIV DNA levels and plasma viraemia control was explored, first by examining the time to virological suppression in all 164 infants. Baseline infant plasma viral load, CD4 percentage and HIV DNA levels independently influenced time to suppression of plasma viraemia, based on univariate Cox regression analyses and a multivariate analysis (Table 4.5). The addition of infant sex, age of cART initiation and PoC testing did not individually influence time to suppression nor improve the fit

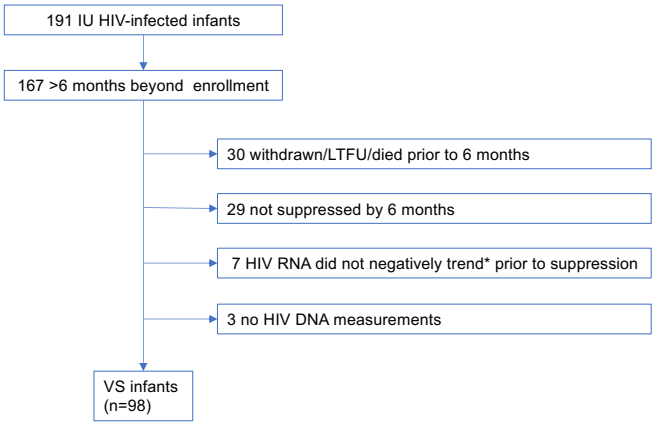


Figure 4.3: Selection of infants with adequate plasma viral suppression for HIV DNA decay analysis. ∗plasma HIV RNA trended in a negative direction i.e. consecutive monthly measurements HIV RNA the same, lower or <1 $\log_{10}$ higher than the previous month. VS: viral suppression. LTFU: loss to follow-up.

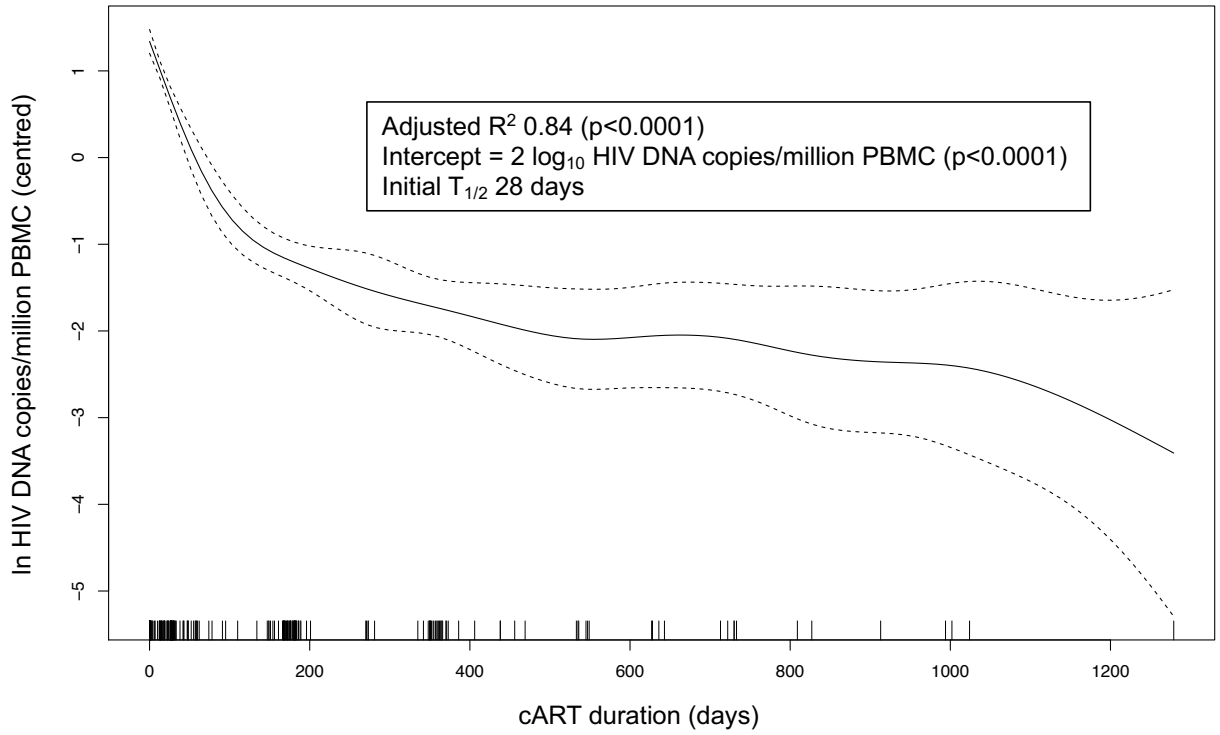


Figure 4.4: HIV DNA decay of viral suppression (VS) infants only. Non-linear (centered) effect of time on cART (in days) on ln HIV DNA copies per million PBMC fitted via the `gam` R-function with individual infant as a random effect (intercept only). 95% confidence interval shown by the dashed lines. The vertical spikes on the x-axis denote timing of observations.

Table 4.4: Covariates influencing HIV DNA decay

Parametric coefficients	**Estimate**	**95%CI**	**p-value**
Intercept ($\log_{10}$ HIV DNA copies per million PBMC)	2.12	1.8 to 2.43	<0.0001
Infant sex	0.004	-0.28 to 0.29	0.98
Blips*	0.12	-0.31 to 0.55	0.6
Documented cART non-adherence**	-0.08	-0.22 to 0.06	0.3
PoC tested	0.16	-0.3 to 0.7	0.5
Approximate significance of smooth terms		**EDF**	**p-value**
cART duration (days)		3.19	<0.0001
Infant age at cART initation (days)		1.00	0.8
Age infant suppressed (weeks)		1.00	0.0003
Plasma viral load over time ($\log_{10}$ HIV RNA copies/mL)		1.00	<0.0001
CD4 percentage over time		2.02	0.3
Adjusted R^2 = 0.86			

Modelled using a Generalised Additive Mixed Model (GAMM), ∗blip was defined as plasma viral load >100 HIV RNA copies per mL on one occasion preceded and followed by an undetectable plasma viral load, or a plasma viral load that increased <1 $\log_{10}$ HIV RNA copies per mL between consecutive monthly measurements during initial plasma viral load decline. ∗∗evidence or reports of missing >2 cART doses. EDF: effective degrees of freedom. Effect of smooth terms shown in graphical form in Appendix IV, page 169.

of the multivariate model. In the final multivariate model, baseline $\log_{10}$ plasma had the strongest influence on time to suppression (HR 0.59, 0.49-0.72, p<0.0001). After this adjustment, baseline HIV DNA level still had a significant effect, where an increase of 1 $\log_{10}$ HIV DNA copies per million PBMC decreased the rate of suppression by 25% (p=0.01, Table 4.5).

Table 4.5: Covariates influencing time to viral suppression

		Univariate			Multivariate	
Variable	**HR**	**95% CI**	**p-value**	**HR**	**95% CI**	**p-value**
Baseline infant HIV DNA ($\log_{10}$ copies/million PBMC)	0.59	0.49 to 0.72	<0.0001	0.75	0.60 to 0.94	0.01
Baseline infant plasma viral load ($\log_{10}$ HIV RNA copies/mL)	0.52	0.43 to 0.63	<0.0001	0.55	0.45 to 0.66	<0.0001
Baseline infant CD4%	1.03	1.01 to 1.05	<0.0001	1.02	1.01 to 1.04	0.008
Male infant	0.87	0.60 to 1.27	0.5	-	-	-
PoC tested	0.82	0.57 to 1.17	0.3	-	-	-
Infant age at cART initiation (days)	1.01	0.98 to 1.04	0.7	-	-	-
Wald test p = <0.0001						

Cox Proportional Hazard Model. HR: hazard ratio.

Next, maintenance of plasma aviraemia on cART was evaluated in the VS infants with regard to a) HIV DNA level at baseline and b) HIV DNA at the timepoint plasma viral RNA suppression was achieved. Where a measurement at suppression was not available, the timepoint was within 3 months of suppression. HIV DNA level was assessed both as a continuous variable, and a categorical variable (above and below the median value). Additionally, both low-level and high-level rebound were investigated. Median time to viral rebound >100 HIV RNA copies per mL was 423 days (95% CI 320 - 666) and 571 days (95% CI 280 – ∞) for >1000 HIV RNA copies per mL. The probability of viral rebound >1000 HIV RNA copies per mL by 12 months was 0.46 (95% CI 0.34 - 0.56). The univariate analyses did not find any significant relationship between HIV DNA level at baseline nor at the time of suppression, and time to viral rebound for either definition (Table 4.6). The best fit multivariate model for time to viral rebound >1000 HIV RNA copies per mL was statistically significant, but older infant age at viral suppression, and not HIV DNA level at baseline or at the timepoint plasma viral RNA suppression was achieved, was associated with a slower rebound rate

(HR 0.93, 95% CI 0.88 - 0.98 p=0.009, Table 4.6). The best fit multivariate model for time to low level viral rebound was not statistically significant.

Table 4.6: Covariates influencing time to viral rebound

	Viral rebound >100 HIV RNA copies/mL						Viral rebound >1000 HIV RNA copies/mL					
	Univariate			Multivariate			Univariate			Multivariate		
Variable	**HR**	**95% CI**	**p-value**	**HR**	**95% CI**	**p-value**	**HR**	**95% CI**	**p-value**	**HR**	**95% CI**	**p-value**
Infant HIV DNA												
(log_{10} copies/million PBMC)												
Baseline high vs low**	1.12	0.67 to 1.87	0.7	1.82	0.98 to 3.4	0.06	0.89	0.5 to 1.59	0.7	-	-	-
Baseline continuous	1.05	0.79 to 1.4	0.8	-	-	-	0.99	0.72 to 1.36	0.9	1.37	0.91 to 2.06	0.1
*Suppression high vs low**	0.92	0.47 to 1.79	0.8	-	-	-	0.72	0.34 to 1.55	0.4	-	-	-
*Suppression continuous	1.05	0.67 to 1.64	0.8	-	-	-	0.89	0.56 to 1.42	0.6	-	-	-
Male infant	0.88	0.52 to 1.5	0.6	-	-	-	0.78	0.42 to 1.43	0.4	-	-	-
SoC tested	0.69	0.41 to 1.15	0.2	0.60	0.35 to 1.03	0.07	0.29	0.33 to 1.02	0.06	0.57	0.31 to 1.01	0.05
Infant age at viral suppression (weeks)	0.97	0.94 to 1.01	0.1	0.95	0.91 to 0.99	0.02	0.95	0.91 to 1	0.047	0.93	0.88 to 0.98	0.009
CD4% at suppression	1.01	0.99 to 1.03	0.5	-	-	-	1.01	0.98 to 1.03	0.5	-	-	-
				Wald test p = 0.06						**Wald test p = 0.02**		

∗HIV DNA measurement at the timepoint viral suppression is achieved or within 3 months. ∗∗above and below the median value. Cox Proportional Hazard Model. HR: hazard ratio.

4.5 Discussion

This is the largest study of HIV DNA dynamics in very early-treated IU HIV-infected infants. Baseline infant peripheral total HIV DNA levels were low, in some cases even below the limit of detection, and this was associated with maternal antenatal cART use. However, there was no evidence to suggest that for infants receiving ART as prophylaxis, such as is the standard of care internationally, that there was expansion of the peripheral latent HIV reservoir in the first 3 weeks of life, nor that earlier initiation resulted in faster HIV DNA decay on cART. Point-of-care diagnosis and the subsequent blood sampling within hours of birth enabled a rare insight into clinical factors potentially influencing HIV DNA *in utero*. Higher HIV DNA levels were demonstrated in female infants and those born to acutely infected mothers. Lower HIV DNA was found in infants with lower plasma viral load and higher CD4 percentages, but it unknown whether these correlations were causal or consequential. Plasma viral load and age of suppression of viraemia were significantly associated with HIV DNA levels over time, but this decay was not further influenced by blips or documented non-adherence.

Although lower infant HIV DNA levels were associated with a decreased time to suppression of plasma viraemia, they were not associated with maintenance of aviraemia, suggesting that there are immunological, virological or behavioural factors that override any potential biological advantage of low latent HIV reservoirs in early treated IU HIV-infected infants in the first years of life.

This study showed higher infant HIV DNA levels at birth compared to the only two other studies of HIV DNA in IU HIV-infected PMTCT-exposed infants in the first days of life (median 2.8 vs 2.1 and 2.2 $\log_{10}$ HIV DNA copies per million PBMC, Garcia-Broncano et al. 2019; Veldsman et al. 2019), although this may be related to the other studies' small sample sizes (n=9 and 7). HIV DNA levels in the first days of life from all three studies were >1 $\log_{10}$ copies per million PBMC lower as compared to a study of IU HIV-infected infants not exposed to PMTCT (Scott-Algara et al. 2010). Maternal cART use and infant ART prophylaxis in this study were common. Thus, this substantial infant exposure to ART transplacentally and postnatally as prophylaxis prior to cART initiation, is lowering or restricting infant latent HIV reservoir size.

From these low levels, HIV DNA decayed quickly with an initial HIV DNA half-life of 28 days, similar to that reported by Garcia-Broncano and colleagues in their analysis of infants with similar ages of cART initiation and plasma viral load suppression (Garcia-Broncano et al. 2019). IU HIV transmission is estimated to occur at a median of 14 days prior to delivery (Rouzioux et al. 1995), and these infants were on average one week post-delivery, thus weeks post-infection, however the HIV DNA decay rate in these infants was still faster than adults treated in the first days of infection (Ananworanich et al. 2016), demonstrating benefits of the tolerogenic neonatal immune system. It was intriguing to note, although HIV DNA decay was significantly associated with plasma viral load, it was not influenced by blips or documented non-adherence (Table 4.4). This raises the possibility that the initial degree and length of plasma viraemia is more crucial in terms of reservoir size, compared with short periods of viraemia later, also shown in analytic treatment interruption studies in adults (Steingrover et al. 2008; Salantes et al. 2018). However, it may have been that 3 monthly

plasma viral load measurements and self-reports of adherence were simply insensitive markers for intermittent viraemia.

In the sub-group of the VS infants, where those infants who either had very low baseline latent HIV reservoir levels, or had blips were excluded, HIV reservoir decay was faster, as expected (half-life 21 days compared with 28 days in the VS group), and HIV DNA levels were slightly lower in those infants starting cART earlier. This is a critical finding, because it indicates that there is a potential advantage to starting cART in the few days of life, but that this effect is not usually realised in the real world, because of the effect of pre-treatment prophylaxis and imperfect ART administration.

As discussed in Chapter 3, due to the different recruitment methods, there may be inherent differences between the PoC and SoC tested infants, who also differ in timing of cART initiation. To minimise the effect of this potential selection bias, the PoC and SoC group were either analysed separately or the analysis was adjusted for by testing group. As previously found, the SoC group had superior maintenance of aviraemia.

Similar to the multivariate model of HIV DNA decay shown here, most paediatric cross-sectional and longitudinal studies have found lower levels of HIV DNA associated with younger infant age at suppression (Martínez-Bonet et al. 2015; Garcia-Broncano et al. 2019; Veldsman et al. 2019; Luzuriaga et al. 2014; Van Zyl et al. 2015; Moragas et al. 2018; Foster et al. 2017; Uprety et al. 2017; Persaud et al. 2012; Bitnun et al. 2019; M. McManus et al. 2016; Kuhn et al. 2018). In this study, the HIV DNA level increased with age of plasma viraemia suppression (Table 4.4, Appendix IV), suggesting increasing benefit for decreasing age of suppression. The key difference for this study was there was no association between age of cART initiation and plasma viral suppression. This is probably because previous studies analyzed age of cART initiation in the order of months, compared to the narrow window of age of initiation (21 days) in the UL study, and perhaps this study had higher rates of suboptimal ART administration. Interestingly, the time to plasma viral rebound was faster in those UL infants who suppressed plasma viraemia earlier, despite on average, lower HIV DNA

levels. This may have been due to more effective HIV-specific immune responses in those infants who were exposed to plasma viraemia for longer, delaying rebound, as shown previously (Zanchetta et al. 2008). However, anti-HIV immune responses for these infants still requires investigation. Alternatively, this finding may instead be due to behavioural factors. It is possible, for example, that the mothers of infants who were taking longer to virally suppress received the most attentive adherence support, addressed central issues such as disclosure, and developed robust cART administration skills. By contrast, those mothers who quickly achieved suppression, or whose infant had an undetectable viral load from birth, did not receive as much attention and therefore may not have acquired the skills to overcome the many barriers to sustaining cART adherence.

Two studies have shown correlations between lower peripheral total HIV DNA levels and superior maintenance of plasma aviraemia in children (Kuhn et al. 2018; Tagarro et al. 2018). This finding certainly has biological plausibility and it was surprising the same result was not found here. Compared to the other studies, the UL children were younger and had a much higher probability of plasma viral load rebound (0.46 at 12 months compared with 0.01 by Kuhn and colleagues), likely due to the significantly higher rates cART non-adherence in infancy (Meyers et al. 2011; Teasdale et al. 2018). It is possible that lower HIV DNA levels are useful in the context of occasional missed doses, but not for the higher levels of non-adherence seen in younger children. With regards to inducing HIV remission with very early treatment in the way of lowering reservoirs, in examining the three descriptions of paediatric post-treatment control, rebound occurred the earliest (after 27 months off therapy) in the Mississippi Baby who had undetectable HIV DNA levels (Persaud et al. 2013), whereas the VISCONTI child, has not rebounded despite an HIV DNA level of 158 copies per million PBMC eleven years off therapy (Frange et al. 2016). Therefore, there are other, unmeasured variables that assist with plasma virological control, not just latent reservoir size.

The findings of this study are relevant to settings like South Africa, with well implemented PMTCT programmes, including antenatal cART, extensive neonatal pro-

phylaxis and HIV testing at birth. Although I did not find any benefit in terms of latent HIV reservoir size for initiating cART in the first hours versus days of life, it is important to make the point that this should not be used as an argument against PoC testing for EID. In KZN, only 25% of infants return for confirmatory HIV testing following an initial SoC positive test (Smith et al. 2019), and although this may be a gross underestimation due to the lack of unique patient identifiers, it is likely that there are significant numbers of infants lost to follow-up, who do not initiate cART. PoC or near-PoC for EID in Africa, decreases test turnaround time and increases rates of cART initiation (Bianchi et al. 2019; Technau et al. 2017a). This focus on reducing morbidity and mortality, as opposed to the theoretical advantage of inducing HIV remission, should rather drive public health decisions for EID implementation. Furthermore, the innate immunological benefits of cART initiation in the first days of life have been shown (Garcia-Broncano et al. 2019), which is enabled by PoC testing. Although it should be noted that these benefits were described in comparison to infants initiated on cART aged 3-4 months of age, and the difference in immune function resulting from delaying treatment from days to weeks remains to be investigated.

This HIV DNA decay analysis benefited from a large sample size, but its generalizability may be limited by excluding those infants with prolonged time to suppression and higher baseline plasma viral load who may have had intrinsically different viral or immune characteristics. However, inadequate plasma viral suppression for those infants was more likely secondary to cART non-adherence, a scenario for which HIV DNA decay is less relevant.

The infant blood sample volumes were small, which at times restricted the HIV DNA sensitivity. As many as 14.6% of HIV DNA measurements were below the limit of detection, and, when this coincided with low cell input, thus high limit of detection, may have overestimated estimates of HIV DNA. Although this issue was minimized by excluding undetectable samples with high limits of detection, resulting in the average limit of detection for undetectable HIV DNA being 5 copies per million PBMC, it was

impossible to discern HIV DNA decay beyond this point, reducing the sensitivity of the analyses.

Akin to other paediatric HIV studies, total peripheral blood mononuclear cell HIV DNA was measured as a surrogate marker for latent HIV reservoir. By measuring total HIV DNA, defective HIV DNA and non-integrated HIV DNA forms, such as 2-LTR circles were included, over-representing that able to contribute to plasma viraemia and viral propagation. Although integrated replication competent HIV DNA correlates to total HIV DNA, the ratio may differ between infants, diminishing the relationship between HIV DNA and variables in the analyses. However, defective and unintegrated HIV DNA can still trigger inflammation and contribute to disease progression (Imamichia et al. 2016), so measurement of total HIV DNA still has clinical relevance. Small blood samples precluded sorting CD4+ T cells in order to measure HIV DNA in this cellular compartment. There was an inverse relationship between CD4+ T cell percentage and HIV DNA, therefore in the case of low CD4+ T cell count there would have been an even higher concentration of HIV DNA per CD4+ T cell than was measured. Because the CD4+ T cell counts were measured on whole blood, and the HIV DNA was measured on PBMC, it was not possible to adjust the raw HIV DNA counts by CD4+ T cell count. Although there were technical limitations to the HIV DNA measurements, these were counteracted by the large number of measurements analysed.

In conclusion, where PMTCT programmes are implemented well, and the standard of care can achieve HIV diagnosis in the first 3 weeks of life following birth testing, the priority for IU HIV-infected infant cART initiation should be timed to achieve maximal caregiver adherence education and support, rather than aiming for HIV remission in the way of lower HIV DNA.

Chapter 5

Increasing diagnostic uncertainty for *in utero* HIV infection

The majority of this work has been published in: **Millar, J. R.**, Mvo, Z., Bengu, N., Fillis, R., Sprenger, K., Matthews, P.C., Archary, M., Ndung'u, T., Adland, E., Puertas, M.C., Martinez-Picado, J., Goulder, P. (2019) Increasing Diagnostic Uncertainties in Children With In Utero HIV Infection. *PIDJ* 38, pp. e166-e168. https://doi.org/10.1097/inf.0000000000002315

5.1 Introduction

In South Africa, with the increasing use of ART as maternal and infant prophylaxis, HIV diagnosis following *in utero* MTCT is becoming increasingly problematic. Until 2010, PMTCT in South Africa consisted only of single dose NVP for both the mother and infant at delivery, after which time AZT at 14 weeks gestation or cART for those women with CD4+ T cell counts <350 cells per μL was introduced. In 2013 cART became available for all women, regardless of CD4+ T cell count, throughout pregnancy and breastfeeding, and in 2015 this was extended to lifelong cART for all HIV-infected individuals (Republic of South Africa National Department of Health 2015b) along with dual prophylaxis (AZT and NVP) for 12 weeks for infants at high risk of infection.

Umbilical cord-to-maternal serum drug level ratios for commonly used maternal cART regimens are close to one (McCormack and Best 2014), therefore it is no surprise that

HIV-infected infant plasma viral loads at birth pre-cART are decreasing with expanding antenatal cART coverage (Haeri Mazanderani et al. 2018b). As described in Chapter 3, even infant plasma viral loads below the limit of detection are being reported in IU HIV-infected infants at birth (Garcia-Broncano et al. 2019; Haeri Mazanderani et al. 2018b; Strehlau et al. 2019). Not only are infant plasma viral loads at birth seen today lower than previously, but so are HIV DNA levels, making HIV detection in DBS or whole blood samples in the first weeks of life more difficult (Burgard et al. 2012; Balasubramanian et al. 2017; Connolly et al. 2013). The number of EID indeterminate results in KZN are increasing, with 0.58% of all HIV tests in infants <16 weeks of age indeterminate in 2015 compared to 0.36% in 2013 (Smith et al. 2019). In 2015 there were 3000 indeterminate infant HIV PCR tests in South Africa, half of which were confirmed HIV-infected (Haeri Mazanderani et al. 2017).

The impact of NVP, even single dose at birth, is to reduce plasma viral load by a median of 1.3 $\log_{10}$ HIV RNA copies per mL in the first days of life (Goulder et al. 2004). The current doses for the two infant prophylactic ARVs in South Africa are effectively indistinguishable from therapeutic doses (Cressey et al. 2017; Republic of South Africa National Department of Health 2019b,a), with no evidence that two is better than three ARVs in initial viraemia decline in infants. Therefore, the older the neonate is, the longer the duration of significant ART exposure, increasing the risk of false negative confirmatory test following an initial HIV TNA PCR test positive shortly after birth (Haeri Mazanderani et al. 2014, 2018a).

5.2 Hypothesis and aims

Following the retrospective discovery of a case from the Ucwaningo Lwabantwana cohort where there was a failure to diagnose an infant *in utero* HIV-infected at birth, every effort was put into closely investigating and monitoring instances in the study of infants with discordant or borderline positive HIV PCR results. The aim of this chapter is to describe these cases and discuss how their occurrence might influence the

design of an EID screening and confirmatory test algorithm in South Africa, a setting of high antenatal HIV prevalence and extensive ART use.

5.3 Chapter specific methods

5.4 Results

5.4.1 Case report

In 2016 at one of the UL study hospitals, a female baby was born via vaginal delivery to an HIV-positive mother. In her first pregnancy in 2011 the mother had initiated cART and delivered an HIV-uninfected female. During this second pregnancy the mother did not attend antenatal clinic and reported taking her cART haphazardly. The mother presented to hospital in labour at 36 weeks gestation. At delivery she had an absolute CD4 count of 452 cells per μL (24%), and a plasma viral load of 12 000 HIV RNA copies per mL (NucliSens, bioMérieux). The baby received NVP prophylaxis at 2 hours of age. At 19 hours of age the baby tested HIV positive using a PoC TNA PCR on whole blood (GXP, Cepheid). The Ct value measured 41.3, just below the positive cut-off value of 42.0. Blood for plasma viral load testing (NucliSens, bioMérieux) and a dried blood sample for laboratory-based HIV TNA PCR (CAP/CTM, Roche) were drawn before infant cART, comprising of AZT, 3TC and NVP, was initiated at 20 hours of age. The mother exclusively breast fed.

The infant's absolute CD4+ T cell count was 1226 cells per μL (36%), below the 10th centile for an HIV-uninfected newborn. However, the plasma viral load was lower than the detectable limit (<20 HIV RNA copies per mL) and HIV TNA PCR on dried blood was negative. On day 10 these tests were repeated, and the results were unchanged.

In the absence of a confirmatory HIV test, cART was switched to AZT and NVP as prophylaxis against postpartum transmission. At 3 months the child returned to clinic and both the child's plasma viral load (NucliSens, bioMérieux) and dried blood HIV TNA PCR (CAP/CTM, Roche) remained undetectable. The absolute CD4+ T cell count and percentage remained low. Prophylaxis was stopped after this. When the

child next attended clinic aged 5 months, HIV TNA on dried blood was now detectable by PCR (CAP/CTM, Roche) and the subsequent plasma viral load was $>10^7$ HIV RNA copies per mL (COBAS 6800/8800 HIV-1 Test, Roche) (Figure 5.1A). At this time the mother's plasma viral load had risen to 212 000 HIV RNA copies per mL (COBAS, Roche) and she was still breastfeeding. cART was reinitiated for the infant and by 18 months of age, her plasma viral load was almost suppressed (120 HIV RNA copies per mL) although the absolute CD4+ T cell count and percentage remained low at 1446 cells per μL (19%).

Initially it was hypothesised that this child had been HIV-infected via breastfeeding. In order to distinguish between *in utero* or postpartum infection, HIV DNA was measured in the child's stored samples using ddPCR. The total HIV DNA viral load was measured at 40.7 (95% CI 13.6-88.1) and 48.5 (17-106.7) copies per million PBMC, from day one and ten of life respectively (Figure 5.1B). HIV *gag* was amplified and sequenced from plasma stored from the mother and from proviral DNA from the child from day one of life, showing phylogenetic clustering of the sequences (Figure 5.1C), confirming *in utero* infection of the child.

LC-MS/MS detected FTC, TDF and EFV in the mother's plasma from day one postpartum.

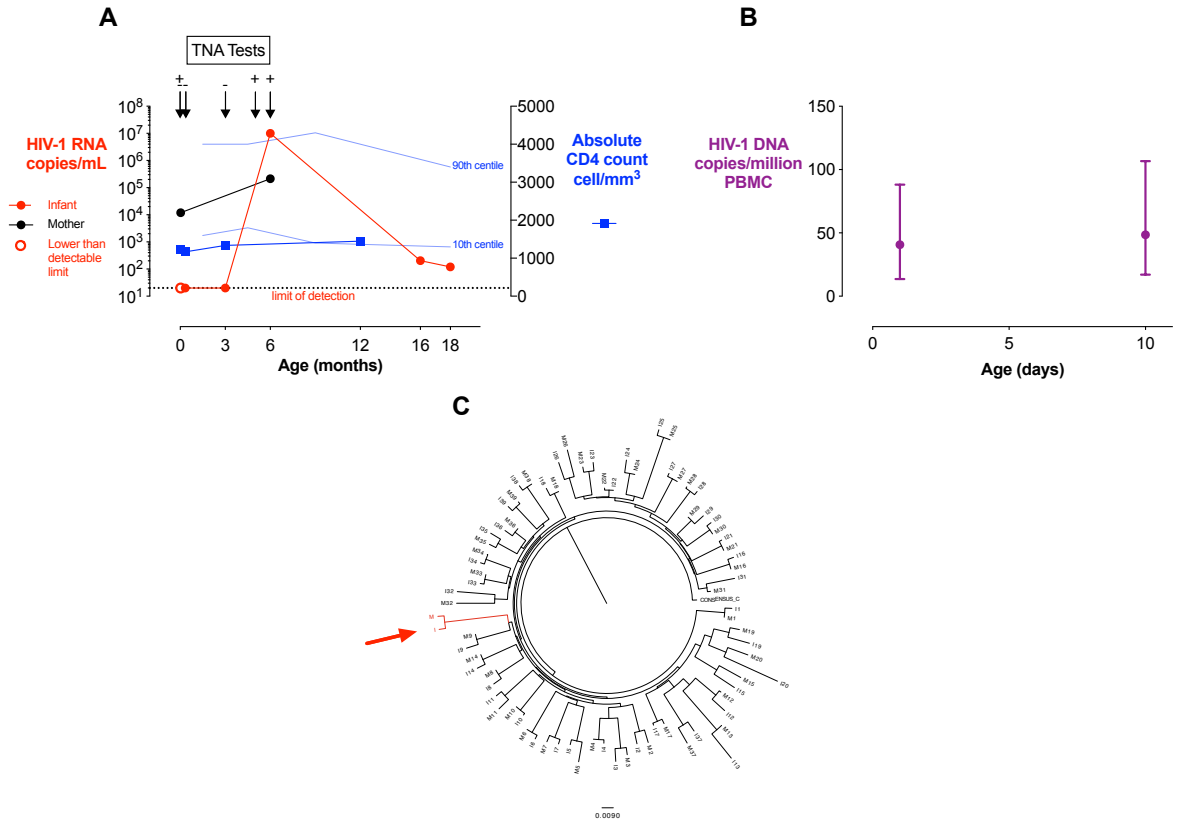


Figure 5.1: Infant and maternal blood test results. (A) Summary of the infant's and mother's plasma HIV RNA viral load, the infant's absolute CD4 count and the HIV TNA PCR tests (+ indicates a positive test and - indicates a negative test). The dotted lines represent the absolute CD4+ T cell count 90th and 10th centiles for healthy HIV unexposed infants. (B) Detectable HIV DNA as measured using ddPCR, 95% CI shown. (C) Phylogenetic clustering of mother-infant pairs. A maximum likelihood phylogenetic tree was constructed using full-length *gag* sequences. Population sequences were generated by Sanger sequencing from 40 mother-infant pairs from the UL study cohort and rooted on a subtype C consensus sequence. The mother-infant pair that is the subject of this case is shown by the red arrow. The legend indicates phylogenetic distance between mother-infant pairs and is expressed as nucleotide substitutions per site.

5.4.2 Prevalence of diagnostic uncertainty

21/191 (11%) of the IU HIV-infected infants enrolled on the UL study had a baseline plasma viral load lower than the detectable limit (below 100 HIV RNA copies per mL in 7/21 cases, and below 20 HIV RNA copies per mL for 14 cases). For five of these 21, (24%), there was difficulty confirming infection using standard TNA PCR, including the first case described (Figure 5.2).

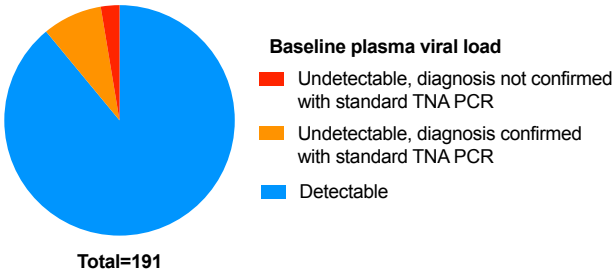


Figure 5.2: Baseline infant plasma viral load detectability. TNA: total nucleic acid.

5.4.3 Undetectable baseline plasma viral load

By comparing those infants that had a undetectable baseline plasma viral load (the "undetectables"), compared with those who had a detectable baseline plasma viral load (the "detectables"), the effect of PMTCT on infant viral load can be observed. In terms of antenatal (transplacental) ART, the "undetectables" all had mothers who initiated cART prior to the onset of labour, which was significantly more likely than for the "detectables" (p=0.009), were less likely to have a mother who reported cART non-adherence during pregnancy (p=0.051), and had mothers with significantly lower plasma viral loads around the time of delivery (p<0.0001, Table 5.1). In terms of the effect of infant prophylactic ART postnatally, infants were more likely to be undetectable if they were older at blood draw (10 days compared with 6 days, p=0.03), and in keeping with this older age, were also less likely to have been PoC tested. Relating the infant characteristics to diagnostic uncertainty, the "undetectables" were more likely to have a non-positive (negative or indeterminate SoC TNA PCR at birth, p=0.001) and had significantly lower HIV DNA in the same sample as the plasma viral load (142 vs 923 p<0.0001). In connection to possible benefits to the overall health

Table 5.1: Comparison of infants with and without a detectable baseline plasma viral load

	Detectable (n=170)		Undetectable (n=21)		
	n/median	%/IQR	n/median	%/IQR	p-value
Sex male	62	36.5%	11	52.4%	0.16
Birth weight (kg)	2.8	2.3 - 3.1	2.9	2.5 to 3.2	**0.048**
Gestation (weeks)	38	36 - 39	38	36 to 40	0.60
Indeterminate or negative SoC	11	6.5%	6	28.5%	**0.001**
Dual prophylaxis	161	65.3%	16	76.2%	0.65
Breastfeeding	135	79.4%	16	76%	0.78
PoC tested	82	48.2%	5	23.8%	**0.038**
Age infant at blood draw (days)	6.5	1 - 11	10	6 - 15	**0.034**
Infant CD4+ T cell count (cells/μL)	2120	1352 - 2648	2367	1685 - 2814	0.39
Infant CD4+ T cell percentage	43	34 - 51	45	40 - 53	**0.030**
Infant CD8 + T cell count (cells/μL)	1142	741 - 1619	1055	765 - 1350	0.17
Infant CD8+ T cell percentage	24	19 - 32	21	18 - 31	0.13
Infant CD4:CD8	1.72	1.07 - 2.57	2.4	1.31 - 3.26	0.10
Infant HIV DNA (copies/million PBMC)	923	291 - 2712	142	23 - 318	**<0.0001**
Timing maternal infection chronic	56	32.9%	5	23.8%	
Timing maternal infection acute	50	29.4%	3	14.3%	**0.02**
Timing maternal infection unknown	64	37.6%	13	61.9%	
Maternal cART prior to labour	130	76.4%	21	100%	**0.009**
Days maternal cART in pregnancy	99	12 - 231	138	92 - 182	0.23
Self-reported maternal cART non-adherence*	81	62.3%	9	42.9%	**0.051**
Maternal CD4+ T cell count (cells/μL)	461	316 - 640	484	379 - 650	0.73
Maternal CD4+ T cell percentage	24	18 - 31	24	22 - 31	0.72
Maternal CD8 + T cell count (cells/μL)	957	720 - 1270	1001	728 - 1358	0.61
Maternal CD8+ T cell percentage	52	43 - 59	53	48 - 58	0.70
Maternal CD4:CD8	0.46	0.3 - 0.70	0.49	0.35 - 0.64	0.53
Maternal plasma viral load (HIV RNA copies/mL)	5450	957 - 49 000	110	20 - 490	**<0.0001**

$*$ of those who initiated cART prior to labour.

of the infant, the "undetectables" had higher CD4+ T cell percentage (p=0.03) and birth weight (p=0.048).

In accord with the rest of the cohort (as discussed in Chapter 3), the rate of plasma viral rebound for those infants with undetectable plasma viral load at baseline was high (Figure 5.3), with a median time to plasma viraemia of 306 days (95%CI 90-∞), excluding the planned treatment interruption in the first case report described. Four of the 13 infants without prescribed treatment interruption had only an isolated blip or spike in plasma viral load before returning to plasma aviraemia, while the rest had

significant periods of viraemia.

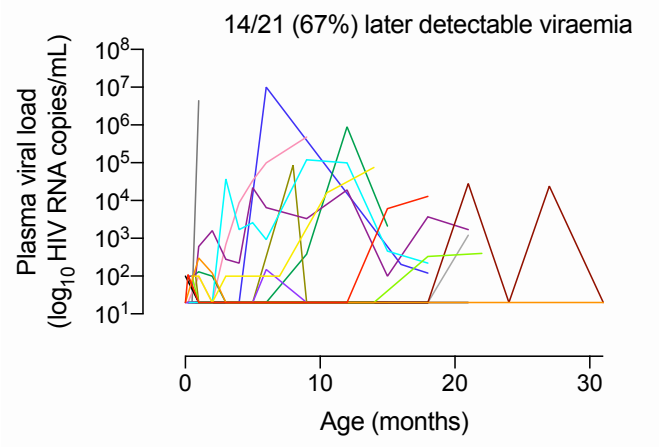


Figure 5.3: Infants with an undetectable baseline plasma viral load. A summary of subsequent plasma viral load measurements for all 21 infants. 14 infants had subsequent viraemia >100 HIV RNA copies per mL of which only one had a prescribed treatment interruption (in dark blue, as described in the case report above).

5.4.4 Difficulty confirming diagnosis

The clinical characteristics and HIV diagnostic test results for the five infants who were unable to be confirmed infected using standard clinical TNA PCR tests are summarised in Table 5.2, where Case 1 is that already described.

For Case 2, the mother had a detectable plasma viral load at delivery, despite 5 months of cART. Almost identical to Case 1, the infant had a borderline positive PoC test (Ct 41.1) at birth and two negative SoC TNA PCR tests, but HIV DNA was detectable, and HIV *gag* was clustered with the mother on a phylogenetic tree (see Figure 6.2, on page 120: M12 and C12). The infant had a viral blip aged 6 months without a prescribed treatment interruption.

Case 3, was an unwell mother, who presented to antenatal booking at 27 weeks gestation with a CD4 count of 25 cells per μL, at which time she was initiated on cART. The infant's birth PCR was indeterminate and follow up PoC PCR was positive, but with a high Ct value (38.9). On the same day, HIV DNA was negative (95% CI 0-15 copies per million PBMC), and following amplification by PCR *gag* was not detected. On day 28 the HIV DNA level was just detectable (13 copies per million PBMC, 95%

CI 7-42). The child rebounded to a plasma viral load of 1200 HIV RNA copies per mL aged 21 months.

Case 4 was a mother known to be HIV-infected prior to pregnancy and admitted to missing cART doses antenatally. SoC PCR at birth was positive, but the child was not able to be recalled back to clinic until day 20, at which time the repeat SoC PCR was indeterminate - although very close to positive (Ct 32.7, RFI 4.1). HIV DNA was detectable (22 copies per million PBMC 95% CI 6-57) but following amplification by PCR, *gag* was not detected. This child has not rebounded after 2 years despite reports from the caregiver of missing some cART doses.

Case 5 (also described in Sub-section 7.3.8 on page 147) was a mother who HIV seroconverted in the last weeks of pregnancy and only started cART 1 week prior to delivery. The initial positive SoC PCR at birth was only followed by an indeterminate SoC PCR on day 14, however diagnosis was confirmed with positive HIV DNA and HIV *gag* which phylogenetically clusters with maternal HIV. This child has not had detectable plasma viral load after 2 years despite significant periods without cART.

Table 5.2: Infants unable to be confirmed HIV-infected with standard TNA PCR

	1	2	3	4	5
MOTHER					
Estimated timing HIV seroconversion	unknown	3m prior to pregnancy	7 years prior	unknown	3rd trimester
Weeks of cART during pregnancy (%)	36 (100%)	20 (52%)	9 (25%)	40 (100%)	1 (3%)
cART regimen	EFV/FTC/TDF	EFV/FTC/TDF	EFV/3TC/ABC	EFV/FTC/TDF	EFV/FTC/TDF
Self-reported cART non-adherence	true	false	false	true	false
CD4 count at delivery (cells/μL)	452	231	73	774	783
CD4:CD8 at delivery	0.50	0.42	0.22	0.56	0.79
Plasma viral load at delivery (HIV RNA copies/mL)	12000	370	<20	180	1100
INFANT					
Infant sex	female	male	female	male	male
Birth weight (kg)	3.1	2.8	2.1	2.8	3.4
Gestational age at birth (weeks)	36	38	36	40	38
Mode of delivery	vaginal	C/S	C/S (abruption)	vaginal	C/S
Infant prophylaxis	NVP	AZT+NVP	AZT+NVP	AZT+NVP	AZT+NVP
Method of feeding	breast	breast	formula	formula	breast
INITIAL TESTING					
Birth PoC HIV PCR Ct	41.3	41.1	-	-	-
Birth SoC HIV PCR Ct (RFI)	negative	negative	37.3 (1.59)	30.5 (6.52)	28.4 (9.6)
Additional PoC test Ct	-	-	38.9 (day 12)	-	-
INFANT CONFIRMATORY TESTING					
Age infant (days)	1	2	12	20	7
CD4 count (cells/μL)	1220	1263	1751	4136	2796
CD4%	36	40	52	61	53
CD4:CD8	1.16	1.65	3.44	2.61	2.40
Repeat SoC test	negative	negative	34.7 (3.29)	32.7 (4.1)	-
Plasma viral load (HIV RNA copies/mL)	<20	<20	<20	<20	<20
HIV DNA copies/million PBMC (95%CI)	41 (14-88)	199 (53-530)	0 (0-15)	22 (6-57)	25 (6-66)
Viral or proviral gag sequence c.f. mother	related	related	PCR negative	PCR negative	related
INFANT SUBSEQUENT TESTS					
	-	-	HIV DNA: 13 (7-42) day 28	-	SoC PCR: 35.5/2.44, day 14
Age in months at detectable plasma RNA (value in HIV RNA copies/mL)	5 (>10^7)*	6 (150)	21 (1200)	no viraemia by 2y	no viraemia by 2y

Green cells represent a negative test, yellow for indeterminate, and red for positive. Grey cells are where the test was not undertaken. $*$ this infant is the only one of the five that had a planned treatment interruption. C/S:Caesarean section, Ct: cycle threshold, RFI: relative fluorescence index, c.f: compared with.

5.4.5 False positive tests

As noted in Figure 3.1, page 57, there were eight infants enrolled onto the UL study who had a false positive HIV diagnosis; half following a positive PoC test, and the other half following a SoC test, as summarised in Table 5.3. All infants were switched from cART to dual prophylaxis for 12 weeks following the initial negative confirmatory testing, except for FP8 who remained on cART until 6 months as the mother's plasma viral load remained >4 $\log_{10}$ HIV RNA copies per mL throughout the breastfeeding period. Two of the infants falsely diagnosed following a PoC test had relatively low Ct values. The amplification curves for these tests were examined by the Cepheid technical support team, who could not offer any explanation for their occurrence. All the false positive SoC tested infants were re-tested at 10 days or older, thus after 10 days of prophylaxis, adding to the uncertainty. Two of these infants were unable to be followed up, so the true diagnosis is not confirmed. One of the infants not followed up (FP4) had a detectable HIV DNA (38 HIV copies per million PBMC, 95% CI 8-99) on day 10, but this was undetectable at 1 month. However, for this infant the possibility of a mislabelled specimen cannot be excluded, as demonstrated by another child (FP6) who was proven uninfected after remaining negative months after stopping prophylaxis, who had an improbably high HIV DNA at 10 days, compared to 1 month, suggesting the first sample did not belong to the child.

Table 5.3: False positive infant HIV diagnoses

	Birth testing		Confirmatory testing				Follow up		Retrospective HIV DNA	
Infant	PoC (Ct)	SoC	Age (days)	CD4%	Plasma viral load (HIV RNA copies/mL)	SoC	Followed until 6m?	Remained negative off prophylaxis?	Birth	1 month
FP1	positive (41)	negative	2	69	<20	negative	yes	yes	-	-
FP2	-	positive	10	67	<20	negative	no	?	-	-
FP3	positive (41)	negative	2	60	<20	negative	yes	yes	-	-
FP4	-	positive	10	49	<20	negative	no	?	38 (8-99)	0 (0-49)
FP5	positive (30)	negative	1	54	<20	negative	yes	yes	-	-
FP6	-	positive	10	64	<20	negative	yes	yes	24449 (23252 - 25682)	0 (0-19)
FP7	positive (36)	negative	1	59	<20	negative	yes	yes	0 (0-30)	0 (0-120)
FP8	-	positive	12	47	<20	negative	yes	yes	0 (0-22)	0 (0-24)

5.5 Discussion

With the increased use of ART as prophylaxis, peripheral blood viral dynamics of infant HIV infection no longer look like they once did (see Figure 1.8, page 17). Because infant diagnosis relies on viral amplification, this dramatic reduction in measurable virus has increased the complexity of HIV diagnosis at birth. The UL study provided the opportunity to explore possible false diagnoses in more depth than was feasible with the standard South African testing algorithm and assays. The conclusion is that diagnostic uncertainty in IU HIV infection is common, with 11% of IU HIV-infected infants having an undetectable plasma viral load in the first days of life. Furthermore, 2-3% of the HIV-infected infants were unable to have their diagnosis confirmed using standard clinical HIV TNA PCR tests, however, this phenomenon was less common than the 4% (8/191) of infants falsely diagnosed HIV-infected from the initial test.

Today in South Africa, baseline infant plasma viral loads for IU HIV-infected infants are low, which is beneficial as it reflects little to no active HIV replication in peripheral blood, but is also potentially risky as it increases the chance of having undetectable plasma HIV RNA, thus interpreted as the absence of infection. Because of this potential clinical implication, the "undetectables" were investigated as a group, rather than using plasma viral load as a continuous variable (Table 5.1). The only unifying feature of the "undetectables", was that they were all exposed to antenatal cART, however the converse was not true; most of the detectables were also exposed to maternal cART. Futhermore, there was no relationship between duration of maternal cART and an undetectable infant plasma viral load, likely due to maternal cART non-adherence or HIV drug resistance masking the relationship. There also could have been unmeasured effects, such as infant viral fitness at play. Maternal plasma viral load at delivery, a good measure for maternal cART ingestion, was the strongest predictor for an undetectable infant plasma viral load. But again, this was not a hard and fast rule, as in Case 1, where the mother had a very high plasma viral load of 12 000 HIV RNA copies per mL at delivery. Nevertheless, the mother's plasma viral load was a $\log_{10}$ lower at delivery than 6 months later, suggesting that just prior to delivery, the mother was

taking enough cART for partial treatment, further demonstrated by the ART detected in her plasma at the time of viral load measurement. Presumably it was this cART that lowered her own and therefore her infant's viral load.

The chances of an undetectable plasma viral load also depends on laboratory techniques. The NHLS in South Africa are more experienced at running HIV PCR assays compared with the private laboratory used by the UL study, and with this experience comes a more sophisticated choice of assay and interpretation of results. NHLS report HIV plasma viral load as either a) target detected, along with the absolute value, b) target detected but below the limit of quantification (20 copies per mL) or c) target not detected. This stratification can help reduce the chance of a false negative classification when the concentration of HIV is low. The plasma viral load testing done by NHLS might also be more sensitive, either due to the testing platform used (Roche COBAS 6800/8800 HIV-1 Test or Abbott RealTime HIV-1 Viral Load) or by using larger sample volumes (the test is declined unless >1 mL of whole blood is received, compared with the 0.5 mL blood used in the UL study). An example of this was one of the "undetectables" who had two plasma viral load test results <100 HIV RNA copies per mL two weeks apart, but in between, a result from NHLS was reported as 252 HIV RNA copies per mL tested via the Abbott platform. Therefore it is possible, that in the UL study the number of undetectable infants were overestimated. Nevertheless, IU HIV-infected infants with undetectable plasma HIV RNA at birth do exist, therefore the WHO recommendation that EID can be undertaken via plasma RNA PCR should be cautioned.

Lower plasma viral loads at birth also decrease the likelihood of HIV TNA PCR tests detecting true infection, as demonstrated by the lower infant HIV DNA levels along with a higher probability of a negative or indeterminate SoC PCR in the "undetectables". According to manufacturer validation studies, the SoC PCR (CTM/CAP, Roche) on DBS has a similar sensitivity and limit of detection to the whole blood PoC TNA PCR (GXP, Cepheid), with 95% probability limits of detection 278 (95% CI 253 – 304) and 221.8 (95% CI 195.6 - 260.5) HIV RNA copies per mL respectively

(Roche Molecular Diagnostics 2015; Cepheid 2017). As with the cases described, and as is becoming increasingly common, when the copy number of HIV is lower than the limit of detection of the test, false negative results ensue. There is some suggestion from these cases that the PoC PCR test might be more sensitive than the SoC PCR test, with Cases 1 and 2, diagnosed positive by PoC but negative by SoC testing. This study was not designed to evaluate the PoC and SoC test performance, however a field test on infants in South Africa found the Cepheid PoC and Roche SoC perform similarly, but the PoC specificity compared to the SoC test is 99.9%, suggesting that false positives are more likely with the PoC (Cepheid) test (Technau et al. 2017a). Although it should be noted the field test used the Roche SoC test on whole blood, not DBS as was the case for the UL study.

The phenomenon of repeated negative plasma HIV RNA PCR testing on prophylaxis followed by a positive result after prophylaxis discontinuation, has been described before (Connolly et al. 2013), but the difference here in Case 1, was that the initial low-level infection was captured using the sensitive PBMC ddPCR assay and MTCT at birth was shown using viral sequencing. A recent report from South Africa (Strehlau et al. 2019) described a similar case of an infant with an indeterminate SoC HIV PCR at birth, followed by two further indeterminate tests a week apart, then persistently negative tests. The child stopped prophylactic ART before two months of age, and remained HIV negative for another three months. At a year of age the child presented symptomatic with HIV infection around the time of weaning. Nested HIV DNA PCR from the infant's samples at birth and in the first month of life were positive on multiple occasions, proving IU HIV infection. Maternal ART was detected in the infant's plasma during the period of negative HIV TNA tests and breastfeeding, but at levels lower than considered therapeutic. The authors conclude that this infant's delayed rebound was due to the immunomodulatory and HIV-inhibitory factors in breastmilk, hence the rebound at weaning. Breastfeeding in the UL study was common and there were several infants with undetectable latent HIV reservoirs, who had a plasma viral rebound, even while breastfeeding continued. It is unlikely that breastfeeding can

confer a full protective effect from HIV reactivation. Perhaps for the case described by Strehlau and colleagues, the reactivation occurring at weaning was a coincidence, or perhaps breastfeeding, along with other favourable features, such as a small, low viral fitness reservoir, minimal gut mucosal inflammation, and a strong innate immune response can indeed suppress viraemia.

A criticism of the published case report describing Case 1, was that the infant's positive HIV PCR tests at birth were actually detecting HIV DNA in maternal cells, and did not represent true infection (Lou and Zhou 2019). Although maternal cells do cross into foetal circulation, in order to achieve the levels of 41 and 49 HIV DNA copies per million PBMC that were observed in the Case 1 child on days one and ten of life, respectively, it would require larger transfusions of maternal blood into the baby than arise in nature. A recent study, using DNA methylation to detect maternal contamination of foetal blood, showed that the levels of contamination were too low to be detected in 80-90% of cases, and, where detected, amounted to between 10^{-5} to 10^{-4} of cells (Morin et al. 2017). Assuming a maternal HIV DNA level of 1000 copies per million PBMC, 1 million PBMC per mL of maternal blood and an infant total blood volume of 240 mL with 5 million PBMC per mL, it would require 20% of total infant blood volume to be maternal, compared to the level of <0.001% that is typically observed in reality (ibid.).

One study found that 60% (8/14) of HIV-infected infants classified as having intrapartum HIV infection (HIV RNA and DNA negative at birth but positive at 4-6 weeks) had detectable HIV DNA in PBMC by ddPCR at birth (Bryson et al. 2019). The authors of this study did not provide information regarding infant PMTCT exposure, which could explain the low-level HIV in peripheral blood. The alternative is that the infants were infected in early labour, and given that latent HIV reservoirs are established <3 days after infection (Whitney et al. 2014), HIV DNA is just detectable at birth. If this is the case, Bryson and colleagues demonstrate the arbitrary distinction between *in utero* and intrapartum HIV transmission, and raises the possibility that

some of the "undetectables" reported here, might be very early in infection, possibly with the highest cure potential. For two of the five difficult to diagnose cases, the infants had documented cART non-adherence with no plasma viraemia detected, furthering this idea of cure potential.

5.5.1 Public health implications

The screening test

In the face of low HIV RNA and DNA levels, one approach to avoid diagnostic uncertainty and false negatives, would be to increase the sensitivity of the infants' first (screening) test, however, the danger in doing this is losing test specificity. Given the declining prevalence of infant HIV infection, maintaining test specificity is imperative in order to avoid false positive tests. For example, even with an HIV EID test specificity of 99.9%, an HIV prevalence of 1% gives a positive predictive value (PPV) of 91%, which translates to 9% of test results being falsely positive. Because of this high risk of false positives, the indeterminate range was introduced by NHLS in South Africa.

Indeterminate results

As explained in Chapter 1, on page 18, NHLS has developed a definition for an indeterminate HIV PCR result. It is estimated that this indeterminate range can capture 93% of false positive results (Luo et al. 2019), however this is balanced against the documented increased risk of loss to follow-up and death in the delay to definitive diagnosis (Technau et al. 2017b). No indeterminate range has been validated for the Cepheid PoC test. If it had, the two false positive infants (FP1 and FP3) who had a Ct value of 41, might have been in that indeterminate range, triggering further testing without the initiation of cART.

The confirmatory test

With a high level of false positives occurring, confirmatory tests are imperative. Omitting confirmatory testing would result in a significant number of infants unnecessarily

committed to the potential toxicity and burden of lifelong cART, as would have occurred for the eight false positive infants described here. Furthermore, the financial cost of unnecessarily committing a child to lifelong cART far outweighs the cost of confirmatory testing (Dunning et al. 2017). For these reasons, WHO does not recommend reliance on a single initial positive EID HIV test (WHO 2018). However, as demonstrated by Case 1 and 2, when the HIV copy number in peripheral blood is low, there is a chance that the confirmatory test is falsely negative. The optimal confirmatory test should be more sensitive and specific than the original screening test, to truly confirm infection. The sensitivity of the ddPCR assay has a variable limit of detection, depending on the number of cells used, but in the UL study ranged from 2-25 HIV DNA copies per million PBMC, therefore is much more sensitive than the standard TNA PCR tests, enabling the confirmation of *in utero* infection. However, although accurate at quantification, ddPCR has been reported to have a higher risk of false positive results compared with semi-nested quantitative PCR, with 2-3 positive droplets in a third of negative control wells (Kiselinova et al. 2014). Thus some researchers recommend against using this method for discriminating between the presence or absence of HIV DNA (Rutsaert et al. 2018). Therefore, very low level HIV DNA (<3x the limit of detection) by ddPCR on only one occasion, should not be used as the sole confirmatory test. Furthermore, there are multiple steps between phlebotomy and the read out from ddPCR, with many opportunities for samples being muddled, as demonstrated by the error in HIV DNA results for infant FP8. Thus, even a high-level HIV DNA level by ddPCR needs to be repeated on a separate sample for confirmation. More sensitive tests such as ddPCR and viral sequencing are more expensive and require a higher level of expertise than standard TNA HIV PCR. However, in South Africa, confirmatory testing remains cost-effective unless the confirmatory test exceeds $400 USD (Dunning et al. 2017). It may also be reasonable to limit the more sensitive confirmatory testing to those initial test results that fall within a borderline or indeterminate range.

Point-of-Care testing

Not only how, but also when this confirmatory testing occurs in EID is imperative. With the viral suppressive effect of infant prophylaxis accumulating with time, the sooner the confirmatory test occurs after birth, the more likely a higher HIV RNA level will be detected, avoiding diagnostic confusion (Mazanderani SAMJ 2014). Although earlier testing did not help for Cases 1 and 2, perhaps earlier re-testing for Cases 4 and 5, who went from a positive to an indeterminate SoC PCR after 2-3 weeks on prophylaxis, could have reduced diagnostic uncertainty. Infants in South Africa have been shown to have lower HIV PCR Ct values when tested <7 days of age, compared to after 7 days (Ct 23.8 vs 27.3, Haeri Mazanderani et al. 2018a) and HIV DNA levels can become undetectable in as little as 6 days of cART (Veldsman et al. 2018). This earlier testing confirmatory testing can be facilitated by PoC testing.

The bottom line

In the absence of more sensitive or PoC testing, as is the case currently for the South African public health system, repeat testing, most importantly after prophylaxis cessation is a safe option. In cases where cART has already been started, it should be continued until breastfeeding cessation and a supervised treatment interruption can be conducted. Treatment interruption may cause a viral rebound which could expand the latent viral reservoir irreversibly (Martínez-Bonet et al. 2015), but this is balanced against the physical and emotional harm of committing the child to an incorrect life-long diagnosis.

5.5.2 Implications for individuals

When planning an EID programme, implications at an individual level should also be considered. The gravity of maternal anxiety and guilt around infant HIV diagnosis must not be underestimated. The most common reason for refusing consent for PoC testing in the UL study was mothers who had just delivered and did not feel ready for their child's potential diagnosis; wanting more time to enjoy their "HIV negative"

baby. This is not an argument against PoC testing, but instead is a concern that could be addressed by better preparing expectant mothers for the testing that will occur at birth, and what the results will mean. Another issue found at the patient level was trying to explain indeterminate diagnoses. Even the concept of an undetectable plasma viral load was a challenging concept to convey. One mother of an "undetectable" interrupted treatment when the child was 21 months of age - just to check for herself whether or not treatment was really necessary. An associated risk is inadvertent re-testing. With limited medical records shared across health facilities in South Africa, children on cART are accidentally re-tested for HIV. If this occurs when the child is successfully suppressed, this can result in the caregiver being told the child is HIV negative. This scenario occurred twice in the UL study, and of course the mothers were looking for any hope that their child might not be HIV-infected, so this false information was difficult to backtrack from. In summary, the timing of EID and the way the results are communicated must take into account the patient or caregiver experience, with strong emphasis on giving clear and consistent explanations.

5.6 Conclusion

This case-based discussion demonstrates that prevention of MTCT strategies involving ART in pregnancy and during the breastfeeding period, whilst highly successful in reducing transmission, also can complicate diagnosis of *in utero* HIV infection in infants. The crossover in test results between false positive and false negatives infants highlights the infallibility of standard clinical HIV PCR in PMTCT exposed infants. On a public health level, the important messages are firstly to recognise that now with a lower prevalence of *in utero* HIV infection, the positive predictive values of standard HIV PCR tests are reduced. Therefore, confirmatory EID tests following a positive or indeterminate result are imperative. Second, given the rapidly declining infant plasma viraemia, if feasible, the confirmatory test should occur early, which can be facilitated by PoC testing, and be a more sensitive test such as a HIV DNA PCR on PBMC, particularly if the initial test result is in the indeterminate range. In the absence of

more sensitive tests, standard tests should be repeated frequently and most crucially, after infant prophylaxis cessation. In summary, there is a blurred line between an HIV-exposed uninfected infant and an HIV-infected infant, but as long as prophylaxis continues and follow-up tests occur, the distinction is not necessarily urgent, although must be clearly explained to the caregiver.

Chapter 6

Second generation mother-to-child HIV transmission

6.1 Introduction

With improvements in HIV treatment and care, the number of perinatally HIV-infected children surviving to adulthood is increasing (UNAIDS 2019b; Slogrove et al. 2018b). In 2016 there were approximately half a million perinatally HIV-infected adolescents worldwide. Pregnancy outcomes for perinatally HIV-infected women have been reported from various regions worldwide, including the Americas (Crane et al. 1998; Levine et al. 2006; Lundberg et al. 2018; Goodenough et al. 2018; Millery et al. 2012; Brogly et al. 2007; S. F. Williams et al. 2009; Badell et al. 2013; Badell and Lindsay 2012; Patel et al. 2020; Trahan et al. 2020; Zorrilla et al. 2003; Cruz et al. 2010), Europe (Elgalib et al. 2011; Byrne et al. 2017; Kenny et al. 2012; Thorne et al. 2007; Calitri et al. 2014) and India (Chibber and Khurranna 2005), but notably absent are publications from sub-Saharan Africa, where most perinatally HIV-infected young women reside (Slogrove et al. 2018b). Findings from these reports include high rates of sexually transmitted infections, immunocompromise, and HIV drug resistance. Most studies have found similar, or lower, MTCT rates compared to non-perinatally HIV-infected women. However, a study undertaken in the United States and Puerto Rico, the largest to date, found a two-fold increased risk of MTCT (Goodenough et al. 2018). Despite these transmissions occurring, the clinical outcomes for this second-generation of perinatally HIV-infected children are unknown.

6.2 Hypothesis and aims

The aim of this case series was to describe the clinical outcomes in occurrences of second-generation mother-to-child HIV transmission in the Ucwaningo Lwabantwana cohort. The ultimate goal was to identify any specific maternal or infant characteristics that need to be addressed in the optimisation of their HIV care.

6.3 Chapter specific methods

6.3.1 Study participants

Based on the history reported by the mothers at enrolment in the UL study before December 31, 2019, HIV transmitting mothers were identified as having themselves been perinatally HIV-infected. This included mothers who reported being told (either at the time or retrospectively) they were HIV-infected as a child, and also had no recollection of sexual abuse. Where possible, the maternal grandmother of the child was invited to be enrolled on the study, provided written informed consent and gave one peripheral blood sample.

6.3.2 HIV diversity analysis

HIV diversity analysis was conducted by Dr Nick Grayson. From the aligned maternal and infant baseline HIV deep sequences (as described in Section 2.4), nucleotide diversity (π) was calculated following the published methods of Zhao and Illingworth (2019) and compared to 22 other transmitting mother-child pairs from the wider cohort for whom NGS data were available.

6.3.3 HLA-typing

Where the maternal grandmother blood samples were available, HLA typing was used in combination with HIV *gag* phylogenetics from the grandmother, mother and infant samples in order to confirm two generational HIV MTCT. DNA was extracted from whole blood using the DNeasy Blood and Tissue Kit (Qiagen) by the HIV Pathogenesis Programme core lab (University of KwaZulu-Natal, South Africa). HLA typing

was performed by Timothy Rostron (Weatherall Institute of Molecular Medicine, University of Oxford, UK) who used the DNA to determine HLA-A/B/C type by using PCR to amplify and sequence Exons 2 and 3 for each locus and comparing results to the IMGT-HLA database (`https://www.ebi.ac.uk/ipd/imgt/hla/`).

6.4 Results and discussion

Six (6/191, 3.6%) IU HIV-infected infants born to mothers, who were themselves perinatally infected with HIV were identified. The clinical and virological characteristics for these six cases are summarised in Table 6.1 and Figure 6.1A-D. Of note, periods of high plasma viraemia in both the mothers and children were frequent (Figure 6.1A,C). In two cases (Cases 4 and 6), access to maternal grandmother blood samples, as well as from the second generation mother-child pair, enabled phylogenetic analysis of HIV *gag* and HLA typing to confirm HIV transmission across the three generations (Figure 6.2, Table 6.2). The circumstances surrounding the difficulties in maintaining plasma viral suppression for both mothers and children in this case series highlight the challenges unique to perinatally infected mothers which act as barriers to cART adherence and are summarised as follows.

Table 6.1: Clinical details of mother-child pairs

MOTHER

CASE	Orphaned (age, years)	Age at diagnosis (years)	Age at disclosure (years)	Age ART initiated (years)	Age delivery (years)	Partner's HIV status	Self-reported ART adherence during pregnancy	CD4+ T cells at delivery (cells/uL)	Plasma viral load at delivery (HIV RNA copies/mL)	Post-delivery DRMs	Effect on plasma viral load >3months after switching to LPVr regimen	Comorbidities
1	yes (6)	10	at testing	10	18	confirmed negative	interrupted	206	12 000	K103N, V108I, M184V	no change	none
2	yes (10)	unknown	13	18	20	refuses testing	interrupted	330	13 000	not tested	NA	none
3	no	unknown	15	15	20	unknown	interrupted	211	400 000	K103N, V106M, M184V	no change	cervical CIS depression
4	no	3	8	18	19	reported negative	no missed doses	648	91	not tested	NA	depression
5	abandoned young	18	at testing	18	18	unknown	poor	50	12000	K103N	NA	none
6	no	12	at testing	12	23	unknown	interrupted	109	40 000	V106M	improved	post-TB bronchiectasis chronic diarrhoea

INFANT

	Sex	Gestational age at birth (weeks)	Birth weight, kg (centile*)	Delivery mode	Age ART initiated (days)	Age virological suppression** (months)	Age first viral rebound (months)	Health concerns	HIV status disclosure issues
1	Female	37	2.2 (5)	Caesarean	1	2	6	none	none
2	Female	39	3.9 (95-97)	Vaginal	1	2	3	none	household not aware
3	Female	39	3 (10-50)	Caesarean	1	4	5	none	family who assist with caregiving not aware
4	Male	40	3.1 (10-50)	Vaginal	10	4	never - LTFU at 6m	none	none
5	Male	38	2.2 (<3)	Vaginal	4	never - died at 3m	-	neonatal sepsis and FFT, later died with acute malnutrition and sepsis	none
6	Male	37	2.55 (10-50)	Caesarean	5	2	12	none	none

LTFU: loss to follow-up, FTT: failure to thrive, CIS: carcinoma in situ, DRMs: drug resistance mutations, *Intergrowth 21st growth chart, **Plasma viral load <20 HIV RNA copies per mL

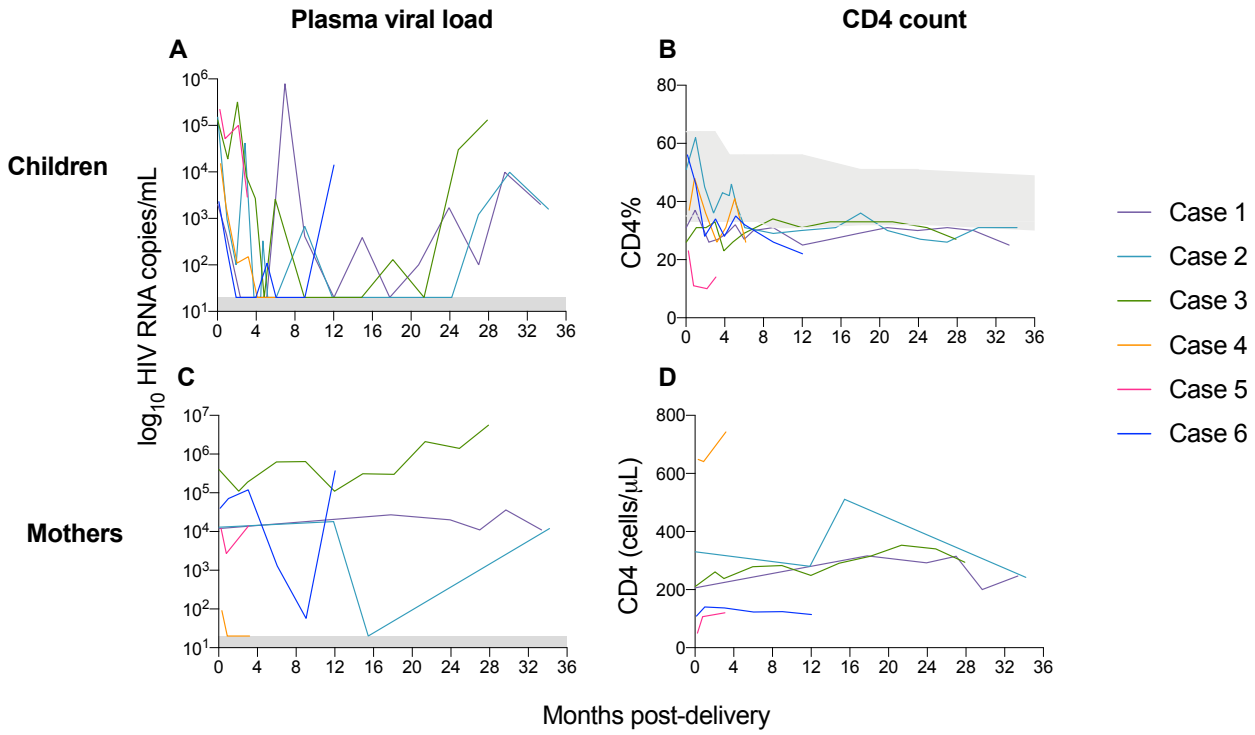


Figure 6.1: Plasma viral load and CD4+ T cell counts for mother-child pairs. Timelines span from delivery until last seen by the study. Case 5 was censored early due to death, and Case 4 due to loss to follow-up. (A) Plasma viral load of the children and (C) of the mothers in $\log_{10}$ HIV RNA copies per mL. (B) CD4+ T cell percentage of the children and (D) absolute CD4+ T cell count of the mothers, in cells per μL. Normal CD4+ T cell percentage range (10th to 90th centile for HIV-uninfected infants) shown in grey shading in panel B.

Table 6.2: HLA Class I typing from the grandmother-mother-infant trios

	HLA-A	HLA-A	HLA-B	HLA-B	HLA-C	HLA-C
Grandmother 4	03:01	66:01	15:03	58:02	02:10	06:02
Mother 4	30:02	66:01	57:02	58:02	18:01	06:02
Child 4	30:04	66:01	44:03	58:02	04:01	06:02
Grandmother 6	23:01	30:01	15:10	15:17	03:04	05:01/03
Mother 6	23:01	30:02	15:10	08:01	03:04	18:01
Child 6	23:01	43:01	15:10	15:03	03:04	18:01

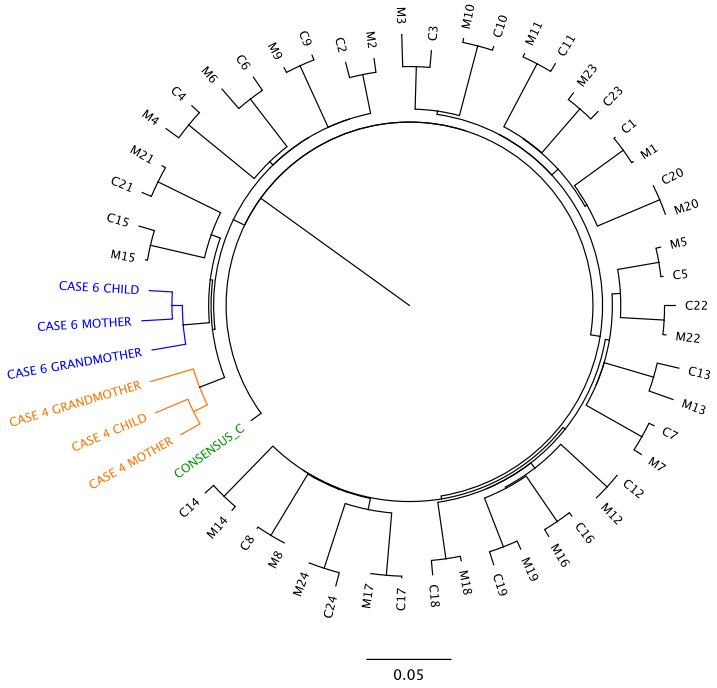


Figure 6.2: Phylogenetic clustering of HIV *gag* for the grandmother-mother-infant trios. Constructed using a Maximum Likelihood phylogenetic tree of 24 mother-infant pairs from the UL study rooted on subtype C consensus sequence. The grandmother-mother-infant trios from Case 4 and 6 are indicated in colour. The legend indicates phylogenetic distance between mother-infant pairs and is expressed as nucleotide substitutions per site.

6.4.1 The survival effect; non-progressor status of mothers

cART only became available through the South African Department of Health in 2004 (Simelela and Venter 2014) and until September 2016, was only initiated according to clinical or immunological criteria (Republic of South Africa National Department of Health 2015b). Born prior to this availability, the perinatally HIV-infected women included in this case report therefore possessed a slow progressing HIV infection phenotype, since they were among the 25% of perinatally HIV-infected children who can survive beyond 10 years without cART (Marston et al. 2011). Most strikingly, in Case 4, the mother had a CD4+ T cell count above 600 cells per μL after 18 years without cART: a long-term non-progressor phenotype. Their own survival without cART may have made it difficult for these young women to realise the life-saving importance of cART for their infants. Case 4 was an example of this, where after 6 months the mother refused to continue cART for her child, since the mother herself had not needed cART as a child.

Despite it being more difficult to navigate adherence counselling in these cases, these mothers may have other advantages. Although HLA type does not predict paediatric slow progression (Adland et al. 2015), there may be other inherited immunological attributes that improve their children's survival (Muenchhoff et al. 2016). Going forward, with better infant diagnosis, earlier treatment initiation and improved cART coverage, this survival effect phenomenon will diminish.

6.4.2 HIV drug resistance

ART experienced mothers have a high risk of HIV drug resistance (Hamers et al. 2018). Even with the relatively limited exposure to cART in these cases, four out of six of the mothers had NNRTI DRMs, and two had the NRTI DRM M184V postnatally. Although, for Cases 1 and 2 who had both NNRTI and NRTI DRMs, switching to a regimen they were susceptible to did not alter their plasma viral load at all, suggesting ongoing non-adherence. It is of concern that mothers in Cases 1, 2, 3 and 6, despite being pregnant and having a history of failing NNRTI therapy, were continued on an NNRTI. There needs to be greater awareness of the almost ubiquitous NNRTI DRMs after viraemia and previous NNRTI use (Gupta et al. 2017), and the urgent requirement to suppress pregnant women's plasma viral load to prevent MTCT. With the increased use of cART in children in South Africa, resistance will only become more of a problem (Pillay et al. 2014). A recent study in Canada found DRMs in all perinatally HIV-infected pregnant women tested, and nine out of eleven, had resistance to three neonatal ART drugs (Trahan et al. 2020). Resistance usually develops from periods of sub-optimal adherence, and this commonly occurs during adolescence (Zanoni et al. 2016).

6.4.3 Viral adaptation

It has been very clearly shown both in mother-to-child and in adult-to-adult that transmission of viruses pre-adapted to the HLA class I molecules expressed by the recipient dramatically accelerates disease progression (Goulder et al. 2001; Carlson et al. 2016). In the examples described here, selection pressure on the virus has

been imposed by two individuals sharing the identical HLA haplotype before being transmitted to a third sharing that same haplotype (illustrated by both cases 4 and 6, Table 6.2).

6.4.4 Adolescence

In Cases 1, 2, 3 and 6, the mothers had initiated cART in late childhood, but stopped taking their treatment during adolescence. The developmental transition from childhood to adulthood is a vulnerable life-stage (Davidson et al. 2015), particularly when partnered with chronic illness (Brady et al. 2017). Specific features that may add complexity to their HIV infection, include stigma from having what is commonly known as a sexually transmitted infection, the burden of daily oral medication, delayed puberty, and subtle cognitive impairment (Flynn and Abrams 2018). Although it is difficult to disentangle how much is due to the disease itself rather than the socioeconomic circumstances linked to HIV risk, perinatally HIV-infected adolescents have also been shown to have high rates of mental disorders, substance abuse, risky behaviours, and reduced cognitive functioning (Abrams et al. 2018). These issues likely contribute to the high rates of virological failure in adolescents compared to adults, seen across many settings (Slogrove et al. 2018a), including South Africa (Zanoni et al. 2016).

6.4.5 Disclosure issues

Adding to this teenage turmoil, for the mother in Case 3 were the circumstances and advanced age (15 years) at which she was informed of her HIV status, and her subsequent depressive symptoms and non-adherence. This is an example of the potentially detrimental psychological effects of delayed disclosure (Stein et al. 2019), and why the local KwaZulu-Natal guidelines recommend full disclosure to have occurred by 10 years of age (Department of Health Province of KwaZulu-Natal 2017). Cases 3, 5 and 6 the mother did not disclose their HIV status to their partner, putting them at risk for HIV infection. Case 2 and 3 did not disclose to their family or the household who regularly care for the child, in fear of losing their support. This non-disclosure limited

the opportunities for the children to receive ART doses, and is a commonly cited reason for sub-optimal adherence (Hodgson et al. 2014). A study in the United States found that children and adolescents who have been aware of their diagnosis for longer have higher rates of disclosure (Santamaria et al. 2011), suggesting that acceptance of HIV diagnosis may have been the key missing factor in these cases. Nevertheless, the pervasive HIV stigma in South Africa, intermixed with poverty and gender power imbalances (Bhatia et al. 2017; Visser et al. 2009), can make disclosure more difficult and dangerous than in other settings, and certainly is not unique to perinatally infected mothers.

6.4.6 Poor maternal health

Cumulative years of virological failure, whether due to non-adherence or resistance, or in Case 5, a late diagnosis, means that perinatally HIV-infected mothers can have impaired immunological function (Trahan et al. 2020; Byrne et al. 2017). Poor maternal health can affect the child in multiple ways: limiting the mother's ability to care for the child, increasing exposure to co-infections, and impairing infant immune development (Jennewein et al. 2017). Case 6 is an example of the former. The mother developed chronic diarrhoea, eventually diagnosed as *Cystoisospora belli* infection. In the delay to diagnosis, the mother had profound weight loss, and became so unwell she could no longer administer cART to her child. Her child had been aviraemic since 2 months of age but rebounded to 14 000 HIV RNA copies per mL at this time.

For Case 5, the mother was severely immunocompromised (CD4+ T cell count 50 cells per μL) at delivery, which likely led to foetal immune dysfunction, manifesting as poor growth, a low CD4+ T cell count, and pancytopenia with suspected sepsis at birth. At 3 months of age the child died from sepsis and malnutrition, in keeping with the increased mortality risk for children born to mothers with low CD4+ T cell counts (Abrams et al. 2017; Newell et al. 2004). In deep sequence analysis of plasma viral HIV, this pair's HIV showed high viral diversity (Figure 6.3), in the mother likely due to the many years of high maternal viraemia. The unusually high viral diversity

observed in this child raises the possibility that more than one viral strain had been transmitted to the child. This would have been a further immunological insult, and likely a manifest of the materno-foetal immunocompromise.

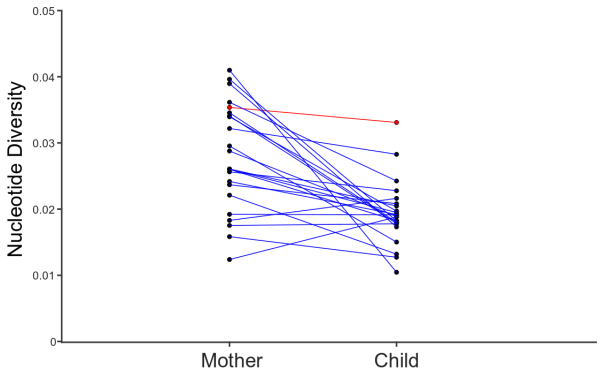


Figure 6.3: Nucleotide diversity of HIV transmitted *in utero*. Nucleotide diversity (π) of deep-sequenced HIV virus from 23 IU transmitting mother-child pairs, within 28 days of delivery. The high nucleotide diversity of virus from the infant in Case 5 is indicated in red.

6.5 Conclusions

Although conclusions are limited by the small sample size, from a population of children with a background high rate of virological failure (see Chapter 3), this case series demonstrates universally poor adherence in the second generation of mother-to-child transmission of HIV. This study was not designed to incorporate a formal maternal psychological assessment, so could not analyse the mothers' experiences in-depth, but I have obtained enough information to prime further qualitative studies. The outcomes for perinatally infected non-transmitting mothers were not analysed, so the IU MTCT rate cannot be determined, nor the occurrence or outcomes for infants infected postnatally. Thus, the true magnitude of the problem of second-generation MTCT remains unknown.

In South Africa, the number of perinatally HIV-infected girls surviving to childbearing age and having children of their own is increasing. Optimising their care, and preventing transmission of HIV to their infants, requires careful consideration and specific insights. The first action must be preventing unplanned pregnancies, and improving

the support for adolescents living with HIV, including early disclosure. If they become pregnant, it must be ensured that these young women get adequately treated to reduce transmission risk and optimise maternal physical and mental health. There should be a low threshold for suspecting ART resistance, and tailored adherence counselling to deal to with issues of stigma, adolescence, orphanhood, and onward disclosure. In the occurrence of second-generation mother-to-child transmission, recognising the profound vulnerability of these children is essential to ensure they can survive and thrive.

Chapter 7

Sex differences in *in utero* HIV infection

The majority of this work has been published in: Adland, E.*, **Millar, J.***, Bengu, N., Muenchhoff, M., Fillis, R., Sprenger, K., Ntlantsana, V., Roider, J., Vieira, V., Govender, K., Adamson, J., Nxele, N., Ochsenbauer, C., Kappes, J., Mori, L., van Lobenstein, J., Graza, Y., Chinniah, K., Kapongo, C., Bhoola, R., Krishna, M., Matthews, P. C., Poderos, R. P., Lluch, M. C., Puertas, M. C., Prado, J. G., McKerrow, N., Archary, M., Ndung'u, T., Groll, A., Jooste, P., Martinez-Picado, J., Altfeld, M., Goulder, P. (2020) Sex-specific innate immune selection of HIV-1 in utero is associated with increased female susceptibility to infection. *Nat Commun* 11, 1767, https://doi.org/10.1038/s41467-020-15632-y *Joint first authors.

7.1 Introduction

Biological sex is defined by the biological differences between males and females, i.e. sex chromosomes, reproductive organs and sex hormones, whereas gender refers to the behaviours and activities that are determined by society or culture in humans (Klein and Flanagan 2016). Incidence and outcome from many infectious diseases, including HIV, are influenced by both sex and gender. By way of natural selection, evolution of the sexes of a species diverges due to their inherently different means of achieving reproductive success. This sexual selection results in sexual dimorphism; excluding the sexual organs, different anatomy and physiology of the sexes of a species, including distinct immune profiles (Zuk 1990). Males and females bias in opposite directions

in the fine balance between immunoreactivity and immunotolerance. Broadly speaking, females mount stronger immune responses than males, resulting in them being less susceptible to infection and cancers, but more prone to immunopathology and autoimmune disease (Klein and Flanagan 2016).

In general, infant mortality is higher in males than females, due to increased male infection susceptibility and prematurity (WHO 2007a). Of note, in China and India, where culturally, sons are preferred to daughters, male infant mortality is lower than females, presumably due to gender-specific health-seeking behaviour. Beyond the infancy period, the sex ratio of under-five mortality varies around the world, due to differential distributions of infectious diseases, vaccination rates and gender roles (Sawyer 2012). However, during childhood, males are usually more susceptible to most infections, particularly protozoal and parasitic (Muenchhoff and Goulder 2014; Klein and Flanagan 2016), although for some pathogens, such as measles and dengue, females have more severe disease (WHO 2007a).

Young girls have enhanced immunoreactivity to childhood vaccinations compared to males (Voysey et al. 2016). Additionally, non-specific benefits from some vaccinations, such as the measles vaccine, are higher in girls (Aaby and Benn 2011).

Female children have higher levels of serum inflammatory markers (C-reactive protein, erythrocyte sedimentation rate and neutrophilia) in acute illness (Casimir et al. 2010) and this stronger immunological response to foreign antigens is also true for self-antigens. Overall, childhood autoimmune disease is more common in girls, although there are specific conditions more common in males, for example, Kawasaki disease (Chiaroni-Clarke et al. 2016).

Adolescent females are 60% more likely to acquire HIV compared to adolescent males but given that the sex ratio of HIV prevalence evens out in older age brackets, there is likely a significant behavioural component driving this difference (UNAIDS 2019b). Gender clearly influences HIV acquisition risk, but may also be masking differential

biological susceptibility, indicated by HIV transmission studies. Adult females are more likely than males to be infected with multiple viruses from a single sexual partner (Long et al. 2000) and are infected with lower fitness viruses, as inferred from viral sequencing (Carlson et al. 2014). Apart from the obvious anatomical differences, it is possible that this increased HIV susceptibility is the consequence of a different immune response.

In untreated HIV infection, women have better virological control, with plasma viral load set points approximately two-fold lower (Gandhi et al. 2002; Napravnik et al. 2002) and elite control five times more likely in women (Yang et al. 2017). Although treated males and females have similar levels of HIV DNA, more low-level viral replication occurs in males (Scully et al. 2019). The cost of enhanced virological control is higher immune activation.

Chronically HIV-infected untreated females have higher levels of CD8+ T cell activation and IFN-1 response after adjusting for plasma viral load (Meier et al. 2009; Chang et al. 2013). Since immune activation is a strong independent risk factor for HIV disease progression (Deeks et al. 2004), in untreated HIV, for a given plasma viral set point, females progress faster to AIDS (Napravnik et al. 2002; Farzadegan et al. 1998). For treated HIV, females have less reduction in key markers of inflammation and immune activation (Mathad et al. 2016) and higher rates of non-AIDS related morbidity and mortality (Triant et al. 2007).

Like adults, girls have better virological control in untreated HIV infection compared to boys (European Collaborative Study 2002; Ruel et al. 2011), although the reverse in true in the first four years of life (European Collaborative Study 2002). Moreover, paediatric elite control has been reported almost exclusively in school-aged females (Vieira et al. 2019).

Albeit understudied, to date there has been no evidence for sex differences in immune activation in paediatric HIV. One study of untreated HIV-infected South African chil-

dren found no difference in CD4+ or CD8+ T cell activation (CD38+ HLA-DR+) between girls and boys (Ruel et al. 2011). HIV uninfected females have higher CD4+ T cell counts and percentage from birth to adulthood (European Collaborative Study 2003; B.-W. Lee et al. 1996; Foca et al. 2006; Mori et al. 2015)), even in untreated HIV infection (Mori et al. 2015; Kiboneka et al. 2008; Ruel et al. 2011; Foca et al. 2006). Furthermore, historically, when criteria had to be met in order to initiate cART, females were more likely to meet clinical criteria before immunological criteria, suggesting that immune dysfunction occurs at higher CD4+ T cell counts in females (Mori et al. 2015; Kiboneka et al. 2008). Following cART initiation or class switch, immune reconstitution is more rapid and complete in females (Mori et al. 2015; Puthanakit et al. 2009; Shiau et al. 2014).

It has been shown that mortality is higher for both treated and untreated HIV-infected girls, compared to boys (Rouet et al. 2003; Foca et al. 2006; Zanoni et al. 2011), but not all studies have found this result, so it may be geographic, age-group specific or gender-driven (Liu et al. 2004; Marinda et al. 2007; European Collaborative Study 2004b).

Several studies have found the odds of female *in utero* HIV infection approximately 1.5-2 times that of males, not explained by other risk variables, as summarised in Table 7.1.

Apart from the studies listed in Table 7.1, most infant transmission studies have not found an increased rate of intrapartum, postnatal or overall female paediatric HIV infection (Piwoz et al. 2006; Taha et al. 2005; Connor et al. 1994; Guay et al. 1999; Breastfeeding and HIV International Transmission Study Group 2004). One large meta-analysis of 4067 paediatric HIV transmissions, found an increased risk for males to be infected via breastfeeding after 4 weeks of age (HR 1.4 [95% CI 1.1-1.6]), however, these results should be interpreted with caution as twice as many infants were excluded from the analysis that were included, because the timing of transmission was unknown (Breastfeeding and HIV International Transmission Study Group 2004). Furthermore, there is a small amount of evidence suggesting that males could be at higher risk

Table 7.1: Paediatric HIV cohorts showing a female bias for infection

Pub.	Setting	Cohort period	HEs tested	M:F HE live births	Infected infants	Infected females	Infected males	OR	aOR
IU INFECTION									
1	Malawi	1994	1102	1:1	92	53	39	1.4 (0.9-2.2)	-
1*	Malawi	1996-2003	606	0.98:1	28	27	11	2.5 (1.3-3.3)	-
2	Zimbabwe	1997-2000	4443	1.03:1	383	229	154	1.6 (1.29-2)	1.53 (1.23-1.91)
3	Malawi	2000-2003	1964	0.97:1	187	126	61	2.14 (1.56-2.95)	2.14 (1.55-2.94)
4	Europe	1986-2004	1086	1.11:1	48	32	16	2.31 (1.3-4.3)	2.14 (1.14-4)
5	Multinational	2014-2019	460	-	54	33	21	-	-
6	Botswana	2015	-	-	40	28	12	-	-
IP INFECTION									
1*	Malawi	1996-2003	606	0.98:1	43	28	15	1.8 (1-3.5)	-
IU + IP INFECTION									
7	Kenya	1991-1992	94	1.04:1	29	20	9	3.2 (1.1-9.2)	-
1	Malawi	1994	292	0.98:1	75	52	23	2.7 (1.5-4.9)	-
8	Italy	1996-2001	1777	1.07:1	82	54	28	2.13 (1.33-3.45)	2.33 (1.4-3.85)

Publications (Pub.): 1. Biggar et al. 2006, 2. Piwoz et al. 2006, 3. Taha et al. 2005, 4. ECS 2004, 5. Persaud et al. 2019, 6. Shapiro et al. 2019, 7. Temmerman et al. 1995, 8. Galli et al. 2005. ∗Twins only. HE; HIV-exposed infant, OR; odds ratio, aOR; adjusted odds ratio, IP; intrapartum.

for HIV infection in older children via the parenteral route. A study from Zaire found iatrogenic HIV infection in children almost exclusively occurred in males (15/16, p=0.004) with no documented difference in the number of injections or transfusions by sex (Mann et al. 1986). More recently, in the 2019 paediatric HIV outbreak in Pakistan following unsafe medical practices, 64% of 591 infected children were male, again with no sex difference in exposure (Mir et al. 2019). Together these data suggest, that if females are more likely to be infected with HIV, that this predominantly occurs *in utero*.

The sex bias in HIV susceptibility more obvious *in utero* suggests that there might be something sex specific that is unique to the transplacental transfer of HIV as compared to mucosal or parenteral entry. The placenta is derived from the same embryonic tissue as the foetus, so too is of a biological sex, thus exhibits sexual dimorphism from the gross down to the transcriptomal level. A transcriptomic study of >300 non-pathological placentas found >140 genes differentially expressed between the sexes, >60% of which were autosomal genes, many involved with cell growth and proliferation, which may explain the larger size of male placentas and infants (Buckberry et al.

2014). It remains to be explored whether there are sex differences in trophoblast IFN-1 release or Hofbauer cells, which are thought to have important roles in the placental transmission of HIV (see page 11). Histological sex differences of the materno-foetal interface from healthy placentas have also been described, although the significance of the specific cellular differences is unclear (Barapatre et al. 2019). Despite having bigger placentas, male foetuses have higher perinatal complications attributed to placental dysfunction, including bacterial infection, pre-eclampsia and intra-uterine growth restriction (IUGR) (Goldenberg et al. 2006). In the occurrence of preterm delivery, there is a male tendency for materno-foetal interface abnormalities such as chronic deciduitis, decidual vasculopathy (M. G. Walker et al. 2012) and maternal immune reaction to foetal tissue (Goldenberg et al. 2006).

One factor contributing the maternal immune response to the male foetus is the H-Y antigen. H-Y antigens are a group of highly antigenic minor histocompatibility antigens encoded for on the Y chromosome (Popli et al. 2014). Secondary recurrent miscarriage is more common if the mother's first child was male, and corresponds to higher maternal anti-H-Y cell-mediated immune responses (Christiansen et al. 2010), suggesting an immune sensitisation process. Similarly, chronic graft-versus host disease is more common in female donor-male recipient haematopoetic stem cell transplants (Stern et al. 2008), and females are more likely to reject allograft kidneys from male compared to female donors (Gratwohl et al. 2008). This histocompatibility reaction between maternal lymphocytes and male foetal H-Y antigens, along with other histopathologic changes, may be alter the transmissibility of free or cell-associated HIV across the placenta.

Another obvious difference between male and females are sex hormones. All of the sex hormones; testosterone, progesterone and oestrogen, influence immune phenotype and function (Klein and Flanagan 2016). There are sex hormone receptors in most immune cells, the binding of which can alter gene expression and cell activity, including the Th1/Th2 cytokine balance for CD4+ T cells. Male foetuses produce testosterone from 8 weeks gestation (Bouvattier et al. 2012), a hormone which has been shown to have

immunosuppressive effects in adults (Furman et al. 2013). Foetal oestrogen levels are higher in females than males (Van De Beek et al. 2004), while progesterone levels are similar (Hagemenas and Kittinger 1973).

Oestrogen has a complex role in immune modulation, but is broadly thought to be pro-inflammatory (Khan and Ahmed 2016). Many of the differentially expressed genes between males and females following stimulation *in vitro* have an oestrogen response element in the promoter, suggesting a role for oestrogen in increasing immunoreactivity (Hewagama et al. 2009). Conversely, oestrogen binding to the oestrogen receptor-1 is a key factor in limiting HIV latency reversal (Das et al. 2018).

Progesterone has broadly anti-inflammatory effects (Klein and Flanagan 2016) and is thought to contribute to the increased maternal HIV susceptibility during pregnancy, when progesterone levels are high (Thomson et al. 2018). There has been some data suggesting that progesterone-based contraception increases HIV acquisition risk (Polis et al. 2016), but a recent large randomised trial found no increased risk (ECHO Trial Consortium 2019).

An additional mechanism for sex hormones to influence immune function is through gene methylation. Many immune cell, sex-specific, differentially expressed genes are found in autosomal regions of the genome, due to differential methylation (Mamrut et al. 2015) which could be mediated by sex hormones.

Along with differential autosomal gene expression, the human transcriptome differs between males and females due to the sex chromosomes, not only the male Y chromosome but also X-linked gene dosage. The X chromosome has 1100 genes, encompassing 5% of the human genome (Libert et al. 2010) and contains 10% of the genome's micro-RNA, which regulate expression of coding genes (Schurz et al. 2019). The X chromosome also encodes many crucial immune-related genes, well demonstrated by the 13 X-linked primary immune deficiency disorders attributed to mutations in 11 genes on the X chromosome (Libert et al. 2010). For females, dosage compensation

occurs, whereby one X chromosome is randomly and permanently inactivated early in embryogenesis. However, this X chromosome inactivation (XCI) is incomplete, with 15% of genes escaping inactivation in a non-random manner (Libert et al. 2010). Therefore, two copies of X-linked immune-related genes can be expressed by females.

XCI escape has been demonstrated with regard to the Toll-like Receptor 7 (TLR7) gene, which has been found to be dually expressed in 30% of peripheral immune cells (Souyris et al. 2018). TLR7's key role is on the phagosomes of plasmacytoid dendritic cells (pDCs) where it recognises single-stranded RNA, such as that of HIV. This TLR7 overexpression in females is thought to contribute to higher IFN-1 response to HIV by females (Meier et al. 2009). The 13 subtypes of IFN-1's play a critical role in restricting viral replication through the induction of host restriction factors, but also contribute to detrimental systemic inflammation, therefore the intricate interaction between IFN-1's and HIV is likely dose, timing and location specific (Utay and Douek 2016), so the direct implication for female HIV infection is unknown.

7.1.1 Hypothesis and aims

The Ucwaningo Lwabantwana study provided the opportunity to test the hypothesis that females have increased susceptibility to *in utero* HIV infection, and consider additional immunological, virological and clinical sex differences, in order to elucidate possible mechanisms.

The overall aim addressed in this chapter was to further explore sex differences in *in utero* HIV infection, specifically by comparing males and females in:

1. Odds of infection, and scenarios where this might be increased or decreased
2. The phenotype of the transmitted founder virus
3. Levels of immune activation
4. Viral burden and dynamics on cART
5. Disease progression

7.2 Chapter specific methods

7.2.1 Study participants

Ucwaningo Lwabantwana Study

Participants and data from the UL cohort up until December 31, 2019 were included. In addition, one sex discordant twin pair who were enrolled in February 2020 were included, but only in the case description.

National Health Laboratory Services birth HIV PCR data

HIV PCR tests results for infants $\leq$7 days of age located in the study site hospital districts; iLembe, uMgungundlovu, uThungulu, and eThekwini for 2017-2018 were provided by NHLS following written request and approval from the KwaZulu-Natal Biomedical Research Ethics Committee. The data were anonymised and provided in an Excel sheet, stating each test result, date of sample, facility name, patient age, patient sex and a laboratory assigned unique identification number for tests with both a similar name and similar date of birth. It should be noted that newborn tests are recorded under the mother's name. Invalid test results, due to insufficient sample or laboratory error were excluded by NHLS prior to being sent.

Cord blood

Cord blood was collected from twin pairs at Kimberley Hospital, Northern Cape, South Africa and PBMC were isolated on site. Informed consent was obtained from all participating subjects. The study was approved by the University of the Free State Ethics Committee, Bloemfontein and the Research Ethics Committee, University of Oxford.

7.2.2 Viral replicative capacity

Viral replicative assays were performed by Dr Emily Adland.

Gag-protease amplification

The *gag-protease* RT amplifications from UL mothers and infants at baseline (see section 2.6) were used in a second round PCR using 100-mer primers that completely matched the NL4-3 sequence (see below), using Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Tables 7.2, 7.3, 7.4). PCR products were purified using the QIAquick PCR purification kit (Qiagen).

Table 7.2: *Gag-protease* PCR primers

Primer	Sequence (5'-3')	Strand	HXB2 coordinates
Gag-Pro F	GACTCGGCTTGCTGAAGCGCGCACGGCAAGAGGCGAGGGGCGGCGACTGG TGAGTACGCCAAAAATTTTGACTAGCGGAGGCTAGAAGGAGAGAGATGGG	Forward	695 to 794
Gag-Pro R	GGCCCAATTTTTGAAATTTTTCCTTCCTTTTCCATTTCTGTACAAATTTCTA CTAATGCTTTTATTTTTTCTTCTGTCAATGGCCATTGTTTAACTTTTG	Reverse	2646 to 2547

Table 7.3: *Gag-protease* PCR mastermix

Reagent	Volume (uL)
H_20	76.6
x10 buffer	10
dNTP	2
Forward primer (10 mM)	2
Reverse primer (10 mM)	2
Hi-Fi Platinum Taq DNA polymerase	0.4
$MgS0_4$ (50 nM)	3
1st round PCR product	4
Total	**96**

Table 7.4: *Gag-protease* PCR conditions

Step		Temperature	Duration
1	Denaturation	94°C	2 min
2	Denaturation	94°C	30 s
	Annealing	60°C	30 s
	Elongation	72°C	2 min
3	Repeat step 2, 39 times		
6	Elongation	72°C	7 min
7	Storage	4°C	

Generation of recombinant *Gag-protease* viruses

A plasmid for the laboratory strain R4 tropic subtype B HIV-1 virus, NL4-3 (Adachi et al. 1986, GenBank ref. AF324493, NIH AIDS Reagent Program), was constructed that lacked the entire *gag-protease* region (Stratagene Quick-Change XL kit) replacing this region with a BstE II (New England Biolabs) restriction site at the 5' end of *gag* and the 3' end of *protease*. To generate recombinant viruses, 10 μg of BstE II-linearised plasmid plus 50 μL of the *gag-protease* amplicon (approximately 2.5 μg) were mixed with 2 million cells of a *tat*-driven green fluorescent protein (GFP) reporter T cell line (GXR 25 cells, Brockman et al. 2006) in 800 μL of R10 medium and transfected by electroporation using the GenePulser II instrument (300 V and 500 uF, BioRad). Following transfection, cells were rested for 45 minutes at room temperature, transferred to 25 mL culture flasks in 5 mL warm R10 and fed with 5 mL R10 on day 4. GFP expression was monitored every 2-3 days by flow cytometry (LSR II, BD), and once GFP expression reached >15% among viable cells, supernatants were harvested and aliquots stored at -80°C.

Viral replicative capacity

The replication capacity of each chimera (VRC) was determined by infection of GXR cells at a low multiplicity of infection (MOI) of 0.003. The mean slope of exponential growth from day 2 to day 7 was calculated using the semi-log method in Excel. This was divided by the slope of growth of the wild-type NL4-3 control included in each assay to generate a normalised measure of replication capacity. Replication assays were performed in triplicate, and results were averaged.

7.2.3 Type I interferon sensitivity

Interferon sensitivity assays were performed by Dr Emily Adland. To determine IFNα2a concentrations required to inhibit virus replication to 80% (IC_{80}), GXR 25 cells were left untreated or cultured in the presence of increasing amounts of IFNα2a (0.00074–5.5 pg/mL), infected with equal amounts of patient specific *gag-pro* chimeric virus (MOI 0.03) and cultured for 7 days. IFN-containing medium was replenished

every 24 hours. Viral replication was measured by GFP expression and the mean slope of exponential growth from day 2 to day 7 was calculated using the semi-log method in Excel. Viral replication was plotted for each IFN concentration as the percentage of viral growth in the absence of IFN. Replication in the untreated cells was used to determine the replicative capacity of *gag-pro* viral isolates.

7.2.4 *In vitro* HIV infectivity

Viral infectivity assays on sex discordant twin cord blood were performed by Dr Emily Adland. PBMC were thawed in RPMI with 20% FCS medium, rested for 1 hour at 37°C, 5% CO_2; and 1 million stained in a total volume of 50 μL staining solution with titrated concentrations of fluorochrome conjugated monoclonal antibodies against cell surface markers (CD3, CD4, CD8, PD-1, HLA-DR, CCR5, CD45RA, CCR7) and near-infrared live/dead marker (Invitrogen) for 30 minutes at room temperature in the dark. Cells were then washed twice in PBS and fixed in 2% paraformaldehyde. Samples were acquired on an LSR II flow cytometer (BD) within 12 hours of staining and analysed using FlowJo v.8.8.6. The remaining PBMC were activated with PHA for 72 hours then infected with an R5 tropic HIV-GFP tagged virus at MOI 0.01 and cultured for 7 days. R10 medium was replenished every 24 hours. Viral infectivity was measured as the amount of CD4+ T cells expressing GFP at day 7 post-infection. The R5 tropic HIV-GFP tagged virus was produced by transfecting 293-T cells (American Type Culture Collection) with the plasmid construct R5-HIV-1-GFP by CalPhos (Invitrogen). After 48 hours, viral supernatant was harvested and stored at -80°C, and the 50% tissue culture infective dose ($TCID_{50}$) was determined on TZM-bl cells (NIH AIDS Reagent Program) (Reed and Muench 1938).

7.3 Results

7.3.1 Susceptibility to *in utero* HIV transmission

Consistent with previous studies (Table 7.1), in the Ucwaningo Lwabantwana cohort, female infants significantly outnumbered males by 1.6:1. In addition to this female preponderance, in all three cases of sex discordant twins enrolled on the study, the

female twin was infected, and not the male (Table 7.5). To compare the odds of infection between males and females, all the infants who were PoC tested for HIV at three study sites over a period of 2 years (n=2275) were examined. The ratio of males to females tested was 1.06:1 and the odds ratio of female infection compared to males was 2.15 (95% CI 1.23 - 3.84, Figure 7.1A). This increased odds was stronger for infants born to mothers acutely HIV-infected in pregnancy compared with chronically infected mothers (OR 2.82 [95% CI 1.11-7.24] versus OR 2.22 [95% CI 0.86 - 5.8] respectively, Figure 7.1A), further exemplified by the diverging female to male ratio of infected infants with increasing recency of maternal infection (Figure 7.1B-C).

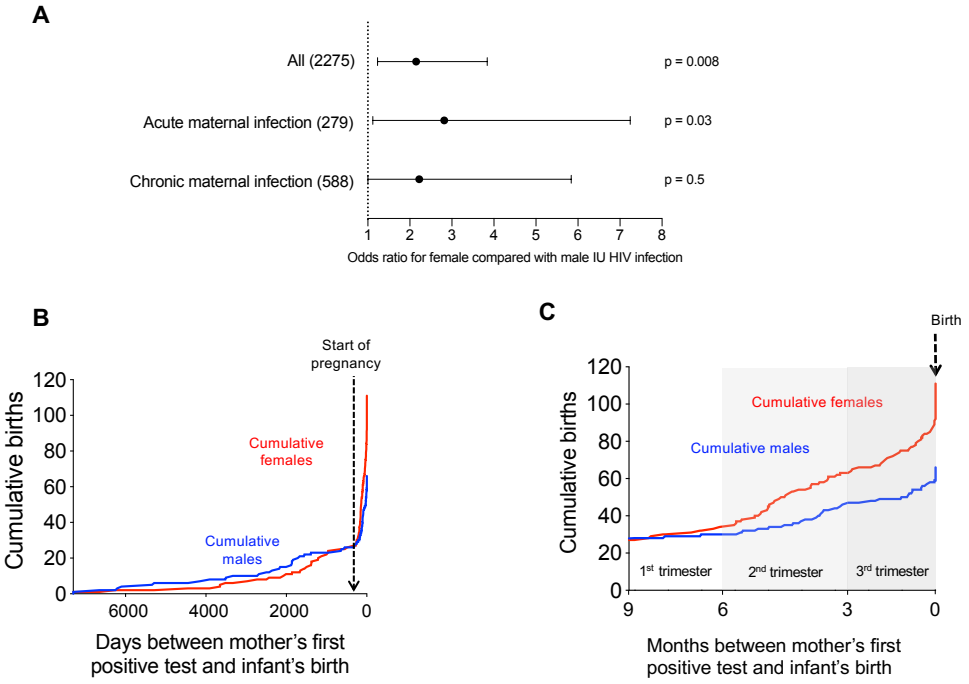


Figure 7.1: Increased odds of female compared to male *in utero* HIV infection, particularly following acute maternal infection. (A) Odds ratio for female to male HIV infection for all HIV-exposed infants PoC tested at three of the hospital sites where the complete data set was available for 2016-2018. Similar numbers of females as males were tested (females 1070, males 1151) but more females tested positive. Acute maternal infection was where the mother had a documented negative rapid HIV antibody test during pregnancy while chronic maternal infection where the mother was known to be HIV-infected prior to pregnancy. P values were calculated using Fisher's Exact Test. (B) Cumulative number of male and female IU transmissions by time (days) between mother's first HIV-positive test and the birth of the infant. Start of pregnancy shown as 280 days prior to delivery for simplicity (some infants were born prematurely). (C) Cumulative number of male and female IU transmissions by time (months) between mother's first HIV-positive test and the birth of the infant in the mothers who first tested positive during the pregnancy. As in (B), start of pregnancy shown as 280 days prior to delivery. (B-C) figures drawn by Dr Nicholas Grayson.

In order to increase power and reduce possible selection bias in those PoC tested, the SoC birth HIV PCR results from the four study site hospital districts from 2017-

Table 7.5: Case summaries of sex discordant twin pairs enrolled

	Twin pair 1		Twin pair 2		Twin pair 3	
MOTHER						
Age (years)	41		32		38	
Number of other children	5		1		2	
Timing of maternal HIV infection	unknown		acute (last 4w)		unknown	
First HIV positive test	in labour		in labour		antenatal booking	
Timing of maternal cART initiation	postnatally		postnatally		early pregnancy	
Maternal cART regimen at enrollment	TDF/FTC/EFV		TDF/FTC/EFV		TDF/FTC/EFV	
Perinatal complications	no antenatal care UPTL		none		UPTL BBA, PIH	
Plasma viral load (HIV RNA copies/mL)	1 100 000		600		27000	
CD4+ T cell count (cells/μL)	137		444		179	
CD4:CD8	0.16		2.78		0.25	
INFANTS						
Gestation at birth (weeks)	32		38		33	
Mode of delivery	Vaginal		Caesarean (for breech)		Vaginal	
Sex	Female	Male	Female	Male	Female	Male
Birth weight (kg)	1.6	1.5	3.1	2.9	2.3	1.9
Birth weight centile (WHO)	37	18	22	9	84	42
Breastfed	yes	yes	no	no	no	no
Neonatal admission	yes	yes	no	no	yes	yes
Postnatal complications	RDS, jaundice, anaemia	RDS, jaundice, anaemia	none	none	none	none
Infant ART prophylaxis	AZT/NVP	AZT/NVP	AZT*	AZT*	NVP	NVP
Standard-of-care TNA PCR at birth	positive	negative	positive	negative	positive	negative
Age of infant at blood draw (days)	1	-	10	-	15	-
Plasma viral load (HIV RNA copies/mL)	54 000 000	-	3300	-	1 100 000	-
CD4+ T cell count (cells/μL)	723	-	2926	-	2303	-
CD4+ T cell percentage	18	-	40	-	44	-
CD4:CD8	0.35	-	2.43	-	1.71	-
Additional tests	-	negative at 18m	-	negative at 6m	-	-

The mother's blood draw was on the same day as the infants. *The additional NVP that was indicated was out of stock in the district. UPTL: unexplained preterm labour, BBA: born before arrival to hospital, PIH: pregnancy-induced hypertension, RDS: respiratory distress syndrome (hyaline membrane disease)

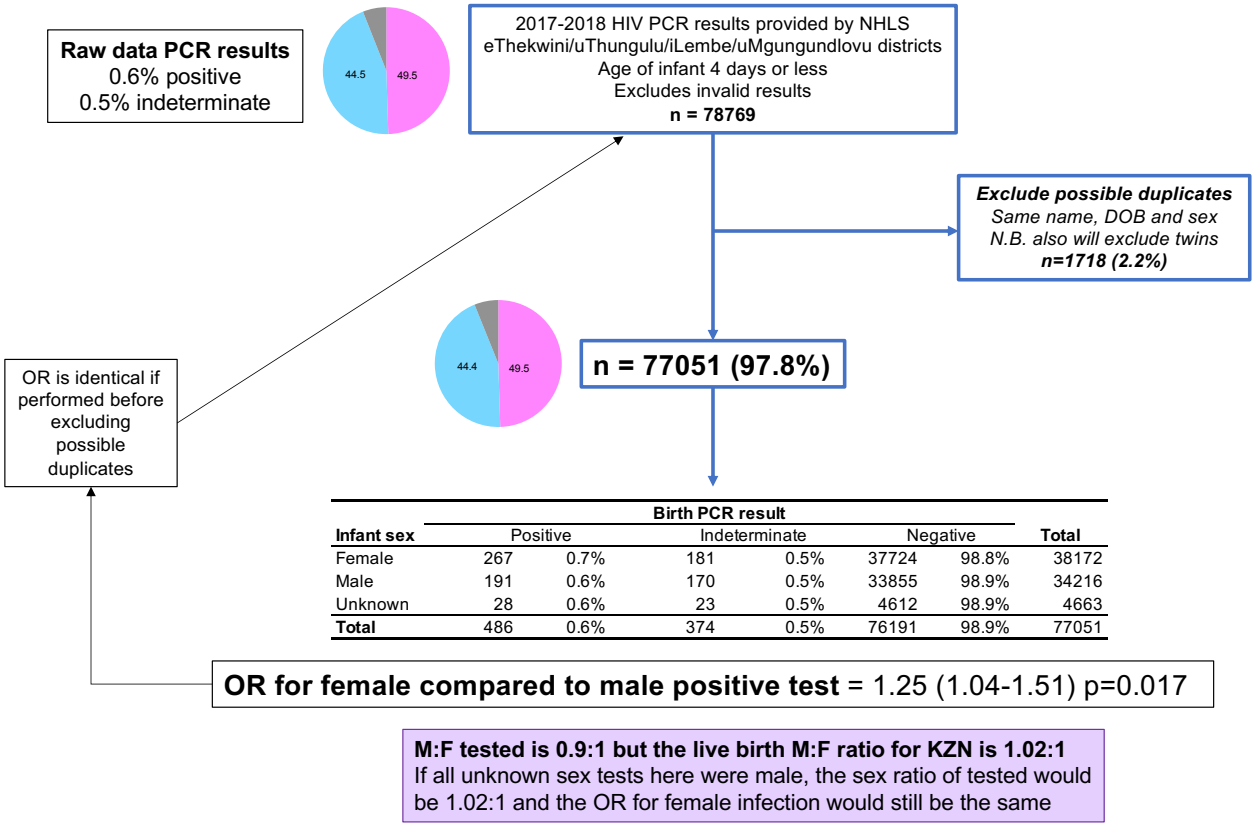
2018 were examined. From the 77 051 HIV PCR tests for HIV exposed infants ≤4 days of life, following removal of possible duplicated results, the odds ratio for female compared to male infection was 1.25 (95% CI 1.04-1.51, p=0.017), however the sex ratio before and after manipulation of the data was 0.9 males to 1 female. Given an HIV exposure rate of 40%, the male to female ratio of live births for HIV uninfected mothers would need to be 1.13:1 to give the overall live birth ratio of 1.02:1 in KZN (Statistics South Africa 2017). This discrepancy was not explained by the number of male early neonatal deaths in KZN (Statistics South Africa 2016a), which may have resulted in recorded live births but untested infants. If all six percent of those tests with an unknown sex were male, then the sex ratio of infants tested would have been 1.02:1 and the odds of female infection would still have been significantly higher than male infection. However, it is likely that not all live born HIV exposed male infants have been accounted for in these data, therefore, although there is a suggestion of increased female susceptibility to HIV *in utero* the results from this specific analysis are inconclusive.

7.3.2 *In vitro* susceptibility to *in utero* HIV infection

Further evidence of female HIV susceptibility is suggested from *in vitro* data. Results showed that the viruses (*Gag-Pro* sequences) transmitted to females *in utero* had significantly lower replicative capacity that those transmitted to males (p=0.0005, Figure 7.3A). Assessment of the replicative capacity of virus from the mothers of infected females indicated that female permissiveness to infection via low replicative capacity of viruses did not occur because of lower replication capacity of viruses to which they were exposed (Figure 7.3B).

7.3.3 Sex specific selection of HIV transmission

There is some evidence that in adults, circulating viruses in chronic infection are predominantly IFN-α-sensitive but the transmitted viruses are IFN-α-resistant (Lyer et al. 2017; Fenton-May et al. 2013). Since females make stronger IFN-α responses to



	Birth PCR result						
Infant sex	Positive		Indeterminate		Negative		**Total**
Female	267	0.7%	181	0.5%	37724	98.8%	38172
Male	191	0.6%	170	0.5%	33855	98.9%	34216
Unknown	28	0.6%	23	0.5%	4612	98.9%	4663
Total	486	0.6%	374	0.5%	76191	98.9%	77051

Figure 7.2: Inconclusive results from National Health Laboratory birth HIV test analysis. HIV PCR tests provided by the South African National Health Laboratory Service (NHLS) for the hospital districts of the four UL study sites (n=78769). The sex ratio of this raw data is shown by the pie graph; pink for girls (49.5%), blue for boys (44.5%) and grey for unknown sex (6%). Because there was no unique patient identifier, an attempt was made to eliminate duplicated patients by excluding tests with the same name, date of birth (DOB) and sex, although this will have also removed same sex twins. The sex ratio and positivity rate was unchanged by this exclusion. Test result by infant sex are displayed in the table, along with the odds ratio (OR) for female compared to male positivity. The male to female ratio of infants tested was very low compared to that documented for the sex ratio of live births in KZN for 2017 (Statistics South Africa 2017), the reason for which has not been identified.

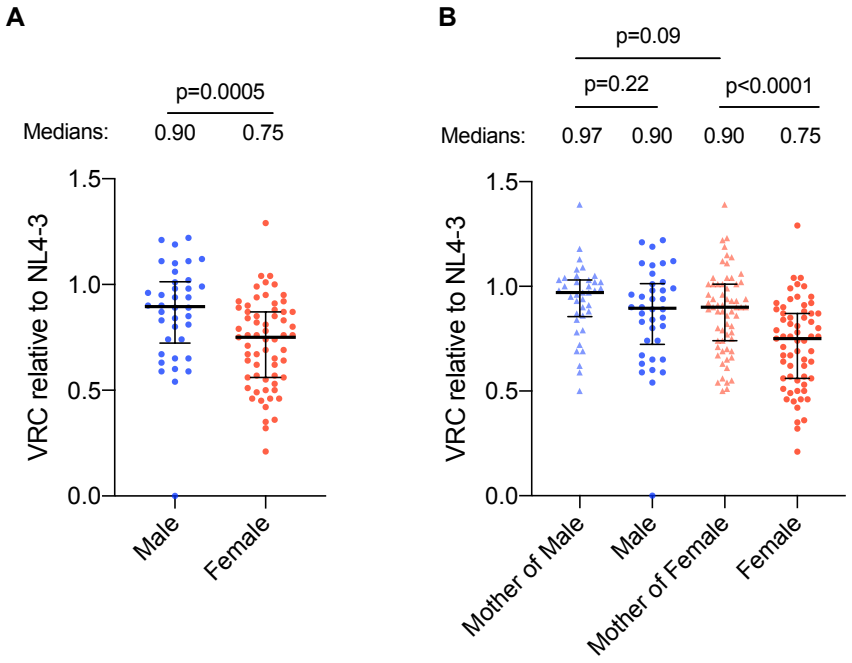


Figure 7.3: Lower viral replicative capacity in female infants at baseline. Viral replicative capacity (VRC) within 21 days of birth from 101 IU infected infants, 63 females and 38 males, and mothers. Data are presented showing medians and interquartile ranges. In panel (A), the statistical test used was the Mann-Whitney test. In panel (B) the statistical tests used were the Wilcoxon matched-pairs signed rank test (comparing males versus mothers of males and females versus mothers of females), and the Mann-Whitney test (comparing mothers of males versus mothers of females).

HIV, it was hypothesised that female foetuses are more susceptible than males to infection via IFN-α-resistant viruses; and, in addition, that recently infected mothers are more likely to harbour IFN-α-resistant viruses than chronically infected mothers. Analysis of *in utero* transmitted viruses in 22 infants at baseline (at a median of 5.5 days of age) showed that viruses transmitted to females are also more IFN-α-resistant than viruses transmitted to males (Figure 7.4A-B). However, although numbers studied were small, viruses harboured by mothers who were recently HIV-infected did not appear more IFN-α-resistant than those harboured by chronically infected mothers (Figure 7.4C); in these mothers, the first positive HIV test was a median of 3 days versus >6 years, respectively, prior to delivery). Thus, the sex bias within IU MTCT being most marked in the setting of acute maternal infection is not explained here by changes in viral IFNα-sensitivity over the course of infection. However, these data show sex-specific selection of the virus, and that mothers transmitting to females are more likely to carry and transmit IFN-α-resistant virus to females, irrespective of how

long they have been infected.

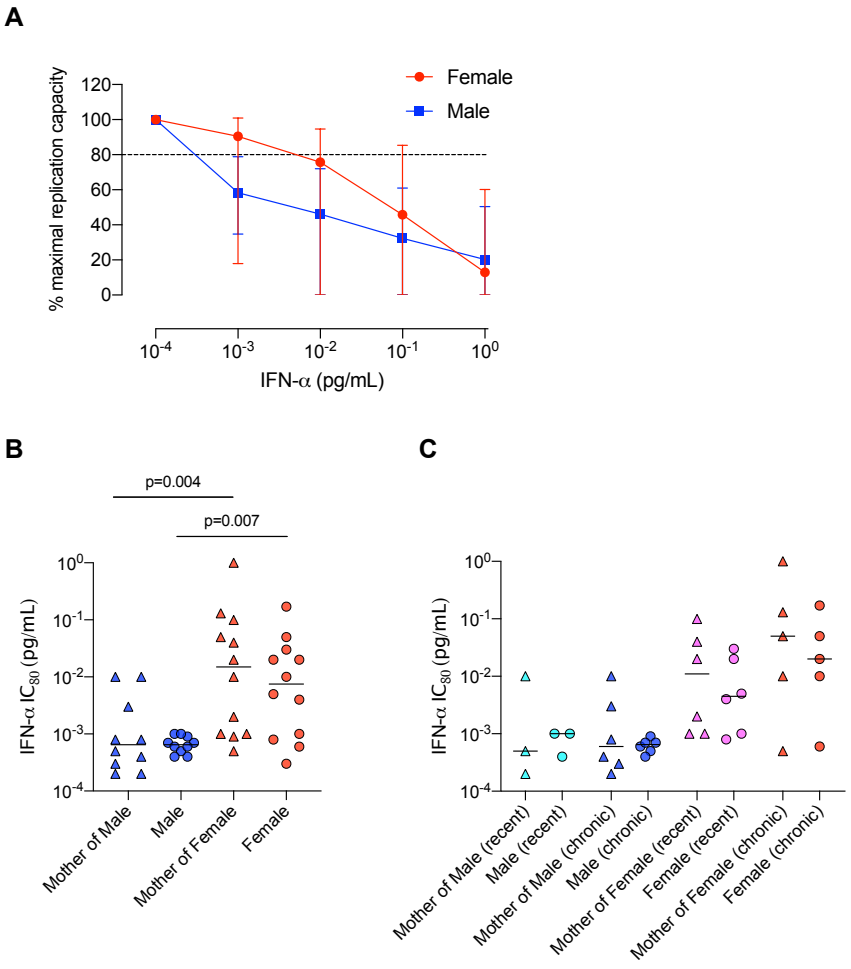


Figure 7.4: *In vitro* Type I interferon sensitivity for baseline mother and infant HIV. IFN-α sensitivity of Gag-Pro-NL4-3 chimeric viruses derived from baseline virus from 22 infants (10 male, 12 female). Raw data for the 22 infants are shown in panel (A), with the median values and range displayed. IC_{80} values and p values from these raw data are shown for the 22 infants and mothers of the infants in panel (B). The statistical test used in each case was the Mann–Whitney test. Medians and interquartile ranges are shown. (C) Data as shown in panels (A, B) but also showing timing of maternal infection categorised : 'chronic': first HIV test prior to pregnancy; or 'recent': first HIV test during current pregnancy. Medians and interquartile ranges are shown.

7.3.4 Immune activation

One possible mechanism for increased female HIV susceptibility *in utero*, particularly in recent maternal infection, is higher immune activation in females leading to increased CCR5 upregulation facilitating cell infection. Pre-*in vitro* infection, using cord blood CD4+ T cells from 19 pairs of HIV-uninfected sex discordant twins, females had higher levels of HLA-DR expression (Figure 7.5A). Females also had higher *in vitro* HIV infection levels than their male twin (Figure 7.5B). In multivariate linear

regression, there was no significant relationship between CD4+ T cell HLA-DR expression pre-HIV exposure, and levels of HIV infection on day 7, even when adjusted for sex (β-coefficient=6.3, p=0.2, adjusted R^2=0.04, p=0.17, not shown), however, there was a trend in the relationship between baseline HLA-DR expression and post-infection CCR5 expression, also adjusted for sex (β-coefficient=8.9, p=0.049) although the deviance explained by this model overall was low (adjusted R^2=0.09, p=0.07, not shown).

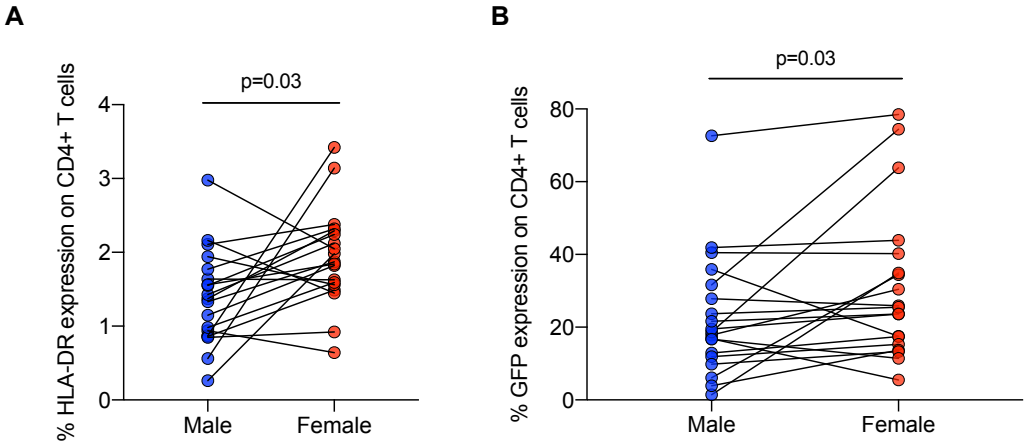


Figure 7.5: *In vitro* HIV infection in uninfected sex discordant twin cord blood. (A) HLA-DR expression in cord blood CD4+ T-cells in 19 sets of HIV-uninfected sex-discordant twins. (B) *in vitro* HIV infection of cord blood CD4+ T-cells from the same 19 pairs of HIV-uninfected sex-discordant twins. The statistical test used for (A) and (B) was the Wilcoxon matched-pairs signed rank test.

7.3.5 Baseline characteristics

Plasma viral load was similar in male and female infants at baseline in the UL cohort (Figure 7.6A), even when adjusted for age of infant and CD4+ T cell count (β-coefficient 0.24 p=0.23, not shown), and also in the PoC sub-group, despite having higher HIV DNA levels (Sub-section 4.4.1, page 80). However, in a matched pair analysis, female infants had significantly higher plasma viral loads compared to their mother, while male infants had plasma viral loads similar to their mothers (p=0.0001 versus p=0.2 respectively, Figure 7.6A). There was no difference in baseline CD4+ T cell percentage between males and females (Figure 7.6B), even when adjusting for infant age and plasma viral load (β-coefficient 0.87, p=0.6, not shown).

Females were heavier at birth (mean birth weight 2.78 kg versus 2.57 kg, p=0.04), although taking into account that males were born on average 1 week gestation earlier (p=0.0009), there was no difference in birth weight between males and females (β-coefficient -0.03, p=0.7). In keeping with younger gestational age, proportionally more male than female infants were admitted into the neonatal unit in the first 28 days of life (32/73 versus 32/118 respectively, p=0.02). There was no difference in the number of infants treated for symptomatic congenital CMV infection (3 males and 4 females, p=1).

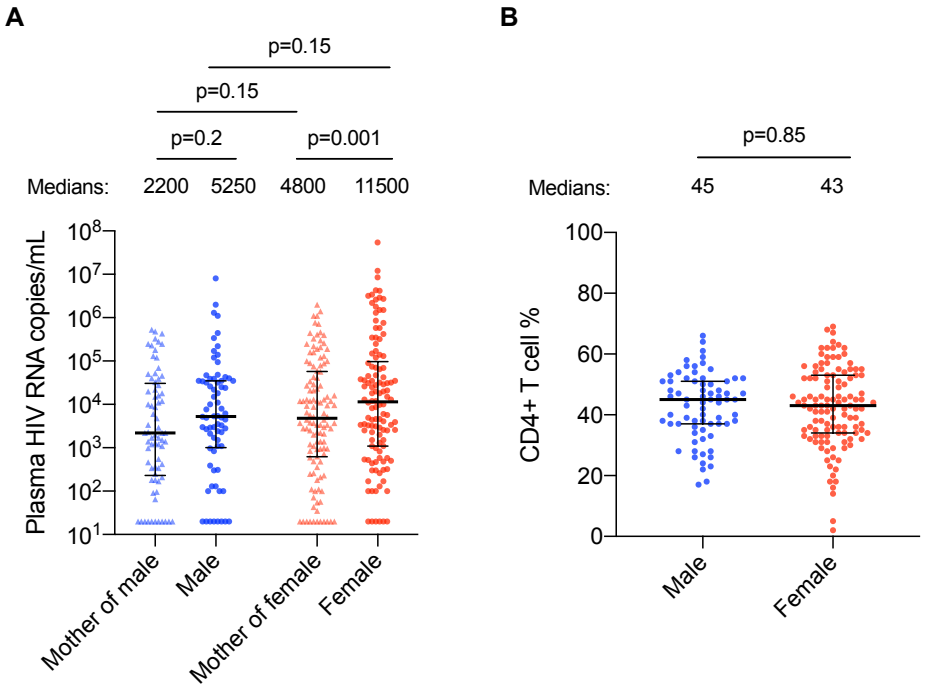


Figure 7.6: Male and female infant baseline plasma viral load and CD4+ T cell count. Plasma viral load (A) and CD4+ T cell percentage (B) for 191 *in utero* HIV infants from the UL cohort at baseline. In (A) infant measurements were compared to maternal baseline plasma viral load using a Wilcoxon matched-pairs signed rank test and for males versus females and mothers of males versus mothers of females using the Mann-Whitney test. In (B) males and females were compared using the Mann-Whitney test.

7.3.6 Viral dynamics

As discussed in Chapter 3, in the UL cohort, infant sex did not significantly influence time to plasma viral load suppression but males did have have a slightly (7%) slower rate to viral rebound compared to females (Table 3.2, page 64). However, in later analyses, with more infants, a longer period of follow-up and adjustment for HIV

DNA, no influence of infant sex on plasma viraemia was found (Sub-section 4.4.3, on page 85). Consistent with this, using a GAMM with participant as a random effect, $\log_{10}$ plasma viral load over time did not differ between males and females (β-coefficient 0.03, p=0.66, not shown).

7.3.7 Disease progression

CD4:CD8 ratio, adjusted for plasma viral load over time, with participant as a random effect, modelled using a GAMM, was on average 0.35 higher in females (p <0.0001, Figure 7.7A), and this was due to both higher CD4+ T cell and lower CD8+ T cell percentages in females, both calculated in a similarly adjusted GAMM (β-coefficients 2.4, -3, respectively, both p-values <0.0001, Figure 7.7B-C). Although these models were highly statistically significant they did not account for high level of variability of the results (adjusted R^2 = 0.15, 0.20 and 0.12 respectively). Infant sex did not influence infant mortality rate (Table 3.2). Moreover, after excluding clearly congenital infections (those diagnosed before hospital discharge following delivery), there was no difference in the time to WHO Clinical Stage 3 or 4 HIV diagnosis (10 males and 8 females, Log-rank p=0.1).

7.3.8 Possible post-treatment control

Although planned cART interruption to assess for post-treatment control was not undertaken in this cohort, it is unfortunately the case that the majority of HIV-infected infants are ART non-adherent at some point, as discussed in Chapter 3. Non-adherence is notoriously difficult to ascertain by history alone (see sub-section 1.5.5), so although males and females did not differ in rate of plasma viral rebound, the extent to which their cART was interrupted is unknown. However, incidentally there were two cases of well-documented treatment interruption as evidenced by a combination of history, returned medication measurement and plasma ART quantification; without HIV RNA or DNA rebound, and in both instances the subjects were male.

207-60-1633-1 was a 15 month old male child who was brought to the clinic for an unscheduled visit. The child had diarrhoea and the mother was concerned this was

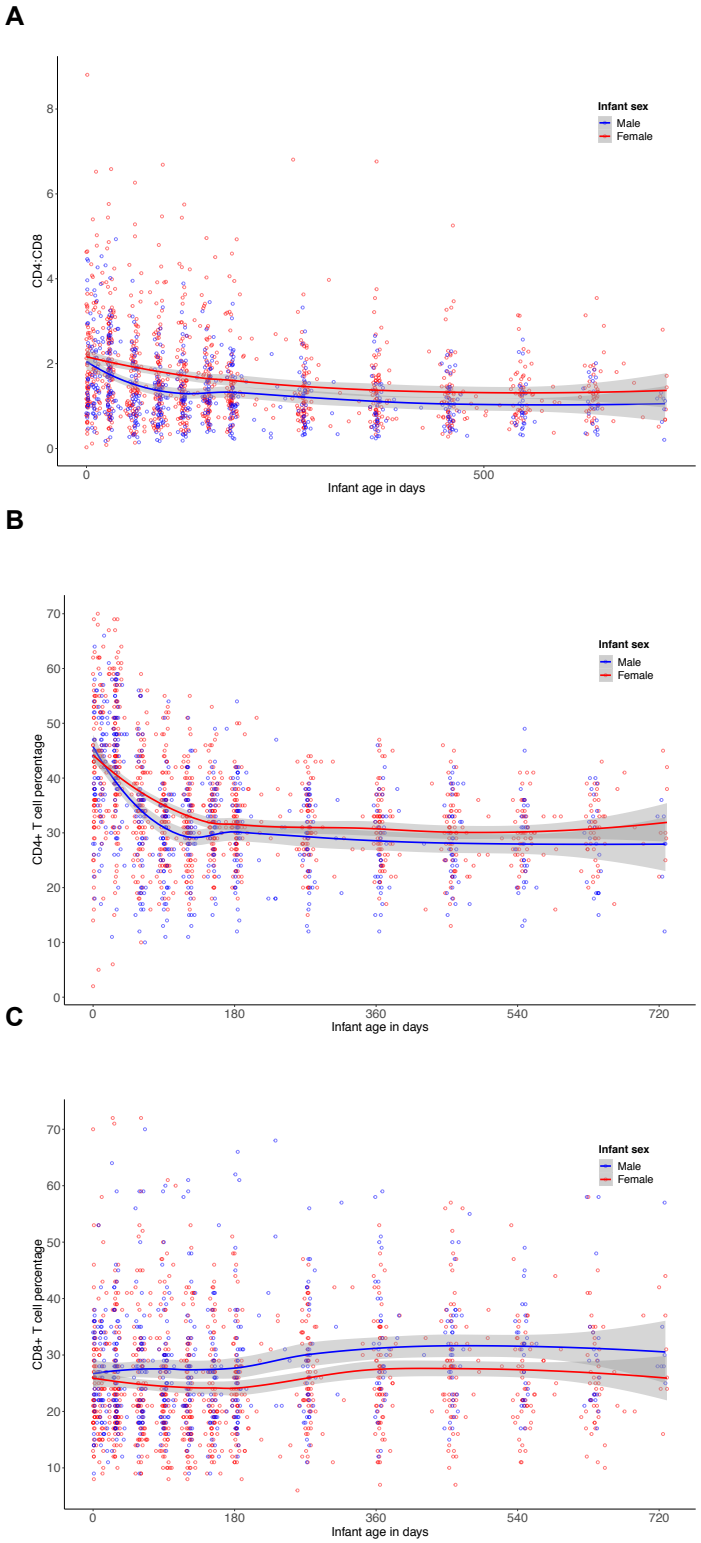


Figure 7.7: Male and female CD4:CD8 ratio over time. Values and Locally Weighted Scatterplot Smoothing (LOESS) line with 95% CI shown for (A) CD4:CD8 ratio, (B) CD4+ T cell percentage and (C) CD8+ T cell percentage. A separate GAMM found after adjustment for infant age and plasma viral load, and with participant as a random effect, significant differences between the sexes remained (not shown).

because the child had not been receiving his cART. Pharmacy records showed no cART had been collected since the mother had returned to work 3 months prior at which time the mother had handed the cART responsibilities over to her sister. Calculated from medication volumes dispensed, it is thought this child received no cART for at least 7 weeks, with no change in plasma viral load or HIV DNA from blood drawn 1 week following cART reinitiation (Figure 7.8A).

207-80-0114-1 was a male child born to a mother who had difficulty disclosing her HIV status to her partner and family, making ART administration difficult. Between 5-12 months of age, the mother often brought back nearly full bottles of ARVs, reported missing doses and prescribed ART was not detected in infant plasma on three separate occasions, during which time the plasma viral load and HIV DNA remained undetectable (Figure 7.8B).

The first question to address, was whether these infants were truly HIV-infected. Both were born to mothers acutely HIV-infected in the third trimester of pregnancy, so the pre-test probability was high. 1633-1 had a positive PoC HIV PCR, but the Ct value was borderline (41.8, positivity cut-off 42). His SoC test was also indeterminate (Ct 33.3, RFI 3). However, plasma viral load on day 1 of life was 1100 HIV RNA copies per mL, which in combination with the indeterminate PCR, according to the National guidelines, was sufficient to diagnose the infant HIV-infected. 0114-1 had a clear positive SoC test on day 1 of life (Ct 28.4, RFI 9.6), but by day 14, this test was indeterminate (Ct 35.5, RFI 2.4) with an undetectable plasma viral load. After consultation with the local paediatric infectious disease team, this child was also diagnosed HIV-infected. Both infants had HIV *gag* sequenced from plasma that showed phylogenetic relatedness to their mothers' (Figure 7.8C) and detectable HIV DNA on one occasion (Figure 7.8A-B).

Although anecdotal, these two cases describe lack of viral rebound in two HIV-infected infants following significant periods of ART non-adherence, and there could be relevance to them both being male.

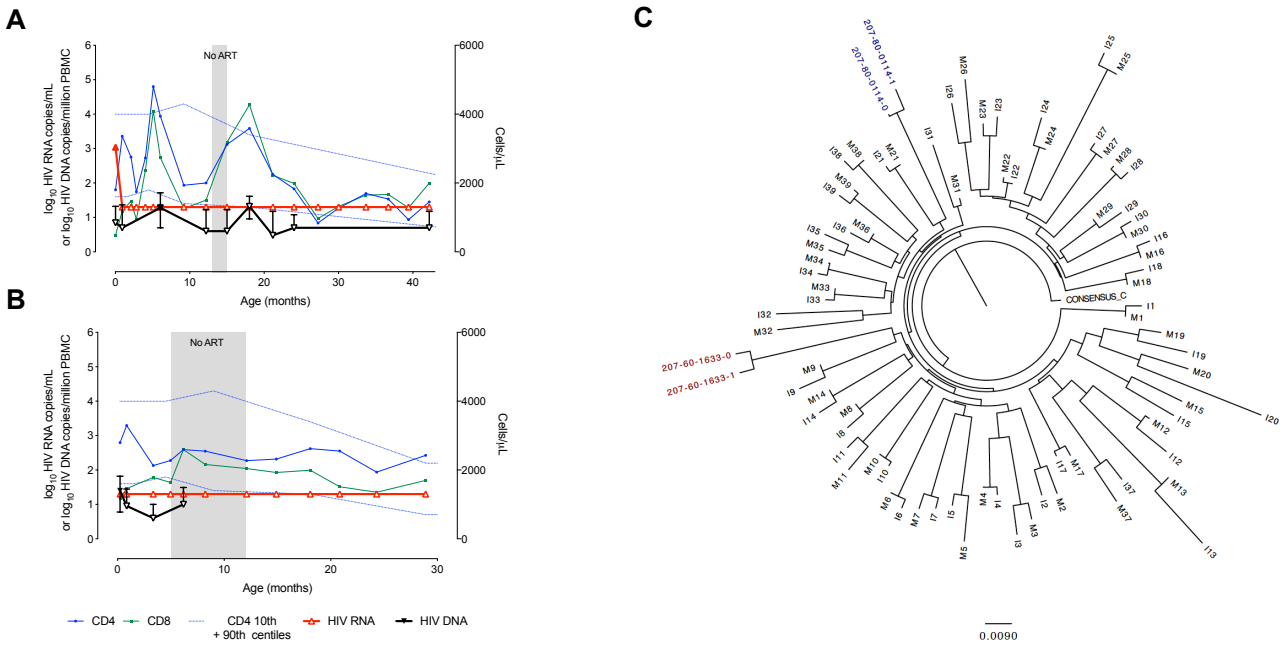


Figure 7.8: Lack of viral rebound in two male subjects following significant periods of ART non-adherence. (A) Male subject 207-60-1633-1 received no cART for at least 7 weeks at 12–15 months' age: pharmacy records show that the aunt of the child did not collect any ART medication for 2 months following the return of the child's mother to work 12 m after the child's birth. cART was restarted 7 days prior to the viral load test at 15 months. Undetectable HIV RNA and DNA values are recorded as an empty triangle at the test limit of detection. (B) Male subject 207-80-0114-1: none of the ARVs prescribed were detected in plasma tested over a 7 month period (age 5–12 months) along with evidence of non-adherence from returned medication. (C) *Gag* was amplified from plasma from the baseline sample from both these mother-child pairs and the sequences determined clustered on a phylogenetic tree, consistent with HIV infection and mother-to-child transmission in each case.

7.4 Discussion

These data, in showing sex-specific immune selection of HIV, associated with an increased female susceptibility to *in utero* HIV infection, demonstrate that immune sex differences exist prenatally. These differences were also reflected at birth, with higher levels of immune activation, HIV DNA and RNA found in females. Although clinical parameters and outcomes for males and females in this ART-treated cohort differed only slightly, if at all, the combination of the aforementioned sex differences could differentially affect chances of HIV remission or cure.

This study has further confirmed that there is indeed a propensity for female HIV infection *in utero*. An obvious explanation for this female HIV bias at birth, would be an excess of HIV-infected male deaths *in utero*, a possibility which is very difficult to exclude with the minimal stillbirth data currently collected by South African government hospitals. A separate, albeit small study tested HIV-exposed stillbirths for HIV and concluded that stillbirths were rarely attributed to HIV infection (Shapiro et al. 2012). Conversely, the unexpectedly low male to female ratio of infants tested for HIV in the NHLS data (Figure 7.2) does raise the possibility that males are dying antenatally or perinatally from HIV infection. However, because IU HIV transmission is rare, males dying from HIV infection *in utero* would have made little difference to the live birth ratio in HIV-exposed infants, so the discrepancy is likely due to males dying from causes in addition or instead of HIV. There is also a high chance this data was biased, due to the exclusion of invalid tests, the possibility of duplicated results and the large proportion of results with unknown sex. It is unfortunate that this much larger NHLS birth PCR data set did not provide firm evidence on the sex ratio for IU HIV infection highlighting the potential utility of WHO recommended unique patient identifiers in this testing programme (WHO 2017a).

A female infant bias does not just occur for HIV. Human T-lymphotropic virus 1 (HTLV-1) is also more common in girls than boys (Ureta-Virdal et al. 1999) and females have twice the *in utero* rate of Hepatitis C Virus compared to males (European

Paediatric Hepatitis C Virus Network 2005; Mast et al. 2005). Furthermore, although the incidence for congenital CMV for males and females is equal, females have 2-3 times the risk of neurological sequelae (Watt et al. 2016; Picone et al. 2005), either due higher female viral burden, as measured by higher amniotic fluid viral load (Picone et al. 2005), or due to the increased neurological immunopathology in response to infection, given that neural damage is proportional to CD8+ T cell infiltration (Gabrielli et al. 2012).

The new observation that the higher risk of female IU HIV infection is found particularly following acute maternal infection brings us closer to determining a mechanism for increased female susceptibility. The higher levels of baseline immune activation in sex discordant twins suggest that higher immune activation in females in response to acute maternal HIV infection could lead to increased HIV susceptibility. Although there was no relationship between CD4+ T cell pre-exposure HLA-DR and post-exposure CCR5, the numbers tested were low, and HLA-DR expression levels were very low so it is possible that flow cytometry of frozen cells was not an accurate enough measurement. Additionally, immune activation might occur within the placenta, and result in the trafficking of cells, both of which would not have been captured by the experiment performed here.

The high rate of females HIV-infected in the sex discordant-HIV discordant twin pair cases (3/3) is consistent with the 7/8 females in similar pairs described by Biggar and colleagues (2006). Admittedly, the numbers are small, and possibly not representative, but the pairs were extremely well matched, with identical HIV exposures and similar genetics, so provide an excellent natural experiment to test the theory that females are more susceptible to HIV *in utero*. The clinical data are also interesting to examine (Table 7.5). In all three cases, the female twin was heavier than the male twin at birth, a difference ranging from 100-400 g or 19-42 centiles (WHO), the opposite trend than typically seen for sex discordant twins (Jahanfar and Lim 2016). In the UL cohort, there was no significant difference between male and female birth weight for gestational age, differing from the ∼200 g excess for males that is expected (Villar et al. 2014).

IUGR is more prevalent in maternal HIV infection and with decreasing socioeconomic status (Dadabhai et al. 2019). These factors, along with high rates of poorly managed pre-eclampsia seen in South Africa (Nathan et al. 2018), may have disproportionately affected male infants. To support this theory, in the third sex-discordant twin case, the mother was diagnosed with pregnancy-induced hypertension and her twins had the greatest birth weight discrepancy (Table 7.5). Admittedly, gestational age was estimated clinically, and the scales used to measure birth weight were not calibrated for research purposes, so there may be inaccuracies in the data, nevertheless, the general trend of smaller than expected male infants indicate sex differential placental functioning. These differences at a cellular level, could contribute to the restriction or selection of transplacental passage of HIV. Further investigation of the sex differences in South African HIV-exposed placentas is warranted.

The observation that females were more permissive to IU HIV infection via low replicative capacity viruses is exactly as described in an adult transmission study in which replicative capacity was inferred by viral sequence analysis (Carlson et al. 2014). The lower viral replicative capacity viruses in female infants seems inconsistent with their relatively higher plasma viral load compared to their mothers. However, the fact that these low replication capacity viruses are also relatively IFNα-resistant, may contribute to the higher viral loads observed initially in infected females. An additional explanation could be host restriction factors. Alternatively, the VRC assay used here did not incorporate viral genes outside of *gag-pro* thus may not represent true *in vivo* viral fitness. However, the assay has been adopted and validated by several groups, and has been shown to have adequate correlation to patient-derived full-length viruses (Prado et al. 2010), patient viral load set point and CD4+ T cell count (K.-H. G. Huang et al. 2011; Miura et al. 2009; Wright et al. 2010). Even if the chimeric viruses were not entirely representative of *in vivo* viral characteristics, the fact that significantly different phenotypes occur between male and female IU transmitted founder virus *gag-pro*, is evidence for the transplacental sexual selection of HIV.

The sex differences in IFN-α-sensitivity found in viruses transmitted *in utero* to males and females, implicates the TLR7-mediated response of pDCs to HIV in this process, as has been well reported in adult HIV infection. The mechanisms underlying sex differences in response to TLR ligands such as HIV include TLR7 escape from XCI as described, as well as potentially epigenetic and hormonal factors. The lack of difference in IFNα-sensitivity between acute and chronically infected mothers is in contrast to some previous studies (Lyer et al. 2017; Fenton-May et al. 2013), but not all (Deymier et al. 2015). The inconsistency in these findings may be in part due to issues with methodology, for example here, where only IFN-α-2a was tested, and the GXR-25 cells used for infection were relatively insensitively to IFN, necessitating the use of IC_{80} rather than IC_{50}. Furthermore, the viral adaptation to IFN might occur outside of the *gag-pro* region. Further experiments taking into account these caveats are currently underway.

The finding here that baseline plasma RNA and proviral DNA loads are somewhat lower in IU HIV-infected males corroborates previous studies showing higher plasma viral loads in females in the first year of life (European Collaborative Study 2002). Also consistent with previous studies (Mori et al. 2015) female CD4+ T cell percentage levels over time were higher than males, although interestingly, not at birth. Additionally, higher CD8+ T cell percentage was found in male infants over time, resulting in lower CD4:CD8 ratios in males. The absolute differences were small (2-3% for CD4+ and CD8+ T cells and 0.3 for CD4:CD8) and since this is also true for HIV-uninfected children (European Collaborative Study 2003; B.-W. Lee et al. 1996; Foca et al. 2006) it is possible that these physiological differences do not represent any differential response to treatment nor immune function. That being said, if CD4+ T cell percentage in the peripheral blood compartment is representative of body-wide proportions, females might have higher density of HIV target cells, which could facilitate spread.

The UL cohort has not been followed up for long enough to draw conclusions with regard to sex differences in disease progression, demonstrated by the low numbers of children manifesting symptoms of immunocompromise (n=18), particularly given

that the background rate of serious bacterial infections and TB is high. However, this important data will be provided as the study continues over the coming years.

In keeping with another South African study, male and female children in the UL cohort had similar rates of plasma virological control on cART (Shiau et al. 2014). Without evidence to suggest otherwise, and one study in support (Simoni et al. 2007), it is presumed that male and female children are exposed to similar rates of ART non-adherence, which is why is should not be ignored that the cohort's best described cases of non-adherence with no documented subsequent plasma viral rebound occurred in males. Although not congruous with the higher rates of female elite control in HIV infection, male post-treatment control in infants may be a consequence of a viral reservoir in males that is not only smaller than in females but also one composed of IFNα-sensitive virus. In the setting of ART interruption, the IFNα-sensitive virus emerging in males from previously latently infected cells is potentially more subject to immune control. Of the three cases of paediatric post-treatment control described in the literature (Violari et al. 2019; Persaud et al. 2013; Frange et al. 2016), two were female, of which, one (the Mississippi Baby) rebounded. Of course there will have been many more undocumented cases so it is difficult to draw conclusions from such tiny numbers. Further studies involving planned analytical treatment interruption, as opposed to unplanned ART non-adherence, would be necessary to evaluate more fully the impact of sex differences on functional cure potential.

The benefit of studying HIV in the mother-to-child setting, is that it excludes the multiple and difficult to measure behavioural factors influencing adult HIV infection and disease. However, given the ontogeny of the immune system and differences between mucosal and transplacental HIV transmission, caution is required in extrapolating these findings to adults. That said, it is important to take into account the potential for increased female susceptibility to HIV infection across all age groups in the development of effective prevention strategies and to ensure this possibility is covered in interventional study design. Most importantly, even if there is a biological component

to the increased risk of adult female HIV infection, the sociocultural influences and gender disparities must continue to be addressed in order to curb the HIV epidemic.

In conclusion, HIV preferentially infects females *in utero*, with a virus phenotypically different from that of males, showing sexual selection in the MTCT of HIV. This may result in IU HIV-infected infants differing in latent HIV reservoir size and composition, which may differentially influence chances of post-treatment control and disease remission between the sexes. Further investigation into these sex differences will elucidate crucial information not only about the immune response early in life, but also mechanisms for HIV transmission across the placenta and subsequent pathogenesis.

Chapter 8

Conclusions

Point-of-care testing to diagnose and treat *in utero* HIV-infected infants in the first hours of life in KwaZulu-Natal, South Africa is feasible. However, this very early treatment initiation has little opportunity to confer benefit, when the rate of infant cART non-adherence is so high. Non-adherence is common because these infants are born to the tiny fraction of women for whom freely available HIV testing and cART during pregnancy was not accessible or acceptable to them. These antenatal barriers to cART usually continue postnatally, compromising infant treatment. Barriers include HIV stigma, poverty, maternal physical and mental health, and sociocultural reasons, all of which are interdependent and self-perpetuating, as demonstrated by second-generation mother-to-child HIV transmission. Most HIV-transmitting mothers do take some cART in pregnancy, and in addition infants are all given ART prophylaxis. This pre-treatment ART exposure limits the benefit of starting combination ART on the latent HIV reservoir in the first hours, rather than the standard days, of life in KZN, as well as increasing the complexity of infant diagnosis. Disproportionate female susceptibility to *in utero* HIV-infection occurs, and further exploration into this could give insights into the pathogenesis of HIV transmission and infection. Although very early cART alone is unlikely to cure *in utero* HIV-infected infants, anecdotal cases of possible remission from this study and others, give hope that there is potential for cure still to be explored.

8.1 What do these studies add?

My key contribution to the scientific community was the generation of, what is now a cohort of over 210 IU HIV-infected infants, the largest contemporary cohort of its type in the world. We currently have a bank of thousands of patient samples, which could provide the immunological and virological keys to move us closer to paediatric HIV cure. Furthermore, some of these children who have been studied in detail and have had long-term viral suppression since birth, could be candidates for HIV cure interventions, if and when they become available. What I have explored in this thesis, and published, are the clinical implications of very early diagnosis and treatment for IU HIV-infected infants in KZN, which are important findings in a practical sense for South African clinicians, as well as highlighting specific issues that can guide the focus of future paediatric HIV research.

I have had people, even respected South African scientists, criticize my conclusion that there is not enough evidence to warrant rushing to initiate cART in the first hours rather than days of life, in KZN. Their argument was, due to the high-rates of non-adherence there was not a fair assessment, and that the study should be repeated in a patient group with better adherence and social circumstances in order to see if remission can be achieved. But no such patient group exists, anywhere you look in the world, paediatric HIV in concentrated in the most poor and marginalised groups, and in its current form, cART is challenging to succeed with in those circumstances. Additionally, for whom are we trying to cure HIV? If it is for those populations most affected, such as in KZN, then that is where the intervention must be successful. This study demonstrated the failure of cART in a significant proportion of a large group of HIV-infected infants, which should motivate changing our approach to paediatric HIV-treatment in these settings. Most of the mothers on the study were returning to clinic even if they were not giving treatment at home, so if long-acting treatments were available, they would be feasible.

Although the UL study had high rates of treatment failure, there was still an opportunity to examine the effect of very early cART on latent HIV reservoir size in a subset of successfully treated infants. The major finding from this study was that no difference in reservoir size or decline could be observed between starting treatment in the first hours or days of life, most likely due to prophylactic ART exposure lowering the initial levels, along with suboptimal adherence and insensitive HIV DNA measures. Using a real world example, this study has refuted the finding that earlier cART is always better, but does not exclude that there are biological benefits of very early cART that either were masked by non-adherence, or unable to be detected due to the relative insensitivity of latent HIV reservoir measurements. Either way, low infant latent HIV reservoirs were not observed to confer protection from plasma viral rebound, likely due to significant episodes of cART non-adherence. Although I whole-heartedly agree that early treatment in HIV-infected infants dramatically reduces morbidity and mortality, I have not been able to find the evidence that extrapolating this to treating infants at birth produces observable benefit for the majority of infants, at least in this setting. I believe, instead, the focus needs to be on sustaining treatment once started.

My demonstration of the increasing diagnostic complexity for IU HIV-infected infants, including infected infants testing negative on multiple occasions, has really challenged the traditional definition of an *in utero* HIV-infected infant. PoC testing at birth can allow early, thus in some cases more accurate diagnosis confirmation, particularly if more sensitive confirmatory tests are undertaken. But in this quest for diagnosis, the declining prevalence thus low positive predictive value of tests in HIV-exposed infants must be kept in mind, as it translates to a high risk of false positive tests. The distinction between an HIV-infected and uninfected infant are blurred. In the absence of changes to tests or testing algorithms, the take home message for clinicians in South Africa, is that infant PCR testing must continue regularly, until well after prophylactic ART is completed, before a definitive diagnosis can be made.

8.2 The future of paediatric HIV cure research

8.2.1 Optimizing paediatric HIV treatment

In South Africa, an average sized HIV-infected 6 month old infant is required to take 17 mL of medication in the form of five separate syrups in the morning and 9.5 mL in three separate syrups at night, along with a pharmacy collection every 28 days. This high medication burden can impair medication adherence. Not only large volumes, but cold chain requirements, toxic excipients and foul-taste all equate to ARV suspensions being a poor choice of formulation. Better formulations are urgently required. Following on from the findings in this thesis, I have led the UL study's collaboration with the University of KwaZulu-Natal's paediatric infectious disease specialist Dr Moherndran Archary in order to put together a European-Developing Countries Clinical Trial Partnership grant submission for the Simplifying Antiretroviral Infant and Neonatal Treatment (SAINT) study, which aims to explore the feasibility, PK and success of solid ART preparations for IU HIV-infected infants in KZN, including a 4-in-1 powder for suspension (LPV, RTV, 3TC and ABC). The hope is that simpler and more palatable ART formulations will counteract at least some of the issues with non-adherence in this population, which is a good first step, but ultimately, long-acting or extended-release formulations are the goal. Advances in development for adult HIV treatment include; drug-releasing implants, transdermal patches, or injectables; either intramuscular long-acting ART or subcutaneous broadly neutralising monoclonal antibodies (bNAbs, Flexner 2019). However, in absolute numbers, children living with HIV are a small population, mostly located in LMICs. For these reasons, pharmaceutical companies have little to financially gain from researching and developing drugs for HIV-infected children. Extra effort needs to be made to ensure children are not left behind with innovative ART technologies, despite the difficulties determining doses with the dramatic weight and PK changes that occur early in life (Nachman et al. 2019). bNAbs are currently the most promising candidate for an HIV cure intervention. This is because bNAbs potentially have the additional benefits of assisting in cell killing and immunomodulation, which in non-human primate studies,

has shown promise for inducing HIV remission (Caskey et al. 2019). A bNAb trial in some of the UL children is planned, but is funding dependent.

8.2.2 Exploring *in utero* HIV sex differences further

The higher HIV susceptibility for females *in utero*, along with sexual selection of transmitted viruses are not just interesting findings. They tell us that immune sex differences in the materno-foetal barrier or foetus itself, are present, and clinically significant. Further exploration into this, might provide key information about how HIV transmits across the placenta and immune response in early life. These findings are also a reminder that any HIV prevention, treatment or cure intervention's efficacy must be demonstrated in both sexes before it can be deemed a success. As an extension of the UL study, I have organised cord blood collection and placental sampling from HIV-exposed and unexposed twin deliveries at the study hospital sites. To date, samples from 50 twin pairs, half of which are sex discordant, have been collected and stored. These samples will provide the opportunity to explore sex differences in *in utero* immune response in-depth, both peripherally and at the matero-foetal barrier.

8.2.3 Addressing cultural barriers to HIV treatment

A third of children who died on the UL study visited a traditional health practitioner in the child's final illness. It is not to say that traditional healing killed the children, but certainly the delay in definitive care contributed to mortality. The majority of black South Africans make use of traditional health practitioners and this can conflict with HIV treatment (Petzer and Mngqundaniso 2008). Dr Krista Dong of the Ragon Institute of MGH, MIT and Harvard recognises the potential for harnessing traditional health practitioners' social influence, in order to deliver HIV care in a more acceptable way. I established a partnership between Krista Dong's iTeach Programme and the UL study. iTeach (The Integration of TB in Eduation and Care for HIV/AIDS, `http://www.ragoninstitute.org/international/iteach/`) have trained and certified traditional health practitioners as HIV counsellors and placed them at a UL study site and surrounding local clinics. The plan is to conduct a mixed-methods study

examining the impact of this new service, which provides HIV care in a traditional and spiritual context.

8.2.4 Addressing social barriers to HIV treatment

Ultimately, the future of paediatric HIV research needs to look upstream. Investing heavily in the treatment and cure for a preventable illness, although currently necessary, needs to simultaneously address the social determinants for incidence and outcome. There is some truth to president Mbeki's infamous statement that it is not HIV but poverty that causes AIDS. HIV is a totally preventable and manageable infection, yet children and adults are more likely to die from HIV the further down the social gradient they are. One way to address this, is to provide South African women better opportunities for education and employment. The Females Rising through Education Support and Health (FRESH) study, recruits HIV negative unemployed women from the uMlazi township in KZN and enrols them on a 9 month life skills and empowerment programme, in which they also receive sexual education and free PrEP (Ndung'u et al. 2018). 80% of FRESH participants are in employment or back in education at the programme's completion, however, tragically, the HIV incidence for the women on the study is the same as the background rate in uMlazi (approximately eight infections per 100 person-years, Dong et al. 2017). Perhaps the benefit from the intervention occurs after the initial 9 month study period, or perhaps giving women education, employment and support, still does not enable women in a South African township to protect themselves from HIV. Men are clearly the missing link in HIV prevention, and given the large proportion of infants in the UL study born to mothers acutely infected during pregnancy, men also play a pivotal role in paediatric HIV-infection. There are future plans for the UL study to explore the role of the mother's male partner in the care of an HIV-infected child, but actually, there might be more success in exploring this antenatally for HIV-uninfected women, before she re-tests positive and infects her child.

8.2.5 Emerging issues

In the year 2020, one cannot complete a discussion, without addressing the SARS-Cov-2 pandemic. At the time of writing, SARS-Cov-2 incidence and mortality in South Africa was still rising, slowly but steadily (Karim 2020). The country has been in a strict lockdown for weeks, and many of the families on the UL study who relied on informal employment have lost their income. One of the study hospitals had a case of Covid-19, so the hospital staff went on strike and shut down not only the hospital but also the HIV clinic that provides ART to the study participants. It is likely that this type of chaos will contribute to treatment failure and morbidity in the UL children. On a larger scale, it has been estimated that if this pandemic interrupts ART delivery for as little as 6 months, HIV MTCT rates in sub-Saharan Africa will double (Jewell et al. 2020). This, on a background of an estimated >10% rise in child mortality from disrupted healthcare services and food insecurity (Roberton et al. 2020) will be catastrophic for child well-being in South Africa. Although SARS-Cov2 infection spares children directly, overall child health and HIV must not be forgotten during these unprecedented times.

8.3 Concluding remarks

Working on this project has taught me more about people than HIV, most of which is not written within these pages, but has been the most invaluable experience of my clinical career. I have been extremely privileged to have had the opportunity to build relationships with the mothers of the study, to watch their children grow, and try and understand their perspective; what their child's HIV diagnosis means to them, their expectations and fears. The majority of the mothers' concerns were due to the stigma associated with HIV, usually the fear of being rejected by their family and partner. To me it is unfathomable, in a population where nearly every second woman is HIV-infected, that the diagnosis can be unspeakable; and it is this silence that is deadly. How we fix this problem, I do not know, but without trying, we never will. We can know everything about a disease's pathogenesis, but without understanding

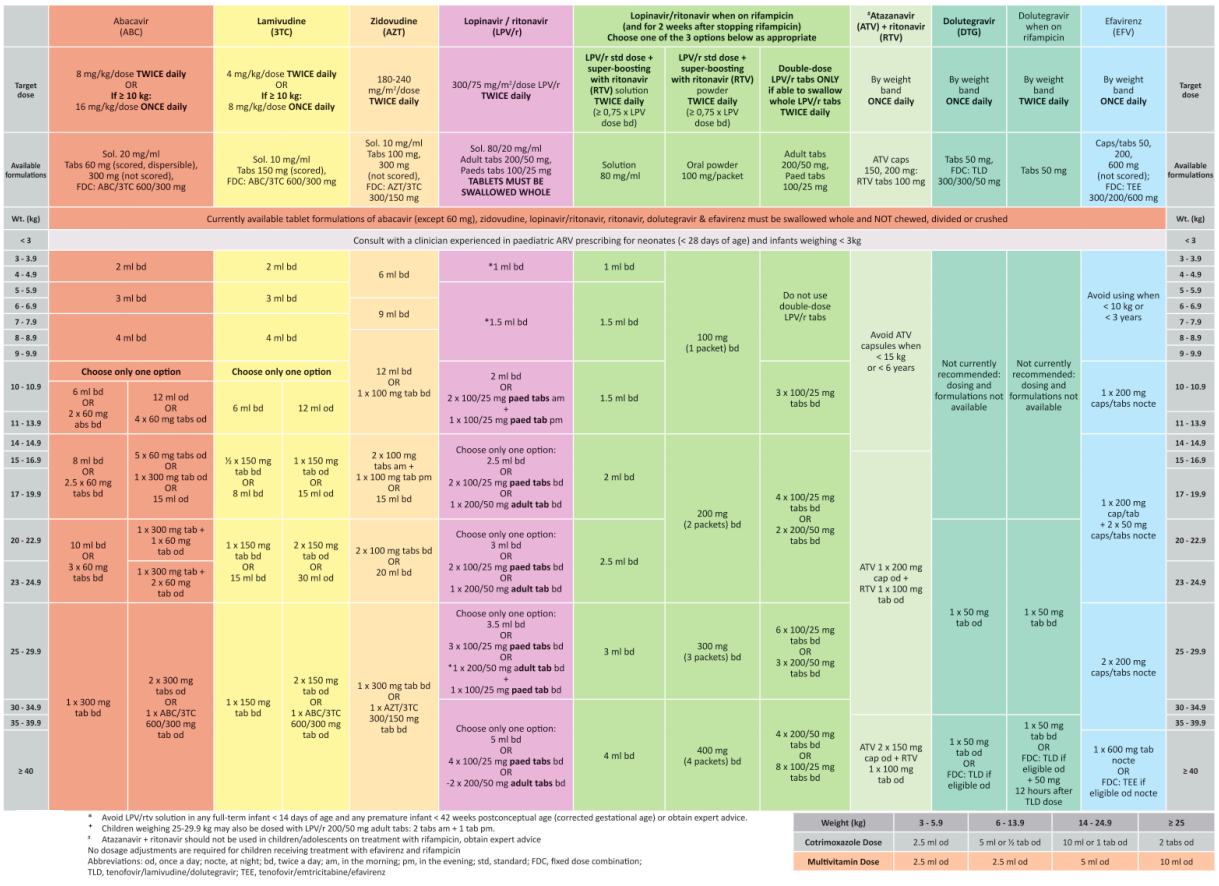
its complex relationship with people's environment, beliefs and behaviours we will be unable to prevent or treat it, let alone cure it.

"It always seems impossible until it's done"

- Nelson Mandela

Appendices

Appendix I: KwaZulu-Natal antiretroviral therapy dosing guidelines



	Abacavir (ABC)		Lamivudine (3TC)		Zidovudine (AZT)	Lopinavir / ritonavir (LPV/r)	Lopinavir/ritonavir when on rifampicin (and for 2 weeks after stopping rifampicin) Choose one of the 3 options below as appropriate			²Atazanavir (ATV) + ritonavir (RTV)	Dolutegravir (DTG)	Dolutegravir when on rifampicin	Efavirenz (EFV)	
Target dose	8 mg/kg/dose **TWICE daily** OR **If ≥ 10 kg:** 16 mg/kg/dose **ONCE daily**		4 mg/kg/dose **TWICE daily** OR **If ≥ 10 kg:** 8 mg/kg/dose **ONCE daily**		180-240 mg/m²/dose **TWICE daily**	300/75 mg/m²/dose LPV/r **TWICE daily**	**LPV/r std dose + super-boosting with ritonavir (RTV)** solution **TWICE daily** (≥ 0,75 x LPV dose bd)	**LPV/r std dose + super-boosting with ritonavir (RTV)** powder **TWICE daily** (≥ 0,75 x LPV dose bd)	**Double-dose LPV/r tabs ONLY if able to swallow whole LPV/r tabs TWICE daily**	By weight band **ONCE daily**	By weight band **ONCE daily**	By weight band **TWICE daily**	By weight band **ONCE daily**	Target dose
Available formulations	Sol. 20 mg/ml Tabs 60 mg (scored, dispersible), 300 mg (not scored), FDC: ABC/3TC 600/300 mg		Sol. 10 mg/ml Tabs 150 mg (scored), FDC: ABC/3TC 600/300 mg		Sol. 10 mg/ml Tabs 100 mg, 300 mg (not scored), FDC: AZT/3TC 300/150 mg	Sol. 80/20 mg/ml Adult tabs 200/50 mg, Paeds tabs 100/25 mg **TABLETS MUST BE SWALLOWED WHOLE**	Solution 80 mg/ml	Oral powder 100 mg/packet	Adult tabs 200/50 mg, Paed tabs 100/25 mg	ATV caps 150, 200 mg: RTV tabs 100 mg	Tabs 50 mg, FDC: TLD 300/300/50 mg	Tabs 50 mg	Caps/tabs 50, 200, 600 mg (not scored); FDC: TEE 300/200/600 mg	Available formulations
Wt. (kg)	Currently available tablet formulations of abacavir (except 60 mg), zidovudine, lopinavir/ritonavir, ritonavir, dolutegravir & efavirenz must be swallowed whole and NOT chewed, divided or crushed													Wt. (kg)
< 3	Consult with a clinician experienced in paediatric ARV prescribing for neonates (< 28 days of age) and infants weighing < 3kg													< 3
3 - 3.9	2 ml bd		2 ml bd		6 ml bd	*1 ml bd	1 ml bd	100 mg (1 packet) bd	Do not use double-dose LPV/r tabs	Avoid ATV capsules when < 15 kg or < 6 years	Not currently recommended: dosing and formulations not available	Not currently recommended: dosing and formulations not available	Avoid using when < 10 kg or < 3 years	3 - 3.9
4 - 4.9														4 - 4.9
5 - 5.9	3 ml bd		3 ml bd		9 ml bd	*1.5 ml bd	1.5 ml bd							5 - 5.9
6 - 6.9														6 - 6.9
7 - 7.9	4 ml bd		4 ml bd		12 ml bd OR 1 x 100 mg tab bd									7 - 7.9
8 - 8.9														8 - 8.9
9 - 9.9														9 - 9.9
10 - 10.9	Choose only one option		Choose only one option			2 ml bd OR 2 x 100/25 mg **paed tabs** am + 1 x 100/25 mg **paed tab** pm	1.5 ml bd		3 x 100/25 mg tabs bd				1 x 200 mg caps/tabs nocte	10 - 10.9
11 - 13.9	6 ml bd OR 2 x 60 mg abs bd	12 ml od OR 4 x 60 mg tabs od	6 ml bd	12 ml od										11 - 13.9
14 - 14.9														14 - 14.9
15 - 16.9	8 ml bd OR 2.5 x 60 mg tabs bd	5 x 60 mg tabs od OR 1 x 300 mg tab od OR 15 ml od	½ x 150 mg tab bd OR 8 ml bd	1 x 150 mg tab od OR 15 ml od	2 x 100 mg tabs am + 1 x 100 mg tab pm OR 15 ml bd	Choose only one option: 2.5 ml bd OR 2 x 100/25 mg **paed tabs** bd OR 1 x 200/50 mg **adult tab** bd	2 ml bd	200 mg (2 packets) bd	4 x 100/25 mg tabs bd OR 2 x 200/50 mg tabs bd	ATV 1 x 200 mg cap od + RTV 1 x 100 mg tab od	1 x 50 mg tab od	1 x 50 mg tab bd	1 x 200 mg cap/tab + 2 x 50 mg caps/tabs nocte	15 - 16.9
17 - 19.9														17 - 19.9
20 - 22.9	10 ml bd OR 3 x 60 mg tabs bd	1 x 300 mg tab + 1 x 60 mg tab od	1 x 150 mg tab bd OR 15 ml bd	2 x 150 mg tab od OR 30 ml od	2 x 100 mg tabs bd OR 20 ml bd	Choose only one option: 3 ml bd OR 2 x 100/25 mg **paed tabs** bd OR 1 x 200/50 mg **adult tab** bd	2.5 ml bd							20 - 22.9
23 - 24.9		1 x 300 mg tab + 2 x 60 mg tab od												23 - 24.9
25 - 29.9	1 x 300 mg tab bd	2 x 300 mg tabs od OR 1 x ABC/3TC 600/300 mg tab od	1 x 150 mg tab bd	2 x 150 mg tab od OR 1 x ABC/3TC 600/300 mg tab od	1 x 300 mg tab bd OR 1 x AZT/3TC 300/150 mg tab bd	Choose only one option: 3.5 ml bd OR 3 x 100/25 mg **paed tabs** bd OR *1 x 200/50 mg **adult tab** bd + 1 x 100/25 mg **paed tab** bd	3 ml bd	300 mg (3 packets) bd	6 x 100/25 mg tabs bd OR 3 x 200/50 mg tabs bd				2 x 200 mg caps/tabs nocte	25 - 29.9
30 - 34.9														30 - 34.9
35 - 39.9														35 - 39.9
≥ 40						Choose only one option: 5 ml bd OR 4 x 100/25 mg **paed tabs** bd OR -2 x 200/50 mg **adult tabs** bd	4 ml bd	400 mg (4 packets) bd	4 x 200/50 mg tabs bd OR 8 x 100/25 mg tabs bd	ATV 2 x 150 mg cap od + RTV 1 x 100 mg tab od	1 x 50 mg tab od OR FDC: TLD if eligible od	1 x 50 mg tab bd OR FDC: TLD if eligible od + 50 mg 12 hours after TLD dose	1 x 600 mg tab nocte OR FDC: TEE if eligible od nocte	≥ 40

* Avoid LPV/rtv solution in any full-term infant < 14 days of age and any premature infant < 42 weeks postconceptual age (corrected gestational age) or obtain expert advice.
+ Children weighing 25-29.9 kg may also be dosed with LPV/r 200/50 mg adult tabs: 2 tabs am + 1 tab pm.
² Atazanavir + ritonavir should not be used in children/adolescents on treatment with rifampicin, obtain expert advice
No dosage adjustments are required for children receiving treatment with efavirenz and rifampicin
Abbreviations: od, once a day; nocte, at night; bd, twice a day; am, in the morning; pm, in the evening; std, standard; FDC, fixed dose combination; TLD, tenofovir/lamivudine/dolutegravir; TEE, tenofovir/emtricitabine/efavirenz

Weight (kg)	3 - 5.9	6 - 13.9	14 - 24.9	≥ 25
Cotrimoxazole Dose	2.5 ml od	5 ml or ½ tab od	10 ml or 1 tab od	2 tabs od
Multivitamin Dose	2.5 ml od	2.5 ml od	5 ml od	10 ml od

KwaZulu-Natal paediatric ART dosing guideline for children born term, >3kg and >28 days of life. Reproduced with permission from the Step by Step Guide For the Management of Children and Adolescents on ART, Department of Health, Province of KwaZulu-Natal, 2017

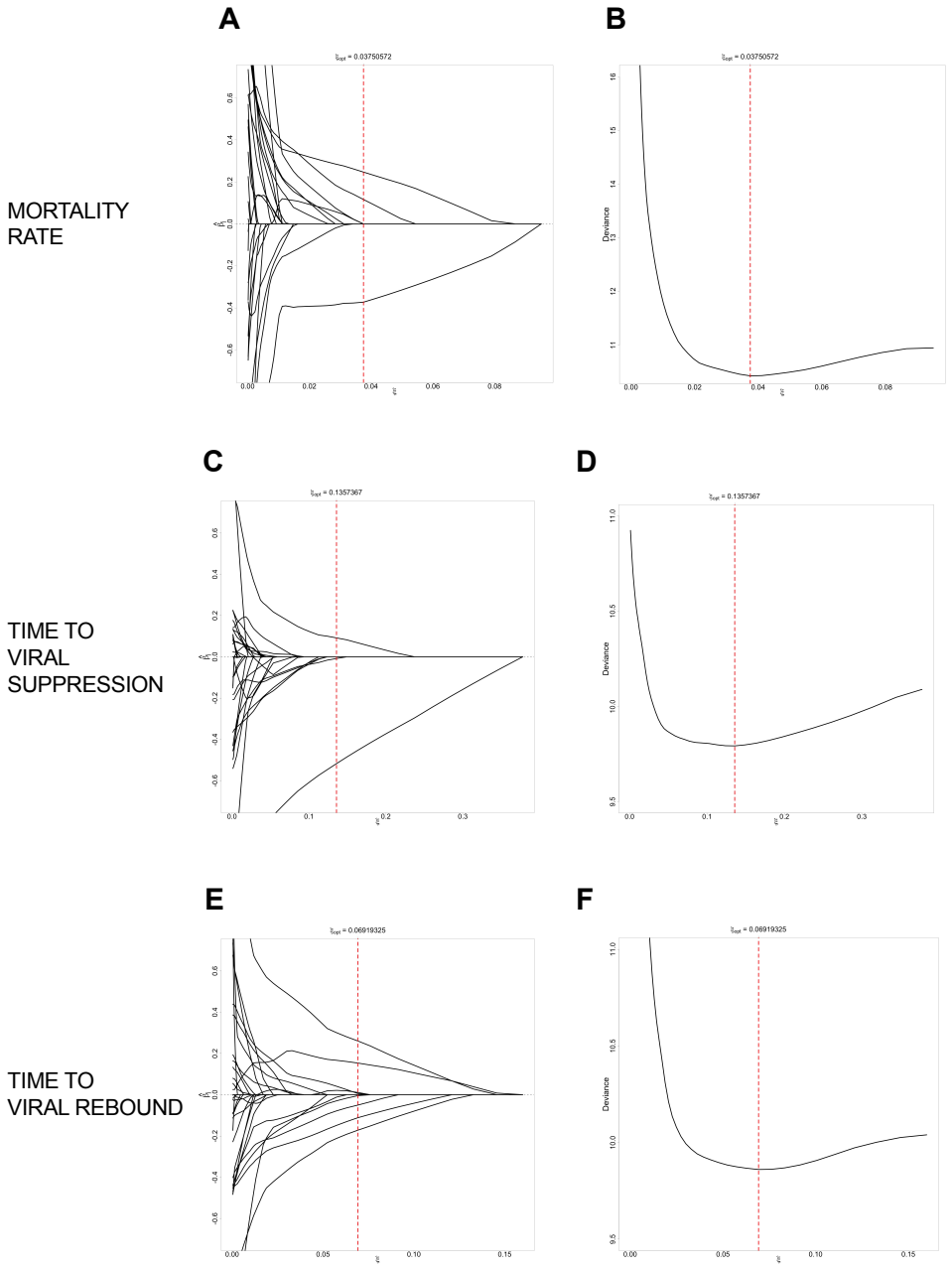
	Lamivudine (3TC)		Zidovudine (AZT)		Nevirapine (NVP)	
Target Dose	*2mg/kg/dose TWICE daily (BD)*		*4mg/kg/dose TWICE daily (BD)*		*6mg/kg/dose TWICE daily (BD)*	
Available formulation	10mg/ml		10mg/ml		10mg/ml	
Weight (kg)	*Dose in ml*	*Dose in ml*	*Dose in ml*	*Dose in ml*	*Dose in ml*	*Dose in ml*
≥ 2.5-<3.0	0.6ml BD	6mg BD	1.2ml BD	12mg BD	1.8ml BD	18mg BD
≥ 3.0-<3.5	0.7ml BD	7mg BD	1.4ml BD	14mg BD	2.1ml BD	21mg BD
≥ 3.5-<4.0	0.8ml BD	8mg BD	1.6ml BD	16mg BD	2.4ml BD	24mg BD
≥ 4.0-<4.5	0.9ml BD	9mg BD	1.8ml BD	18mg BD	2.7ml BD	27mg BD
≥ 4.5-<5.5	1.0ml BD	10mg BD	2.0ml BD	20mg BD	3.0ml BD	30mg BD
≥ 5.5-<6.5	1.2ml BD	12mg BD	2.4ml BD	24mg BD	3.6ml BD	36mg BD

KwaZulu-Natal paediatric ART dosing guideline for infants >2.5kg and <28 days of life. Reproduced with permission from the Step by Step Guide For the Management of Children and Adolescents on ART, Department of Health, Province of KwaZulu-Natal, 2017

DRUG	LAMIVUDINE	ZIDOVUDINE	NEVIRAPINE
Post-natal Age		D0 – D14 D15 – D28	
<30 weeks	2mg/kg BD	*2mg/kg BD*	150 mg/m^2 BD
>30 – 35 weeks	2mg/kg BD	*2mg/kg BD* 3 mg/kg BD	150 mg/m^2 BD

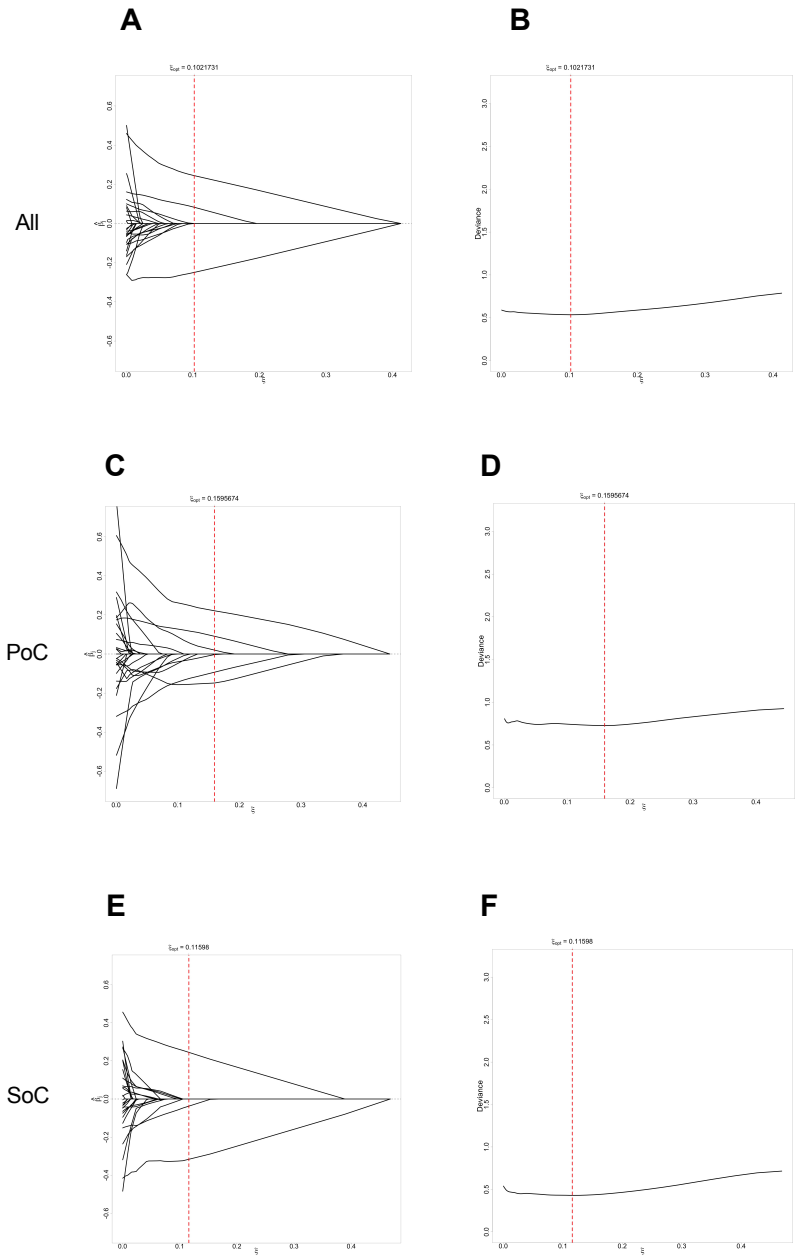
KwaZulu-Natal paediatric ART dosing guideline for infants <2.5kg or <35 weeks gestation at birth. Reproduced with permission from the Step by Step Guide For the Management of Children and Adolescents on ART, Department of Health, Province of KwaZulu-Natal, 2017

Appendix II: Penalised Cox models



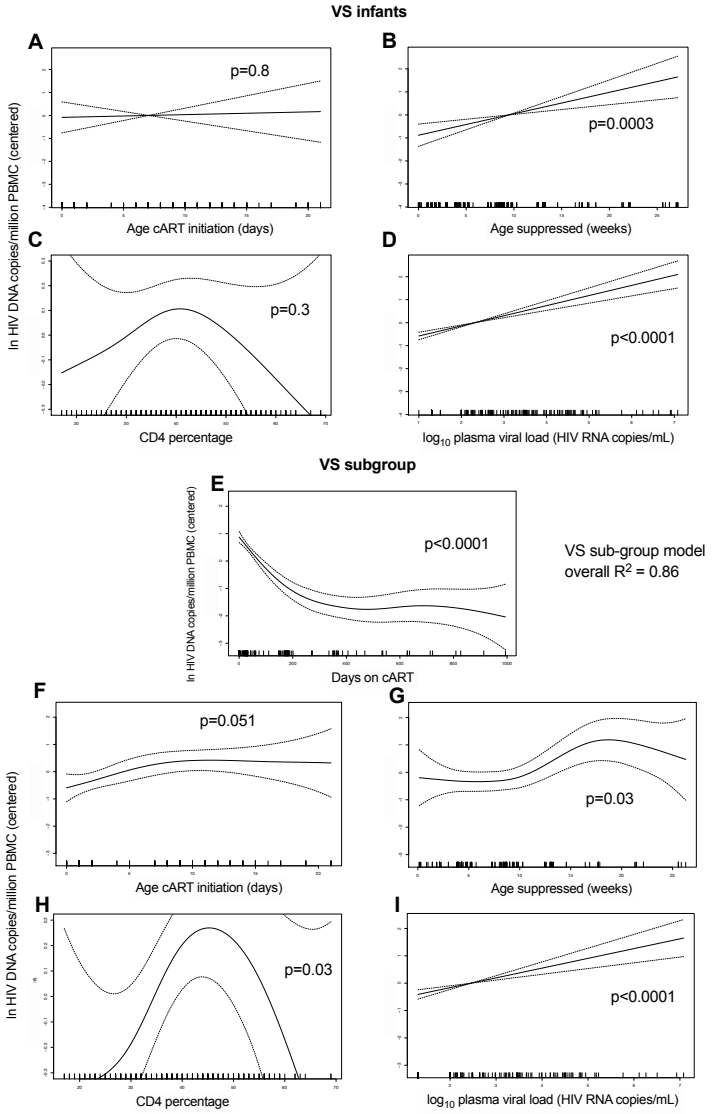
Penalised Cox regression models for the Ucwaningo Lwabantwana cohort. LASSO is a regularized form of regression, which avoids overfitting by shrinking (penalizing) the β-coefficients towards zero and is performed using the R package `glmnet`. (A,C,E) shows the LASSO-penalised Cox model coefficient paths with increasing penalty parameter ξ. The optimal value of the penalty parameter ξ is indicated by the red vertical dashed line. The final magnitude of β coefficient (y-axis) of the relevant covariates are determined by where they intersect the optimal penalty parameter ξ. (B,D,F) shows the cross-validation error (deviance) for 10-fold cross-validation. (A,B) shows data from the penalised Cox model for time to death, while (C,D) and (E,F) show the penalised Cox model for the time to viral suppression and rebound, respectively.

Appendix III: Baseline HIV DNA penalised regression models



Baseline HIV DNA: multivariate regression model. LASSO is a regularized form of regression, which avoids overfitting by shrinking (penalizing) the β-coefficients towards zero and is performed using the R package `glmnet`. (A,C,E) shows the LASSO-penalised linear regression model coefficient paths with increasing penalty parameter ξ. The optimal value of the penalty parameter ξ is indicated by the red vertical dashed line. The final magnitude of β coefficient (y-axis) of the relevant covariates are determined by where they intersect the optimal penalty parameter ξ. (B,D,F) shows the cross-validation error (deviance) for 10-fold cross-validation. (A,B) shows the LASSO-penalised regression model for all 164 infants, while (C,D) and (E,F) show the analysis of the Point-of-Care (PoC) tested infant and Standard-of-Care (SoC) infant subgroups, respectively.

Appendix IV: HIV DNA decay GAMM



Multivariate mixed model of longitudinal HIV DNA. Repeated HIV DNA measurements over time for the VS infants were modelled with a Generalised Additive Mixed Model (GAMM) using the `mgcv` R package, in order to visualise HIV DNA decay and determine the influence of other variables; both parametric (categorical) and smooth (continuous and potentially non-linear) terms. (A-D) show the adjusted influence of the separate variables for the VS infants (the effect of time is shown in Chapter 4, page 86), while (E-I) show the variables for the model with the subset of the VS infants after excluding those with low baseline levels and blips. The specific variable represented by each plot is written on the x-axis. Note in (F), the HIV DNA level is slightly lower for infants initiation cART <5 days of age. All the y-axes show ln HIV DNA copies per million PBMC, and is centred around the average value. GAMM are an excellent way to visualise the effect of a potentially non-linear variable, as they are not constrained to a specific formula. 95% CI intervals are denoted by the dotted lines and the dashes on the x-axis represent an individual measurement. Because of repeated measurements on infants, participant was incorporated as a random effect.

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