PEDIATRIC HIV-1 DRUG RESISTANCE IN BOTSWANA

By

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Thesis presented in fulfilment of the requirements for the degree of

MSc in Medical Virology

in the Faculty of Health Science at

Stellenbosch University

Division of Medical Virology, Department of Pathology

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December 2021

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DECLARATION

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ENGLISH - ABSTRACT

Antiretroviral therapy (ART) treatment in pregnant women and new-borns for prevention of mother-to-child transmission (PMTCT) can result in Human Immunodeficiency Virus drug resistance (HIVDR) in paediatrics; either transmitted or arising de novo after transmission. To our knowledge, there is paucity of data describing patterns of HIVDR in infants in Botswana. The aims of this dissertation were to describe HIVDR patterns in newly diagnosed infants in Botswana and determine the prevalence and role of HIVDR mutations in HIV-1 cell associated DNA (HIV-1 CAD) of early treated <18-month-old infants.

Seventy-eight residual dried blood spots (DBS) from <18-month-old HIV positive children from the Botswana HIV early infant diagnosis (EID) program (2016 - 2019) were available for HIVDR surveillance. Protease and reverse transcriptase regions were amplified and sequenced using ATCC HIV-1 Drug Resistance Genotyping kits and big dye chemistry. Surveillance drug-resistance mutations (SDRMs) were assessed using the Calculated Population Resistance program.

We analysed 257 proviral near full-length sequences (nFLS) obtained from peripheral blood mononuclear cells (PBMCs) of 27 infants in the Botswana early infant treatment (EIT) Study. Sanger sequencing of HIV *pol* was also performed for 22 maternal samples at delivery and at clinical failure in children. All nFLS and HIV *pol* sequences were analysed for the presence of HIVDR mutations using the Stanford HIV Drug Resistance database.

Of the 78 DBS samples, 32 (41%) were successfully amplified and sequenced. The median age was 2 months (IQR: 2-4). Three (9.7%) newly diagnosed infants had detectable SDRMs. Among these infants, one (33%) had a non-nucleoside reverse transcriptase inhibitor (NNRTI) HIV SDRM (K103N) detected and two (67%) had a detectable protease inhibitor (PI) SDRMs (M46L and L23I).

A total of 27 (56.2%) EIT infants had nFLS within one month of life. The median age at enrolment for infants was 2 days [Range: 2-3], with a median digital droplet PCR (ddPCR) HIV-1 CAD load value of 492 units [IQR: 78-1246]. Cell-associated HIVDR mutations were detected in 3/27 (11.1%) infants with intact HIV-1 CAD. A total of 106 (41.3%) intact sequences had at least 1 DRM; 29.2% had NNRTI, 7.5% NRTI, 0.9% PI and none with INSTI associated mutations. A total of 151 (58.7%) defective sequences had at least 1 DRM; 31.8% NNRTI, 15.2% NRTI, 5.3% PI and 15.5% INSTI associated mutations. Higher frequency of

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DRM was detected in defective HIV-1 CAD compared to intact infant HIV-1 CAD, although not statistically significant (p=0.14). Three infants had the same HIVDR mutations detected in their intact HIV-1 CAD and corresponding maternal plasma at delivery. Archived HIV-1 CAD HIVDR mutations were detectable at later clinical rebound on only one occasion.

In conclusion, we report relatively high rates of HIVDR mutations among newly diagnosed and early treated infants with known and unknown PMTCT exposure in Botswana. Our results suggest that exclusion of sequences with defects when interpreting HIVDR mutations from HIV-1 CAD is crucial as these may overestimate true mutations which may impact future clinical outcomes in children.

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AFRIKAANS - OPSOMMING (MAX. 500 WORDS)

Antiretrovirale behandeling (ART) in swanger vrouens en pasgebore babas wat moeder-tot-kind oordrag van menslike immuniteitsgebreksvirus (MIV) voorkom, kan lei tot middelweerstandige MIV in babas wat of oorgedra word of de novo kan ontstaan. Tans is daar min data beskikbaar wat patrone van MIV middelweerstandigheid (MIVMW) in babas van Botswana beskryf. Die doelwitte van hierdie tesis was om MIVMW patrone te beskryf in pas gediagnoseerde babas in Botswana en om die voorkoms en rol van MIVMW-mutasies in MIV-1-sel-geassosieerde deoksiribonukleïensuur (DNS) (MIV-1 SGD) van vroegbehandelde babas jonger as 18 maande te bepaal.

Agt-en-sewentig residuele gedroogde bloedvlekke (GBV) van MIV-gediagnoseerde kinders jonger as 18 maande oud wat by die Botswana program vir vroeë baba-diagnose (VBD) (2016-2019) gewerf was, was beskikbaar vir MIVMW bestudering. Protease- en tru-transkriptase gene is geamplifiseer en volgorde bepaling is gedoen met behulp van ATCC MIV-1MW genotiperings kits en BigDye™ chemie. Middelweerstandige mutasies monitering (SDRMs) is geondersoek deur die Berekende Bevolkingsweerstandsprogram.

Twee honderd sewe-en-vyftig provirale naby-vollengte volgordes (nFLS) van perifere mononukleêre selle van 27 babas, van die studie vir vroeë babas in Botswana, is geanaliseer. Sanger-volgordebepaling van MIV pol is ook uitgevoer vir 22 monsters van moeders met geboorte en wanneer kinders klinies faal. Alle nFLS- en HIV-pol *volgordes* is geanaliseer vir die teenwoordigheid van MIVMW-mutasies deur gebruik te maak van die Stanford HIV Drug Resistance databasis.

Twee-en-dertig (41%) van die 78 GBV-monsters is suksesvol geamplifiseer en die volgorde is bepaal. Die mediaan-ouderdom was 2 maande (IQR: 2-4). Drie (9,7%) pas gediagnoseerde babas het opspoorbare SDRMs gehad. Van hierdie babas het een (33%) 'n nie-nukleosied tru-transkriptase-inhibitor (NNRTI) MIV SDRM (K103N) gehad en twee (67%) het protease-inhibitor (PI) SDRMs (M46L en L23I).

Sewe-en-twintig (56,2%) EIT-babas het binne een maand van hul lewe nFLS gehad. Die mediaan ouderdom by die inskrywing vir babas was 2 dae [Reeks: 2-3], met 'n mediaan digitale druppel PCR (ddPCR) MIV-1 SGD-laadwaarde van 492 eenhede [IQR: 78-1246]. Sel-geassosieerde MIVMW-mutasies is opgespoor in 3/27 (11,1%) babas met ongeskonde MIV-1 SGD. Honderd en ses (41,3%) ongeskonde volgorde bepalings het ten minste een middelweerstandige mutasie gehad; van die 29,2% was NNRTI, 7,5% NRTI, 0,9% PI en geen

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het INSTI geassosieerde mutasies gehad nie. Altesaam 151 (58,7%) defektiewe volgordes het ten minste een middelweerstamdige mutasie gehad; 31,8% was NNRTI, 15,2% NRTI, 5,3% PI en 15,5% INSTI was geassosieerde mutasies. 'n Hoër frekwensie van middelweerstandige mutasies is opgespoor in defektiewe MIV-1 SGD in vergelyking met ongeskonde MIV-1 SGD, alhoewel nie statisties beduidend nie ($p = 0.14$). Dieselfde MIVMW-mutasies is opgespoor in ongeskonde MIV-1 SGD en ook in die ooreenstemmende moeder plasma tydens die geboorte in drie van die babas. Geargiveerde MIV-1 SGD MIVMW-mutasies was optelbaar tydens ‘n kliniese terugslag slegs een keer.

Ter afsluiting, ons rapporteer relatief hoë koerse van MIVMW-mutasies onder pas gediagnoseerde en vroegbehandelde babas met bekende en onbekende blootstelling aan PMTCT in Botswana. Daarom is die uitsluiting van volgordes met defekte is noodsaaklik tydens die interpretasie van MIVMW-mutasies van MIV-1 SGD, aangesien dit ware mutasies kan oorskat wat die toekomstige kliniese uitkomste by kinders kan beïnvloed.

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ACKNOWLEDGEMENTS

I would like to thank God Almighty for his unending grace and giving me the opportunity to do my post graduate studies.

I would like to extend my sincerest gratitude to the following people who have assisted me in preparation and submission of this thesis:

1. **Prof. Gert U VanZyl** at Stellenbosch University who has guided and mentored me throughout my project despite the interruptions due to COVID-19 travel restrictions and otherwise. He has provided excellent wisdom and advice which will remain with me throughout my career as a biomedical researcher. The inspiration Prof VanZyl has given me by showing his in-depth knowledge about HIV virology remains unparalleled.

2. **Dr Sikhulile Moyo** at the Botswana Harvard Partnership (BHP) who has been my mentor since my career debut, with guidance in data analysis, manuscript writing as well as general career advice which will forever be cherished and carried onto my next career associated endeavours. Dr Moyo has also been a great father figure to me with guiding wise words each time I would feel overwhelmed about my project and life in general.

3. **Dr Simani Gaseitsiwe** at the BHP who helped remove my training wheels when I was fresh out of University. He has been my mentor since 2015 and has since guided me in the path toward building my career in HIV and co-infection research. I would like to also thank Dr Simani for assisting me with funding opportunities from Fogarty and SANTHE without which I would not have been able to begin and get to finish my MSc.

4. The BHP Research laboratory team with their undying support across all my projects and assisting me with laboratory work, listening to my presentations and providing valuable feedback as well as helping me with my thesis write up. Special mention goes to Ms Ontlametse Bareng, Mrs Segomotso Maphorisa, Mr Mompati Mogwele, Ms Kwana Lechiile, Mrs Nametso Kelentse, Mr Patrick Mokgethi, Ms Bonolo Phinius, Ms Lynette Bhebhe.

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5. I would like to also acknowledge the BHP management for allowing me the opportunity to do my MSc as well as allowing me to assist with COVID-19 testing which is now hitting our country hard since January 2021. Dr Joseph Makhema, Mr Terence Mohammed, Mr Comfort Maphorisa.

6. The fellow students at Stellenbosch University (Division of Medical Virology) who were always very kind and offering me their assistance including showing me around the laboratory and around Cape Town in general. Special thanks to Kirsten Veldsman, Shahieda Isaacs, Mary Grace Katusiime, Kayla Delaney, Carli Gordjin, Shalena Naidoo, Ian Botha.

7. The collaborators at the T.H Chan Harvard School of public health, Harvard Medical School, Ragon institute and the Brigham and Women's Hospital, who assisted me with the second AIM of my project via the EIT study. Special thanks to Dr Roger Shapiro for allowing me access to the EIT sequence data.

8. I would also like to acknowledge my funders; The Sub-Saharan African Network for TB/HIV Research Excellence (SANTHE), NIH Fogarty and the Poliomyelitis Research Foundation (PRF) without whom this project may have not been possible. SANTHE has provided me with funding since my graduate internship and continues to build my capacity as a biomedical researcher. Special thank you to Prof Thumbi Ndungu and Dr Dennis Chopera.

9. I would like to thank to my mother (Martha R. Moraka) and my sister (Ofentse B. Ntshabele) for their unending support in this very difficult road, they have supported me even when they did not understand what was fully going on and continue to do so every day.

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10. I would like to thank my thesis advisory committee Dr Shahin Lockman, Prof Susan Engelbretcht and Prof Wolfgang Preiser who believed that my project is worth doing and guided me especially in the first lag.

11. A special thank you to Dr Catherine Koofhethile for her mentorship and guidance on my thesis.

12. Last but not least I would like to thank my friends who continue to be my support system throughout my MSc and career in general; Ms Itumeleng Mosweu, Ms Rorisang Oautlwa Mokgwathise and Mr Ogopotse Martin Koofhethile.

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DEDICATION

I would like to dedicate this thesis to my mother **Martha Rita Moraka** and my sister **Ofentse Beverley Ntshabele** who have raised me without any complaints and have always encouraged me to reach further than the stars. My mother as my role model, has always taught me that if you want anything in life you have to work hard for it her patience, encouragement and guidance will always be the best things I have learnt from her. My sister has always been an inspiration to me and always tells me to go for what I love regardless of how hard it may be.

I would also like to dedicate this thesis to two Angels who left this earth too early. My late young sister **Thatoyaone Faith Moraka** and my niece who never got the chance to cross into this world before going back to heaven in 2020, **Wamorena Ntshabele.**

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RESEARCH OUTPUTS AND ACHIEVEMENTS

Below are the research outputs and conferences attended during this MSc project.

Publications:

Moraka N.O, Garcia-Broncano P, Hu Z, Ajibola G, Pretorius-Holme M, Maswabi K, Maphorisa C, Mohammed T, VanZyl G.U, Gaseitsiwe S, Kuritzkes D, Lichterfeld M, Moyo S and Shapiro R L, **Patterns of pre-treatment drug resistance mutations of very early diagnosed and treated infants in Botswana.** AIDS Journal **[submitted, under review]**

International Conference Attendance:

Oral Presentations:

Moraka N.O, MSc Progress Report: **Virologic characteristics of vertically transmitted HIV-1 strains in Botswana.** The Sub-Saharan African Network for TB/HIV Research Excellence (SANTHE) AGM 2020, Virtual.

Moraka N.O, MSc Proposal: **Virologic characteristics of vertically transmitted HIV-1 strains in Botswana.** The Sub-Saharan African Network for TB/HIV Research Excellence (SANTHE). Epidemiology meets Phylogenetics. Durban, South Africa.

Poster Presentations:

Moraka N.O, Garcia-Broncano P, Hu Z, Ajibola G, Pretorius-Holme M, Maswabi K, Maphorisa C, Mohammed T, VanZyl G.U, Gaseitsiwe S, Kuritzkes D, Lichterfeld M, Moyo S and Shapiro R L. **HIV-1 drug resistance mutations in proviruses of early treated infants in Botswana.** IAS 2021, Virtual [submitted]

Natasha O. Moraka, Sikhulile Moyo, Ditiro Setlhare, Mompati Mogwele, Segomotso Maphorisa, Nametso Kelentse, Patrick Mokgethi, Julia Ngidi, Gert U. VanZyl, Simani Gaseitsiwe. **Prevalence of HIV-1 drug resistance mutations in newly diagnosed infants in Botswana.** AIDS2020, Virtual.

Natasha O. Moraka, Sikhulile Moyo, Ditiro Setlhare, Mompati Mogwele, Segomotso Maphorisa, Nametso Kelentse, Patrick Mokgethi, Julia Ngidi, Gert U. VanZyl, Simani Gaseitsiwe. **Prevalence of HIV-1 drug resistance mutations in newly diagnosed infants in Botswana.** Virology Africa, Cape Town, South Africa.

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Trainings:

Below is a list of trainings attended during this MSc project.

Training	**Date & Venue**	**Capacity**
CANTAM Network Scientific writing and publishing	6 September 2020, Virtual	Trainee
1st Virtual AAD 2020: Faculty of Medicine & Health Sciences, Stellenbosch University	26 – 27 August 2020, Virtual	Student/Delegate
H3ABioNet Introduction to Bioinformatics Training 2020	04 May – 31 July 2020, Virtual	Trainee
Epidemiology meets Phylogenetics	9 - 13 September 2019, Durban, South Africa	Trainee
Introduction to Molecular phylogenetics and virus evolution	18-21 February 2019, Gaborone, Botswana	Trainee

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LIST OF ABBREVIATIONS

3TC Lamivudine

A3G APOBEC3G

ABC abacavir

AIDS acquired Immunodeficiency syndrome

ART antiretroviral therapy

ARVs antiretroviral drugs

ATCC American Type Culture Collection

ADR acquired drug resistance

CAD cell associated DNA

CCR5 C-C chemokine receptor type 5

CD4 cluster of differentiation 4 (glycoprotein)

CDC Centers for Disease Control and Prevention

CNS central nervous system

CRFs circulating recombinant forms

CTL cytolytic T lymphocyte

d4T Stavudine

DBS dried blood spot

ddPCR droplet digital polymerase chain reaction

DNA deoxyribonucleic acid

DRM drug resistance mutations

DTG Dolutegravir

EDTA ethylenediaminetetraacetic acid

EFV Efavirenz

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EID early infant diagnosis

EIT early infant treatment

FLIP Full-Length Individual Proviral Sequencing

GALT Gut-associated lymphoid tissue

HAART highly active antiretroviral therapy

HIV human Immunodeficiency virus

HIVDR HIV drug resistance

HLA human leukocyte allele

IN integrase

kb kilobases

KS Kaposi's sarcoma

LPV/r lopinavir-ritonavir

LLV low level viremia

LTNP long-term non progressors

LTR long terminal repeat

MHC major histocompatibility complex

MTCT Mother to child transmission

NK natural killer cells

NRTI nucleoside or nucleotide reverse transcriptase inhibitor

NNRTI non-nucleoside reverse transcriptase inhibitor

NVP nevirapine

PBMC peripheral blood mononuclear cell

PCR polymerase chain reaction

PDR pre-treatment drug resistance

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PHA phytohemagglutinin

PJP Pneumocystis jiroveci Pneumonia

PI protease Inhibitor

PR Protease

PLWHIV people living with HIV

PMTCT Prevention of mother-to-child transmission

RNA ribonucleic acid

RPP30 ribonuclease P/MRP Subunit P30

RT reverse transcriptase

SDRM surveillance drug resistance mutations

SGA single Genome Amplification

SSA sub-Saharan Africa

SIV simian immunodeficiency viruses

TB tuberculosis

TBE Tris/Borate/EDTA

TDR transmitted Drug Resistance

UNAIDS United Nations Programme on HIV/AIDS

UTT universal test and treat

WHO World Health Organisation

WLWHIV women living with HIV

VF virologic Failure

VL viral load

VOA viral outgrowth assay

ZDV zidovudine

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CHAPTER ONE

1.1 INTRODUCTION AND LITERATURE REVIEW

1.1.1The history and epidemiology of HIV

Cases describing the Human Immunodeficiency Virus (HIV) were first reported in the early 1980s(1) where it was identified as an immunosuppressive syndrome that affected homosexual men(2) as well intravenous drug users.(3) By 1984 , it was evident that other individuals were also at risk as the virus was also transmissible through heterosexual contact, blood products such as factor VIII for haemophiliacs, blood transfusion and mother to child transmission.(4, 5) It was called acquired immunodeficiency syndrome (AIDS) as it is acquired, unlike cases of primary immunodeficiency, which are inherited. Although most of the first reported cases of AIDS were reported in the United States of America, the reality was that HIV/AIDS had already been widespread in many parts of the world.(4)

Currently, an estimated 38 million people are living with HIV (PLWHIV) globally, 1.8 million are estimated to be children below the age of 15 years according to Joint United Nations Programme on HIV/AIDS (UNAIDS) estimates in 2019.(6) In 2019 there were 1.7 million new infections and 690 000 HIV related deaths worldwide. Similarly, the number of HIV related deaths in children in 2019 was estimated to be 95 000. East and Sub-Saharan Africa remain the most severely affected regions worldwide, contributing 20.7 million PLWHIV, 15.0 million of which are estimated to be on treatment.

Although Botswana is among the countries with a successful treatment program for people living with HIV, it remains one of the most affected countries in the world.(7) HIV-1 group M subtype C predominates in Botswana. The latest HIV survey carried out in Botswana revealed a prevalence of 18.5% people living with HIV above 1.5 years of age; with an annual incidence of 1.38%.(8) Within this population, 2.2% are infants below 18 months. Despite prevention mother to child transmission of HIV programs (PMTC), HIV positive infants in Botswana remain a national health problem with 9000 children less than 14 years of age living with HIV, 60% of which are receiving treatment.(9)

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1.1.2 HIV classification

HIV belongs to the genus Lentivirus (which means slow virus) included in the Retroviridae family and Orthoretrovirinae subfamily.(10) Retroviral infections have been associated with different disease manifestations in vertebrates including immune deficiencies and several malignancies, anaemia, and autoimmune diseases.(11, 12)

There are two types of HIV namely HIV-1 and HIV-2 which differ in genetic makeup and pathogenesis/virulence(13), a study has revealed that there is only 55% similarity in genetic makeup of the two HIV types.(14) HIV-1 is further divided into three groups namely the major (M), new (N) and outlier (O) groups.(14) Group M is further divided into ten different phylogenetically associated subtypes A, B, C, D, F, G, H, J, K. Recombination of different subtypes, when found in epidemiologically unlinked cases, indicating the circulation of inter-subtype recombinant lineages are referred to as circulating recombinant forms (CRFs).(14)

HIV has entered humans by transmission from a non-human primate host infected with simian immunodeficiency viruses (SIV) which infect non-human primates. Phylogenetic evidence from blood samples from humans and faecal samples from chimpanzees indicate that an SIV of *Pan troglodytes troglodytes* species of chimpanzee (SIV*cpz*) gave rise to HIV-1 infection in humans. Moreover, this chimpanzee species is found in Southern Cameroon and surrounding areas where most HIV-1 groups and the highest HIV diversity are found.(15, 16) The SIV of the sooty mangabey monkey (SIV*sm*) is most closely related to HIV-2 infection in humans and the putative source-species of HIV-2 cross-species transmission.(17) SIV appears to be non-pathogenic in some natural hosts that have coevolved with SIV, such as African green monkeys and sooty mangabeys whereas it causes immune suppression and AIDS in other species of non-human primates.(18, 19) Figure 1 below shows the phylogenetic relationship between HIV and SIV with other primate lentiviruses, indicating a probable link between zoonotic immunodeficiency causing retroviruses in primates and human HIV infection.(20)

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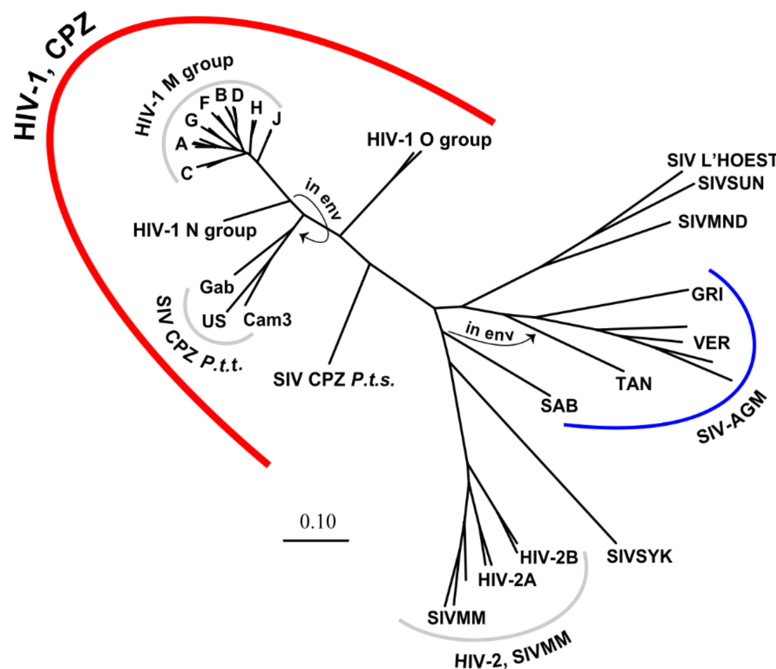

Figure 1: Phylogenetic relationship between human and primate lentiviruses (Source: Kuiken, C., Foley, B., Hahn, B., Marx, P., McCutchan, F., Mellors, J. W., Mullins, J., Wolinsky, S. & Korber, B.(1999). A compilation and analysis of nucleic acid and amino acid sequences. In Human Retroviruses and AIDS. Los Alamos, New Mexico: Theoretical Biology and Biophysics Group, Los Alamos National Laboratory. Public domain image obtained via Wikimedia Commons, https://commons.wikimedia.org/wiki/File:HIV-SIV-phylogenetic-tree.svg, current version dated 20 April 2008)

1.1.3 Structure of HIV

The HIV virion (Figure 2) is spherical in shape of approximately 100nm in diameter.(21) The HIV virion is comprised of the envelope (*env*), which is an outer lipid bilayer made up of the host cell membrane, human leukocyte allele (HLA)/major histocompatibility complex (MHC) components, as well as a glycoprotein of 160 kD (gp160), which is responsible for binding and entry into host cell. The gp160 is cleaved into gp120 and gp41 post translation.(21, 22) The C-terminal subunit, gp41, which can be referred to as the transmembrane protein, contains a cytoplasmic domain which facilitates the conformational change which is necessary for fusion of the virion to the host cell.(22) The inside of gp41 is associated with the matrix protein p17. The N-terminal subunit, gp120, also known as the docking glycoprotein, is found outside the viral membrane and is divided into five conserved regions (C1-C5) combined with five variable regions (V1-V5).(22) The host receptor cluster of differentiation 4 (glycoprotein) (CD4) interacts with residues in the conserved regions of gp120 on either side of V4, and the

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coreceptor C-C chemokine receptor type 5 (CCR5) interacts both with a GPGR/Q motif at the apex of the V3 loop and at its base.(21, 22)

The HIV virion has a cone shaped nucleocapsid core which is made up of the major core protein p24, this core surrounds two copies of the viral ribonucleic acid (RNA) covered by two smaller core proteins p6 and p7 as well as virally encoded enzymes reverse transcriptase (RT), protease (PR) and integrase (IN).(21)

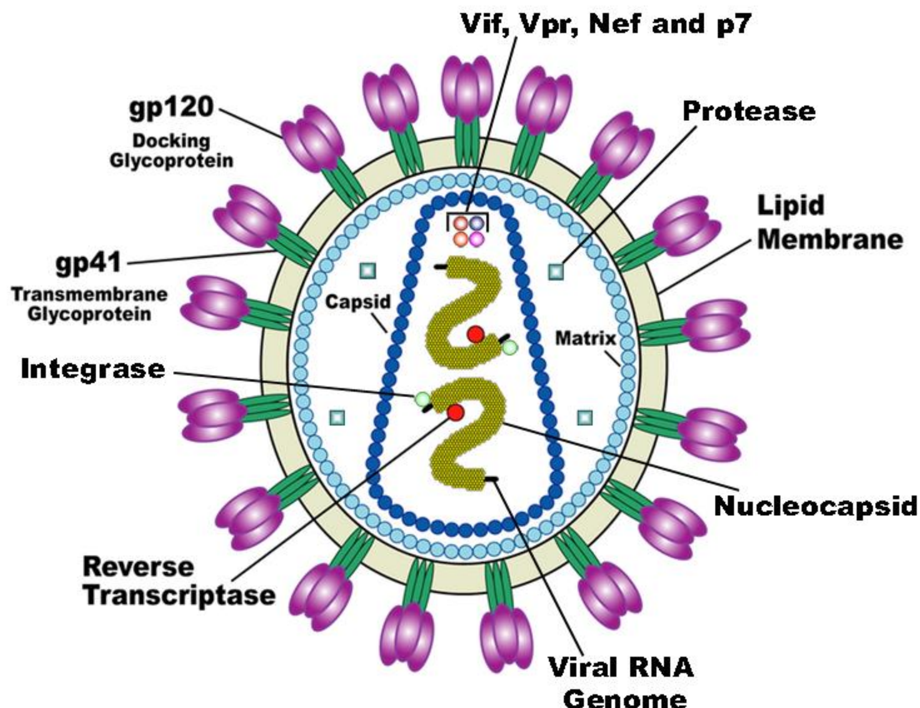

Figure 2: Structure of the HIV virion (Source: US National Institute of Health (redrawn by en:User:Carl Henderson), Public domain image obtained via Wikimedia Commons, https://commons.wikimedia.org/wiki/File:HI-Virion-en.png, current version dated 4 April 2009)

1.1.4 HIV genome

The HIV genome is approximately 9.7 kilobases long single-stranded, positive-sense RNA.(22, 23) The genome has many open reading frames that code for a number of viral proteins which consist of (*gag*, *pol*, and *env*), regulatory genes (*tat*, and *rev*), and accessory genes (*vif*, *vpr*, *nef*, and *vpu*).(21, 22) Figure 3 below shows the HIV genome structure and Table 1 shows the different genes and their functions;

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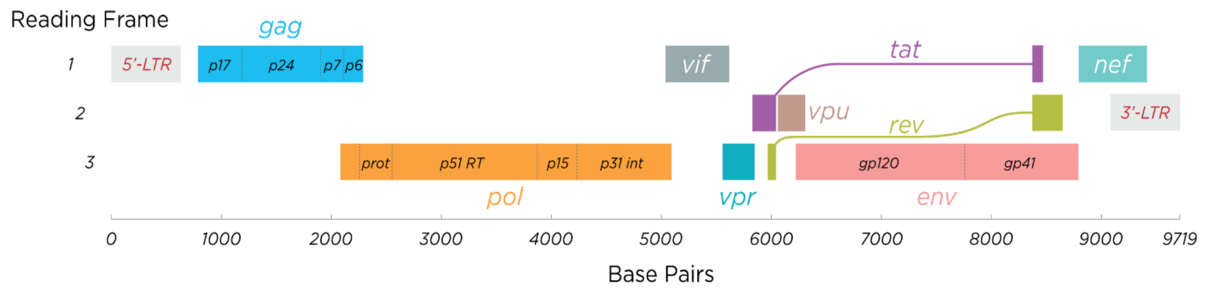

Figure 3: HIV Genome Structure (Source: Obtained from the WikiMedia Commons page for this image: Thomas Splettstoesser (www.scistyle.com), CC BY-SA 3.0 <https://creativecommons.org/licenses/by-sa/3.0>, via Wikimedia Commons)

Table 1: HIV genes and proteins and their functions (Adapted from Levy, 2007)

Proteins	Size (kDa)	Function	Abbreviation
Gag	p24	Capsid (CA), structural protein	CA
	p17	Matrix (MA) protein, myristoylated	MA
	p7	Nucleocapsid (NC) protein, helps in reverse transcription	NC
	p6	Role in viral budding (L domain)	-
Polymerase	p66, p51	Reverse Transcription (RT): RNase H - inside core	RT
Protease	p10	Gag/Pol cleavage and maturation	PR
Integrase	p32	Viral cDNA integration	IN
Env	gp120	Envelope surface protein	SU
	gp41	Envelope transmembrane protein	TM
Tat	p14	Transactivation	Tat
Rev	p19	Regulation of viral mRNA expression	Rev
Nef	p27	Pleiotropic, can increase or decrease virus replication	Nef
Vif	p23	Promotes virion maturation and infectivity	Vif
Vpr	p15	Helps in virus replication, transactivation	Vpr
Vpu	p16	Helps in virus release, disrupts gp160:CD4 complexes	Vpu
Vpx	p15	Helps in entry and infectivity (Only in HIV-2 and SIV)	Vpx

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1.1.5 HIV pathogenesis

1.1.5.1 The HIV Life Cycle

The replication cycle of HIV occurs in several sequential stages. The first step during HIV infection is binding and fusion where the virus fuses with the CD4 cell assisted by the *env* protein with the capsid entering the cell. The binding of gp120 to the CD4 molecule causes conformational change which exposes the coreceptor binding site, a complex is formed between the three components leading to further change in the gp120 and gp41 molecules; this is followed by fusion of lipid bilayer on the host cell with the virus and injection of the viral core into the cell where the nucleocapsid is uncoated and released.(24) Following is the reverse transcription which forms double stranded deoxyribonucleic acid (DNA) which is translocated to the nucleus and integrated into the host cell genome forming what is called a "provirus".(25) The next step involves transcription and translation of the viral genes from the provirus, leading to viral assembly where new viral particles are produced and bud out of the cell.(24) As the reverse transcriptase lacks proofreading ability, HIV replication has a high error rate and hence a high mutation rate. Although many mutations are deleterious for viral survival, a few may offer a survival advantage. The ability of HIV to rapidly evolve in response to pressures issued by the environment has given it the means of continual adaptation, thus it can avoid selective forces such as immune system responses and antiretroviral drug treatment. Figure 4 below shows a detailed diagram of the HIV replication cycle;

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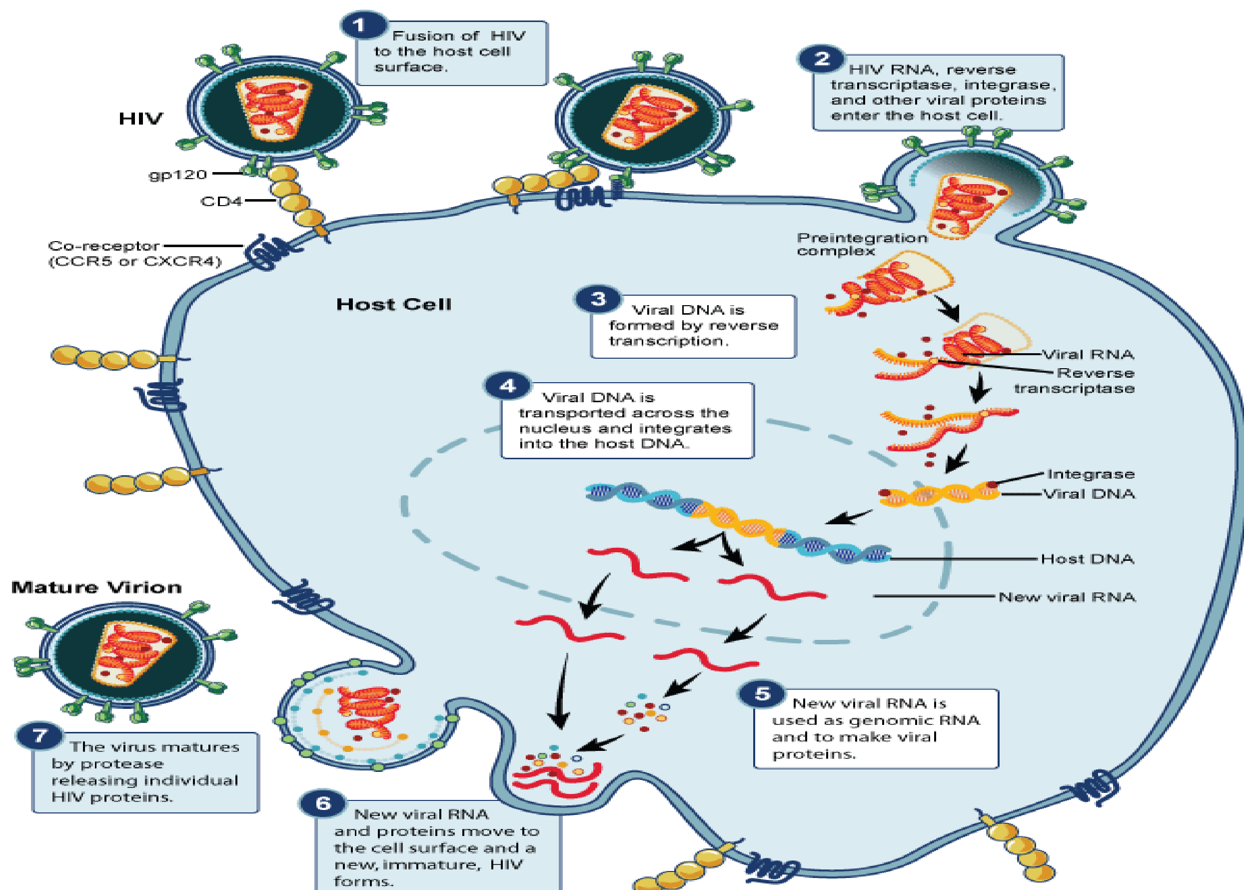

Figure 4: The HIV Replication Cycle (Source: NIAID. Public domain image obtained via Wikimedia Commons, https://commons.wikimedia.org/wiki/File:HI-Virus_Replication_Cycle_(5057022555).jpg, current version dated 1 April 2018)

1.1.5.2 HIV latency and persistence

HIV is able to stay dormant and hidden where its genes remain integrated into the host genome, thus allowing it to persist even with treatment; this is the challenge faced with regards to eradication of HIV, the subset of HIV infected cells that remain dormant is called the "latent reservoir" and is established very early following HIV infection.(21, 26) Latent HIV has been observed in several cell types including but not limited to peripheral blood mononuclear cells (PBMCs) such as monocytes/macrophages, dendritic cells as well as naïve and memory T-cells.(27, 28) The resting memory CD4+ T cells have been shown to be the major HIV reservoir as they have a half-life that goes up to 44 months, compared to only less than 6 hours for HIV circulating in plasma.(29) Following infection, HIV disseminates to the lymph nodes and later to the bloodstream; this facilitates the spread of the HIV-infected cells to different anatomical sites including the gastrointestinal tract, lymphoid tissues such as the spleen, thymus, the gut-

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associated lymphoid tissue (GALT), lungs, liver, kidneys, the central nervous system CNS), and the bone marrow.(29, 30)

The expression and diversity of proviral HIV is different across cell and tissue reservoirs. The increase in frequency HIV infected cells differs through host regulatory processes including homeostatic proliferation or antigen driven proliferation. The decrease of such cells is also driven by mechanisms such as viral cytolysis and activation induced cell death.(31, 32) However, not all cells are able to express enough HIV viral protein to be recognized by the host immune system.(33) Some of these cells expressing low levels of recognizable viral proteins may exist in a quiescent state and hence viral decay of replication-competent HIV cell reservoir is very slow.(34)

The HIV reservoir also includes a widespread diversity of proviral sequences due to several factors including errors in the reverse transcription step of the HIV life cycle, high levels of recombination, APOBEC hypermutations, compartmentalization of the virus, viral quasispecies able to evade detection by host immune mechanisms as well as accumulation of HIVDR mutations.(34-36)

The measurement of the latent reservoir includes measurement of HIV DNA load, HIV gag p24 assays, measuring HIV RNA and sequencing of HIV RNA at limiting dilution, inducible HIV RNA/ HIV viral protein, as well as using quantitative viral outgrowth assays (QVOA/VOA).(34) The gold standard for measuring proviral HIV is the use of VOA, where CD4+ T-cells collected and stimulated with mitogen and then cultured together with phytohaemagglutinin (PHA) activated CD8-depleted PBMCs from HIV-seronegative individuals at limiting dilution, followed by measurement of p24 protein in the latency reversed cells.(37) A few modifications have been made to this assay to reduce the limitations of it being costly and that it requires high number of cells in order to get a suitable limit of detection, these modified assays also give more insight on true measurements of replication-competent HIV in reservoirs.(38-40)

1.1.5.3 Stages of HIV infection and HIV disease progression

HIV infection in the host can generally be categorized into four different stages (Figure 5): primary infection, clinically asymptomatic stage, symptomatic HIV infection, and progression from HIV to AIDS.(41) Primary infection, also called seroconversion illness/acute primary infection, refers to the first two to four weeks of infection. The infected patients undergo

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seroconversion; going from a state of being HIV free to a state of being HIV infected.(41) These seroconverting individuals exhibit flu like symptoms such as fever and sore throat. The viral load is very high in this stage due to high replication rates of HIV and since adaptive immune responses which limit viral replication take a few days to develop. People in this acute stage are highly infectious due to rapid viral replication; it persists until the body has created anti-HIV antibodies within 4-6 weeks of infection.(41) Following the acute stage, the viral replication slows down and reaches a plateau stage called "viral load set point."(22) The clinically asymptomatic stage of HIV infection is also known as the clinical latency stage, where the term latency refers to a period where the virus is present in an infected person without producing visible symptoms.(41) The duration of the asymptomatic phase is related to the viral load set point with higher viral load setpoint associated with faster progression. The virus continues replicating but at a much slower pace than in acute phase. The progression to the third stage is greatly reduced by ART. The clinically symptomatic stage is characterised by the low CD4+ cell counts and high viral load, it is observed when the infected individual's immune system continues to deteriorate due to HIV replication.(41) Furthermore, the CD4+ T cell counts are usually between 200 and 500 cells/mL and mild disease is mostly exhibited as worsening of symptoms such as developing herpes simplex disease, chronic skin conditions and recurrent diarrhoea.

The increase in opportunistic infections due to lowered immunity are the defining symptoms of AIDS. Some examples of the most common opportunistic infections include respiratory infections caused by *Pneumocystis jiroveci* pneumonia (PJP), tuberculosis (TB) and Kaposi's sarcoma (KS).(41) The diagram below (Figure 5) shows the relationship between CD4+ cell counts and viral load in the different stages of HIV infection.

When CD4+ cell counts reach the range of 50-200 cells/mL, the individual is considered to have advanced to AIDS. There are several factors that may affect the rate of HIV infection progressing to fully blown AIDS. These factors may include viral load and viral factors, age, co-infections, genetic variation, route of infection, host immunity, overall nutrition, pregnancy, and lifestyle (drug use).

In the absence of treatment, different individuals have different rates of disease progression. There are individuals who are termed rapid progressors, where development of AIDS is within 3 years of infection; intermediate progressors, individuals who exhibit slow AIDS development between 3 to 10 years after seroconversion; long-term non progressors (LTNPs), who are very

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rare and constitute about 0.5% of the entire HIV infected population and maintain low level viremia (LLV) and stable CD4 counts above 500copies/ml exceeding ten years. A subset of individuals is also able to maintain high CD4+ and CD8+ T-cell counts and undetectable viral loads while remaining therapy naïve, termed “elite/natural controllers”.

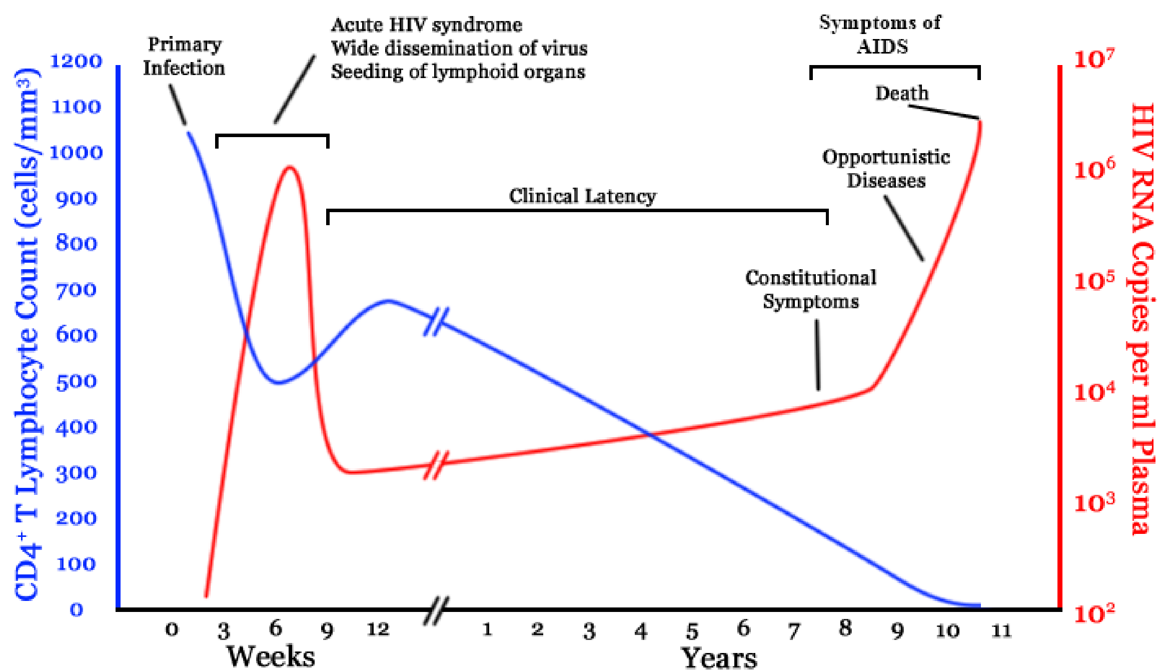

Figure 5: Stages of HIV Infection and disease progression (Source: Jurema Oliveira. Public domain image obtained via Wikimedia Commons, https://commons.wikimedia.org/wiki/File:Hiv-timecourse.png, current version dated 4 June 2011)

1.1.5.4 Host defence against HIV

Following HIV infection, the human immune system usually develops antibodies to HIV within 3 months. The cytotoxic CD8+ T cells are also able to reduce the number of HIV infected cells through production of cytokines and chemokines with antiviral properties.(42) Similarly, neutrophils and natural killer (NK) cells also all have the ability to kill HIV infected cells through several mechanisms. There are some cellular restriction factors including the human cytidine deaminase APOBEC3G (A3G), tetherin and TRIM5α which are able to inhibit HIV.(43, 44) During reverse transcription, A3G deaminates dC to dU bases in the HIV cDNA negative sense strand and causes accumulation of G to A substitutions in the proviral positive-strand cDNA which in end disrupt transcription; tetherin prevents budding of new virions from

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the cell by binding the host cell plasma membrane and the HIV viral envelope together; TRIM5α disturbs the uncoating process before reverse transcription may occur.(44, 45) There are several host genetic defences against HIV. Some individuals of northern or central European ancestry have the delta32-CCR5 deletion, which is a 32 base pair deletion in the CCR5 gene (CCR5-delta32) which is located on chromosome 3 in humans; this deletion results in a non-functional CCR5 co-receptor and therefore immunity against infection by R5 tropic viruses.(46) Similarly polymorphisms in the CCR5 gene has been described in Africa, and may influence HIV CCR5 coreceptor binding.(47, 48)

1.1.6 HIV treatment

HIV does not have a cure. Before the availability of combination antiretroviral therapy in African settings, the only available options were prophylaxis or treatment of opportunistic infections. However, since combination antiretroviral drugs (ARVs) have become available this has become the mainstay of treatment. These drugs inhibit viral replication and allow at least partial recovery of the immune function. Antiretroviral therapy (ART) is the only way of preventing or partially reversing disease progression of HIV/AIDS. Treatment success is defined viral suppression (undetectable viral loads).(49) Antiretroviral therapy is also the most successful available biomedical intervention to reduce HIV transmission. A range of ARVs that are used as ART belong to six major drug classes: nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), fusion inhibitors, integrase inhibitors and co-receptor binding inhibitors also known as entry inhibitors.(49) To date a combination of three of these drugs, known as highly active antiretroviral therapy (HAART), have been used in order to target several genes at a time to slow down disease progression and also allow the immune system to recover and prevent opportunistic infections leading to AIDS.(49)

The mechanism of action of ARVs is to target several stages of the HIV life cycle to block replication. Drugs that limit entry target two steps attachment and fusion: CCR5 receptor antagonists and fusion inhibitors.(50) The CCR5 antagonists block viral attachment to the CD4+T-cell by targeting binding of the virus to the CCR5 -receptor.(51) The fusion inhibitors bind to the gp41 molecule and prevent the conformational change necessary for fusion with the host cell.(50, 51) The NRTI and NNRTI drugs interrupt the reverse transcription step of the

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HIV life cycle; the NRTIs block reverse transcriptase (RT) activity competing with the natural substrate dNTP triphosphates and by acting as chain terminators in the synthesis of proviral DNA. The NNRTI do not compete with the natural substrate, but instead block the RT by binding to distinct sites disrupting the conformational change of its catalytic site.(49, 51)

Below is a table showing examples of ARVs with their abbreviations according to drug class;

Table 2: Antiretroviral Medications

NRTI	**PIs**
Abacavir, ABC (Ziagen) Didanosine, ddl (Videx, Videx EC) Emtricitabine, FTC (Emtriva) Lamivudine, 3TC (Epivir) Stavudine, d4T (Zerit) Tenofovir alafenamide, TAF (Descovy) Tenofovir disoproxil Fumarate, TDF (Viread) Zidovudine, AZT/ZDV (Retrovir)	Atazanavir, ATV (Reyataz) Darunavir, DRV (Prezista) Fosamprenavir, FPV (Lexiva) Indinavir, IDV (Crixivan) Nelfinavir, NFV (Viracept) Ritonavir, RTV/r (Norvir) Saquinavir, SQV (Invirase) Tipranavir, TPV (Aptivus)
NNRTI	**INSTIs**
Delavirdine, DLV (Rescriptor) Efavirenz, EFV (Sustiva) Etravirine, ETR (Intelence) Nevirapine, NVP (Viramune, Viramune XR) Rilpivirine, RPV (Edurant) Doravirine, DOR (Pifeltro)	Bictegravir, BIC (Bictarvy) Dolutegravir, DTG (Tivicay) Elvitegravir, EVG (Vitekta) Raltegravir, RAL (Isentress)
Fusion Inhibitor	
Enfuvirtide, ENF/T-20 (Fuzeon)	
Entry Inhibitors	
Maraviroc, MVC (Selzentry) Ibalizumab (Trogarzo)	

Globally, there have been several attempts made at eliminating the detrimental effects of the HIV pandemic, one of the latest strategy was deployed by UNAIDS; commonly known as the "Treat All" strategy or the UNAIDS 90-90-90 goal/strategy.(52) This strategy was coined in 2014, the goals set here were that by the year 2020; 90% of all people living with HIV know their HIV status; 90% of people diagnosed with HIV be started on ART; and that 90% of all people who are on ART achieve viral suppression indicated by undetectable HIV viral load in their blood.

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The Botswana National ART Programme, also known as the "Masa" programme, was implemented in 2002 and has successfully operated for the past 18 years, significantly reducing mortality rates caused by HIV-1 infection.(8, 53) With over 220,000 infected patients on ART and an estimated 87% coverage of those in need at the end of 2013, Botswana's citizens continue to receive free high-quality ART, care and support. Today, Botswana is among the first African countries to implement the treat all strategy and implement use of Dolutegravir (DTG) as part of first line therapy for all people living with HIV and is well within grasp of achieving the 90-90-90 goal with overall 70.2% (95% CI 67.5-73.0) of people living with HIV-infected exhibiting virological suppression, which is very close to the UNAIDS target of 73%.(54)

1.1.7 Mother to child transmission (MTCT) of HIV

HIV-1 transmission can occur through blood and body fluids; including blood transfusions, unsterile intravenous needles drug users, sexual intercourse, and mother-to-child transmission/vertical transmission.(55) The transmission of HIV can occur from a mother living with HIV to their child during gestation in the uterus, peripartum or post-partum during breastfeeding.(56) MTCT is the leading cause of paediatric HIV worldwide, constituting 90% of all child infections.

Without breastfeeding it is approximated that 60-70% of infected children become infected intrapartum (during delivery), the rest of the 30-40% are assumed to be in utero, in the last stages of pregnancy; infections in the first and second trimester are estimated to be lower than 5%.(57-60) The added risk of MTCT via breastfeeding has been estimated to be 15% and in some settings 40% of MTCT cases have been due to breastfeeding alone or mixed with formula feeding.

There are several risk factors for HIV MTCT(61); these include maternal health and HIV stage, maternal HIV viral load, the level of cell-free virus in genital secretions and cervical or vaginal HIV-DNA.(62-65) Chorioamnionitis, preterm delivery, low birth weight and premature rapture of membranes are some of the foetal and obstetric factors associated with HIV MTCT.(62, 65-67) Furthermore, maternal virus phenotype may increase risk of HIV transmission of viruses with high replicative capacity and cytopathogenicity.(66) Similarly, HIV-1 subtype C has been shown to be transmitted vertically above other subtypes.(67) Behavioural factors such as illicit drug use, unprotected sexual intercourse leading to sexually

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transmitted infections, smoking during pregnancy have also shown to be high risk factors for HIV MTCT.

Some factors have been associated with reduction of HIV MTCT in the absence of ART; these include HIV-1 specific immune responses as well as innate immunity of both mother and child, including secretor leucocytes, cytokines and chemokines in bodily fluids such as saliva and breastmilk.(61, 68, 69)

Furthermore, HIV-specific maternal neutralizing antibodies, CD4+ T cell responses and cytotoxic T-lymphocyte (CTL) responses also reduce risk of transmission.(70) NK cells also have a role in natural host defence against HIV infected cells, have been demonstrated to be protective against MTCT of HIV.(71) Mode of delivery has been shown to be in favour of caesarean section before labour and instead of vaginal (natural) delivery despite maternal viral load as prolonged rapture of membranes is a risk factor for MTCT.(72)

1.1.8 Prevention of Mother-To-Child Transmission (PMTCT) of HIV

The fight against transmission of HIV between mothers and their infants has been at the forefront of eliminating HIV for many years. PMTCT has been implemented worldwide since the ACTG 076 ANRS 024 study in 1993 which revealed a 68% reduction in risk of perinatal transmission after giving mothers zidovudine (ZDV) from the 14-34th week of gestation, during labour and delivery. The infants were also given ZDV orally the first 6 weeks.

Currently PMTCT is administered in the form of combination ART where mothers are given a combination of drugs in which NNRTI or PI are is administered together with NRTIs(73). Several studies have revealed that ART started in pregnancy through 6 months of breastfeeding reduces MTCT to lows of 1%, thus allowing breastfeeding to be an option for lactating mothers living with HIV.(56, 73)

There has been an evolution of PMTCT programmes to meet several treatment strategies recommended by the World Health Organization (WHO) which have been implemented over the past years. These included Option A, where eligible (based on CD4 count) pregnant women were given Zidovudine (ZDV) from 14 weeks gestation, then a single dose of nevirapine (sdNVP) when beginning labour; these women were then given a combination of ZDV and Lamuvudine (3TC) until 1 week postpartum. In option A, the HIV-exposed infant was given

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daily oral nevirapine (NVP) from birth until the end of breastfeeding.(74) In Option B, the criteria were implemented for women who were not eligible for ART where they were given a triple dose regimen from 14 weeks until the end of the breastfeeding period with daily oral doses of NVP or ZDV given to the HIV exposed infant for the first 6 weeks of life. Currently, the WHO recommends Option B+, where lifelong ART is given to pregnant women living with HIV (WLWHIV) regardless of CD4 count or clinical stage of the disease. Option B+ has also given most countries a good baseline for policy making and implementation with roll out of ART to the general public as in the context of "Treat all" or universal test and treat (UTT).(74)

Botswana was one of the first countries to implement PMTCT to all expecting WLWHIV since early 2000, with a coverage in the public sector above 95%, and mother-to-child transmission rates below 2%.(75),(76) The current national HIV clinical care guidelines indicate that Botswana has implemented Option B+ for all pregnant WLWHIV and recommends that all be given FTC/TDF (Truvada) with DTG regardless of pregnancy stage. All exposed infants are given ZDV regardless of PMTCT exposure immediately after delivery within 72 hours.(76) Of note, there are no published estimated numbers of averted MTCT due to successful PMTCT in Botswana for the year 2020.

1.1.9 Paediatric HIV

Despite tremendous efforts to reduce vertical (and horizontal) transmission of HIV, a subset of children is still living with HIV to date. Generally, children progress much faster to AIDS than adults, the majority of children develop symptoms within the first few months and these do not disappear as seen in adults.(77) The age at which vertically infected children progress to AIDS is bimodal, there are children who can control the virus for years and those who develop AIDS early within the first year of life; where if untreated most infants progress to AIDS or death(78, 79) Some of the most common signs and symptoms of paediatric AIDS include lymphadenopathy, splenomegaly, very chronic cough, persisting diarrhoea, infant tuberculosis as well as encephalopathy.(80-83) Moreover, the progression of disease in children slows down after 1 year and is relatively slow between 5 and 10 years of age. Studies have shown that 2% to 16% of children are also able to suppress HIV viral load and maintain stable CD4+ T cell counts without treatment; as LNTPs and elite controllers.(83) This bimodal progression is similar across Western and African regions, however, paediatric HIV associated mortality in

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African settings (especially sub Saharan Africa) is much higher. A meta-analysis done in 2004 revealed mortality rates of 35.2% by one year of age and 52.5% by the age of two.(84)

Progression of HIV in infants has been shown to be influenced by several factors including timing of infection, maternal HIV stage during pregnancy, maternal CD4+ T cell count, maternal viral load and overall infant and maternal immunity; infants infected earlier in the uterus progress faster and have more severe symptoms.(81, 82)

Treatment options for children living with HIV have improved over the years. PMTCT programs have introduced an early infant diagnosis (EID) approach, where HIV exposed infants are screened for HIV within 6 weeks of life using polymerase chain reaction (PCR) assays from dried blood spot (DBS) samples. The current shift in treatment of these infants is very early ART initiation in order to not only improve their outcomes but to minimize viral reservoir development from an early age.(85, 86)

One of the most referenced cases which indicated the possible impact of very early initiation of ART on the viral reservoir is the case of the Mississippi baby, who received ART from as early as 30 hours after birth for 18 months, and later exhibited delayed HIV-1 viral rebound for 27 months after cessation of ART.(87) This child showed an initial steep decline in presence of short-lived virus-producing cells, indicating a rapid decay of the viral reservoir.

Paediatric HIV viral load suppression in resource limited settings such as sub-Saharan Africa (SSA) unfortunately remains challenging. A meta-analysis of 72 studies carried out in 2015 revealed that children less than 18 years in low- and middle-income countries has VL suppression rates ranging from 64.7% to 74.7% within 12 months.(88, 89) This was different from children in high income settings such as UK and Ireland who reported 92% suppression rate within 12 months of ART initiation.(90) Similarly, rates of VF among children in SSA are high, where approximately 64% of treatment experienced children and adolescents fail treatment.(91-93) A large percentage of children and adolescents have been shown to fail treatment in several SSA countries with VF rates up to 64% in Senegal in 2019, 51.6% in Togo in 2016 and 30.6% in Zimbabwe in 2015.(91-93)

The major causes of adverse virologic outcomes in children include poorly tolerable paediatric formulations (palatability, drug toxicity etc), dosing difficulties due to variable infant pharmacokinetics, changing body weight.(94) However, the major cause of VF in children is adherence, it may be caused by these factors as well as other psychosocial issues; lack of

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adherence may lead to worsened outcomes including paediatric HIV drug resistance and thus VF and progression to AIDS.

1.1.10 HIV drug resistance

Generally, HIV-1 is characterized by its high genetic diversity as a result of high levels of viral replication together with the high level of mutations in its genome; this is caused by the inability of the RT to proofread when transcribing RNA into DNA during the HIV replication cycle. Therefore, this leads to presence of "quasispecies" within infected individuals; where many circulating viruses in the individuals are genetically related but distinct from one another.(95) The quasispecies which have adapted well to the host environment will survive better than those that have not; in the event where the environment changed, for example where there is introduction of ART, the quasispecies with mutations will hinder the effectiveness of the drugs.(95) Most of the transmitted viruses are those that have HIV drug resistance (HIVDR) mutations which do not severely affect the fitness, therefore most drugs used for ART have to have good "genetic barrier" which is the lowest threshold where ART drug resistance may develop.

Some NNRTIs such as nevirapine and NRTIs like efavirenz only require one non-synonymous mutation for emergence of pronounced phenotypic drug resistance, implying a low genetic barrier.(96, 97) However, drugs such as zidovudine (NRTI) and etravirine (NNRTI) development of drug resistance is slower as high level resistance usually require a few non-synonymous mutations.(97). For all boosted PIs there is high genetic barrier and these drugs need several mutations to be present in order to confer clinical drug resistance.(98, 99) First generation integrase inhibitors such as raltegravir have low genetic barrier as well but resistance toward second generation integrase inhibitors such as dolutegravir occurs very slowly or does not occur at all. It is also important to note that mutations selected during therapy with a particular drug may cause multi-drug resistance across the same drug class.(100, 101)

There are two types of HIV drug resistance. Resistance can be "acquired" (acquired drug resistance- ADR), or "transmitted" (transmitted drug resistance- TDR) from one person to the next. ADR occurs as a result of an evolutionary process that involves successive steps that include selection of pre-existing low frequency drug resistant variants with subsequent sequential mutations or recombination of different drug resistant variants and further selection

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of the most resistant lineages, which occurs when there is ongoing viral replication in the presence of selective drug pressure. However, Pre-treatment Drug resistance (PDR) refers to drug resistance in individuals initiating ART irrespective of whether it is TDR or acquired as a result of prior (often undisclosed) ART exposure.

Vertically transmitted HIV drug resistance remains a major problem in treatment of infants who become infected from their mothers.(102,103) The use of antiretrovirals (ARVs) for treatment in mothers and as prophylaxis in new-borns for PMTCT may drive the development of HIVDR in infants infected with HIV, either when drug resistant variants are being transmitted from the mother or arise due to selection pressure in the infant after transmission.(103) Women failing ART during pregnancy are at higher risk of transmitting HIVDR strains to their unborn infants. Furthermore, it has been shown that children are twice more likely to experience VF than adults with heterosexually transmitted HIV-1 after 5 years on ART.(104-106)

The presence of HIVDR strains in naïve populations threatens the effectiveness of ART, and TDR increases with the greater coverage of ART. There has been a reported 34-99% resistance level reported in children failing first and second line treatment containing NNRTI and NRTI regimens thus further limiting treatment options for this population(105); therefore, since 2013 the WHO has recommended use of PI (lopinavir-ritonavir - LPV/r) based ART regimens for children less than 3 years of age, encouraging phase out of NNRTI use with introduction of DTG as part of first line PMTCT.(107, 108)

Previous studies have reported TDR levels ranging from 0% to high rates of 33.3% resistance to NNRTIs, 16.6% resistance to NRTIs and 18.2% to protease inhibitors (PIs) in infants with perinatally acquired HIV-1 infection.(102),(109) In SSA, a meta-analysis including data from 13 different countries, reported a pooled PDR prevalence of 42.7% among PMTCT-exposed children and 12.7% among PMTCT-unexposed children in 2016.(109)

Currently the WHO reports that in SSA, newly diagnosed infants are prone to resistance to efavirenz as well as nevirapine and the levels of PDR to NRTIs in this population (with or without PMTCT exposure data) are greater than 10% among some countries including Eswatini, South Africa, Malawi, Togo, Mozambique, Nigeria, Cameroon and Uganda.(107) PDR to abacavir and lamivudine was also high in a few of these SSA countries. To our knowledge, the prevalence of HIVDR mutations among HIV infants in Botswana has not been reported. Moreover, HIV treatment guidelines for ARV in paediatric infection recommend

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HIVDR genotyping prior to initiation of ART, however in resource limited countries this may not be routinely done. In Botswana, the HIV clinical care guidelines indicate that infants above 1 month of age be started on a PI containing regimen, however, HIVDR genotyping is only done when the infant fails the first line regimen.

Other factors that contribute to HIVDR include poor treatment adherence, where interruptions in taking ART may lead to low concentration of ARVs allowing HIV to replicate more freely and produce more diverse quasispecies. Poor absorption may also lead to development of drug resistance, sometimes this is aided by drug toxicity and illnesses caused by side effects such as vomiting and diarrhoea in patients receiving ART. Another factor is varying pharmacokinetics, which means the extent at which a drug is absorbed, broken down and removed from the body. Drug-drug interactions in the body may cause complications in drug absorption; for example, the combination of NRTI tenofovir and PI atazanavir inhibits the absorption of atazanavir in the blood and thus a booster such as ritonavir is needed to boost atazanavir in the bloodstream. Some programmatic factors also exist at population level that drive the emergence and persistence of HIVDR; these include drug stock-outs and poor drug supply chain and management, especially in low-middle income settings.

1.1.11 HIV drug resistance Testing

The presence of HIVDR mutations in some individuals may be detected by genotypic or phenotypic drug resistance testing.

Genotypic assays are used to detect the presence of specific HIVDR mutations in specific HIV genes. There are several definitions for mutations that confer resistance to HIV drugs, usually these definitions include the following conditions; 1) the mutation confers a level of phenotypic resistance in cells when it is introduced by site-directed mutagenesis into a drug-sensitive strain of HIV in vitro, 2) the mutation is selected by serial in vitro passage of the virus in the presence of a specific antiretroviral drug with increasing observed phenotypic resistance and 3) mutations appear in samples from individuals with virologic failure during clinical therapy. Briefly, genotyping involves sequencing specific HIV gene regions and comparing the sequences to a reference strain. The sequencing involves conversion of HIV RNA to cDNA (in cases of sequencing cell free virus) followed by amplification through PCR where the DNA sample is combined with a master mix containing DNA polymerase enzyme, primers specific

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for the gene target, deoxyribonucleotide triphosphate (dNTPs) and buffers in a thermocycler and subjected to a series of temperature changes that lead DNA to denaturation, primer binding/annealing and then the amplification/elongation of the target gene of each DNA strand; this is repeated several times until the desired copy number is achieved and then sequencing can be done.(110) When using the classic Sanger sequencing method, where instead of dNTPs, a mixture of cellular dNTPs and low concentration ddNTP are added which terminate the chain elongation amplification step due to the lack of a 3' OH group, when they are randomly incorporated. This results in multiple strands with variable length which are separated using gel electrophoresis; in the case of automated Sanger sequencing each different ddNTP (A.T, G or C) is marked with a different fluorophore. Capillary gel electrophoresis allows the separation of stands by a length difference of a single base with each strand represented by the colour of the fluorescent ddNTP.(110) The sequence of fluorescence therefore represents the order of nucleotides in the sequence and is recorded as an electropherogram. There are several software packages available that can translate electropherogram data into a DNA sequences that shows the average sequence end product. The end product of Sanger sequencing is an average/aggregate of all sequences produced that will form what is known as a "consensus" sequence. This consensus sequence is then translated into its corresponding amino acid sequence which is then compared with the sequence of a wild-type consensus wild-type amino acid sequence. The differences observed in the test sequence and the reference generate a list of mutations which are reported using the one-letter code for the wild-type reference amino acid, followed by the amino acid position, followed by the one-letter code for the amino acid mutation found in the sequence(111), an example using reverse transcriptase position 184 is that rather than having methionine (M) at this position, there is a valine (V) and this is annotated as "M184V". When there is a mixture of resistant or wild-type variants as major populations (>20%) in the sample, different amino acid substitutions are represented by showing both variants for example "M184/M/V"(111). There are a few commercially available sanger sequencing HIV genotyping kits used in clinical settings, the most commonly used are the ViroSeq ™ HIV-1 genotyping system (ViroSeq; Abbott GmbH, Wiesbaden, Germany) , the TruGene HIV-1 genotyping kit (TruGene; Siemens Healthcare Diagnos-tics GmbH, Eschborn, Germany) as well as the ATCC® HIV-1 Drug Resistance Genotyping Kit developed American Type Culture Collection (ATCC), Centers for Disease Control and Prevention (CDC) and Thermo Fischer Scientific.(112-114) These assays use plasma, whole blood, and DBS samples. However, several "in-house" assays have also been used for genotyping, especially in research settings.

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Recent advances have been made to sequencing protocols and there are new next generation sequencing (NGS) assays and platforms which can produce many short reads of every sequence simultaneously, bioinformatic tools are then used to analyse and to combine these fragments by comparing these reads to the reference sequence.(115) The use of NGS is particularly better than Sanger sequencing as these assays are able to sensitively detect even low abundance variants that are present at frequencies below the minimum threshold of detection using Sanger sequencing, these variants are detected in low frequencies as low as <1% of the viral population; termed minor variants.(116, 117) It has been observed that HIV minor variants may be selected in future and form the predominant pool of circulating variants; although the impact of these variants is still under debate.(116, 118) Only a few NGS assays are used in clinical settings.(119-121) Of note, through massive parallel sequencing of multiple sections across a genome, NGS can also be used for more efficient sequencing of whole genomes.(115) Recently with the development of novel long read-length third generation sequencing, it has now become possible to sequence whole viral genomes in one read. However, the disadvantage of NGS sequencing is its impracticality in low-income countries due to high input costs of most platforms and the requirement for special knowledge of bioinformatics to clearly retrieve accurate data from the sequence data; thus, Sanger sequencing remains the gold standard in clinical care.(112, 113)

Phenotypic resistance assays are conducted to measure the extent to which viral replication is disrupted/inhibited by a specific antiretroviral drug. This is done using recombinant virus assays (RVAs) where the key viral gene sequences are inserted into a reference strain of HIV. These RVAs are grown in the presence of different concentrations of the target drug and subjected to measurements of fold-change in a patient's virus and response to treatment while taking account of mutations present with fold-change in drug susceptibility. The measure of the response is usually represented by the drug concentration that inhibits 50% of viral replication, this is known as the inhibitory coefficient IC_{50}.(122, 123)

HIV latent reservoirs may harbour archived HIVDR mutations which may not be detectable when using standard Sanger sequencing from plasma. The latent reservoir has a subset of cells that have genome defective proviruses which have several genetic alterations that may include APOBEG G-to-A hypermutations, small insertions/deletions (indels) that disrupt open reading frames, and large internal deletions in the terminus of the gene sequences.(37, 124, 125) It is important to note that although about 88% of proviruses are considered to be defective, the clonal expansion of the virally infected cells may harbour intact cell associated DNA (CAD)

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that may in future re-start production of new infectious virions.(126) Most proviral sequencing using NGS platforms have migrated to single genome amplification/sequencing (SGS/SGA), which uses Poisson statistics to dilute a template to an endpoint where 30% of all replicates are assumed to have 80% chance that amplification is a result of a single genome cDNA molecule.(127)

The use of NGS to determine HIVDR mutations in HIV-1 CAD has assisted in the initial detection of transmitted drug resistance (TDR) as the viral reservoir is established very early after infection.(128) Similarly for treatment naïve patients, the use of cell associated DNA as a template for HIVDR genotyping has revealed significantly higher rates of mutations in DNA than in plasma RNA; however the opposite observation is made in treatment experienced adults.(129) Some studies have shown that the HIVDR mutations observed in HIV-1 CAD sequences may lead to future virologic failure thus implicating treatment options for patients even during suppressive ART.(130, 131)

The sequencing of HIV CAD has evolved over the years, initial attempts were able to get more insight on reservoir maintenance and distribution.(132, 133) However, most assays were unable to determine the virus replication capacity due to sequencing of only sub-genomic regions thus missing viruses that have large deletions within their primer binding sites.(134) Similarly, these assays were unable to differentiate between the intact viruses and the defective "graveyard", overestimating the size of the fully replication-competent reservoir.(134)

Near full-length HIV-1 proviral sequencing assays have since been developed using amplification of sub-genomic regions via Sanger sequencing followed by assembly to get the much-needed differentiation of intact or defective HIV-1 CAD.(37, 126) Because this method had several limitations including cumbersome use of multiple primers which often had mismatches to target genome regions, further development of even more sensitive assays that use NGS sequencing for the same aim have been employed including the Full-Length Individual Proviral Sequencing (FLIPS) assay.(135) The FLIPS is used to amplify and sequence near full-length (intact or defective) HIV-1 CAD limiting the number of primers used and thus decreasing capturing CAD sequences that have unintentional introduction of defects into their viral sequence. The assay is performed in 6 steps: "1) lysis of HIV-1 infected cells, 2) amplification of single HIV-1 CAD via nested PCR performed at limiting dilution using primers specific for the highly conserved HIV-1 5' and 3' U5 LTR region, 3) purification and quantification of amplified products, 4) library preparation of amplified CAD for NGS, 5)

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NGS, and 6) de novo assembly of sequenced HIV-1 CAD to obtain contigs of each individual HIV-1 CAD sequence.(128-131, 135)Another similar assay, matched integration site and proviral sequencing (MIP-Seq), has been developed to allow for more precise assessment of proviruses and their corresponding chromosomal integration sites.(136),(34)

There are a few commercially available assays for NGS genotyping from PBMCs, these include the GenoSure Archive® test from Monogram Biosciences. This assay was designed to provide HIVDR data for patients with undetectable viral loads, which are difficult to test using standard genotyping methods. The GenoSure assay provides a list of "archived" mutations and gives information on susceptibility of the proviral strains to different ARV drugs. It is important to note that this assay does not give information on the intactness of the proviruses being genotyped.

Recently the WHO has recommended that infants being screened for HIV in EID programmes also be screened for SDRMs.(137) In this approach the RT, PR and IN regions of the HIV-1 *pol* gene are to be sequenced using Sanger sequencing methods from residual DBS samples. The WHO recommends that DBS samples be stored at room temperature for less than 14 days after collection where they are then processed for genotyping or stored at -20°C or below for long term use. DBS sample storage (temperature and humidity) is imperative for sequencing success, some studies have shown that storage of DBS specimens at high humidity and temperature reduced their amplification and sequencing success.(138-140) The use of DBS samples for HIV genotyping has been proven to be efficient and cost effective especially in low-middle income settings where there is lack of infrastructure for collection and transportation of blood, plasma, or serum samples.(141)

Although DBS are cost effective and good specimens to collect in infants, they have proven to be difficult in production of good quality full length sequences using Sanger based methods; furthermore, due to low volume of blood specimen, sequences from DBS have shown to be as good as plasma sequences using NGS based methods for proviral DNA sequencing.(142) However, these sequences are not able to yield information on proviral replication competent viruses especially in settings of low proviral load.(143) Therefore, the use of HIV DNA from other reservoir sites such as PBMCs (144, 145) may also be an option for screening for HIVDR in newly diagnosed infants, this may also give better insights on regimen choice for infants which are already severely limited.

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1.2 STUDY RATIONALE/ SIGNIFICANCE OF THE STUDY

Some of the factors associated with HIV MTCT in high PMTCT coverage settings such as Botswana include: late presentation and delayed ART initiation during pregnancy, inadequate adherence to ART, and infection with drug resistant HIV strains. The children who become infected, especially after PMTCT failure, are at a particular risk of HIV drug resistance (HIVDR) as a result of exposure to antiretroviral drugs administered to the mother and infant.

Despite the major success of combination ART in suppressing viral replication and preventing disease progression, it is unable to eradicate HIV-1 infection. There are limited ARVs which are specifically formulated for the HIV infected paediatric population. Regardless of evidence that lopinavir/ritonavir based antiretroviral therapy regimens are more durable in children than non-nucleoside reverse transcriptase inhibitor-based regimens, the poor tolerability of protease inhibitor liquid formulations, complicates adherence. Furthermore, due to a high risk of toxicity, lopinavir is contraindicated during the first two weeks of life or until a prematurely born child reaches a corrected age of 42 weeks. These challenges with regimen tolerability and the requirement for life-long therapy increase the risks for development of drug resistance. Surveillance of possible HIVDR is necessary to ensure that regimens that are recommended for children have a low risk of treatment failure. Although data has been shown in South African paediatric populations, revealing high percentages of NNRTI drug resistance, the patterns of HIVDR in infants with or without maternal PMTCT history have not been described in the Botswana paediatric population.

Furthermore, there is strong evidence of persistence of replication-competent viruses in different anatomical sites (reservoirs) of prolonged ART in adults. Several studies have shown a sharp initial decline in HIV reservoirs in children who are initiated early on ART, especially in peripheral blood mononuclear cells (PBMCs). The general diversity and evolution of viral reservoirs in infants has been assessed in very few studies, including in Botswana, which illustrate that early treatment generally reduces the size of the reservoir and improves HIV-1–specific T cell response with reduction of abnormal T cell immune activation. Recently, the use of single-genome near full-length proviral sequencing has allowed characterization of the evolution of proviral reservoir cells in great detail. This technique can distinguish the small proportion of genome-intact proviruses in CAD from the much larger number of defective proviruses, which are typically regarded as fossils of the replicative history of HIV-1 in a given patient, and not as an active viral reservoir. Single-genome near full-length proviral sequencing

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therefore provides for a possibility to evaluate antiretroviral HIVDR mutations that selectively occur in genome-intact CAD.

1.3 AIMS AND OBJECTIVES

MAIN AIM

To understand the patterns of HIV drug resistance in early diagnosed infants in Botswana.

SPECIFIC OBJECTIVES

1. To describe and characterize surveillance drug resistance mutations (SDRMs) from dried blood spot (DBS) samples in early diagnosed <18-month-old infants in Botswana.

2. To investigate the prevalence and role of HIV associated drug resistant mutations in intact versus defective HIV-1 cell associated DNA (CAD) of early treated <18-month-old infants.

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CHAPTER TWO

2.0 METHODOLOGY

2.1 AIM 1 Methodology

AIM 1: To describe and characterize surveillance drug resistance mutations (SDRMs) from dried blood spot (DBS) samples in early diagnosed <18-month-old infants in Botswana.

2.1.1 Study Design and population (Early Infant Diagnosis Cohort)

For the first aim of this study, samples from the Botswana early infant diagnosis (EID) program were used. The EID program screens for HIV in HIV exposed infants within 6 weeks of birth using qualitative real time PCR from dried blood spot (DBS) samples. For this study, these samples were collected in a retrospective cross-sectional approach, selected by consecutive census selection approach (selecting any consecutively available samples by date of collection until we reached the target sample size) to determine HIV-1 drug resistance in children prior to initiation of combination antiretroviral therapy.

2.1.2 Sample type and sample size

Dried blood spot (DBS) samples from <18-month-old HIV positive children who were diagnosed as part of the Botswana public sector HIV infant diagnosis program have been collected from January 2016; samples were stored at -20 degrees Celsius. A total of 78 samples were successfully retrieved and stored at -20 degrees Celsius for testing.

2.1.3 Methods, Data Collection, and Analysis

2.1.3.1 Total Nucleic Acid (TNA) Extraction

One spot from each of the DBS samples was diluted in 500µL of Nuclisens Lysis Buffer and moderately spun for about 4 hours at room temperature. This was followed by extraction using the NucliSENS® easyMag® Extraction (biomérieux, Marcy I'Etoile, France).

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2.1.3.2 Amplification and Sequencing

The protease and reverse transcriptase regions were amplified and sequenced using the ATCC HIV-1 Drug Resistance Genotyping kits (method below) and big dye chemistry. HIV *pol* amplicons were sequenced using a 16 capillary 3130 XL ABI Prism Genetic Analyzer sequencer (Applied Biosystems, Foster City, Canada). Sequencher version 5.0 premier DNA software were used to edit raw chromatograms manually and form fasta contigs. Bidirectional sequences that span the pro-RT and integrase regions were then analyzed for the presence of HIVDR mutations to all drug class regimens using the Stanford HIV Drug Resistance database for analysis (hivdb.stanford.edu). SDRMs were also assessed using the Calculated Population Resistance program (http://cpr.stanford.edu/cpr.cgi) from Stanford. A neighbour joining tree was constructed to check for relatedness and contamination using Mega version 7.0 software with1000 bootstrap replicates.

Table 3: RT-PCR (ATCC Protocol)			
	Volume		
1. Master mix	**1 rxn**	**12 rxns**	-
RT-PCR Master Mix	19.5μL	234 μL	
SuperScript™ III One-Step RT-PCR with Platinum™ Taq High Fidelity Enzyme	0.5μL	6μL	
Template volume	5μL		
Total Reaction volume	**25μL**		
2. Cycling conditions			
Step	**Temperature**	**Time**	**Cycles**
Reverse transcription	50°C	45 minutes	1
Enzyme inactivation	94°C	2 minutes	1
Denature	94°C	15 seconds	40
Anneal	50°C	20 seconds	40
Extend	72°C	2 minutes	40
Final extension	72°C	10 minutes	1
Hold	4°C	Max 18hrs	

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Table 4: NESTED PCR (ATCC Protocol)			
1. Master mix	**Volume**		
	1 rxn	**12 rxns**	
Nested PCR Master Mix	47.5 µL	570 µL	
AmpliTaq Gold™ LD DNA Polymerase	0.5 µL	6 µL	
Template volume	2 µL		
Total Reaction volume	**50 µL**		
2. Cycling conditions			
Step	**Temperature**	**Time**	**Cycles**
Initial denaturation	94°C	4 minutes	1
Denature	94°C	15 seconds	40
Anneal	55°C	20 seconds	40
Extend	72°C	2 minutes	40
Final extension	72°C	10 minutes	1
Hold	4°C	Max 18hrs	

Table 5: Purification (ATCC Protocol)

1. Master mix	**Volume**
ExoSAP-IT™ PCR Product Cleanup Reagent	4 µL
Nested PCR product	10 µL
Total Reaction Volume	**14 µL**

2. Cycling conditions

Step	Temperature	Time
Digest	37°C	15 minutes
Heat deactivation	80°C	15 minutes
Hold	4°C	Hold

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Table 6: Cycle Sequencing (ATCC Protocol)

1. Master mix	Volume
F1, F2, F3, R1, R2, R3 Sequencing Mixes	18 µL
Purified nested PCR product	2 µL
Total Reaction Volume	**20 µL**
pGEM Sequencing Control	20 µL

2. Cycling conditions

Step	Temperature	Time	Cycles
Denature	96°C	10 seconds	25
Anneal	50°C	5 seconds	
Extend	60°C	4 minutes	
Hold	4°C	Maximum of 18 hours	

Table 7: Sequence Clean Up (ATCC Protocol)

1. Master mix	Volume per 96 well plate
SAM™ solution	9.9 mL
BigDye Xterminator Solution	2.2 mL
Total Reaction Volume	**12.1 mL**

2.2 AIM 2 Methodology

AIM 2: To investigate the prevalence and role of HIV associated drug resistant mutations in intact versus defective pro-viruses of early treated <18-month-old infants.

2.2.1 Study design and study population (Early Infant Treatment Cohort)

HIV-exposed infants <96 hours of age were screened for HIV in the Gaborone and Francistown regions of Botswana as part of the Botswana Harvard Partnership early infant treatment (EIT) Study (ClinicalTrials.gov NCT02369406). EIT study has been described elsewhere.(146) Briefly, participants were recruited during pregnancy from five hospital maternity wards (Princess Marina Hospital in Gaborone, Scottish Livingstone Hospital in Molepolole, Deborah Reteif Memorial Hospital in Mochudi, Nyangabgwe Referral Hospital in Francistown, and

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Selebi Phikwe Government Hospital in Selebi Phikwe), and in surrounding maternity clinics. Participants were enrolled if mother/guardian was 18 years of age and above, able to give informed consent, gestational age at birth ≥35 weeks, infant birth weight of ≥2000g and infant age <96 hours after birth. Participants initiated antiretroviral therapy (ART) within 7 days after birth and were eligible for ART through the Botswana government programme (which provides ART for pregnant or breastfeeding citizens with HIV infection regardless of CD4 cell count).

The study was approved by the by ethical review boards in Botswana (Health Research Development Committee) and Boston (Harvard T.H. Chan School of Public Health Office of Human Research Administration).

2.2.2 Sample processing

Blood samples from neonates and infants were collected using venipuncture; samples from adults were obtained by venipuncture. Viable PBMCs were isolated and cryopreserved using standard Ficoll-Paque density gradient centrifugation following the ACTG/IMPAACT Cross-network PBMC procedures.(147)

2.2.3 Infant Droplet digital PCR and Next Generation Sequencing from PBMCs

PBMCs were subjected to DNA extraction using commercial kits (QIAGEN All Prep DNA/RNA Mini Kit or QIAGEN DNeasy kit) according to the manufacturer’s instructions.

Total HIV-1 DNA was amplified using the QX100 Droplet Digital PCR System (ddPCR; Bio-Rad) using primers and probes described previously(148) (sequences shown in Table 8 below) [127– base pair (bp) 5′-LTR–gag amplicon; coordinates 684 to 810 in HIV-1 reference strain HXB2] and normalized to the RPP30 gene. Table 8 below shows primer sequences with HXB2 coordinates.

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Table 8: ddPCR primer sequences

	Sequence	**HXB2 coordinates**
HIV DNA (LTRgag)		
Forward Primer	5'-TCTCGACGCAGGACTCG	684-700
Reverse Primer	5'-TACTGACGCTCTCGCACC	793-810
Probe	5'-/56-FAM/CTCTCTCCT/ZEN/TCTAGCCTC/31ABkFQ/	772-789
Reference Gene (RPP30)		
Forward Primer	GAT TTG GAC CTG CGA GCG	NA
Reverse Primer	GCG GCT GTC TCC ACA AGT	NA
Probe	/56-FAM/CTGACCTGA/ZEN/AGGCTCT/31ABkFQ/	NA

HIV DNA, human immunodeficiency virus deoxyribonucleic acid; LTR, long terminal repeat; RPP30, ribonuclease P/MRP Subunit P30; ZEN, a dark quencher used to reduce background fluorescence and increase signal by emitting heat instead of light, allowing it to be used with multiple reporter dyes; 31ABkFQ, 3′ lowa Black® FQ quencher

Briefly, HIV LTR-gag/RPP30 mastermix was made according to the mastermix template (Table 9) for the number of wells needed + 2 wells.

Table 9: General Mastermix for ddPCR (rare event)		
Mastermix	**Volume**	**GAPDH**
ddPCR supermix 2X	11µL	11µL
Forward primer 20µM	1µL	1µL
Reverse primer 20µM	1µL	1µL
Probe 20 µM	0.35µL	0.35µL
H_2O	5.65µL	7.65µL
Total	19µL	21µL

19µL of HIVgag (for the target samples) or 21µL RPP30 (for the reference) were added to the mastermix to a well in regular 96 well PCR plate while making sure there is enough mastermix for replicates for negative control (water), positive control (8e5 cell line(149), and samples. Wells with mastermix were covered and vortexed followed by a quick spin on the eppendorf, then 3µL of DNA samples were added in HIVgag mastermix wells and 1µL of (1:100) in RPP30 mastermix wells (22µL total volume). Very slowly, 20µL of each column

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of the 96 well plate that has sample and mastermix were transferred into a ddPCR droplet cassette (small, middle wells) using electronic multichannel. 70µL of droplet generation oil was transferred into the bottom wells of cassette using sterile basin and manual multichannel for each cassette. The cassette and gasket were put onto ddPCR droplet generator. Once droplets generated, droplets were very slowly transferred from top well of the cassette to eppendorf 96 well PCR plate.

Plates were then placed in C1000 Touch thermocycler ASAP, because droplet generation is time sensitive before PCR, and run on the following protocol.

1. 95°C 10 min
2. 95°C 30 sec
3. 60°C 1 min

*repeat steps 2-3 45 times

4. 98°C 10 min

After PCR program, droplets are stable at 4C

Plates were then transferred to ddPCR droplet reader and set up a new run on Quantasoft program on attached laptop. Quantasoft software was set to RED (rare event detection), ddPCR supermix for probes on supermix and to measure FAM in channel 1 and VIC in channel 2.

For single genome amplification (SGA), 1st PCR and 2nd PCR mastermix (Invitrogen Platinum Taq #11304-029) were made according to the mastermix template for the number of wells you needed + 2 wells as shown below:

Table 10: 1st Round PCR Mastermix for SGA

Mastermix 1st PCR	**Vol**(µL)/rxn	**Final [ ]**
10xBuffer	2	1x
$MgSO_4$ (50mM)	0.8	2mM

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dNTP (10mM)	0.4	0.2mM
Forward primer (20µM)	0.4	0.4 µM
Reverse primer (20µM)	0.4	0.4 µM
Taq (5U/µL)	0.2	1U/rxn
H_2O	14.8	
MasterMix	19	
DNA	1	
Total	20	

Table 11: 1st Round PCR Mastermix for SGA

Mastermix 2nd PCR	**Vol**(µL)/rxn	**Final [ ]**
10xBuffer	2	1x
$MgSO_4$ (50mM)	0.8	2mM
dNTP (10mM)	0.4	0.2mM
Forward primer (20µM)	0.4	0.4 µM
Reverse primer (20µM)	0.4	0.4 µM
Taq (5U/µL)	0.2	1U/rxn
60% Sucrose	2	1x
H_2O	12.8	
MasterMix	19	
DNA	1	

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Total	20

DNA samples were diluted to keep the ratio 1:3 [1 copy of HIV per 3wells (Poison Distribution)], and the mix prepared (for example, for 30 copies in 90 wells if you have a sample with 2.54 copies/µL, you would need: 1710µLof mastermix + 11.83µL of DNA + 78.17µL H20). 20µL of mix was then added to a well in regular 96 well PCR plate and controls added (19µL of mastermix + 1µL of H20 or 8E5 DNA (66 copies/µL) respectively). After a brief vortex, plates were placed into a C1000 Touch thermocycler, and run on the following protocol:

1. 92ºC 2 min

2. 92ºC 10 sec

3. 60ºC 30 sec

4. 68ºC 10 min

*repeat steps 2-4 9 times

5. 92ºC 10 sec

6. 55ºC 30 sec

7. 68ºC 10 min

*repeat steps 5-7 19 times

8. 68ºC 10 min

9. 4ºC ∞

After PCR program, keep at 4ºC until 2nd run

For the 2nd PCR, 19µL of 2nd master mix was added in the same number of wells (considering the controls wells) and 1µL of the 1st PCR product transferred to each of them (total volume per well 20µl). After brief vortex, plates were placed in the C1000 Touch thermocycler, and run on the same PCR conditions of 1st PCR. Products were run on a 0.7% agarose gel and run using 3µL per sample.

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PCR products were purified using AMPure XP magnetic beads and individually submitted for next-generation sequencing on the Illumina MiSeq platform.(150) Amplification products were subjected to Illumina MiSeq sequencing at the Massachusetts General Hospital (MGH) DNA Core facility, using an in-house standard protocol for library construction using primers spanning HXB2 coordinates 638-9632.(150, 151) Resulting short reads were de novo assembled using Ultracycler v1.0 and aligned to HXB2 to identify large deleterious deletions (<8000 bp of the amplicon aligned to HXB2), out of-frame indels, premature/lethal stop codons, internal inversions, or 5′-LTR defect (≥15 bp insertions and/or deletions relative to HXB2), using an automated in-house pipeline written in R scripting language. Presence/absence of APOBEC3G/3F–associated hypermutations was determined using the Los Alamos HIV Sequence Database Hypermut 2.0 program. Viral sequences that lacked all mutations listed above were classified as "genome-intact." Multiple sequence alignments were performed using MUSCLE Viral sequences were deposited in GenBank.

Table 12: SGA Sequencing Primers

	Sequence
1st Round PCR	
Forward Primer – U5-638F	5'-GCGCCCGAACAGGGACYTGAAARCGAAAG
Reverse Primer – U5-547R	5'- GCACTCAAGGCAAGCTTTATTGAGGCTTA
2nd Round PCR	
Forward Primer – U5-623F	5'-AAATCTCTAGCAGTGGCGCCCGAACAG
Reverse Primer – U5-601R	5'-TGAGGGATCTCTAGTTACCAGAGTC

2.2.4 Maternal HIV genotyping from plasma

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We genotyped HIV from a total of 22 mothers using Sanger sequencing. The viral RNA was extracted from 140-280µL patient plasma according to the QIAamp viral RNA Mini kit protocol (Qiagen; Valencia, CA), eluted in a final volume of 60µL and stored at -80ºC for later use. The reverse transcription and first-round PCR were performed using the Superscript IV One-Step RT-PCR System with Platinum High Fidelity Taq (Invitrogen; Carlsbad, CA). In order to minimize possible resampling, the RT-PCR reactions were performed in triplicate for each sample in 25µL reactions. 3µL of the extracted viral RNA as template was added into 12.5µL of 2x buffer, 1.0µL of SuperScript IV RT/Platinum Taq HiFi mix, 0.5µL of 10µM of forward and reverse primers: PF1,5'-AAAGGGCTGTTGGAAATGTGG-3' and PR1,5'-CCCCAATATGTTGTTATTAC-3' and 7.5µL of distilled water. For plasmid DNA from reference viruses, 2ng of template DNA was used in each PCR. The RT-PCR conditions were as follows: (1) an initial reverse transcription step of 55°C for 30 min; (2) 2 min at 94°C for DNA denaturation and Taq DNA polymerase activation; (3) 25 cycles of 94°C for 30 sec for DNA melting, 56ºC for 30 sec for annealing and 68ºC for 3.5 min for elongation followed by final extension at 68ºC for 5 min. Triplicate reactions for each sample were combined and mixed well to use as template for the second-round PCR reaction, which was carried out in 3 separate 25µL reactions using High Fidelity DNA Polymerase kit (Life Technologies, US) with forward and reverse primers: PF2, 5'- AAGGAAGGACACCAAATGAAAGA-3' and PR2, 5'-TGGGATGTGTACTTCTGAACTTA-3'. The 25µL PCR reaction contained 2.5µL of 10x HF buffer, 0.5µL of 10mM dNTP, 1.0µL of 50mM Mg2SO4, 0.2µL of Platinum Taq High Fidelity, 0.5µL of 10µM of forward and reverse primers, 2µL of the RT-PCR amplicon, and 17.8µL of distilled water. Amplification was carried out with the following conditions: at 94°C for 2 min, then 32 cycles of at 94°C for 30 sec, at 58°C for 30 sec and at 68°C for 3 min followed by final extension at 68°C for 5 min. The triplicate PCR reactions were combined and the presence of an approximately 3.1 kilobases (kb) amplicon was verified by agarose gel electrophoresis. Amplification products were subjected to Sanger sequencing at the Massachusetts General Hospital (MGH) DNA Core facility. Sequence alignments were performed using Geneious, presence/absence of HIVDR mutations was determined using Stanford University-HIV Drug Resistance Database (https://hivdb.stanford.edu/)

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2.2.5 HIV Drug Resistance Analysis

All near full-length HIV-1 CAD (from infant PBMCs) and HIV *pol* (from maternal plasma) sequences were analyzed for the presence of HIVDR mutations using the Stanford HIV Drug Resistance database for analysis (hivdb.stanford.edu). Sequences were subtyped by on-line tools REGA HIV-1 subtyping tool ver. 3 and Context-based Modeling for Expeditious Typing (COMET HIV-1).(152, 153)

2.2.6 Statistical Considerations

Descriptive statistics were analyzed using SAS software. Microsoft excel was used to sort and determine frequencies of HIVDR mutations present. We determined the difference in proportion of HIVDR mutations present in intact vs defective HIV-1CAD by two sample Z-test. Because of the small sample size, comparisons were descriptive in nature. Proportion comparison of HIVDR mutations detected in intact vs defective HIV-1 CAD sequences was determined using two proportion Z test. For purposes of this analysis, we assumed that all identified resistance mutations in the first HIV-1 CAD sample available within 1 month were TDR rather than de novo mutations. Statistical analysis was conducted using GraphPad Prism version 9 for Mac (GraphPad Software, San Diego, California USA, www.graphpad.com) and Stata version 14 (StataCorp LP, College Station, Texas).

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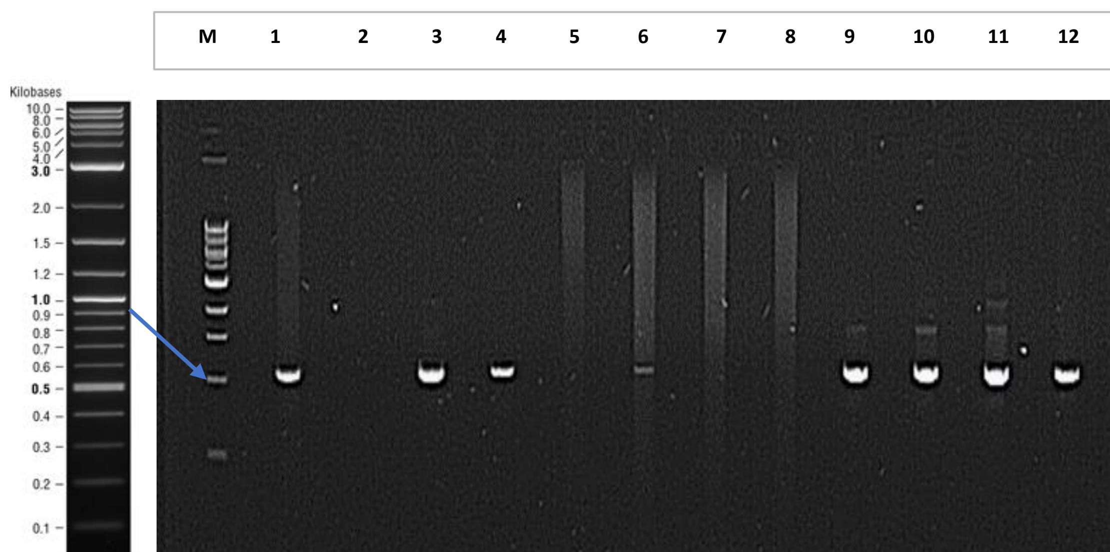
CHAPTER THREE

3.0 RESULTS

3.1 AIM 1 RESULTS

We used 78 DBS samples from the Botswana EID programme. The DBS samples were from <18-month-old recently HIV diagnosed children collected from 2016 to 2018; samples were stored at -20ºC. A total of 78 samples were successfully retrieved and stored at -20ºC for testing. The median age of all infants was 2 months (IQR: 2- 4).

Of the 78 DBS samples available, 32 (41%) were successfully amplified and sequenced. Figure 6 below shows an example of a 1X Tris/Borate/ ethylenediaminetetraacetic acid (EDTA) (TBE) gel of the nested PCR product (2nd round PCR product). Wells 1, 3, 4, 6, 9, 10, 11 and 12 show bands of approximately 1.1kb which had successful 2nd round PCR amplification. Wells 2, 5, 7 and 8 show samples that failed amplification.



M = Biolabs Inc 1kb Plus DNA ladder

Figure 6: Example of 1X TBE gel electrophoresis of the Nested PCR product (approx. 1.1kb)

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A total of three [9.7%, 95% CI: 2.2-25.6] newly diagnosed infants had detectable SDRMs. One sample was excluded due to APOBEC-induced mutations G68R, G112K, W212*.

Although PMTCT coverage is >95% in Botswana, PMTCT exposure data was not available for all infants. Among these infants, one (33%) had a NNRTI HIV SDRM (K103N) detected and two (67%) had a detectable PI SDRMs (M46L and L23I) (Table 13).

Table 13: Patient characteristics and detected SDRMs in <18-month-old EID infants

Sequence ID	Year of sample draw	Patient Age (months)	NRTI SDRMs	NNRTI SDRMs	PI SDRMs
NOM879597	2017	18	None	None	L23I
NOM428094_2	2018	5	None	None	M46L
NOM308293_2	2019	4	None	K103N	None

NRTI, nucleoside reverse transcriptase inhibitors; NNRTI, non-nucleoside reverse transcriptase inhibitors; PI, protease inhibitors

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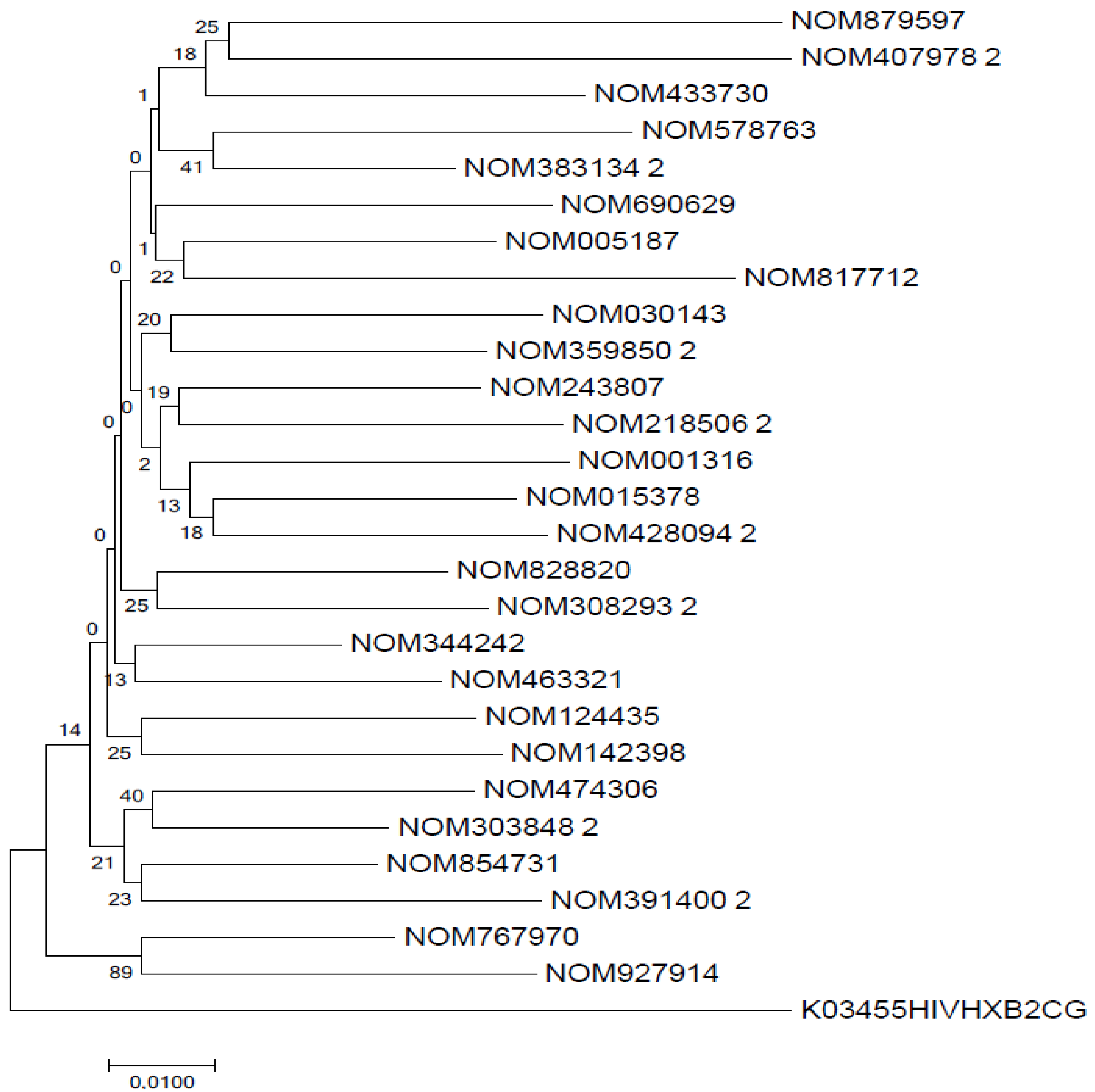

Figure 7: Neighbour joining tree constructed from 32 HIV-1C *pol* (protease and reverse transcriptase) sequences using bootstrap values of 1000

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3.2 AIM 2 RESULTS

A total of 27/42 (64.3%) of infants from the EIT study were included in this analysis, median age at enrolment for these infants was 2 days. The median age at PBMC sample collection was 2 days (range: 2-32 days), with a median digital droplet PCR HIV-1 CAD load value of 492 (IQR: 78, 1246) copies/mL. Maternal and infant HIV regimen was available for all infants. All 27 infants were started on zidovudine (ZDV)/lamivudine (3TC)/nevirapine (NVP), with transition to ZDV/3TC/lopinavir-ritonavir (LPV-r) at 2-5 weeks. Twenty-two (52.3%) mothers of median age 28 years had plasma genotyped for HIVDR mutations. Mothers were receiving either efavirenz (EFV)/emtricitabine (FTC)/tenofovir (TDF) (36.4%), dolutegravir (DTG)/FTC/TDF (13.6%), lopinavir/ritonavir/FTC/TDF (4.5%), or no ART (40.9%) (Table 14).

Table 14: Baseline demographics of EIT infants and mothers of infants at enrolment

Maternal Characteristics (N=22)	
Maternal Age (years), Median (IQR)	28 (23, 30)
Maternal Absolute CD4 Count (cell/µl), Median (IQR)	377 (220, 634)
Maternal Viral load (log10), Median (IQR)	4.4, (3.3, 4.9)
Maternal Marital status, n (%)	
Cohabiting	1 (4.5)
Single	21 (95.5)
Maternal Highest education level, n (%)	
Primary	1 (4.5)
Junior Secondary	12 (54.5)
Senior Secondary	7 (31.8)
Tertiary	2 (9.1)
Household Electricity, n (%)	
No	7 (31.8)
Yes	15 (68.2)
Household Car, n (%)	
No	18 (81.8)

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Yes	4 (18.2)
Maternal ARV Regimen, n (%)	
None	9 (40.9)
EFV+FTC+TDF	8 (36.4)
TRU+DTG	3 (13.6)
TRU+ LPV/r	1 (4.5)
Maternal Mode of Delivery, n (%)	
SVD	16 (72.7)
C-section	6 (27.3)
Enrolment Site, n (%)	
Francistown	10 (45.5)
Gaborone	12 (54.5)

Infant Characteristics (N=27)	
Gender, n (%)	
Female	19 (70.4)
Male	8 (29.6)
Age at enrollment (days), Median (IQR)	2.0 (2.0, 3.0)
Congenital abnormalities, n (%)	
No	27 (100.0)
ZDV after birth, n (%)	
No	1 (3.7)
Yes	26 (96.3)
sdNVP after birth, n (%)	
No	4 (14.8)
Yes	23 (85.2)
Feeding method immediately after delivery, n (%)	
Breastfeeding only	7 (25.9)
Formula feeding only	20 (74.1)
ART regimen initiated, n (%)	

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ZDV-3TC-NVP	27 (100.0)
Birth weight (kilograms), Median (IQR)	3.0 (2.7, 3.2)
Gestational age at birth (weeks), Median (IQR)	39 (36, 40)
Absolute CD4 Count (cells/ul), Median (IQR)	1727 (1538, 2177)
Infant Viral load (log10), Median (IQR)	3.5 (3.0, 4.5)
Cell associated HIV DNA_ ddPCR (copies/mL), Median (IQR)	492 (78, 1246)

ARV, antiretroviral drugs; ART, antiretroviral treatment; ddPCR, digital droplet PCR; DTG, dolutegravir; EFV, efavirenz; FTC, emtricitabine; IQR, interquartile range; NVP, nevirapine; LPV/r, Lopinavir/Ritonavir; sdNVP, single dose nevirapine; SVD, spontaneous vaginal delivery; TDF, tenofovir disoproxil fumarate; 3TC, lamivudine; TRU, Truvada (FTC/TDF), ZDV, zidovudine

HIVDR mutations in infants by 1 month of life

We analysed a total of 257 sequences from 27 infants with successful NGS HIV-1 CAD genotyping in the first month, with a median of 24 (Q1, Q3: 11,47) sequences per infant. All sequences were HIV-1 subtype C (100%). HIVDR mutations from HIV- 1 CAD were detected in 9/27 (33.3%) infants. Table 15 shows the overall HIVDR mutations detected in these 9 infants, as well as the mutations identified in the plasma of 22 mothers by population-based Sanger sequencing.

Among infants with HIVDR mutations detected most common mutations detected (overall) were NNRTIs and PIs (88.9%). PI major mutation D30N was detected in 5/8 infants with PI mutations in their overall HIV-1 CAD. NNRTI mutations were detected in 6/9 (66.7%) infants, with M230I mutation detected in 6/8 infant with NNRTI mutations. The most common NRTI mutation detected was M184I (5/6); M184V, K65R and Y115F were all detected in 1 infant each contributing 16.7% prevalence each among infants with NRTI mutations. A total of 7/9 (77.8%) infants had detectable major INSTI mutations in their pro-viral DNA; G140R was the most common major INSTI mutation observed.

Overall mutations were detected in infants exposed to maternal ART (66.7%) as well as those whose mothers were not receiving any ART at delivery (33.3%). Among infants exposed to NNRTI regimens in pregnancy (EFV/3TC/TDF), 5/6 had NNRTI mutations detected in their overall HIV-1 CAD within 1month of life. When looking at only intact HIV-1 CAD sequences, 2/3 infants with NRTI and NNRTI (EFV+FTC+TDF) exposure during pregnancy had HIVDR mutations associated with these regimens. One third of these infants had K103N mutation

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without prior exposure to ART. The single infant whose mother received a PI containing regimen did not have any PI mutations detected. All PI and INSTI mutations, detected without prior ART exposure, were in defective HIV-1 CAD and none of them in intact HIV-1 CAD.

Of the 22 mothers with successful plasma genotypes, 5 (22.7%) had detectable HIVDR mutations. None of the mothers were genotyped for INSTI HIVDR mutations. The most commonly occurring mutations were to NNRTIs (K103N, E138A and A98G) detected in 4 (18.1%) of the mothers. Only 1 mother had a detectable PI accessory mutation in plasma. When the genotyped mothers were compared with their infants, 3 of the 5 mothers with detectable plasma mutations may have transmitted these to their infants, based on detectability in intact HIV-1 CAD within 1 month. Of these, 3 pairs had at least one matched NNRTI mutation detected (including K103N, V106I/M, A98G and E138K), and one pair had the same NRTI mutations detected (K65R, Y115F and M184V). INSTI mutations in children could not be evaluated for TDR because of limited maternal sampling.

Table 15: Overall HIV-1 CAD drug resistance mutations detected in infant PBMCs within 1 month of life and maternal plasma at enrolment

HIV DRMs	Frequency, (%) (All infants genotyped, N=27)	Frequency, (%) (All mothers genotyped, N= 22)
Any DRM	9/27 (33.3)	5/22 (22.7)
PI mutations		
Any PI mutation	8/9 (88.9)	1/5 (20.0)
D30N	5/8	0
M46I	3/8	0
G48R[a]	5/8	0
G73S[a]	7/8	0
G48E[a]	1/8	0
L23LFIV[a]	0	1/5 (20.0)
NRTI mutations		
Any NRTI mutations	6/9 (66.7)	2/ (40.0)
M184I	5/6	0
M184V	1/6	1/5
K65R	1/6	1/5

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Y115F	1/6	1/5
NNRTI mutations		
Any NNRTI mutations	8/9 (88.9)	4/5 (80.0)
E138K	4/8	0
E138A	0	1/5
M230I	6/8	0
A98G	1/8	1/5
K103N	2/8	2/5
F227L	1/8	0
V106I/M	2/8	0
INSTI mutations		
Any INSTI mutations	7/9 (77.8)	
G140R	6/7	NA
E138K	1/7	NA
R263K	1/7	NA
G163R[a]	5/7	NA
G140E[a]	1/7	NA
G140K[a]	2/7	NA

ARV, antiretroviral drugs; HIV DRMs, Human immunodeficiency virus associated drug resistance mutations; INSTI, integrase strand transfer inhibitors; NRTI, nucleoside reverse transcriptase inhibitors; NNRTI, non-nucleoside reverse transcriptase inhibitors; PI, protease inhibitors`

a – accessory mutations, NA- data not available

Early HIVDR mutations in defective vs intact infant HIV-1 CAD

Figure 8A shows overall HIVDR mutations detected in intact and defective sequences (n= 257) from 9 infants with detectable HIV-1 CAD HIVDR mutations, where each dot represents a sequence with a detectable DRM. Figure 8B shows the number of intact and defective HIV-1 CAD sequences with frequency of resistance mutations (per drug class) among the 9 infants who had detectable HIVDR mutations. We observe overall lower frequencies of mutations within intact infant HIV-1 CAD (33 [31.1%] of 106 sequences) as compared with defective HIV-1 CAD [(39.7%) of 151 sequences] (Figure 8A), p = 0.14. Among the intact HIV-1 CAD sequences with HIVDR mutations detected, 29.2% had NNRTI, 7.5% NRTI, 0.9% PI and none

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had INSTI associated mutations. Among the defective HIV-1 CAD with HIVDR mutations detected, 31.8% had NNRTI, 15.2% NRTI, 5.3% PI, and 16.6% had INSTI associated mutations (Figure 8 B).

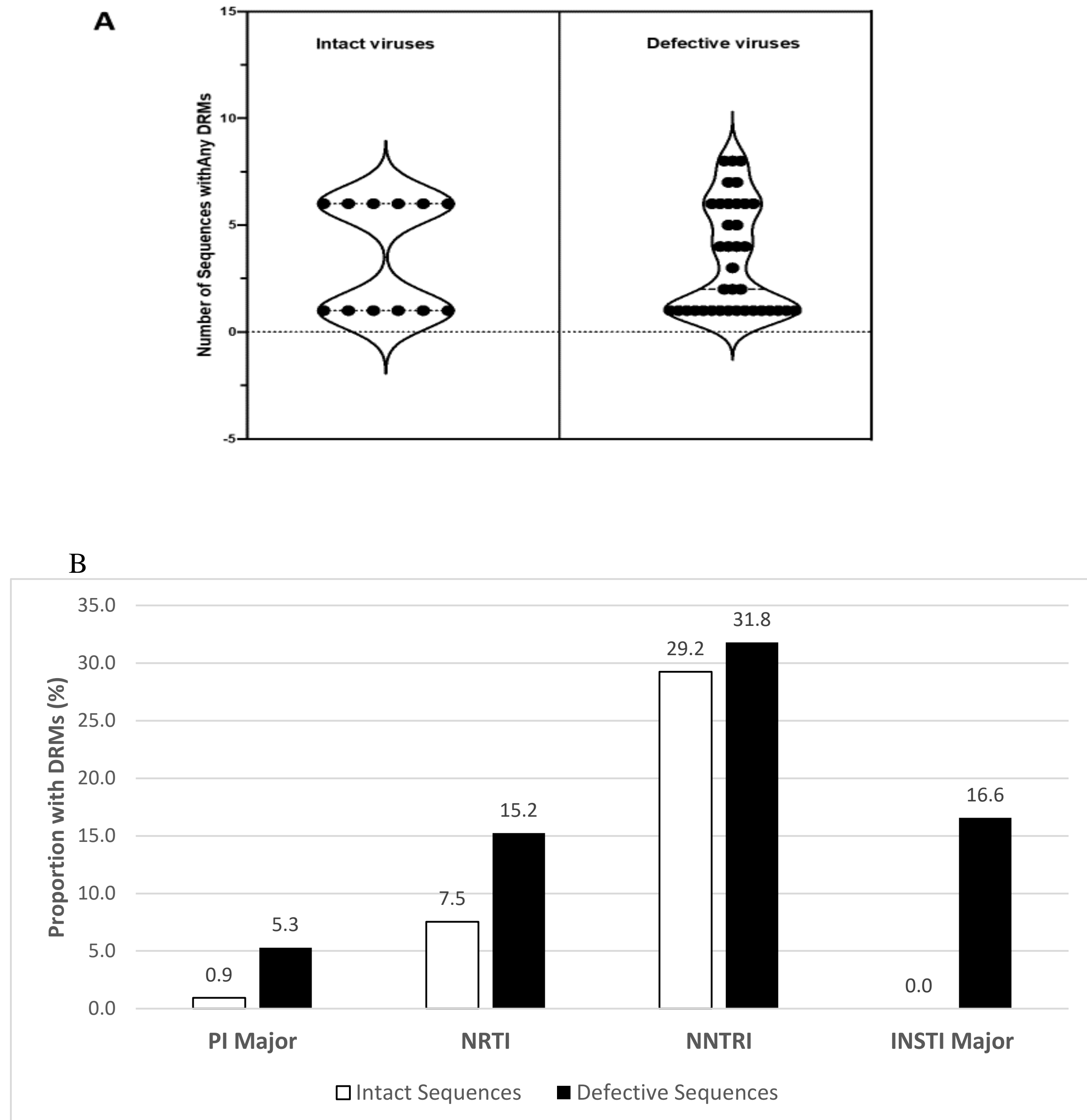

Figure 8: Frequency of any drug resistance mutations between intact and defective HIV-1 CAD of infants within 1 month of life

Figure 8 (A) is a violin plot that summarizes the differences in distribution of total HIV HIVDR mutations detected in intact and defective sequences of 9 EIT neonates (determined by single-genome, near–full- length next-generation sequencing from PBMCs) within 1 month of life, where each dot represents a sequence. Defective sequences were either: hypermutated, had a premature stop codon, internally inverted with large deletions or harboured large deletions. Figure 8 (B) further represents total number of HIVDR mutations detected in intact vs defective sequences by drug regimen.

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HIVDR mutations in intact HIV-1 CAD sequences of infants by 1 month of life

The results in Table 16 below show HIVDR mutations detected only in intact HIV-1 CAD of infants within 1 month of life. We show that all intact infant sequences harboured the exact same mutations, majority of which were HIVDR mutations associated with NNRTIs. Table 16 also compares these mutations with those found in maternal plasma at baseline using population-based sanger sequencing; indicating a high concordance to what is found in infant HIV-1 CAD within 1 month of life. Of these matched pairs, NNRTI mutations detected among both infants and mothers included K103N, V106M and A98G and one pair had the same NRTI mutations detected (K65R, Y115F and M184V).

Table 16: HIVDR mutations detected among intact HIV-1 CAD sequences of infants and maternal HIV RNA in plasma

Mothers*					**Infants#**					
Mother ID	**PI**	**NRTI**	**NNRTI**	**INSTI**	**Infant ID**	**No. of intact sequences**	**PI**	**NRTI**	**NNRTI**	**INSTI**
M-104	None	None	A98G	NA	AP-104	4	None	None	A98G	None
M-109	None	K65R, Y115F, M184V	K103N, V106M	NA	AP-109	10	None	K65R, Y115F, M184V	K103N, V106M, F227L	None
M-119	None	None	K103N	NA	AP-119	22	None	None	K103N	None

HIVDR, Human immunodeficiency virus associated drug resistance; INSTI, integrase strand transfer inhibitors; NA, not applicable (not tested); NRTI, nucleoside reverse transcriptase inhibitors; NNRTI, non-nucleoside reverse transcriptase inhibitors; PI, protease inhibitors

* Generated using population-based sanger sequencing of HIV pol from plasma

Generated using NGS FLIP sequencing from PBMCs

Note: All mutations were found across all 36 infant sequences

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CHAPTER FOUR

4.0 DISCUSSION

The main aim of this study was to determine the patterns of HIV-1 drug resistance among early diagnosed and treated infants living with HIV in Botswana.

Sequencing of HIV-1 from DBS

The first aim of the paper utilized dried blood spot (DBS) samples from early HIV diagnosed infants. This is the first study to do a surveillance of drug resistance mutations in infants in Botswana. Our results show a sequencing success rate of only 41% out of a small sample of 78 infants at initial screening. Of note, similar studies have observed sequencing success rates up to 100% using DBS stored samples.(154, 155) Because we are certain that the sample storage for the DBS cards was optimal, the low sequencing success rates observed in this study may have been due to lack of viral load data for the infants. The ATCC genotyping has been validated for plasma and dried blood spot sample types with HIV viral loads of at least 1000 copies/mL, there is therefore possibility that the samples that failed amplification belonged to infants with low HIV viral loads.

HIVDR in EID infants in Botswana

We observed relatively high levels of surveillance HIVDR mutations among 32 early diagnosed infants in Botswana between the years of 2016 and 2019. The total prevalence of PDR in this sample was 9.7% (95% CI: 2.2-25.6) from three infants out of 32. Among the three infants, one (33%) had NNRTI HIV SDRM (K103N) detected and two (67%) had a detectable PI SDRMs (M46L and L23I). These results were in concordance with a recent study in Cameroon that showed 31.6%, 26.3% and 15.8% resistance to NNRTI, NRTI and PI respectively in treatment naïve infants.(156) Although resistance to PIs was higher than expected, lack of PMTCT exposure data may be a contributing factor. A large number of infants who present VF even on PI containing regimens have been found to be exposed to PIs during PMTCT or harbour vertically transmitted mutations from mothers failing PMTCT.

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HIVDR in PBMCs of early treated infants

This study reports HIVDR mutations in HIV-1 CAD of early treated infants with HIV DNA levels similar to those recently reported in paediatric populations with suppressed viral loads.(157, 158) We report a relatively high overall HIVDR mutation prevalence (33.3%) in HIV-1 CAD of early treated infants within 1 month of life, with higher frequency in defective proviruses. When considering only intact HIV-1 CAD, HIVDR mutations were detected in 11.1% of the participants. The advantage of this whole-genome sequencing approach allowed us to detect defective viruses using all genomic regions of the virus other than just the HIV *pol* gene commonly used in population-based Sanger sequencing. The findings in this study emphasize the importance of taking into account defective viruses when using HIV-1 CAD sequences from sample types such as dried blood spots and PBMCs.

Generally, the most commonly occurring HIV-1 CAD mutations in infants were to NNRTIs and PIs. However, when looking at only intact HIV-1 CAD, there were no PI or INSTI associated resistance mutations in these infants. Maternal and infant mutations to NNRTIs occurred in 100% of matched pairs, supporting our assumption that the HIVDR mutations identified in intact PBMC HIV-1 CAD sequences represented TDR. Mismatched drug resistance mutations between maternal-infant pairs were not common when assessing HIVDR mutations in intact HIV-1 CAD, we observe only one mismatch between mother and child HIVDR mutations and believe this is likely to represent TDR of minor variant F227L (rather than an early infant de novo mutation) as the infant PBMCs were drawn within 2 days of life.

A study looking at adult patients with low-level viremia (50-1000 copies/mL) characterized HIVDR mutations from PBMCs of 468 patients comparing them to those obtained in plasma.(159) They revealed high proportions of HIVDR mutations in PBMCs; (30.6%) NRTI associated mutations, followed by (22.2%) NNRTI associated mutations, (14.1%) PI associated mutations and (4.9%) IN associated mutations. 20.1% of the patients had mutations shown in the PBMC sequences only(159), supporting the hypothesis that the use of HIV-1 CAD for detection of HIVDR mutations may provide additional information and may reveal hidden archived mutations which may lead to future viral rebound. However, the limitation of this study is that they did not report on whether these HIV-1 CAD sequences represent replication competent viruses. Another study that assessed the presence of HIV associated mutations from DBS reported high rates of NRTI (26%) and NNRTI (40%) resistance associated mutations in children aged 9.9 (IQR 7.6 -13.4) years in Mali, who were on suppressive ART. However,

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approximately 37% of these children had defective HIV-1 CAD genomes.(157) Although these results were obtained by bulk sequencing, they show that reporting resistance data from samples such as PBMCs and DBS may overestimate the prevalence of HIVDR mutations from HIV-1 CAD.

HIVDR in intact vs defective HIV-1 CAD sequences of early treated infants

The second aim of this study was to investigate the frequency of HIV associated drug resistance mutations among intact and defective HIV-1 CAD sequences from very early treated infants. In our analysis we observed higher frequencies of HIVDR mutations within HIV-1 CAD with defects, although not statistically significant ($p = 0.14$). Most mutations that were observed in the defective HIV-1 CAD sequences were APOBEC induced hypermutations, which is expected when using HIV-1 CAD. The mutations observed in defective HIV-1 CAD sequences from these children correlated with what was observed in maternal plasma at delivery on only one occasion, the child had M184I mutation detected in one defective sequence, the matching maternal *pol* sequence at delivery had mutation M184MR. The mutation D30N was the most common PI major mutation in defective HIV-1 CAD, this is a well-known APOBEC induced mutation and was not observed in any of the intact HIV-1 CAD. Similarly, the NNRTI E138K as well as INSTI E138K mutations are common known APOBEC induced mutations and commonly occur in high frequencies when assessing mutations from HIV-1 CAD.(160) This highlights expected findings that HIVDR mutations would occur more frequently in defective viruses as these have altered genotypic and phenotypic characteristics that impact their functionality. Furthermore, we observed INSTI mutations only in HIV-1 CAD sequences with defects, none of the infants with these mutations were exposed to integrase containing regimen in utero or post-partum. Although there is a possibility of children developing resistance mutations without prior ART exposure(161), HIVDR mutations found in defective viruses may have little to no bearing on future clinical outcomes as these are most likely to be observed in genomes that do not contribute to the pool of replication competent viruses. This finding also emphasizes consideration of defective viruses when reporting HIVDR data, especially in settings where HIV-1 CAD from cells, whole blood or DBS are used.

When considering only intact HIV-1 CAD, the most common mutations observed were NRTI and NNRTI mutations. Both the infants and their mothers in this cohort were on NRTI and NNRTI containing regimens. A large number of studies have revealed TDR of NNRTIs to be

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one of the predictors of virologic failure in infants on ART, similarly resistance to NVP and EFV have been reported in treatment experienced children.(107, 137, 162) There is a possibility that the HIVDR mutations detected in intact HIV-1 CAD before one month of age may have had an impact on future clinical failure in this cohort. This is because the mutation A98G was observed across all intact HIV-1 CAD of the infant who had a viral rebound after 84 weeks, with the same clinical genotype detected by Sanger sequencing at failure, indicating that it was likely to be clinically relevant (data not shown). While this child supports the possibility that early archived mutations in children may be implicated in future clinical failure(131), there were also two children with only novel mutations at time of clinical failure. A study conducted by Simonetti *et al*., revealed that a 58-year-old African-American man diagnosed with HIV-1 in 2000 had a replication competent provirus in an expanded cell clone which matched a single viral variant present and detectable during ART.(163) Other studies have supported this finding, showing that intact proviruses are able to produce infectious virions which later lead to adverse treatment outcomes, even in the presence of long-term suppressive ART.(37, 164) Similarly, a study by Katusiime *et al*. 2020, showed that intact HIV-1 CAD were detected in South African children (aged 7 to 9 years) who initiated ART after approximately 2 months of age, these replication competent HIV-1 CAD were maintained by clonal expansion prior to ART initiation.(165) This finding supports our hypothesis that intact HIV-1 CAD are the main drivers for future viral rebound even in the presence of suppressive ART, especially if they harbour early archived HIVDR mutations. In Botswana, a study by Koofhethile *et al*. 2020, quantified the proviral reservoir in 15 perinatally infected adolescents who were receiving ART for over 13 years. This study revealed that despite lifelong ART which was started within the first year of life, neither CAD nor inducible replication-competent proviruses were eliminated.(166)

There were several limitations to this study; these included lack of maternal regimen data for the EID infants who were screened for surveillance drug resistance. There was high sequence failure rate using DBS samples for HIV genotyping, this may have been also due to lack of viral load data for these infants that may have had impact on assay used. None of the mothers were genotyped for INSTI HIVDR mutations and thus comparisons between maternal and infant INSTI HIVDR mutations could not be done in the EIT cohort. We were also not able to show that sequence-intact HIV-1 CAD genomes were actually representative of replication competent and thus definitive proviral reservoirs. Our sample size was very small within both cohorts, precluding statistical comparisons.

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Strengths of our study included use of recent residual DBS samples which were stored in optimal conditions from the EID programme. We also had the advantage of using a carefully followed cohort and the ability to evaluate HIV-1 CAD sequences in early PBMC samples. Most prior studies have traditionally used either plasma or dried blood spots to describe TDR among infants. However, with very early treatment it is increasingly difficult to sequence and genotype HIV from plasma using current methods, and the availability of early PBMCs was an advantage. These archived HIV-1 CAD sequences are very likely to represent TDR, given current knowledge of the early establishment of the latent proviral reservoir following infection -including in utero infection.

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4.1 CONCLUSION

As objectives of this study were set, we investigated the frequency of surveillance SDRMs among newly diagnosed infants in the Botswana EID program as well as the frequency of HIVDR mutations among intact and defective HIV-1 CAD sequences from very early treated infants (EIT). We report relatively high and regionally comparable rates of SDRMs among newly diagnosed infants with unknown PMTCT exposure in Botswana which may suggest that most transmissions occurred in mother-baby pairs who did not access PMTCT services. Although the current Botswana clinical guidelines follow what the WHO currently recommends, our results reveal the importance of genotyping HIV diagnosed infants for HIVDR prior to initiation of first line ART for better lifelong treatment and care.

In the early treated EIT cohort, we observed relatively high rates of HIV drug resistance mutations detected in infant HIV-1 CAD within 1 month of life. Higher rates of these mutations were observed in defective HIV-1 CAD, indicating that the use of whole blood as well as DBS for genotyping in infants may provide an overestimation of what is truly archived and may lead to future viral rebound.

Given high potency of new regimens coupled with EIT, the use of Sanger sequencing in infants with LLV may not yield accurate results, therefore sequencing from cell associated DNA may be the only way to accurately determine HIVDR mutations in cases of MTCT of HIVDR mutations. Our results show that excluding defective HIV-1 CAD sequences from HIVDR analysis yields more accurate drug resistance results. All mutations detected in intact HIV-1 CAD sequences from the EIT infants were also observed in their maternal plasma by Sanger sequencing at delivery, this observation supports the hypothesis that early mother to child HIV transmission of drug resistance may be determined by genotyping HIV-1 DNA from samples such as DBS and PBMCs. Moreover, mutations observed in infant HIV-1 CAD may also provide more information on treatment options for children.

A high proportion of the mutations observed in defective HIV-1 CAD sequences in this study were associated with APOBEC 3-G hypermutation. Therefore, it is pivotal that when performing drug resistance testing on cells that contain HIV-1 CAD, bioinformatic pipelines that exclude defective proviruses should precede any interpretation of drug resistance.

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In conclusion, this study is the first to report drug resistance data in early HIV-1 diagnosed and treated infants in Botswana. Larger studies with higher numbers of participants are recommended to further investigate the impact of drug resistance in Batswana infants for optimal treatment and care.

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ADDENDA

Addendum 1

Table A1a: Drug resistance report for EIT infants <1 month, per sequence

Infant ID	intact/defective	PI Major	PI Accessory	NRTI	NNRTI	INSTI Major	INSTI Accessory
AP-103	Defective	None	G48R, G73S	M184I	M230I	G140R	G163R
AP-103	Defective	None	G48R,G73S	M184I	M230I	None	G140E,G163R
AP-104	Defective	None	None	None	A98G,E138K	NA	NA
AP-104	Intact	None	None	None	A98G	None	None
AP-104	Intact	None	None	None	A98G	None	None
AP-104	Defective	None	None	None	A98G	None	None
AP-104	Defective	None	None	None	A98G	NA	NA
AP-104	Defective	None	None	None	A98G	None	None
AP-104	Intact	None	None	None	A98G	None	None
AP-104	Defective	None	None	None	A98G	NA	NA
AP-104	Defective	None	None	None	A98G	None	None
AP-104	Intact	None	None	None	A98G	None	None
AP-105	Defective	D30N	G48R,G73S	M184I	M230I	None	G140K,G163R
AP-105	Defective	None	G48R,G73S	None	M230I	G140R	None
AP-105	Defective	None	None	None	None	R263K	G140K
AP-105	Defective	None	G73S	M184I	M230I	E138K,G140R	None
AP-105	Defective	None	G48R	None	M230I	None	G140K,G163R
AP-109	Intact	None	None	K65R,Y115F,M184V	K103N,V106M,F227L	None	None
AP-109	Intact	None	None	K65R,Y115F,M184V	K103N,V106M,F227L	None	None
AP-109	Defective	None	None	K65R,Y115F,M184V	K103N,V106M,F227L	None	None
AP-109	Defective	None	None	K65R,Y115F,M184V	K103N,V106M,F227L	None	None
AP-109	Defective	None	G73S	K65R,Y115F,M184V	K103N,V106M,F227L,M230I	None	None
AP-109	Intact	None	None	K65R,Y115F,M184V	K103N,V106M,F227L	None	None
AP-109	Intact	None	None	K65R,Y115F,M184V	K103N,V106M,F227L	None	None
AP-109	Intact	None	None	K65R,Y115F,M184V	K103N,V106M,F227L	None	None
AP-109	Defective	None	None	K65R,Y115F,M184V	K103N,V106M,F227L	None	None

Infant ID	intact/defective	PI Major	PI Accessory	NRTI	NNRTI	INSTI Major	INSTI Accessory
AP-109	Intact	None	None	K65R,Y115F,M184V	K103N,V106M,F227L	None	None
AP-109	Intact	None	None	K65R,Y115F,M184V	K103N,V106M,F227L	None	None
AP-109	Defective	None	G48R	K65R,Y115F,M184V	K103N,V106M,F227L	G140R	None
AP-109	Intact	None	None	K65R,Y115F,M184V	K103N,V106M,F227L	None	None
AP-109	Defective	D30N, M46I	None	K65R,Y115F,M184V	K103N,V106I,E138K,G190E,F227L,M230I	E92K	G140E
AP-109	Defective	None	None	K65R,Y115F,M184V	K103N,V106M,F227L	None	G140K,G163K
AP-109	Defective	None	None	K65R,Y115F,M184V	K103N,V106M,F227L	None	None
AP-109	Intact	None	None	K65R,Y115F,M184V	K103N,V106M,F227L	None	None
AP-109	Intact	None	None	K65R,Y115F,M184V	K103N,V106M,F227L	None	None
AP-109	Defective	None	None	K65R,D67E,Y115F,M184V	K103N,V106M,F227L,M230I	None	None
AP-109	Defective	None	None	K65R,D67E,Y115F,M184V	K103N,V106M,F227L,M230I	None	None
AP-109	Defective	None	None	K65R,Y115F,M184V	K103N,V106M,F227L	None	None
AP-110	Defective	None	G48R,G73S	M184I	None	E138K,G140R,R263K	None
AP-110	Defective	None	G48R,G73S	M184I	M230I	G140R	G163R
AP-110	Defective	None	None	None	None	G140R,R263K	None
AP-110	Defective	None	G73S	None	M230I	G140R	None
AP-110	Defective	None	None	M184I	M230I	G140R	G163R
AP-110	Defective	None	None	None	M230I	None	None
AP-110	Defective	None	None	None	M230I	G140R	G163R
AP-110	Defective	None	None	None	M230I	None	None
AP-110	Defective	None	G48R	M184I	M230I	G140R	None
AP-110	Intact	None	None	None	None	None	None
AP-110	Defective	M46I	G48E	M184I	None	G140R	G163R
AP-110	Defective	None	None	None	None	G140R	None
AP-110	Defective	None	None	None	None	G140R	None
AP-110	Defective	None	G48R	M184I	M230I	G140R	G163R
AP-119	Defective	None	None	None	K103N	None	None
AP-119	Intact	None	None	None	K103N	None	None
AP-119	Intact	None	None	None	K103N	None	None
AP-119	Defective	None	G73S	None	K103N,M230I	R263K	None
AP-119	Intact	None	None	None	K103N	None	None

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Infant ID	intact/defective	PI Major	PI Accessory	NRTI	NNRTI	INSTI Major	INSTI Accessory
AP-119	Defective	None	None	M184I	K103N,M230I	G140R	G163R
AP-119	Intact	None	None	None	K103N	None	None
AP-119	Intact	None	None	None	K103N	None	None
AP-119	Defective	None	None	None	K103N	None	None
AP-119	Defective	None	None	None	K103N,M230I	G140R	None
AP-119	Intact	None	None	None	K103N	None	None
AP-119	Defective	None	None	None	K103N	None	None
AP-119	Defective	None	None	None	K103N	None	None
AP-119	Intact	None	None	None	K103N	None	None
AP-119	Intact	None	None	None	K103N	None	None
AP-119	Defective	D30N, M46I	G48E	None	K103N,G190E	E138K	G140K,G163R
AP-119	Defective	None	None	None	K103N	G140R	None
AP-119	Intact	None	None	None	K103N	None	None
AP-119	Defective	None	None	None	K103N	NA	NA
AP-119	Defective	None	None	None	K103N	None	None
AP-119	Intact	None	None	None	K103N	None	None
AP-119	Intact	None	None	None	K103N	None	None
AP-119	Intact	None	None	None	K103N	None	None
AP-119	Defective	None	None	None	K103N	None	None
AP-119	Intact	None	None	None	K103N	None	None
AP-119	Intact	None	None	None	K103N	None	None
AP-119	Defective	None	None	None	K103N	None	None
AP-119	Defective	None	None	None	K103N	None	None
AP-119	Intact	None	None	None	K103N	None	None
AP-119	Intact	None	None	None	K103N	None	None
AP-119	Defective	None	G73S	M184I	K103N	G140R	G163R
AP-119	Defective	None	G73S	M184I	K103N,M230I	G140R	G163R
AP-119	Intact	None	None	None	K103N	None	None
AP-119	Intact	None	None	None	K103N	None	None
AP-119	Intact	None	None	None	K103N	None	None
AP-119	Defective	None	None	None	K103N	None	None

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Infant ID	intact/defective	PI Major	PI Accessory	NRTI	NNRTI	INSTI Major	INSTI Accessory
AP-119	Defective	None	None	None	K103N	None	None
AP-119	Defective	None	G48E	M184I	K103N,E138K	None	G163R
AP-119	Defective	None	None	None	K103N	None	None
AP-119	Intact	None	None	None	K103N	None	None
AP-119	Defective	None	None	M184I	K103N,M230I	G140R	None
AP-119	Defective	None	None	None	K103N	None	None
AP-119	Intact	None	None	None	K103N	None	None
AP-119	Intact	None	None	None	K103N	None	None
AP-119	Defective	None	None	M184I	K103N,M230I	None	None
AP-130	Defective	D30N	G48R,G73S	None	E138K	G140R	G163R
AP-134	Defective	M46I	None	None	None	NA	NA
AP-134	Defective	M46I	None	NA	NA	NA	NA
AP-138	Defective	D30N	G73S	NA	NA	NA	NA
AP-138	Defective	None	None	M184I	M230I	None	G140K,G163R

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Table A1b: Drug resistance report for EIT mothers at baseline

Maternal ID	PI Major	PI Accessory	NRTI	NNRTI
M-112	None	None	None	E138EA
M-110	None	None	None	None
M-105	None	None	None	None
M-109	None	None	K65R, Y115F, M184V	K103N, V106M
M-120	None	None	None	None
M-117	None	None	None	None
M-125	None	None	None	None
M-103	None	None	M184MR	None
M-115	None	None	None	None
M-118	None	None	None	None
M-123	None	None	None	None
M-124	None	None	None	None
M-121	None	L23LFIV	None	None
M-111	None	None	None	None
M-107	None	None	None	None
M-116	None	None	None	None
M-122	None	None	None	None
M-106	None	None	None	None
M-102	None	None	None	None
M-104	None	None	None	A98G
M-119	None	None	None	K103N
M-101	None	None	None	None