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UNIVERSITY OF THE WITWATERSRAND

MASTERS DISSERTATION

Resistance profiles in HIV-1 positive patients failing protease inhibitor- and dolutegravir-based treatment in the South African public sector

Hendrik Josephus Coetser

A Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine in the Department of Molecular Medicine and Haematology.

Johannesburg, South Africa, 2025

Declaration of Authorship

I, Hendrik Josephus COETSER, declare that this dissertation titled, "Resistance profiles in HIV-1 positive patients failing protease inhibitor- and dolutegravir-based treatment in the South African public sector", and the work presented in it is my own. I confirm that:

- This work was done wholly while in candidature for a research degree at the University of the Witwatersrand.
- Where I have consulted the published work of others, this is clearly attributed.
- I have acknowledged all main sources of help.
- Where this dissertation is based on work done by myself, jointly with others, I have made clear exactly what was done by others and what I have contributed myself.

Signed: 

Date: 07 April 2025

I dedicate this work to Dr. Riffat Munir and Ms. Lara Noble.

You saw potential in me from the very beginning and allowed me to pursue this journey. Your unwavering support and encouragement throughout my master's degree have meant the world to me. I am forever grateful for the trust you placed in me and the invaluable relationship we share.

Presentations and Publications

Presentations

1. 30th International Workshop on HIV Drug Resistance and Treatment Strategies (Cape Town)

   HJ Coetser, L Hans, D Magubane, L Gaelejwe, E Letsoalo, K Steegen (2023) The use of atazanavir limits cross resistance to darunavir in the South African Public Sector. *Oral*

2. University of the Witwatersrand Faculty of Health Sciences Research Day (Johannesburg)

   HJ Coetser, D Magubane, L Gaelejwe, E Letsoalo, S Currin; K Steegen (2024) Dolutegravir drug-exposure testing as a triaging tool to identify patients at the highest risk for HIV drug resistance during antiretroviral therapy for an HIV-1 infection. *Oral*

   HJ Coetser, L Hans, D Magubane, L Gaelejwe, E Letsoalo, K Steegen (2024) The use of atazanavir limits cross resistance to darunavir in the South African Public Sector. *Poster*

3. Conference on Retroviruses and Opportunistic Infections (CROI) 2025 (San Francisco, CA, USA)
   HJ Coetser, D Magubane, L Gaelejwe, E Letsoalo, D Legg-E'Silva, L Hans, S Currin, K Steegen (2025) Plasma Dolutegravir Exposure as a Triaging Tool for HIV Drug Resistance Testing by ONT Sequencing. *Poster*

UNIVERSITY OF THE WITWATERSRAND

Faculty of Health Sciences

Abstract

MSc(Med) Molecular Medicine and Haematology

by Hendrik Coetser

INTRODUCTION: South African antiretroviral therapy (ART) guidelines recommend dolutegravir (DTG)-based regimens, though some individuals remain on protease inhibitor (PI)-based ART during the transition. We evaluated resistance profiles for people living with HIV (PLWH) failing PI- or DTG-based regimens. Additionally, we assessed plasma DTG (pDTG) drug-exposure (DE) testing for predicting DTG resistance and investigated nanopore sequencing for HIV drug resistance (HIVDR) testing.

METHODS: HIV sequences from PLWH who failed PI regimens in 2022 and DTG regimens from 2019–2022 were analysed. Nanopore sequencing using the SQK-NBD114.96 kit and pDTG DE testing through liquid chromatography tandem mass spectrometry were performed on DTG cohort specimens.

RESULTS: In the PI cohort (n=1244), the LPV/r group showed higher cross-resistance to DRV/r compared to the ATV/r group (14.5% vs 8.9%, p=0.013). In the DTG cohort (n=97), 19.5% had DTG resistance, with 8.5% developing resistance within 24 months. Undetectable pDTG (54.6%) accurately predicted the absence of DTG resistance (negative predictive value: 94.2%, 95% CI: 85.1-97.9). Sanger and nanopore sequencing demonstrated 99.6% (range: 88.9-100.0) and 97.9% (range: 75.6-100.0) identity for integrase and protease-reverse transcriptase regions, respectively.

DISCUSSION: Our findings support ATV/r as the preferred PI in the South African public sector. DTG resistance in 19.5% of failures underscores the need for early identification of PLWH at risk for resistance. pDTG DE testing can reduce HIVDR testing costs by triaging samples for HIVDR testing. As DTG resistance developed within 24 months, earlier resistance testing may be warranted. Nanopore sequencing is a reliable alternative to Sanger sequencing for HIVDR testing.

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Abbreviations

Terms and phrases

3TC	Lamivudine
AARMS	Academic Affairs and Research Management
ABC	Abacavir
ADR	Acquired Drug Resistance
AIDS	Acquired Immunodeficiency Syndrome
ALD	Abacavir-Lamivudine-Dolutegravir
ART	Antiretroviral Therapy
ATV/r	Atazanavir/ ritonavir
AZT	Zidovudine
CAB	Cabotegravir
cART	Combination Antiretroviral Therapy
cDNA	Complimentary DNA
CI	Confidence Interval
CMJAH	Charlotte Maxeke Johannesburg Academic Hospital
d4T	Stavudine
DE	Drug Exposure
ddNTP	Dideoxynucleotide Triphosphate
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
DOR	Doravirine
DRM	Drug Resistance Mutation
DRV/r	Darunavir/ ritonavir
dsDNA	Double-stranded DNA
DTG	Dolutegravir
EDTA	Ethylenediaminetetraacetic Acid
EFV	Efavirenz
ETR	Etravirine
EVG	Elvitegravir
FDA	Food and Drug Administrator
FDC	Fix Dose Combination
FTC	Emtricitabine
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
HIVDR	HIV drug resistance
HREC	Human Research Ethics Council
IN	Integrase
InSTI	Integrase Strand Transfer Inhibitor
IQR	Inter quartile Range
LC	Liquid Chromatography
LC – MS/MS	Liquid Chromatography Tandem Mass-spectometry
LMIC	Low to Middle Income Country
LPV/r	Lopinavir/ ritonavir
LTR	Long Terminal Repeat
mRNA	messenger RNA

NDoH	National Department of Health
NGS	Next Generation Sequencing
NHLS	National Health Laboratory Services
NRTI	Nucleoside Reverse Transcriptase Inhibitor
NNRTI	Non-nucleoside reverse Transcriptase Inhibitor
NVP	Nevirapine
ONT	Oxford Nanopore Technologies
pDTG	Plasma DTG
PEPFAR	The U.S. President's Emergency Plan for AIDS Relief
PCR	Polymerase Chain Reaction
PI	Protease Inhibitor
PLWH	People Living with HIV
RAL	Raltegravir
RNA	Ribonucleic Acid
PR	Protease
QC	Quality Check
RT	Reverse Transcriptase
SD	Standard Deviation
TAF	Tenofovir alafenamide
TB	Mycobacterium tuberculosis
TDF	Tenofovir Disoproxil Fumarate
TDM	Therapeutic Drug Monitoring
TDR	Transmitted Drug Resistance
TEE	Tenofovir- Emtricitabine- Efavirenz
TGS	Third Generation Sequencing
T_h	Helper T Cell
TLD	Tenofovir-Lamivudine-Dolutegravir
UNAIDS	United Nations Programme on HIV/AIDS
USD	United States Dollar
UV	Ultra Violet
VL	Viral Load
vs	Versus (as opposed to)
WHO	World Health Organisation
XTC	lamivudine (3TC) or Emtricitabine (FTC)
ZLD	Zidovudine-Lamivudine-Dolutegravir

Symbols and Units of Measurement

~	Approximately
°C	Degree Celsius
%	Percent
x g	Times Gravity
bp	Base Pair
fmol	Femtomoles
IC_{50}	Half-maximal inhibitory concentration
kb	Kilobase
µL	Microlitre
µg	Microgram
µM	Micromolar
mL	Millilitre
V	Volts

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Chapter 1

Introduction and Literature Review

The human body maintains two distinct defence barriers against external pathogenic infections, the innate- and adaptive immune systems. The adaptive immune response, further divided into humoral- and cell-mediated immunity, takes over should the first-line innate immunity fail to confine the infection. [1] The humoral immune response relies on Darwinian principles to combat disease i.e. selective pressure on rapid diversifying mutations. [2] A conventional reaction of the adaptive immune system to infection is characterised by the initial activation of naïve B cells which proliferate in germinal centers of secondary lymphoid organs. These B cells mature and differentiate into memory B cells and antibody-secreting plasma cells which target the acute pathogenic infection. The activation process of the naïve B cell requires additional assistance from a helper T (T_h) cell which binds to the major histone-compatibility complex II on the naïve B cell. [1–3]

However, true to the law of evolution, one lentivirus meticulously shaped its genetic blueprint to evade this systemic reaction and ensure its survival. The human immunodeficiency virus (HIV) specifically infects and destroys T_h cells which renders the immune system defenceless against future pathogenic infections, resulting in a condition known as acquired immunodeficiency syndrome (AIDS) if left untreated. [4] Chronic HIV infection escapes total eradication from the body by the immune system in two broad ways: first ensuring rapid diversification of antigens and secondly creating latent viral reservoirs, both of which are undetectable to immune cells. [5–7]

1.1. Human immunodeficiency virus

1.1.1. Viral classification and genomic structure

The first cases of HIV/AIDS were reported in 1981, however it's estimated that the immunodeficiency virus evolved from non-human primate species to humans during the 1920s in central and west Africa. [8] Modern phylogenetic analysis clusters HIV under the family Retroviridae, subfamily Orthoretrovirinae. The two major types (HIV-1 and HIV-2) are further divided into groups, subtypes, and recombinant forms (derived from various subtypes). [6] The modern AIDS pandemic is mainly driven by the spread of HIV-1 which is divided into four groups; M (Major), O (Outlier), N (non-M, non-O), and the most recent P-group. [9] Moreover,

as the name suggests, group M is responsible for most new infections and includes nine subtypes (A-D, F-H, J, K). In addition to these group M subtypes, 20% of group M viruses are circulating recombinant forms. On the other hand, HIV-2 contains nine groups (A-I) and is mostly confined to West Africa because this type is less infectious. [6,9]

The ~9.6 kilobase (kb) HIV-1 genome contains three reading frames with nine genes, encoding for 15 viral proteins (Figure *1.1*). [6,10] The *pol* gene encodes the enzymatic proteins, i.e. integrase (IN), reverse transcriptase (RT), and protease (PR). [11] These are essential during the viral replication cycle.

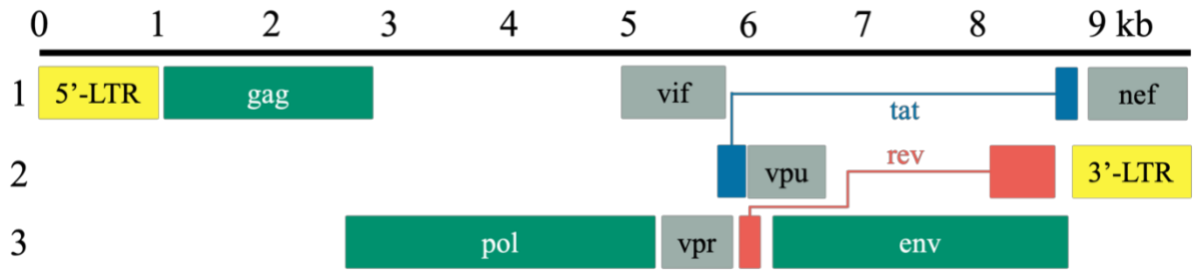


Figure 1.1: Structural organisation of the HIV-1 genome. The three major genes include *gag, pol*, and *env*. *Gag* codes for structural proteins (Capsid, Matrix, Nucleocapsid, and p6), *pol* codes for enzymes (Integrase, Reverse transcriptase, Protease), and *env* codes for envelope proteins (GP120, GP41). There are two regulatory proteins (*rev, tat*) and four accessory proteins (*Nef, vif, vpr, and vpu*). The genome also includes long terminal repeats (LTR) at the 5' and 3' ends. *Figure adapted from Li et al* [10] *and Kirchhoff* [11]

1.1.2. HIV replication cycle

The replication cycle of HIV is complex and divided into two phases, the early phase starts with the attachment of the virion to the T_h cell (however this attachment is not exclusive to one type of immune cell) and ends with proviral deoxyribonucleic acid (DNA) integration (Figure *1.2*). HIV recognises CD4 receptors expressed on immune cell surfaces, the CCR5 and CXCR4 chemokines serve as co-receptors for binding. Infection is initiated with the conformational change that happens when viral GP120 binds with the cellular CD4 receptors. This conformational change allows GP120 to interact with either co-receptors, causing additional conformational changes. The result is the first direct contact between the virus and host cell mediated by GP41 which inserts a hydrophobic phusion peptide into the cell membrane. The trimeric GP41 then develops a helical structure that pulls the two entities together allowing HIV to release its genetic material into the host cell. [11] The HIV genome consists of two single-stranded RNA molecules that must be converted to double-stranded DNA (dsDNA), suitable

for integration into the host's genome. During reverse transcription by the RT enzyme, the capsid is disassembled after which the dsDNA is transported across the nuclear membrane. The IN enzyme subsequently incorporates the proviral DNA into the host genome where the cell can either enter the second phase or stay latent for years. (11)

The late phase involves the assembly process and maturation of virions (Figure *1.2*). Cellular Pol II polymerase facilitates the transcription of proviral DNA. Initially, this process is slow as the viral transactivator protein, tat, is required for efficient elongation. Alternative splicing can produce 25 different messenger ribonucleic acids (mRNA) in three different size classes to translate the necessary proteins (Table *1.1*). Viral particles then gather at the cell membrane for assembly. Shortly after budding, viral PR becomes active to cleave *gag* and *gag-pol* precursors into their respective mature components rendering the mature virus infectious. (11)

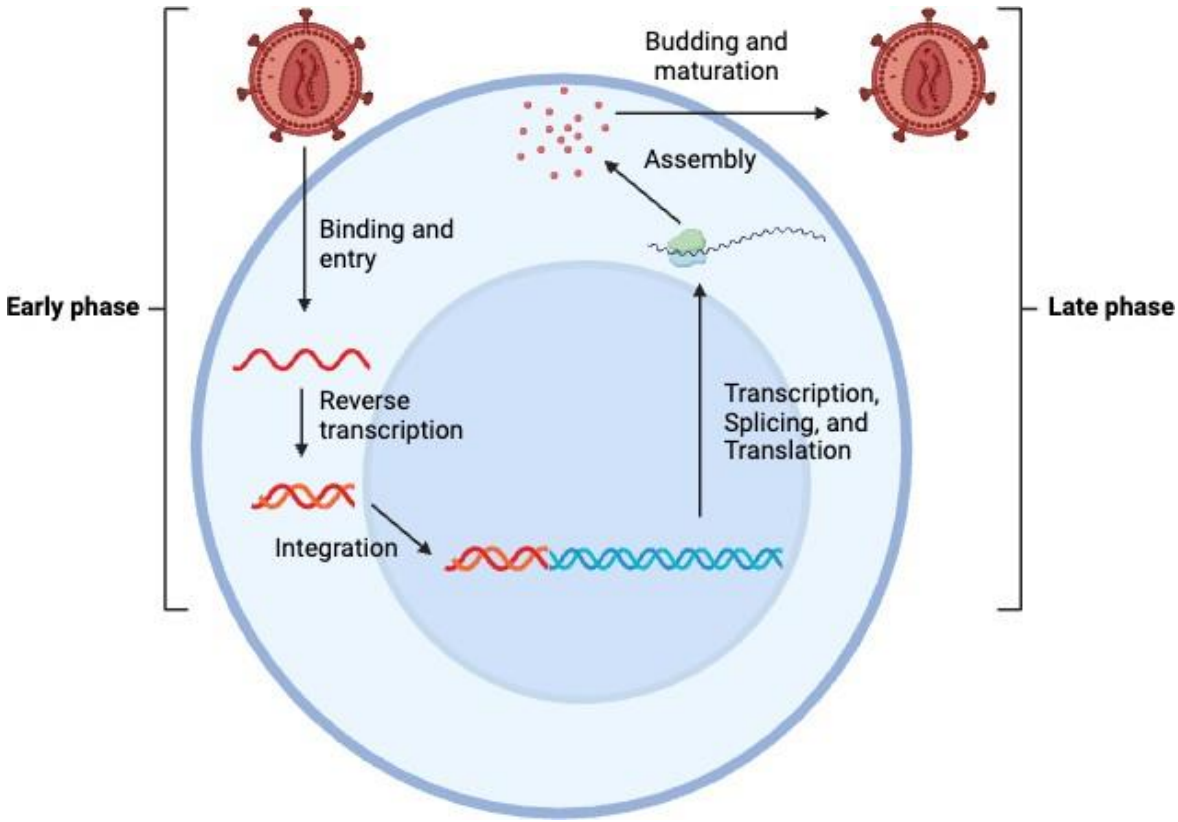


Figure 1.2: HIV replication cycle. The virion attaches and enters the host cell, and reverse transcription of viral RNA into dsDNA occurs next whereafter the dsDNA is integrated into the host genome. This is known as the early phase of the cycle. During the late phase, viral DNA is transcribed and translated into viral proteins which accumulate at the cell membrane, assembles, and buds into mature virions. *Image generated using Biorender.com*

Table 1.1: Proviral alternative spliced mRNA sizes and products

mRNA size	**Product after translation**
Unspliced (9 kb)	*Gag* and *Gag-Pol* precursors
Single spliced (4 kb)	*Vif, Vpr, Vpu*, and *Env*
Fully spliced (2 kb)	*Tat, Rev*, and *Nef*

Kb: Kilobase; mRNA: Messenger ribonucleic acid

1.1.3. Human immune system evasion

One of the leading strategies in which HIV-1 eludes the human immune system is by integrating its pro-viral DNA into the host's genome. An infected T_h cell has either one of two fates; the first being transcriptionally active, this will produce viral antigens which can be recognised by the immune system during the early phase of infection. Alternatively, the cell can stay in a latent phase and transcriptionally inactive without the production of viral antigens, these cells are indiscernible from normal healthy cells to natural killer cells which are actively involved in the destruction of infected cells. [12] These latent cells form the HIV latent reservoir and it is the major barrier preventing total eradication of HIV-1 by current antiretroviral therapy. [13]

Although latent viral reservoirs can be found throughout the body, some tissues are considered hotspots. Lymph nodes and the gut-associated lymph tissue hold most of the inactive provirus followed by the liver, genital tract, and brain. [13,14] There are several strategies developed to combat these latent reservoirs, however, these are still in the experimental phases rendering an HIV-1 infection without a cure. [12]

1.1.4. Natural history of HIV-1 infection

Briefly, there are three main stages of an HIV-1 infection without any treatment interventions (Figure *1.3*). Initial inoculation of HIV is characterised by a high-level viremia and a reduction in total CD4 cell count. The human immune system responds and CD4 cell counts almost return to baseline levels (>500 counts/μL). Thereafter, the patient progresses into an asymptomatic phase where viral transcription is moderately low and a slight decrease in CD4 cell counts is observed, this stage can last for years. Finally, when a patient has progressed into AIDS their CD4 cell count further declines with a sharp increase in the viral load allowing opportunistic infections to set in and ultimately cause death. [6,15] Despite this, people living with HIV (PLWH) on the correct antiretroviral therapy (ART) regimen with suppressed viral

loads can stay in the asymptomatic phase for decades and lead relatively normal and healthy lives. [16]

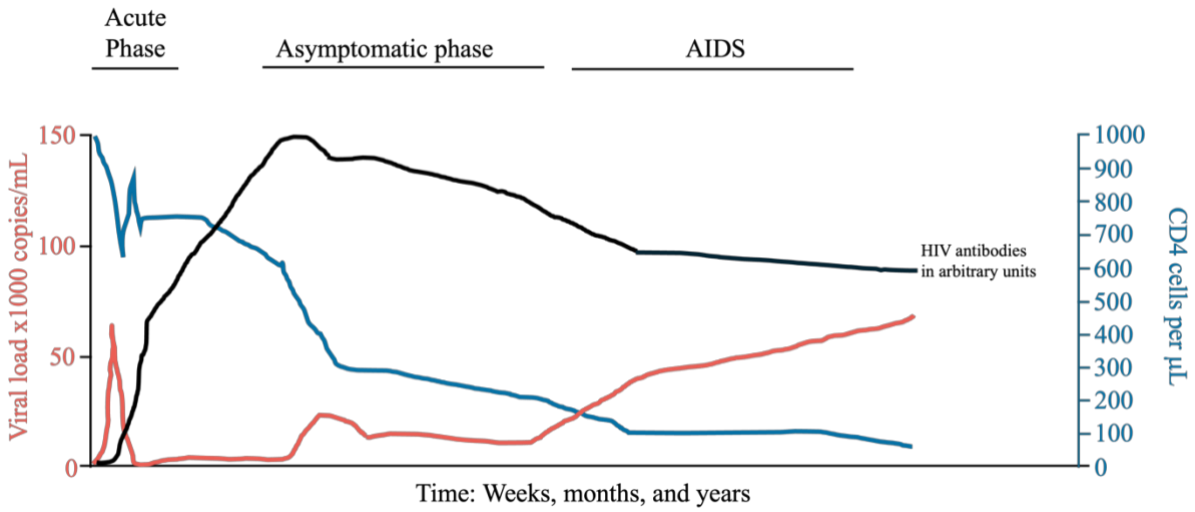


Figure 1.3: HIV-1 infection stages. The initial acute phase is characterised by an increased viremia and decreased CD4 cell count which increases shortly after the immune response. Followed by an asymptomatic phase with a slight decrease in CD4 cell count and low viral replication. AIDS sets in during stage three where the CD4 cell count continues to fall allowing opportunistic infections to settle in and ultimately leading to death. *Figure adapted from German Advisory Committee Blood* [6]

1.1.5. The HIV/AIDS epidemic

In 1996 the joint United Nations Programme on HIV/AIDS (UNAIDS) was established to provide strategic coordination to combat the HIV/AIDS epidemic globally stratified down to local leadership. Their 95-95-95 target for 2025 could be within reach: 95% of PLWH know their HIV status, 95% of those who know their status must be on ART, and 95% of those on ART must be virologically suppressed. As of July 2024, 86% of PLWH know their status, of whom 89% are on ART and 93% of those on ART are virally suppressed. [17] Data from 2023 indicates that South Africa is close to reaching the first (94%) and third target (92%), but still needs to improve on linking PLWH to care as only 77% of those who know their status are on ART. [18] Sub-Saharan Africa has been the epicentre of the HIV/AIDS epidemic for over 40 years, however, recent data show that the decline in new infections is more rapid in this region compared to the rest of the world. [17,19] Nevertheless, the financial impact associated with HIV/AIDS in these low to middle-income countries (LMICs) remains a major burden. [20] One way to reduce financial demand on LMIC public health service entities is to investigate the suitability of alternative testing methods requiring less monetary support, making HIVDR testing more affordable and accessible globally.

1.2. Antiretroviral therapy

Shortly after the first cases of HIV/AIDS were reported a nucleoside reverse transcriptase inhibitor (NRTI) named zidovudine was approved as the first drug to treat PLWH. Later, highly active antiretroviral therapy (HAART), or combination antiretroviral therapy (cART) was introduced which yielded a better success rate. The daily dosing of these therapies was complex with several side effects. However, in 2006 fixed-dose combinations (FDC) made a breakthrough by improving adherence through lower pill burden. [16] The coherent principle of these therapies is to lower the viral load of a patient into the undetectable range (<50 copies/mL) to prevent the progression of HIV into subsequently severe stages and ultimately AIDS. [7,16] As of 2023, around 30.7 million PLWH received ART globally with the South African HIV program providing treatment to 5.9 million individuals. [17] UNAIDS has recently reported a huge rebound in average life expectance of PLWH in South Africa, 65.9 years in 2023 vs 53.6 years in 2004. [17] This can be attributed to the exponential increase in ART roll-out over the years in Southern and Eastern Africa with a current HIV treatment budget of USD 9.3 billion for the region. [17]

1.2.1. ART drug classes and regimens

Current treatment options include eight ART drug classes that target and inhibit specific steps during the HIV replication cycle (Figure *1.2*). [7,21] The first drug class to be approved by the US Food and Drug Administration (FDA) was the NRTIs. This class inhibits the RT enzyme by incorporating the intracellular phosphorylated metabolites into the DNA analogue causing chain termination. The RT enzyme can also be inhibited by the non-competitive inhibitor class; non-nucleoside reverse transcriptase inhibitors (NNRTIs) which results in a conformational change that decreases the enzyme activity. Protease inhibitors (PIs) prevent proteolytic assembly of virions into mature infectious viral particles, these are usually boosted with ritonavir to enhance the pharmacokinetic profile of the active PI. [22] Integrase strand transfer inhibitors (InSTIs), exhibit their pharmacologic action by preventing the formation of covalent bonds between viral DNA and the host chromosome, during the integration process. [7,16] Fusion inhibitors bind to the GP41 protein to prevent the conformational change during binding and entry of the virion. [7] Capsid inhibitors intervene in the capsid assembly during the final stages of the HIV infection cycle. [23] Finally, the CCR5 antagonist drug class and post-attachment inhibitor drug class selectively bind the CCR5 coreceptor, expressed on certain human immune cells, which actively blocks the interaction with GP120 on the HIV capsid. Additionally, within

this class, the CXCR4 coreceptor is also targeted by post-attachment drugs to prevent attachment with HIV. [7,24] Although all of the drugs mentioned above are available in the Western world, only some of the NRTIs, NNRTIs, PIs, and InSTIs are available in the South African public sector (Table *1.2*). Early on, clinicians and scientists agreed that antiretroviral (ARV) monotherapy proved ineffective in suppressing the viral load. [7] Therefore, ART regimens typically included two NRTIs as the regimen backbone and an NNRTI, PI, or InSTI anchor drug. [7,25]

Table 1.2: Current ARV drugs available in the South African public sector

Drug Class	Drug	Drug abbreviation
Nucleoside reverse transcriptase inhibitors	Abacavir	ABC
	Emtricitabine	FTC
	Lamivudine	3TC
	Tenofovir disoproxil fumarate	TDF
	Zidovudine	AZT
Non-nucleoside reverse transcriptase inhibitors	Efavirenz	EFV
	Etravirine	ETR
	Nevirapine	NVP
Protease inhibitors	Atazanavir/ritonavir	ATV/r
	Darunavir/ritonavir	DRV/r
	Lopinavir/ritonavir	LPV/r
Integrase strand transfer inhibitors	Dolutegravir	DTG
	Raltegravir	RAL

Current South African treatment guidelines suggest an NRTI backbone (Tenofovir and Lamivudine) with dolutegravir (DTG) as the anchor drug for all PLWH (Table *1.3*). This regimen is known as TLD and is available in a single tablet, taken once daily, providing the individual with a decreased pill burden and better gastrointestinal tolerability. [26] An alternative DTG-based regimen, with abacavir instead of tenofovir, is recommended for children younger than 10 years and weighing less than 20 kg. This is a significant change from guidelines before 2019 when first-line and second-line regimens did not include InSTIs as these were reserved for personalised third-line regimens. [26,27] This change was sparked when Dolutegravir was welcomed by the World Health Organisation (WHO) in 2019 as the preferred ARV drug due to sufficient data supporting its safety and efficacy, especially at a time when increasing pre-treatment resistance to NNRTIs was observed in LMICs. [28]

Table 1.3: South African ART guideline changes

		Adults (≥ 15 years)	Children and adolescents
2013 - 2019	**1st – line**	TDF + 3TC (or FTC) + EFV Provided as FDC if opted for FTC	**(< 10 years):** ABC + 3TC + LPV/r or EFV [a] **(10-15 years):** ABC + 3TC + EFV [b] / TDF + 3TC/FTC + EFV [c]
	2nd – line	AZT + 3TC + LPV/r [d]	AZT or ABC + 3TC + LPV/r [e]
2019 - 2023		**Adults and adolescents (≥ 35kg; ≥ 10 years old)**	**Children (≤ 10 years old)**
	1st – line	TDF + 3TC + DTG provided as FDC	ABC + 3TC + DTG [f]
	2nd – line	AZT/TDF + 3TC/FTC + ATV/r or DTG [g]	ABC/AZT/TDF + 3TC/FTC + EFV/ NVP or LPV/r or ATV/r or DTG [h]

[a] Children <3 years or older children weighing < 10kg: LPV/r; Children 3-10 years and >10kg: EFV. [b] Weight <40 kg or age <15 years. [c] Weight ≥40 kg and age ≥15 years. [d] Switch LPV/r to ATV/r if dyslipidaemia or diarrhoea associated with LPV/r. [e] Failed first-line (ABC + 3TC + EFV (or NVP)): 3TC; failed first-line (d4T + 3TC + EFV (or NVP)): ABC. [f] Neonates (≤ 2.5kg, < 4 weeks old: AZT + 3TC + NVP); Infants and children (3kg – 20kg, > 4 weeks old: ABC + 3TC + LPV/r). [g] Specific for individuals on a failing NNRTI-based first-line regimens. [h] NNRTI/PI/InSTI specified regimen; 3TC Lamivudine; ABC: Abacavir; ATV/r: Atazanavir/ritonavir; AZT: Zidovudine; d4T: Stavudine; DTG: Dolutegravir; EFV: Efavirenz; FDC: Fix Dose combination; FTC: Emtricitabine; InSTI: Ontegrase Strand Transferase Inhibitor; LPV/r: Lopinavir/ ritonavir; NVP: Nevirapine; NNRTI: Non-nucleoside Reverse Transcriptase Inhibitor; PI: Protease Inhibitor; TDF: Tenofovir;. *Table adapted from NDoH.* (21,26,27,29)

1.2.2. Treatment monitoring and outcomes

Regular viral load (VL) testing, in combination with clinical and immunological monitoring, is used to assess treatment outcomes. (30,31) In South Africa, the first two VL tests are recommended at three and 12 dispensing cycles after initiating ART. A dispensing cycle refers to the number of days an individual would have treatment available if provided with a standard 'monthly' supply of tablets. Thereafter viral load is monitored annually provided the viral load remains suppressed (< 50 copies/mL). A thorough assessment needs to be conducted if the VL exceeds 50 copies/mL which may be caused by adherence challenges, co-infections, incorrect ART dosage, drug interactions, or development of drug resistance. In this case, enhanced adherence support is offered, and a repeat VL is requested after three months with three possible outcomes. If the follow-up VL is suppressed, then the patient returns to routine annual VL monitoring with no additional intervention. If the follow-up VL is between 50 – 999 copies/mL, another round of intensive adherence support is provided and another VL is requested three months later. Lastly, when two consecutive viral loads are above 1000 copies/mL (known as

confirmed virological failure), the patient is considered for a treatment switch, depending on the treatment exposure. [21] If a person living with HIV presents with confirmed virological failure and these VLs were taken at least two years after DTG-based (or PI-based) regimen initiation, while adherence issues have been addressed, the patient might be eligible for resistance testing as explained in Figure *1.4*.

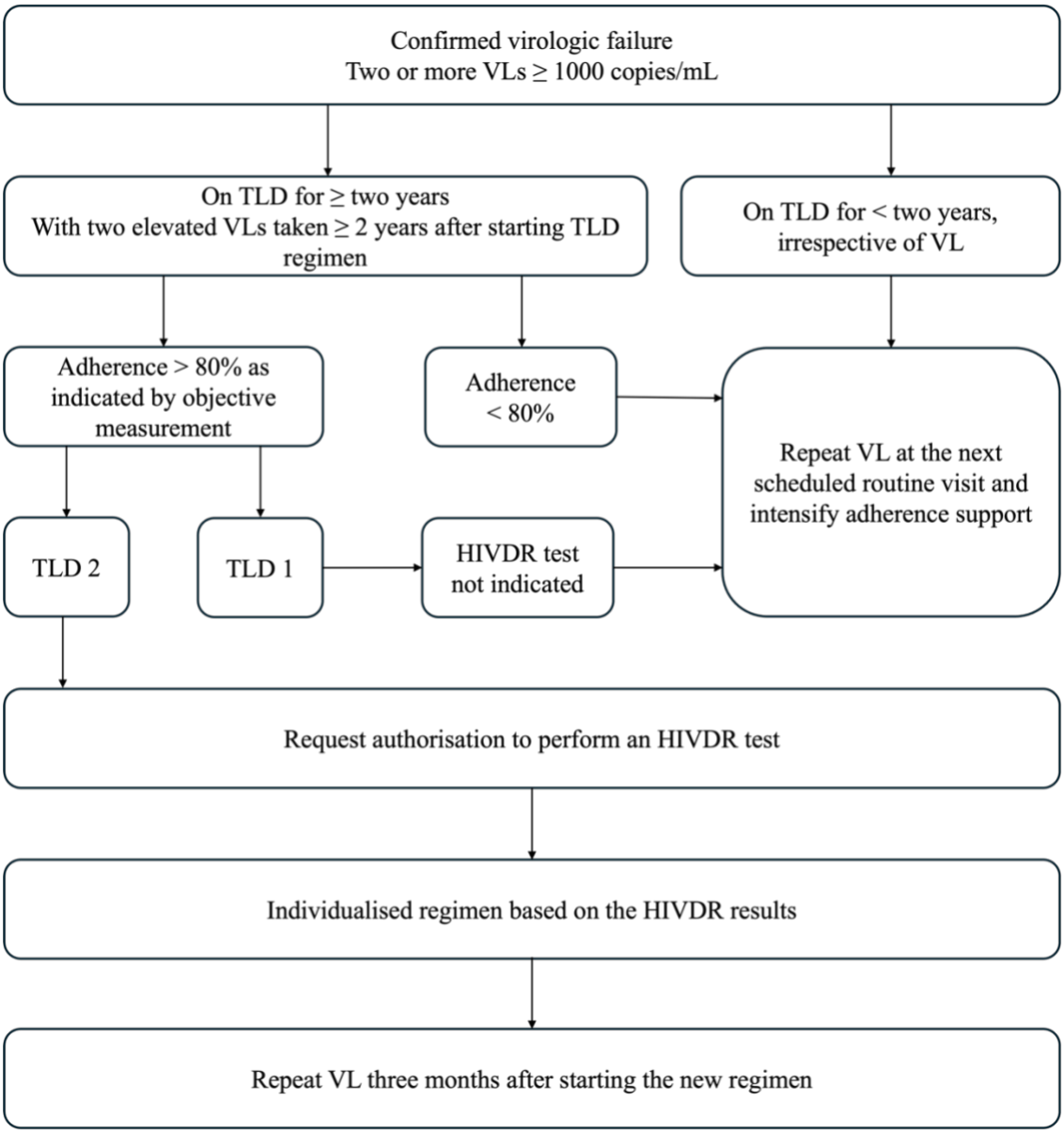


Figure 1.4 Flow diagram on the management of confirmed virologic failure. HIVDR – HIV drug resistance; TLD – Tenofovir, Lamivudine, Dolutegravir; TLD 1 – first-line regimen; TLD 2 – Second-line regimen; VL – Viral load. *Diagram adapted from NDoH* [21]

1.2.3. Antiretroviral drug resistance

Since HIV creates latent virus reservoirs, ART is a life-long commitment and suboptimal adherence to ART has dire consequences. The RT enzyme has one of the highest mutation rates

recorded in any biological system, resulting in a diverse viral population within a single patient. [7,12,32] When a patient is taking ART, selective pressure acts on the viral population and intermittent adherence or temporary discontinuation of the therapy will most likely result in viral rebound and ultimately virologic failure. Given the high error rate during replication, mutations that can evade the treatment regimen will be selected and present in the bulk replicating virus. The selection of resistant mutations under drug pressure is known as acquired drug resistance (ADR). [7,33]

Individual factors can hinder adherence to ARV. To start with, some ARV medications can cause intolerable gastrointestinal toxicity which can lead to the omission of the unfavourable drug leading to unintended suboptimal treatment, or cessation of the ART treatment entirely. [34] Psychiatric side effects of ARVs such as depression are also prevalent in PLWH. [35] While self-reported stigma around HIV/AIDS, pill burden, and forgetfulness also add to the problem. [36] Additionally, PLWH in South Africa may face structural challenges that make it even more difficult for them to adhere to ART. This includes poverty-related barriers such as inadequate funds to access transport to a medical facility, health facilities that might not be able to consistently ensure adequate medical services, and political or cultural factors such as low-level health literacy and gender inequality. [37] However, it is important to note that an ARV naïve patient can also present with HIV drug resistance (HIVDR) when they have been infected by a virus that already carries drug-resistant mutations (DRMs), i.e. transmitted drug resistance (TDR). This poses a significant challenge for public health as pre-treatment HIVDR testing is unattainable in LMICs and hinders the success of initial ART. [38] Finally, pretreatment HIV drug resistance defines any HIVDR present in patients before initiating ART, whether it be an ARV drug-naïve patient or someone reinitiating ART. Both TDR and ADR can form part of this distinct group, the latter includes women exposed to ARV drugs during mother-to-child transmission of HIV prevention, people with previous exposure to pre-exposure prophylaxis, and those reinitiating ART after a treatment interruption. [33]

1.3. HIV drug resistance testing

A peer-reviewed study showed that PLWH have a significantly higher chance of suppressed viremia if their ARV regimens are tailored according to their unique HIVDR profiles. [39] Therefore, the value of HIVDR testing is undeniable, this standard-of-care approach to disease management is evident in the wide implementation in resource-rich countries where HIVDR testing is requested before initiating treatment. [40] However, this approach is neither feasible to

be implemented on a public health scale nor routinely recommended for PLWH in LMICs such as South Africa and is only available under strict guidelines. [41]

Resistance can be assessed through phenotypic and genotypic assays. Phenotypic assays work on the principle to measure ARV susceptibility *in vitro*, which is the ARV concentration that inhibits 50% of HIV-1 replication (IC_{50}). A patient's IC_{50} is then compared to a drug-susceptible reference strain and expressed as a ratio or fold change. However, these assays require costly and time-consuming processes rendering them unsuitable in a public health setting. Therefore, genotypic assays are the preferred method, with a much shorter turn-around time, lower costs, and the availability of commercial kits and laboratory-developed methods. Genotyping involves direct sequencing through either Sanger or next-generation sequencing (NGS) of the viral genome, often specifically focusing on the *pol* gene and determining resistance profiles on a rules-based algorithm. [41] When ART is interrupted the selective pressure favouring mutant virions will be lost and the replicating viral population will revert to wild-type. However, these mutant virions may still be present as low-abundance variants, and therefore more sensitive technologies might be beneficial. [42]

1.3.1. Sanger sequencing

Population-based Sanger sequencing is still the preferred method for HIVDR testing in many clinical and research settings. [43,44] This chain-termination technique utilises chemical analogue monomers of the DNA strand mixed with the DNA template. Deoxynucleotides (dNTPs) are required for extending the DNA strand and dideoxy nucleotides (ddNTPs), which lack the 3' hydroxyl group, terminate elongation when incorporated into the growing strand resulting in different chain lengths. Each type of ddNTP is labeled with a unique fluorophore and during elongation each DNA strand terminates with one ddNTP at a specific length. These DNA strands are then sorted by size as they move through the sequencing capillary resulting in a constructive sequence. [45] The HIVDR principle is to reverse-transcribe viral RNA from plasma into complementary DNA (cDNA) which is sequenced after polymerase chain reaction (PCR) amplification. By using an HIV reference genome one can determine specific point mutations which might result in drug resistance. To date, sequencing of the protease and reverse transcriptase regions of the *pol* gene is standard and a special request by the clinician should be made to include the integrase region for drug-resistant mutations (DRMs) screening. [41] However, Sanger sequencing is limited in its sensitivity as it only accurately detects variants

present in at least 20-25% of the viral population. [44] Further limitations include the high cost of reagents and lab equipment rendering an HIVDR test relatively costly in LMICs. [41]

1.3.2. Next generation sequencing

A huge breakthrough was seen in sequencing technologies with the introduction of massive parallel sequencing, later known as NGS. The genome is essentially broken into smaller fragments and sequenced multiple times concurrently. [46] Recent advances in NGS have dramatically increased sequencing resolution and sensitivity to detect low-abundance mutations between 1-20%. [44] NGS in the HIV research field was initially only used to identify HIV quasispecies, but most NGS platforms are now been implemented for HIVDR testing. [47] Given the higher sensitivity to detect low-abundance variants, NGS has the potential to better predict virologic failure due to DRMs in low quantities at a threshold of >2%. [48,49] It may indicate the early emergence of this DRM, the final phase of its disappearance, or issues related to adherence. Next-generation sequencing can be time efficient and possibly reduce cost because of the high batched sample capacity. [47] Implementation of NGS for HIVDR testing in a public health setting remains challenging in many LMICs due to the need for highly skilled staff, significant capital expenditure for equipment, limited access to distributors and service providers, and complicated validation procedures. [43,44]

Currently, two platforms dominate the HIV NGS sequencing market, the Illumina MiSeq platform and the Ion Torrent Personal Genome Machine manufactured by ThermoFisher. Although both instruments are short-read sequencing platforms they differ in detection methods with variable data outputs (Table 1.4). [43] Briefly, the Illumina MiSeq system utilises specific fluorophores for each nucleotide base, the flow cell is flooded with a single nucleotide at a time whereafter an image of the flow cell is taken, if the base is incorporated it will illuminate upon excitation and recorded in the sequence. [43,50] Whereas the Ion Torrent Personal Genome Machine's method of detection depends on the change in pH of the solution upon the release of a hydrogen ion when a specific base is incorporated. [43,51]

Table 1.4: NGS platforms currently used in HIVDR genotyping

Instrument	Detection	Data output	Maximum read length	Reported accuracy [a] /error Rate	Sequencing time
MiSeq	Fluorescence	0.3–15 Gb; 2–50 million reads	2 × 300 bp	Mostly > Q30/0.8%	4–55 h
Ion Torrent PGM	Semi-conductor	0.03–2 Gb; 0.4–5.5 million reads	400 bp	Mostly > Q20/1.7%	2–10 h

[a] Incorrect base-calling: Q30 – probability of 1 in 1000; Q20 – probability of 1in 100. PGM – Personal Genome Machine. *Table adapted from Ávila-Ríos et al.* [43]

Oxford Nanopore Technologies (ONT) has taken the principle of NGS even further and is now considered a third-generation sequencing (TGS) platform. [46] It is the first ultra-long-read sequencing platform utilising α-hemolysin nanopores embedded in a solid-state graphene membrane. The nucleotide bases are suspended in an electrolyte and pulled through the nanopores when a current is applied to the system. The electrical impendence of the channel increases as the nucleotide enters the nanopore, the particles are then detected and identified as current drops, unique to each specific nucleotide base. [52–54] This technology is more cost-effective than traditional NGS platforms, and the instrument's portability makes it more accessible to infrastructurally challenged settings. [55] A few studies evaluated nanopore-sequencing as a possible substitute for conventional HIVDR testing. One of the first studies was conducted by Gonzalez et al. [56] to evaluate the use of a minION flow cell and two older chemistry ligation sequencing kits (SQK-NSK007 and SQK-NSK108) in an HIVDR setting. They found that nanopore-sequencing has a good positive predictive value (100%) to estimate drug resistance mutations, 74% of sequences were identical with a greater than 95% percentage of identity for the other sequences. [56] Ode et al. [55] demonstrated a high concordance of HIVDR results between nanopore- and sanger-sequencing, with a DRM detection accuracy of 99.9% by nanopore sequencing. The HIV-1 pangenome libraries were prepared using the native barcoding kit (SQK-NBD114.24) sequenced on a minION flow cell. A recent study also deemed nanopore sequencing a strong alternative to Sanger sequencing in terms of reproducibility and economical suitability for routine diagnostic care. [57]

1.4. Protease inhibitor resistance

Before the radical change in ART regimens in 2019, Lopinavir/ritonavir (LPV/r) was the preferred PI in second-line regimens after failure with an NNRTI-based first-line regimen. Patients were only switched to Atazanavir/ritonavir (ATV/r) if they developed dyslipidaemia or gastrointestinal toxicity, two of the most common side effects associated with LPV/r. [27,34,58] The only other available PI in the South African public sector is the second-generation PI Darunavir/ritonavir (DRV/r) which is exclusively reserved for third-line regimens. DRV/r has a higher genetic barrier than other PIs and limited cross-resistance. [59] Even though the current South African guidelines recommend using DTG-based regimens across the board, some patients might still have to stay on their PI-based regimens during the transition period. Patients are eligible for an immediate switch to DTG if their VL is < 1000 copies/mL. In contrast, patients on a PI-based regimen and a VL ≥ 1000 copies/mL and with good adherence, or children younger than 10 years require a resistance test done before the switch (Table 1.5). LPV/r is prescribed for patients co-infected with *Mycobacterium Tuberculosis* (TB) where it is usually super boosted to counteract sub-therapeutic levels. [21] However, this intensifies the gastrointestinal toxicity symptoms such as nausea and vomiting which can decrease its ability to suppress the VL. [34,58] ATV/r is now the preferred PI with its low pill burden and better gastrointestinal tolerability, all of which increase its ability to suppress a patient's viral load. [34,56,58,60] The resistance profiles of these three PIs are quite similar except for the N88S DRM which only impacts ATV/r susceptibility and lack of resistance effect of DRMs at positions 47 and 76 to ATV/r, which also contributes to the preferred use of ATV/r (Figure *1.5*). [34,61] The virus also requires the acquisition of multiple PI mutations to become resistant to DRV/r, proof of its high genetic barrier. [61]

Table 1.5: Viral load dependent protease inhibitor regimen switches to dolutegravir-containing regimens

VL Consideration	Current regimen	Criteria for switch	Regimen if change indicated
VL < 1000 c/mL	LPV/r or ATV/r regimen > 2 years	**Switch to DTG** VL < 50 c/mL in 12 months Repeat VL after 3 months	TLD or ABC/3TC/DTG
	Adult/adolescent on LPV/r or ATV/r and adherence < 80%	**Switch to DTG** Unlikely to have a PI resistance mutation Switch to FDC to support adherence	
Two or more VL ≥ 1000 c/mL taken two or more years after starting PI regimen	LPV/r or ATV/r and adherence > 80%	Patients with confirmed virological failure and > 80% adherence require an authorized HIVDR test and **DO NOT** qualify for same-day switch to DTG	
	Child < 10 years, or weight < 30 kg on any LPV/r or ATV/r regimen	Patients do not yet qualify for TLD and may require a HIVDR test	

3TC: Lamivudine; ABC: Abacavir; ATV/r: Atazanavir/ritonavir; DTG: Dolutegravir; HIVDR: HIV drug resistance; LPV/r: Lopinavir/ritonavir; PI: Protease inhibitor; VL: Viral load; FDC: Fixed-dose combination. *Table adapted from NDoH* [21]

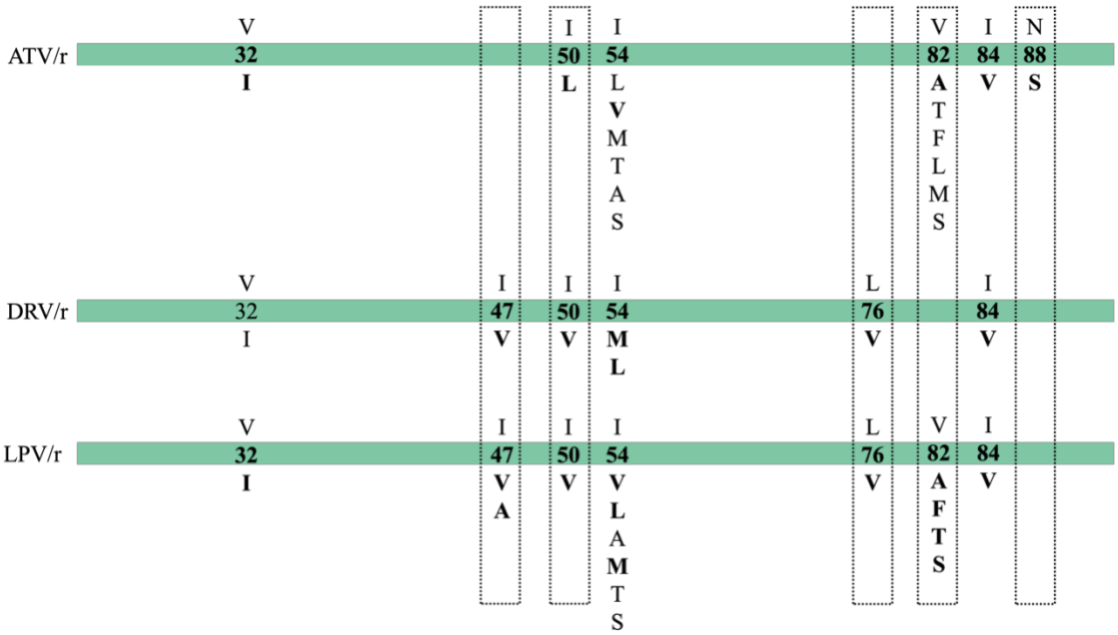


Figure 1.5: Mutations in the protease gene conferring resistance to protease inhibitor (PI). This diagram illustrates the major mutations (highlighted in bold) that can contribute to PI resistance with the major differences between each drug indicated by the dash lines; ATV/r: Atazanavir/ritonavir; DRV/r: Darunavir/ritonavir; LPV/r: Lopinavir/ritonavir. *Figure adapted from Wessing et al.* [61]

1.5. Dolutegravir drug resistance

As previously described, DTG is a second-generation InSTI that prevents viral DNA integration by directly competing for the binding site on the target DNA which blocks strand transfer. [62] Dolutegravir's recommended use is due to its high antiviral potency and high genetic barrier against selecting DRMs. [63] DTG has four properties that suggest this high genetic barrier: slow dissociation rate from the integrase complex, long plasma half-life, robust pharmacokinetics/dynamics interactions, and high plasma inhibition quotient. [63] Nevertheless, DRMs can still be selected within the integrase region that confers resistance to DTG (Figure *1.6*). [61] The nonpolymorphic G118R mutation is often seen, early on the mutational pathway, in patients failing DTG regimens and causes more than 10-fold resistance to DTG. [64] Mutations at position 140 (G140S/A/C/R) are non-polymorphic mutations that have minimal effects on InSTI susceptibility when present alone. However, in combination with Q148 mutations they are associated with intermediate resistance to DTG. [65] By themselves, the nonpolymorphic mutations Q148H/K/R have minimal effects on DTG susceptibility but resistance increases when in combination with E138A/K and G140 mutations. [64,66] Finally, R263K is also a nonpolymorphic mutation that reduces DTG susceptibility. [64,66]



Figure 1.6: Resistance profile of Dolutegravir. Major mutations are highlighted in bold; DTG: Dolutegravir. *Image adapted from Wessing et al.* [61]

There are several recent studies published that describe InSTI drug resistance among PLWH with different treatment scenarios (Table 1.6). Their overall conclusion supports the wide use of DTG due to the minimal development of DRMs. [67–79] A recent meta-analysis of studies by Kanters et al. [80] which assessed the efficacy, safety, and tolerability of DTG during first-line ART, concluded that none of the patients developed DTG resistance, this indicates that most failures are due to suboptimal treatment adherence and HIV disease factors such as malabsorption in critically ill patients. [81]

Paton et al. [78] conducted the NADIA trial in three African countries with two major questions; is DTG non-inferior to DRV/r, and is tenofovir, recycled in second-line ART, non-inferior to switching to second-line zidovudine? They concluded that DTG was non-inferior to DRV/r and individuals should remain on tenofovir in second-line treatment. [78] Additionally, Loosli et al. [82] determined that pre-existing resistance to NRTIs is associated with an increased risk of developing DTG DRMs which is a major hurdle for LMICs. It is important to note that this does not necessarily contradict the findings of the NADIA trial as the observed outcome was different. Loosli et al. identified risk factors for DTG DRM selection in patients failing DTG-based regimens, whereas the NADIA trial measured virologic failure. [78,82]

To a lesser extent, the viral subtype can also influence DTG resistance. Subtype C demonstrates a low enzymatic cost with mutations such as G118R which might be a risk factor for PLWH in South Africa. [83,84] DTG plasma levels can also be reduced due to drug-drug interactions, most notably with Rifampicin which is an antibiotic often prescribed to people with a TB infection. [21] Other drug-drug interactions include polycations found in both antacids and multivitamins, and anticonvulsants for the treatment of epilepsy; all of which can increase the risk for the development of resistance. [21]

Initially, most countries, including South Africa, took a conservative approach when switching NNRTI-exposed patients to a second-line DTG-based regimen. This switch was initially only recommended for patients with a suppressed VL. [21] The NADIA trial, conducted in sub-Saharan African countries, demonstrated that Tenofovir and Lamivudine can be safely recycled in a second-line treatment with DTG as the new anchor drug. [78] The need for VL testing before DTG switching was also challenged by a study conducted in Malawi where 97.9% of participants were virologically suppressed after switching to DTG, regardless of VL result before the switch. [72] Studies such as these have changed South African ART guidelines since 2019 and TLD is now the recommended ARV drug regimen, should the individual meet the criteria as outlined by the guidelines. [21,26]

Despite the high genetic barrier of DTG, resistance is still reported. One risk factor is prior ART exposure with first-generation InSTIs, because of their low genetic barriers to resistance and partial cross resistance with dolutegravir. [64] The risk of developing DTG resistance also seems to increase in PLWH who have failed previous regimens, possibly due to suboptimal adherence that may not have been addressed prior to treatment switch. [85,86] In a recent meta-

analysis, resistance to DTG was assessed over 96 weeks. This analysis estimated that less than 0.1% of all ART naïve patients initiating DTG-based treatment developed resistance. [87] Moreover, only 2.1% of patients failing an NNRTI-based regimen that switched over to a second-line DTG developed resistance. [87] The U.S. President's Emergency Plan for AIDS Relief (PEPFAR) has conducted resistance surveys among patients failing DTG-based treatment in a few countries and reported DTG resistance between 3.9 - 8.6% for patients failing DTG-based regimens and up to 19.6% among treatment-experienced patients that switched to DTG while having a detectable VL. [33]

1.6 Dolutegravir drug exposure testing

The extensive laboratory requirements and cost of HIVDR testing are limiting factors for routine testing in LMICs. Because the expected prevalence of InSTI resistance remains low, especially in patients failing DTG-based treatment as a first-line regimen, performing HIVDR testing on all patients failing DTG-based regimens is unlikely to be cost-effective. [80] Drug exposure testing has recently been proposed as an objective measure to identify patients at the highest risk for virological failure and or acquisition of resistance. [88] A retrospective study in patients failing PI-based regimens demonstrated that almost half of the HIVDR tests performed in South Africa could be avoided if ARV drug levels were used as part of the ART management guidelines. [88] Based on the high negative predictive value found in this study (95%) a qualitative drug-level test could be useful in determining which patients would benefit most from drug resistance testing. The lack of detectable drug levels in many PLWH and non-suppressed viral loads indicated that sub-optimal treatment adherence is the leading cause of viral rebound. [89] Hermans et al. [90] evaluated the ability of a point-of-care test for tenofovir detection in urine samples to predict viral rebound and drug resistance during ART. They concluded that qualitative drug exposure testing may be sufficient to identify patients who would benefit from resistance testing. However, these immunoassays still require thorough validation before clinical use. [90,91]

The most challenging aspect of monitoring treatment outcomes is to objectively measure adherence. [92] The current South African guidelines require adherence to be evaluated by pharmacy refill records, attendance of scheduled clinic visits, and if available to the clinician; detection of tenofovir in urine or the presence of DTG in blood plasma. [21]

Table 1.6: Drug resistance studies for different treatment scenarios

Treatment Scenario	Study	Regimen under investigation VS Comparator regimen	Conclusion
Treatment naïve	SINGLE [67]	DTG–ABC–3TC vs EFV–TDF–FTC	DTG–ABC–3TC has a superior safety and efficacy than EFV–TDF–FTC
	SPRING-2 [68]	TDF-3TC-DTG vs TDF-3TC-RTG	Once-daily DTG was non-inferior to twice-daily RAL
	FLAMINGO [69]	DTG vs DRV/r; with investigator-selected TDF-FTC or ABC-3TC	Once-daily DTG was superior to once-daily DRV/r. Once-daily DTG with FDC NRTIs represents an effective new treatment option for treatment-naive patients.
	ADVANCE [70]	TAF-FTC-DTG vs TDF-FTC-DTG vs TDF-FTC-EFV	High viral suppression observed across three WHO recommended regimens with no resistance to DTG
	NAMSAL [71]	DTG-3TC-TDF vs EFV-3TC-TDF	DTG first-line use in treatment naïve PLWH is supported by its non-inferior efficacy and non-emergence of DTG resistance. PLWH on DTG-based regimens achieved viral suppression quicker
Transition from current first-line	Schramm et al. [72]	DTG-3TC-TDF	Optimal VL suppression one year after TLD initiation supports the unconditional transition strategy in Malawi
Treatment experienced: InSTI naïve	ARTIST [73]	DTG-3TC-TDF vs TDF-FTC-EFV	Switching patients from a failing TEE regimen to TLD is supported by the high genetic barrier of DTG and the NRTI resistance mutations that impair viral fitness
	DAWNING [74]	DTG vs LPV/r; plus two investigator-selected NRTIs (with one being fully active)	DTG in combination with two NRTIs is superior to LPV/r and suitable for second-line treatment
	SAILING [75]	DTG vs RAL; with investigator-selected background therapy	Once-daily DTG with two other ARV drugs is well tolerated with superior virologic suppression compared to twice-daily RAL
	IMPAACT P1093 [77]	DTG plus an optimized background regimen in age-defined paediatric cohorts	DTG plus an optimized background regimen seemed safe, well tolerated, and efficacious
	NADIA [78]	DTG vs DRV/r; in combination with either TDF-3TC or AZT-3TC	DTG is non-inferior to DRV/r. Tenofovir and lamivudine can be continued in second-line therapy
InSTI experienced	VIKING [79]	For 7 days: DTG while continuing their failing regimen (without RAL or EVG), then optimized with ≥1 fully active drug and DTG continued	DTG 50mg twice daily dose is effective in high InSTI treatment experienced patients with InSTI DRMs

3TC: Lamivudine; ABC: Abacavir; ARV; Antiretroviral; AZT: Zidovudine; DRM: Drug Resistant Mutation; DRV/r: Darunavir/ritonavir; DTG: Dolutegravir; EFV: Efavirenz; EVG: Elvitegravir; FDC: Fix Dose Combination; FTC: Emtricitabine; InSTI: Integrase Strand Transferase Inhibitor; LPV/r: Lopinavir/ritonavir; NRTI: Nucleoside Reverse Transferase Inhibitor; PLWH: People Living with HIV; RAL: Raltegravir; TAF: Tenofovir alafenamide; TDF: Tenofovir Disoproxil Fumarate; TEE: Tenofovir-Emtricitabine-Efavirenz; TLD: Tenofovir-Lamivudine-Dolutegravir ; VL: Viral Load; WHO: World Health Organisation

Drug-level testing is commonly performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS), which combines chromatographic separation and mass determination of compounds for quantitative and qualitative analysis. [93] Compounds in a liquid mixture are first separated chromatographically through their interaction with a stationary phase and mobile phases that isolate the compound of interest from the rest of the mixture. These analytes then move to the mass-spectrometer that measures the mass-to-charge ratio of particles, which are generated by various ionisation techniques. The compounds are further distinguished based on characteristic fragmentation patterns that are generated within the mass-spectrometer. This versatile method of testing is considered a gold standard due to its analytical sensitivity and specificity, and is often superior to immunoassays or conventional high-performance liquid chromatography. [93]

Therapeutic drug monitoring (TDM) is a tool to quantitatively measure drug concentrations at specific time intervals to optimise the therapeutic effect on an individual. [94] It was believed that TDM could aid in treatment optimisation for PLWH but was deemed unsuitable for the HIV world. [95] Despite the lack of evidence for TDM, qualitative and quantitative drug levels may still be useful in certain scenarios for PLWH, including the identification of those at the highest risk of resistance. [80,88–92,96–98]

1.6. Study objectives

The overall aim of this study is to identify HIV-1 antiretroviral drug-resistant mutations in patients failing treatment. The following objectives will achieve this:

1.1 To retrospectively describe PI-resistance profiles in a cohort of patients failing either ATV/r or LPV/r-based treatment.

1.2 To retrospectively analyse HIV resistance mutations in patients failing DTG-based treatment, who had drug resistance testing performed for individual patient management.

1.3 To retrospectively perform integrase genotypic resistance testing on patients failing DTG-based treatment, but who only had protease and reverse transcriptase resistance testing done between 2019 and 2022, as integrase testing was not clinically indicated (DTG exposure < 2 years)

1.4 To retrospectively detect drug-resistant low-abundance variants in stored samples (objectives 1.2 and 1.3) using third-generation sequencing for the protease – reverse transcriptase (PR-RT) and integrase (IN) regions and compare findings with results obtained from Sanger sequencing

1.5 To correlate qualitative plasma DTG drug levels with the absence or presence of InSTI resistance mutations

Chapter 2

Materials and Methodology

2.1. PI-based regimen cohort

2.1.1. Study design and participants

The National Health Laboratory Services (NHLS) HIV molecular laboratory at Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) completed 1663 HIVDR tests in 2022 of which 1244 were done for patients failing a PI-based (either ATV/r or LPV/r) regimen, data from all 1244 tests were included. Retrospective data analysis of routinely obtained HIV drug resistance data was approved by the Human Research Ethics Committee (HREC Medical) at the University of the Witwatersrand (certificate number: M221081) (Appendix A: Figure *5.1*).

2.1.2. Genotypic data

Sanger sequences for the PR-RT region of the *pol* gene were available for every sample and obtained through routine testing following the same method as described in sections 2.2.2 - 2.2.3 below.

2.2. DTG-based regimen cohort

2.2.1. Study design and participants

This cross-sectional retrospective study included remnant samples after routine HIVDR testing. Remnant samples were obtained from patients failing DTG-based treatment who had an HIVDR test done between January 2019 and December 2022 at the NHLS HIV Molecular Laboratory at CMJAH. To be included in this study, samples had to meet all the inclusion criteria (Table *2.1*). Relevant demographic and clinical information (age, sex, latest VL, treatment history, duration of treatment) was recorded for each participant. Ethical approval was obtained from the HREC (Medical) of the University of the Witwatersrand (certificate number: M230611) (Appendix A: Figure *5.2*). Approval to use the remnant samples was also obtained from the NHLS Academic Affairs and Research Management (AARMS) (Appendix A: Figure *5.3*). The study was carried out under the declaration of Helsinki.

Table 2.1: Participant inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
Leftover sample is from a patient failing DTG-based treatment (based on information provided on the laboratory request form)	Current treatment exposure is not available on the laboratory request form
Blood specimens have been sent for routine HIVDR testing between January 2019 and December 2022	No HIVDR result could be obtained for routine testing due to PCR or sequencing failure
Remnant sample is available and contains at least 500 µL of plasma	Remnant sample volume is < 500 µL
Latest HIV VL result is ≥400 copies/ml	Latest HIV VL result is <400 copies/mL

DTG: Dolutegravir, HIVDR: HIV Drug Resistance, PCR: Polymerase Chain Reaction

The number of samples necessary to estimate the proportion of HIVDR is 674. This estimate is derived with an error size of 5% and a two-sided 95% confidence interval (CI) of alpha=0.05, assuming that the proportion of HIVDR in the population (among those with viral loads >400 copies/mL) is 50%. Unfortunately, this sample size is not feasible due to the limited samples available for testing and budget restrictions. We therefore used a convenient sample size instead; including all available samples (n=126).

Between January 2019 and December 2022, the NHLS HIV Molecular Laboratory at CMJAH received HIVDR testing requests for 287 patients who were receiving DTG-based treatment. Forty- six (46) specimens were rejected due to a VL <400 copies/mL or poor specimen quality. For another 33 samples, the routine HIVDR test was unsuccessful. For 58 samples, we did not have any remnant material left, and 24 specimens were excluded because they were obtained from patients who received raltegravir-based treatment.

For 59 specimens, InSTI HIVDR testing was not routinely performed but was performed for the project using an in-house validated RT-PCR and Sanger sequencing method. All available specimens (n=126), with sufficient remnant volume, underwent NGS for the protease, reverse transcriptase, and integrase regions. Protease, reverse transcriptase sequencing was performed to assess whether the presence or absence of PI and (N)NRTI mutations contribute to the presence of InSTI resistance (Figure 2.1).

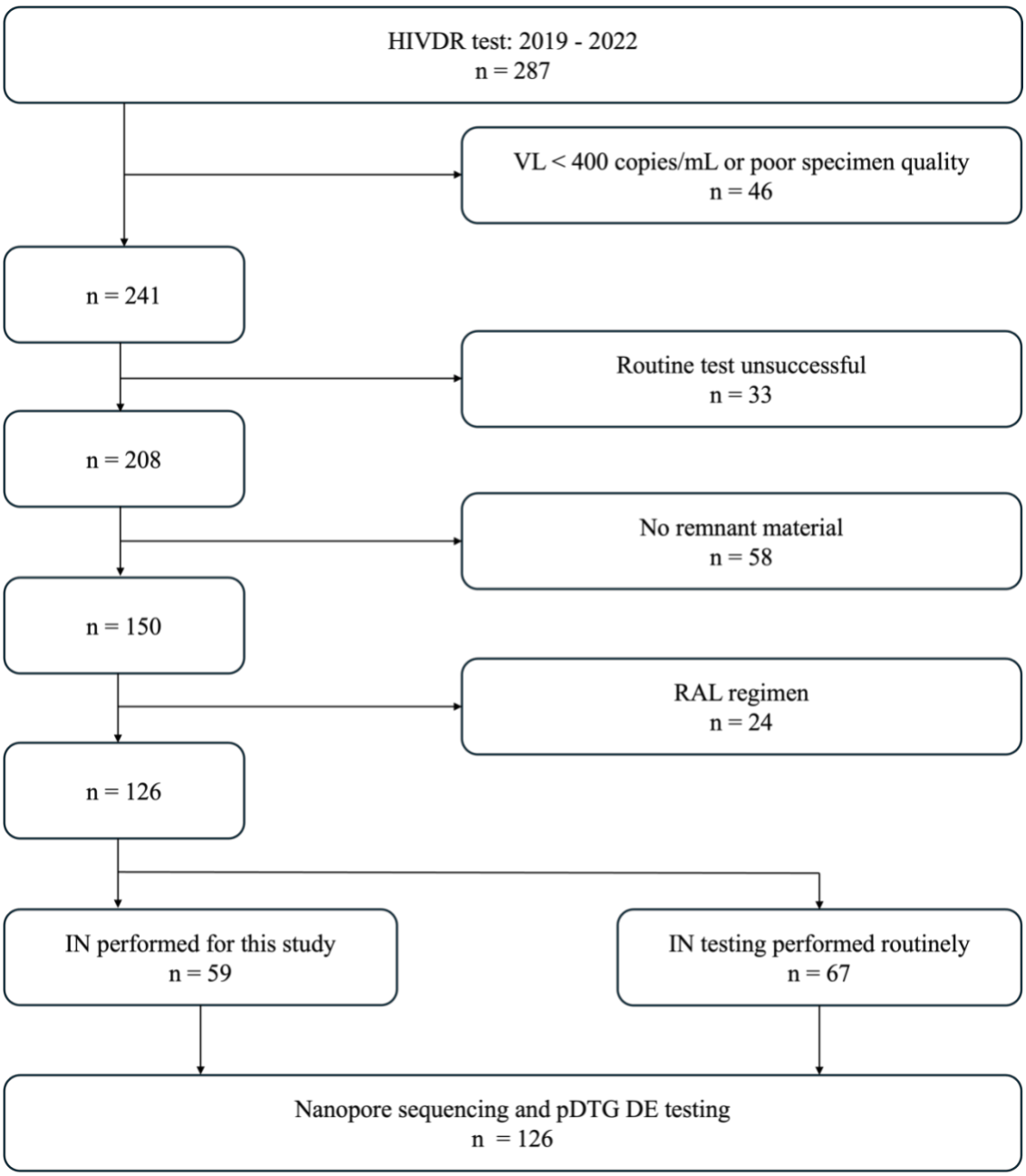


Figure 2.1: Flow diagram representing the sample size which follows the inclusion and exclusion criteria. HIVDR: HIV drug resistance; IN: Integrase; pDTG DE: Plasma dolutegravir drug exposure; RAL: Raltegravir VL: Viral load.

2.2.2. HIV-1 *pol* amplification

Total viral RNA was extracted from human plasma samples using a semi-automated NucliSens EasyMAG (bioMerieux, Marcy-l'Étoile, France) system as per the manufacturer's instructions. Plasma samples were vortexed and an input volume of 500 µL was used, the semi-automated system dispensed lysis buffer in each sample and incubated for 10 minutes to lyse

the viral particles and inactivate all RNases and DNases present in the sample. Thereafter, 50 µL of NucliSens EasyMAG magnetic silica beads were manually added to each sample and thoroughly mixed. The system was allowed to run for 40 minutes to produce total nucleic acid in an eluate of 25 µL which was either used immediately or stored at -80 °C for downstream applications.

The HIV-1 *pol* amplification was then divided into two separate assays: The PR-RT was amplified using the HIV-1 Genotyping Kit Amplification Module (ThermoFisher, Waltham, MA, USA) and the IN region using a validated in-house method based on van Laethem et al. [99] Both methods are briefly explained below.

Protease – Reverse transcriptase

To synthesise cDNA and generate the 1st round of PCR product, 10 µL of viral RNA was added to 39 µL RT-PCR master mix and 1 µL SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity enzyme provided by the HIV-1 Genotyping Kit Amplification Module (Applied Biosystems, Waltham, MA, USA).

An in-house low-positive control (<10 000 copies/mL) and the commercial HIV-1 negative control were included in each run. The RNA template was first incubated at 65°C for 10 minutes before the master mix was added. The RT-PCR was run in the GeneAmp thermocycler for 40 cycles according to the conditions in Table *2.2* and products were stored at -20°C.

Table 2.2: RT-PCR conditions for Superscript III ™ RT enzyme (PR-RT region)

	RT-PCR (PR-RT)	
RNA denaturation	65°C for 10 minutes	
Reverse transcription	50°C for 45 minutes	
Initial denaturation	94°C for 2 minutes	
Denaturation	94°C for 15 seconds	
Annealing	50°C for 20 seconds	40 cycles
Extension	72°C for 2 minutes	
Final extension	72°C for 10 minutes	
Hold	4°C for ∞	

PR-RT: Protease – Reverse Transcriptase, RT-PCR: Reverse Transcriptase Polymerase Chain Reaction

Nested PCR master mix (47.5 µL) and AmpliTaq Gold™ LD DNA Polymerase (0.5 µL) were added to 2 µL RT-PCR product, both reagents supplied by the HIV-1 Genotyping Kit Amplification Module (Applied Biosystems, Waltham, MA, USA). The GeneAmp thermocycler was programmed according to the conditions in Table *2.3* and products were stored at -20°C for downstream applications.

Table 2.3: Nested PCR conditions for Platinum Taq High Fidelity (PR-RT region)

	Nested PCR (PR-RT)	
Initial denaturation	94°C for 4 minutes	
Denaturation	94°C for 15 seconds	40 cycles
Annealing	55°C for 20 seconds	
Extension	72°C for 2 minutes	
Final extension	72°C for 10 minutes	
Hold	4°C for ∞	

PCR: Polymerase Chain Reaction, PR-RT: Protease – Reverse Transcriptase

Amplicons were visualised on a 2% agarose gel (2 mg agarose in 100 mL 1X TAE (Tris/ Acetic acid/ EDTA) buffer (Appendix B.1) containing 10 µL 10 000X GelRed (Biotium, Fremont, CA, USA). PCR products (5 µL) were mixed with 5 µL 6x DNA loading dye (Thermo Scientific, Waltham, MA, USA) and loaded into the wells of the gel. The gel was run at 100 volts (V) for 45 minutes. The gel was viewed under ultraviolet (UV) light with the BIORAD ChemiDoc Imaging System. Ten microliters of a 10 000 bp molecular weight ladder (Thermo Scientific, Waltham, MA, USA; 103 ng/µL) was included to confirm amplification of the correct fragment, 1.1 kb for a PR-RT amplicon.

Utilizing the GeneJET ™ PCR Purification system (ThermoFisher, Waltham, MA, USA) PCR products were purified. Equal parts of the remaining amplification product and binding buffer were added to a purification column and centrifuged for one minute at 12000 x g in the Mikro 185 centrifuge (Hettich, Tuttlingen, Germany). Next 700 µL wash buffer was added and centrifuged for one minute at 12000 x g, followed by an additional minute to ensure complete removal of ethanol from the wash buffer. Finally, the column was placed in a clean Eppendorf tube; 50 µL of elution buffer was added to the column, and centrifuged for one minute at 12000 x g to obtain purified DNA which was stored at -20°C.

Integrase

cDNA and 1st round PCR product were synthesised using the SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity enzyme (Invitrogen, Waltham, MA, USA) in a one-step reverse transcriptase polymerase chain reaction (RT-PCR). The final reaction volume of 50 µL contained 25 µL 2x reagent mix buffer, 10 µL distilled water, 10 µL RNA template, 2 µL of each forward (0.4 µM) and reverse (0.4 µM) primer (Appendix B: Table *5.1*), and 1µL SuperScript III One-step RT-PCR system with Platinum Taq High Fidelity polymerase enzyme (Invitrogen, Waltham, MA, USA).

An in-house low-positive control (<10 000 copies/mL) and a commercial HIV-1 negative control (Roche Molecular Systems, Pleasanton, CA, USA) were included in each run. The RNA template was first incubated at 65 °C for 10 minutes before the master mix was added. The RT-PCR was run in the GeneAmp PCR system (Applied Biosystems, Waltham, MA, United States) for 30 cycles according to the conditions in Table *2.4* and products were stored at -20°C.

Table 2.4: RT-PCR conditions for SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity enzyme (IN region)

	RT-PCR (IN)	
RNA denaturation	65°C for 10 minutes	
Reverse transcription	55°C for 30 minutes	
Initial denaturation	94°C for 2 minutes	
Denaturation	94°C for 15 seconds	
Annealing	53°C for 30 seconds	30 cycles
Extension	68°C for 2.5 minutes	
Final extension	68°C for 5 minutes	
Hold	4°C for ∞	

IN: Integrase, RT-PCR: Reverse Transcriptase Polymerase Chain Reaction

Nested PCR on the first-round product followed as per Sigma Aldrich Expand High Fidelity^{PLUS} kit ™ (Sigma Aldrich, St. Louis, MO, USA) instructions. The final PCR volume included 70 µL distilled water, 10 µL 10x Expand HF^{PLUS} buffer 3, 8 µL $MgCl_2$ (2 mM), 5 µL first round PCR product, 2 µL dNTPs, 2 µL of each forward (0.2 µM) and reverse (0.2 µM) primer (Appendix B: Table *5.1*), and 1 µL Expand HF^{PLUS} DNA polymerase enzyme. The GeneAmp thermocycler was programmed according to the conditions in Table *2.5* and products were stored at -20°C

for downstream applications. The same agarose gel protocol was used to visualise the 1.2 kb amplicon for the integrase region to confirm correct amplification, and purified using the GeneJET ™ PCR Purification system (ThermoFisher, Waltham, MA, USA).

Table 2.5: Nested PCR conditions for Expand HF^{PLUS} DNA polymerase (IN region)

	Nested PCR (IN)	
Initial denaturation	94°C for 2 minutes	
Denaturation	94°C for 15 seconds	10 cycles
Annealing	53°C for 30 seconds	
Extension	72°C for 1.5 minutes	
Denaturation	94°C for 15 seconds	30 cycles*
Annealing	53°C for 30 seconds	
Extension	72°C for 1.5 minutes	
Final extension	72°C for 7 minutes	
Hold	4°C for ∞	

* Add 5 seconds to each cycle; IN: Integrase, PCR: Polymerase Chain Reaction

2.2.3. Sanger sequencing

The two amplicons were sequenced using the HIV-1 Genotyping Kit Cycle Sequencing Module (ThermoFisher, Waltham, MA, USA) for PR-RT and the in-house method based on van Laethem et al. [99]

Protease – Reverse transcriptase

The HIV-1 Genotyping Kit Cycle Sequencing Module (ThermoFisher, Waltham, MA, USA) includes six sequencing master mixes (three forwards and three reverse), 2 µL of purified nested PCR product was added to 18 µL of each sequencing master mix. The thermocycler was programmed according to the conditions in Table *2.6* and products were stored at -20°C for downstream application.

Table 2.6: Cycle sequencing conditions (PR-RT region)

	Cycle sequencing	
Denaturation	94°C for 10 seconds	25 cycles
Annealing	50°C for 5 seconds	
Extension	60°C for 4 minutes	
Hold	4°C for ∞	

PR-RT: Protease – Reverse Transcriptase

Integrase

Four sequencing primers were used (Appendix B: Table *5.1*), 2 µL of undiluted PCR product was added to each primer well which included 4.5 µL distilled water, 1.5 µL 5x Big dye buffer (Applied Biosystems, Waltham, MA, USA), 1 µL Big dye terminator v3.1 (Applied Biosystems, Waltham, MA, USA), and 1 µL of the respective primer (5 pmol/µL). The thermocycler was programmed according to the conditions in Table *2.7* and products were stored at -20°C for downstream application.

Table 2.7: Cycle sequencing conditions (IN region)

	Cycle sequencing	
Initial denaturation	96°C for 1 minutes	
Denaturation	96°C for 10 seconds	25 cycles
Annealing	50°C for 5 seconds	
Extension	60°C for 4 minutes	
Final extension	72°C for 10 minutes	
Hold	4°C for ∞	

IN: Integrase

Purification and sequencing

Cycle sequencing products were purified by adding 80 µL of 75% Isopropanol to each 10 µL product, vortexed and incubated at room temperature for 15 minutes. Centrifugation followed at 2000 x g for 45 minutes in the Eppendorf 5810 centrifuge (Eppendorf, Hamburg, Germany). The plate was then left to air dry in the dark for 10 minutes the DNA pellet was resuspended in 10 µL of HiDi formamide (Applied Biosystem, Waltham, MA, USA). The samples were finally loaded onto the 3730XL ABI genetic analyser (ThermoFisher, Waltham, MA, USA).

Sanger sequences were aligned and analysed using ReCall version 2.28 (British Columbia's Centre for Excellence in HIV/AIDS Research). MEGA software (Pennsylvania State University) was used for phylogenetic analysis and quality control of the samples relating to cross-contamination. [100] These sequences were then submitted to the Stanford HIVdb (https://hivdb.stanford.edu/) to generate resistance reports. [101]

2.2.4. Nanopore sequencing

We utilised ONT to perform NGS using their Native Barcoding Kit 96 V14 (SQK-NBD114.96). The ligation sequencing for amplicons protocol provided by ONT was used to prepare the libraries for sequencing allowing up to 96 samples to be barcoded in one experiment for demultiplexing. The flongle flow cell was used for sequencing and required ~200 fmol of starting DNA material. The protocol has three major steps: End-prep, native barcode ligation, and adapter ligation; both the native barcoding and adapter ligation steps are accompanied by a clean-up procedure (Figure 2.2). [102]

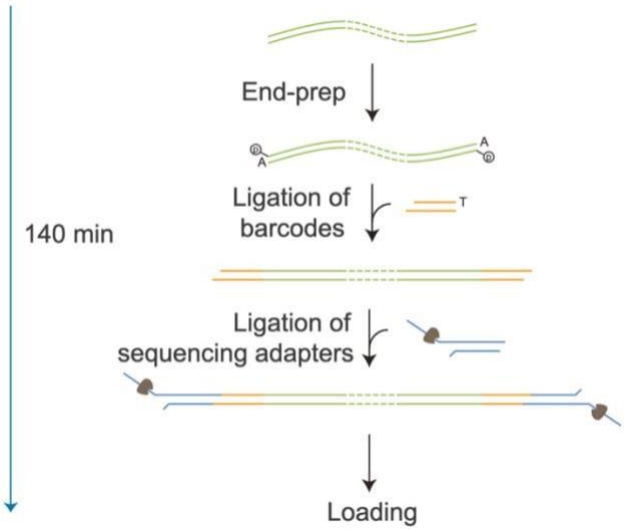


Figure 2.2: Workflow overview for nanopore sequencing. Phosphate groups are exposed at the ends of the amplicons to prepare them for barcode ligation. When the barcodes are ligated, sequencing adapters are finally added to anchor onto the flow cell tethers which facilitate the DNA strands entry into the sequencing pore. *Image extracted from ONT* [102]

In brief, DNA starting material was quality-checked (QC) using Nanodrop 2.0 to meet ONT sample quality requirements (260/280 ~ 1.80; and 260/230 ~2.0-2.2). [103] The DNA molecular weight of each sample was determined with the Qubit dsDNA High Sensitivity Assay Kit

(ThermoFisher, Waltham, MA, United States) and Qubit 4 Fluorometer (Invitrogen, Waltham, MA, United States).

The PR-RT and IN amplicons of each sample were prepared separately using Ultra II End-prep reaction buffer (NEB inc., Ipswich, MA, United States), Ultra II end-prep enzyme mix (NEB inc., Ipswich, MA, United States) and DNA control sample (ONT, Oxford, United Kingdom) that were added in a Twin-Tec PCR plate (Eppendorf, Hamburg, Germany) and incubated at 20°C and 65°C for five minutes at each temperature using the GeneAmp PCR system 9700 (Applied Biosystems, Waltham, MA, United States).

Next followed separate barcode ligation for each amplicon, the Native barcodes (ONT, Oxford, United Kingdom) were ligated to the end-prepped DNA using Blunt/TA Ligase Master Mix (NEB inc., Ipswich, Massachusetts, United States) at room temperature for 20 minutes. The reaction was stopped using EDTA (ONT, Oxford, United Kingdom) and all samples were pooled in a DNA LoBind 1.5 mL tube (Eppendorf, Hamburg, Germany). The mixture was cleaned using AMPure beads (ONT, Oxford, United Kingdom) and 80% ethanol and the eluate was discarded. To elute the DNA from the AMPure beads 35 μL of nuclease-free water was added and incubated for 10 minutes at 37°C in a Thermomixer compact (Eppendorf, Hamburg, Germany), which was then QC using the Qubit 4 system.

The Native Adapter was then ligated using the NEBNext Quick Ligation Reaction Module (NEB inc., Ipswich, MA, United States) at room temperature for 20 minutes. The library was cleaned using AMPure Beads and Short Fragment Buffer to enrich for all fragment sizes and eluted in 15 μL of Elution Buffer (ONT, Oxford, United Kingdom) after a 10 minute incubation at 37°C in a Thermomixer compact (Eppendorf, Hamburg, Germany). Final QC followed using the Qubit 4 system and the final library was made up to 10 – 20 fmol.

Five microliters of the library were added to 15 μL Sequencing Buffer (ONT, Oxford, United Kingdom) and 10 μL sequencing beads (ONT, Oxford, United Kingdom). The Flongle flow cell was primed using the flow cell flush (ONT, Oxford, United Kingdom) and flow cell tether (ONT, Oxford, United Kingdom) and loaded with the sequencing library.

The MinKNOW software (version 24.02.6) which controls all ONT devices has a default QC score of 9.0 for data obtained from Dorado basecalling software (version 7.3.9) and generates

a run report for analysis. [104] Sequencing data was analysed to produce resistance reports using the stand-alone version 0.4.4 of NanoRecall. NanoRecall utilises the Stanford HIVdb to generate resistance reports, sequencing depth and mixture threshold can be modified as needed for a specific analysis. [101,105]

2.2.5. DTG drug exposure testing

Plasma DTG drug levels were determined with LC-MS/MS using a validated method at the Department of Chemical Pathology, University of Witwatersrand. The instrument consists of the Waters Acquity UHPLC System (Waters, Milford, Waltham, MA, USA) and the Micromass Quattro micro API Mass Spectrometer (Waters, Milford, Waltham, MA, USA). All DTG calibrators and controls are produced in-house and initially made in dimethyl sulfoxide (DMSO) using a DTG standard reference material (Toronto Research Chemicals Inc., North York, Canada). This mix is then spiked into drug-free human serum to produce a six point calibration curve ranging from 0.039 – 0.833 μg/mL.

The samples were prepared by adding liquid Chromatography (LC) grade Acetonitrile (Supelco Inc., Bellefonte, PA, USA) for protein precipitation and deuterated raltegravir (Chromsystems, Gräfelfing, Germany) which serves as an internal standard. The supernatant is then injected into the ultra-high-pressure liquid chromatography system. The compounds are separated using two mobile phases (inorganic mobile phase A: 0.1% LC grade Formic acid in Milli-Q ultra-pure water (Merck, Darmstadt, Germany); organic mobile phase B: 0.1% LC grade Formic acid in LC grade Acetonitrile; which run over a gradient using an Acquity HSS T3 1.8 μm column (Waters, Milford, Waltham, MA, USA).

DTG is analysed using tandem mass-spectrometry by ionising the separated compounds through positive electrospray ionisation. The parent DTG compound is broken down into daughter fragments with the following quantifier and qualifier transitions: 420.4>377.1, 420.4>136. Results are then quantified using the response of the sample versus the calibration curve, which is corrected based on the recovery of the internal standard.

Data was analysed using Masslynx Version 4.1 as central operating system. DTG was considered to be detectable in any sample with a concentration of ≥ 0.02 μg/mL, which is above the method's lower limit of quantification.

2.3. Data analysis

All analyses were conducted with STATA version 18 (STATA Corp., College Station, TX, USA). The Shapiro-Wilk test was used to test if continuous data was normally distributed, however, none of the data sets were normally distributed. Medians with corresponding interquartile ranges (IQR) for not normally distributed continuous variables were used to summarise data and proportions were used for categorical variables. We used the Mann-Whitney U test to determine statistically significant differences between non-parametric data, and the chi-square (χ^2) test (or Fisher's exact test for data entries < 5) for categorical data sets. A p-value <0.05 was considered statistically significant. Demographic and clinical data as described in section 2.1 were included in these aforementioned statistical analyses.

The following comparisons and correlations were made:

- Demographic and clinical variables between regimen-specific subgroups
- DRM profile within regimen-specific subgroups
- Clinical variables between males and females
- Analysing and summarising DRM profiles as determined by the Stanford HIVdb
- Correlating pDTG DE to HIVDR
- Correlating Nanopore sequences to Sanger sequences

Sequencing data generated either through Sanger- or nanopore sequencing are analysed by the Sanford HIVdb which generates a resistance report. [101] Each mutation detected within the relevant region contributes to an overall score which is used to determine the level of resistance to each ARV drug. These levels range from one to five; one – susceptible, two – potential low-level resistance, three – low-level resistance, four – intermediate resistance, and five – high-level resistance. To simplify analysis these five levels are grouped into a three-level system: levels one and two – susceptible, levels three and four – intermediate resistance, and level 5 remains high-level resistance, with clinically relevant resistance defined as low-level, intermediate and high-level resistance (Figure *2.3*).

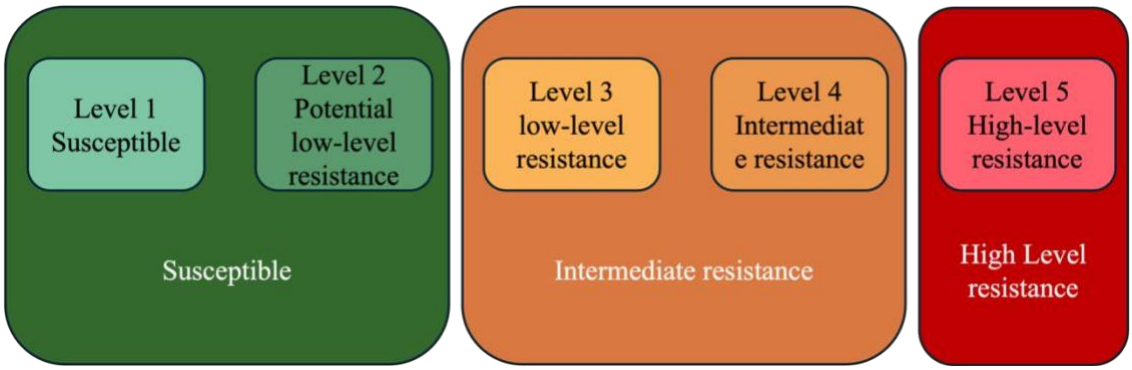


Figure 2.3: Sandford HIVdb resistance levels

A percentage pairwise mutation identity analysis with hamming distance between Nanopore- and Sanger sequencing was done using equation 2.1 for each sample pair. The mean and standard deviation were then calculated for each region (IN and PR-RT). Acceptance criteria for similarity assessment were set at 97% for the PR-RT region and 93% for the IN region based on in-house validation criteria. There were four analyses:

1. A minimum coverage of 400X as defined by NanoRecall (to be comparable to Sanger sequencing) at a 25% mixture threshold [105,106]
2. A minimum coverage of 250X at a 25% mixture threshold
3. Maximum coverage at a 10% mixture threshold
4. Maximum coverage at a 5% mixture threshold

$$100 - ((\frac{Major\ DRM\ differences}{Total\ major\ DRM\ positions}) \times 100)$$

Equation 2.1: Percentage pairwise identity calculated with Hamming distance; DRM: Drug Resistant Mutations

Chapter 3

Results

3.1. Resistance profiles in patients failing protease-inhibitor-based regimens.

A total of 1662 tests were performed by the NHLS CMJAH HIV molecular laboratory in 2022, for individuals failing a PI-based regimen. After excluding samples without a sequence or with missing demographic information, this retrospective data analysis included 1244 HIVDR sequences. The sample population consisted of 62% (n=769) females, with a median age of 39 [IQR: 22-48] and median VL at the time of HIVDR testing of 4.6 $\log_{10}$ copies/mL (Table 3.1). LPV/r-based regimen failure accounted for most of the study population (76.4%). Within the ATV/r exposed group, 62.1% had recorded previous LPV/r exposure.

Table 3.1: Baseline demographic and clinical characteristics

	Population N = 1244	**LPV/r-based regimen n = 951 (76.4%)**	**ATV/r-based regimen n = 293 (23.6%)**	**p-value**
Age	39 [22-48]	40 [21-49]	38 [24-47]	0.266
Sex				
Males % (n)	37.9 (472)	38.4 (364)	37.0 (108)	0.673
Females % (n)	62.0 (769)	61.4 (585)	63.0 (184)	
Viral load (Log_{10}copies/mL)	4.6 [3.9-5.2]	4.6 [3.9-5.2]	4.6 [3.8-5.1]	0.199
Previous LPV/r exposure % (n)	-	-	62.1 (182)	-
≥3 major PI mutations per sequence % (n)	18.5 (230)	20.4 (194)	12.3 (36)	**0.002**
Full ARV drug susceptible profiles % (n)	15.5 (193)	14.6 (139)	18.4 (54)	0.115
DRV/r cross-resistance % (n)	13.2 (164)	14.5 (138)	8.9 (26)	**0.013**

*Treatment duration was not available. Results are presented as median values [lower quartile; upper quartile] for skewed data, and % (n) for categorical variables; ATV/r: Atazanavir/ritonavir; ARV: Antiretroviral; DRV/r: Darunavir/ritonavir; LPV/r: Lopinavir/ritonavir

Figure *3.1* illustrates the proportion of fully PI-susceptible profiles which was similar between the two groups (ATV/r: 72% vs LPV/r: 73%, p=0.538). When considering the presence of three or more major PI DRMs per sequence, the LPV/r group had a significantly higher proportion (20.4%) compared to the ATV/r group (12.3%, p = 0.002). The LPV/r group had a significantly higher number of patients with intermediate to high-level cross-resistance to DRV/r compared to the ATV/r group [14.5% (n=138) vs 8.9% (n=26); p=0.013]. Only three patients in the ATV/r group and four in the LPV/r group presented with high-level cross-resistance to DRV/r.

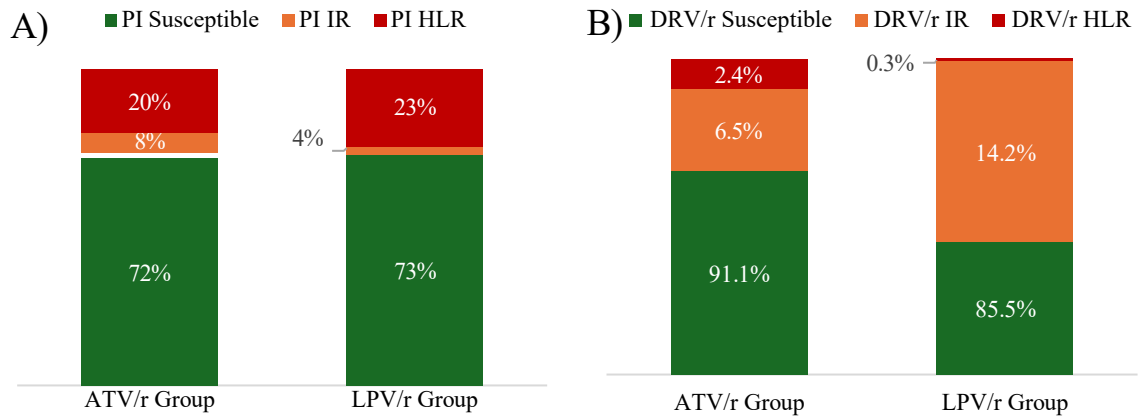


Figure 3.1: PI resistance profiles in each group. **A)** Proportion (%) of PI resistance in both groups with no significant difference. **B)** Proportion (%) of DRV/r cross-resistance in both groups, LPV/r group has significantly more patients with DRV/r resistance (IR and HLR) compared to the ATV/r group (138 vs 26; p = 0.013); ATV/r: Atazanavir/ritonavir; DRV/r: Darunavir/ritonavir; HLR: High level resistance; IR: intermediate resistance; LPV/r: Lopinavir/ritonavir; PI: Protease Inhibitor.

We examined all the resistance profiles in Table 3.2. The V82A mutation was most abundant in the sample population (n=231). The most common combination consisted of four mutations (V82A-M46I-I54V-L76V) followed by a three-mutation combination (V82A-I54V-M46I). The resistance profiles of seven patients with DRV/r cross-resistance were analysed and compared in Table 3.3. Although not a major contributor to PI resistance, the M46I mutation was detected in all seven individuals with the I84V major mutation, and mutation variants at position 54 of the PR region. Considering the drug-specific DRMs, ATV/r-specific mutation V32I was detected in all four patients on ATV/r-based treatment and one individual had N88S. One individual on LPV/r had the I47V mutation specific to LPV/r exposure. However, two patients in the ATV/r group had the L76V mutation exclusively selected under LPV/r drug pressure, confirming prior LPV/r exposure.

Table 3.2: Resistance profiles in people living with HIV and failing a protease inhibitor-based regimen

V82A Pathway (n=231)	**M46I Pathway (n=183)**	**Other (n=64)**
V82A (12)	M46I (11)	D30N (1)
V82A + I54V (29)	M46I + I84V (2)	V32I (2)
V82A + I54A (2)	M46I + N88S (1)	M46L (5)
V82A + M46I (2)	M46I + I50V (1)	I47A (2)
V82A + I50V (2)	M46I + I50L (1)	I47V (1)
V82A + I84V (1)	M46I + I54V (2)	I54L (5)
V82A + I54V + M46I (54)	M46I + L76V (1)	I54V (3)
V82A + I54V + M46L (14)	M46I + V82L (1)	L76V (1)
V82A + I54V + L76V (14)	M46I + I84V + L76V (12)	V82L (1)
V82A + I54V + I48V (1)	M46I + I84V + V32I (1)	I84V (3)
V82A + I54V + L90M (4)	M46I + I84V + I47A (1)	N88S (4)
V82A + I54V + I47V (1) *	M46I + I84V + N88S (1)	L90M (6)
V82A + I54A + G48A (2)	M46I + I54V + L76V (2)	I50L +L90M (3)
V82A + M46I + I84V (2)	M46I + I54V + V82S (1)	L90M + I54L (1)
V82A + M46I + I47V (1)	M46I + I54V + V82C (1)	V32L + I47A (1)
V82A + M46I + L76V (1)	M46I + I54M + N88S (1)	I47A + I84V (2)
V82A + M46I + V32I (1)	M46I + I47A + L90M (1)	I47A + I50V (1)
V82A + M46L + I50L (1)	M46I + I84V + L76V + I47V (1)	I54V + V82C (2)
V82A + M46I + I54V + L76V (55)	M46I + I84V + L76V + I54L (2)	I54V + V82T (1)
V82A + M46I + I54V + I50V (5)	M46I + I84V + L76V + I54V (1)	I54V + V82L (1)
V82A + M46I + I54V + I84V (4)	M46I + I84V + I47A + I50L (1)	I54V +V82M (1)
V82A + M46I + I54V + L50L (2)	M46I + I84V + I47A + L90M (1)	I54V + L76V (1)
V82A + M46I + I54V + I84V (4)	M46I + I84V + V32I + I54L (1)	I50L + V82M (2)
V82A + M46I + I54V + L90M (1)	M46I + I54V + L76V + I47V (1)	I54L + V82L (1)
V82A + M46I + I54V + N88S (2)	M46I + I54V + L76V + V82S (1)	I54L + I84V (1)
V82A + M46I + I54V + I47V (2)	M46I + I54V + N88S + L90M (1)	I54L + N88S (1)
V82A + M46I + I54V + V32I (1)	M46I + I84V + L76V + I54V + V32I (2)	M46L + I47A + I84V (2)

Table 3.2: Continued

V82A Pathway (n=231)	**M46I Pathway (n=183)**	**Other (n=64)**
V82A + M46I + I54V + G48A (1)	M46I + I84V + L76V + I54V + I47V (1)	M46L + I47A + V32I (1)
V82A + M46I + I50L + L90M (2)	M46I + I84V + L76V + I54V + V82C (1)	M46L + V82T + I84V (1)
V82A + M46I + I50L + I47V (1)	M46I + I84V + I47V + I50L + L76V (1)	M46L + I50L + V82M (1)
V82A + M46I + L76V + I84V (1)	M46I + I84V + V32I + I54L + N88S (1)	I54V + L76V + V82M (2)
V82A + M46I + I47A + V32I (1)		I54V +V82C + I84V (1)
V82A + I54V + L76V + M46L (2)		V32I + M46L+ I47A +V82C (1)
V82A + I54V + L76V + I50V (1)		M46L +I47A + I50L +L90M (1)
V82A + I54V + L76V + I84V (2)		I50V + I54V + L76V +V82C (1)
V82A + I54V + G48A + I50L (1)		
V82A + I54V + M46L + G48V (1)		
V82A + M46L + V32I + I47A (1)		
V82A + M46L + I50L + L90M (1)		
V82A + I54V + M46I + L90M + I47V (1)		
V82A + I54V + M46I + L90M + I50L (2)		
V82A + I54V + M46I + L90M + I84V (1)		
V82A + I54V + M46I + L79V + I84V (2)		
V82A + I54V + M46I + L79V + I50V (1)		
V82A + I54V + M46I + N88S + I50L (1)		

Table 3.3: Drug resistance mutation comparison of DRV/r resistant patients

Regimen	ID	Previous LPV/r Exposure	Protease amino acid position						
			32	46	47	54	76	84	88
LPV/r	P1	-		M46I		I54L	L76V	I84V	
	P2	-		M46I		I54L	L76V	I84V	
	P3	-		M46I	I47V	I54V	L76V	I84V	
ATV/r	P4	Yes	V32I	M46I		I54LV	L76V	I84V	
	P5	Yes	V32I	M46I		I54V	L76V	I84V	
	P6	No	V32I	M46I		I54IL		I84V	N88S
	P7	No	V32I	M46I		I54L		I84V	

ATV/r: Atazanavir/ritonavir; LPV/r: Lopinavir/ritonavir

3.2. Resistance profiles in patients failing dolutegravir-based regimens.

We included 126 remnant samples from individuals failing a DTG-based ARV regimen that were sent to the NHLS CMJAH HIV molecular laboratory for resistance testing between 2019 and 2022. These samples had a recent VL of ≥ 400 copies/mL and a remnant sample volume of ≥ 500 µL. We had to further exclude 29 samples from the analysis, these samples either failed to produce amplicons during the PR-RT assay (n=13), IN assay (n=1), or both assays (n=9). One sample did produce an amplicon but failed to create a valid sequence. A further two samples were excluded due to sequence similarity which could have resulted from cross-contamination. Three individuals had two HIVDR tests done within the study time frame, samples with a negative drug exposure test and no DRM accumulation were included for each individual. Laboratory request forms were available for all patients (except for two) to extract relevant clinical information.

Our final sample population (n=97) comprised more females (n=68) than males, with a median age of 35 (IQR: 20-44) (Table 3.4). Overall, most individuals were on zidovudine-lamivudine-dolutegravir (ZLD, n=32, 33%) at the time of treatment failure, followed by TLD (n=29, 29.9%) and abacavir-lamivudine-dolutegravir (ALD, n=9, 9.3%) (Appendix C: Table *5.2*). The sample population had a median duration on ART of 70.1 months [IQR: 39-134] with 13.8 months [IQR: 6.1-26.3] on DTG-based therapy. Females had been exposed to DTG-containing regimens for longer (p = 0.051).

Table 3.4: Baseline analysis of demographic and clinical characteristics

Parameter	Sample population (n=97)	Males (n=29)	Females (n=68)	P value
Age (years)	35 [20-44]	39 [17-49]	32 [20.7-40]	0.093
Latest viral load (log_{10} copies/mL)	4.8 [3.8-5.2]	4.9 [3.6-5.2]	4.7 [3.9-5.2]	0.404
ART duration (months)	70.1 [39-134][a]	55.7 [28.2-108.4] [b]	75.7 [48-137.5] [c]	0.116
DTG duration (months)	13.8 [6.1-26.3][a]	9 [3.8-19.5] [b]	17.7 [6.9-29.9] [c]	0.051
Prior ART exposure				0.531
True % (n)	79.4 (77)	72.4 (21)	82.4 (56)	
False % (n)	12.4 (12)	17.2 (5)	10.3 (7)	
Unknown % (n)	8.2 (8)	10.3 (3)	7.4 (5)	
Prior PI exposure				0.829
True % (n)	54.7 (53)	51.7 (15)	55.9 (38)	
False % (n)	37.1 (36)	37.9 (11)	36.7 (25)	
Unknown % (n)	8.2 (8)	10.4 (3)	7.4 (5)	
Prior NNRTI exposure				0.993
True % (n)	62.9 (61)	62.1 (18)	63.2 (43)	
False % (n)	26.8 (26)	27.6 (8)	26.5 (18)	
Unknown % (n)	10.3 (10)	10.3 (3)	10.3 (7)	

Results are presented as median values [lower quartile; upper quartile] for skewed data, and % (n) for categorical variables; Missing data: [a]n = 26, [b]n = 7, [c]n =19; ART: Antiretroviral Therapy; NNRTI: Non-nucleoside Reverse Transcriptase Inhibitor; PI: Protease Inhibitor

3.2.1. Sanger sequencing and HIVDR data

Sequencing data was available for the PR-RT region of all patients together with 67 IN sequences, the remaining 59 patients underwent IN HIVDR testing. Although data for the PR-RT region was available, we had to generate amplicons for those samples for which a remnant PCR amplicon was not available. The generated amplicons were confirmed by determining the amplicon length through gel electrophoresis; 1.1kb for a PR-RT and 1.2kb for the IN amplicon (Figure *3.2*). Obtained Sanger sequences were assessed for sequence similarities using MEGA, the IN group had five sample pairs that clustered together, two of which were due to suspected cross-contamination (Appendix C: Figure *5.4*). However, we included one pair in this analysis for research purposes because these two samples were not close to each other during processing to be subjected to cross-contamination. Moreover, upon further analysis, the resistance profiles were completely different justifying the inclusion (Appendix C: Table *5.3*). The other three

pairs were expected clusters as they were from the same respective patients. The same expected clusters were observed for the PR-RT phylogenetic analysis. Stanford HIVdb reports for all relevant ARV drugs are summarised in Figure *3.3*. [7,21] Major mutations identified by the International AIDS Society (IAS) were detected by Sanger sequencing and summarised in Figure *3.4*– Figure *3.7*. [61]

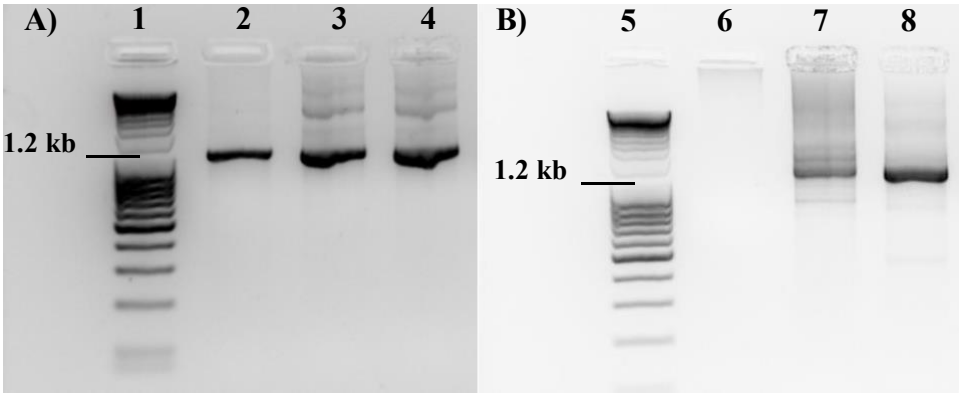


Figure 3.2: Gel electrophoresis for amplicons of the pol gene. **A)** PR-RT amplicons, **B)** IN amplicons. Lanes 1 and 5 represent the molecular weight ladder. Amplification was considered successful if PR-RT size was ~1.1 kb and IN size was ~1.2 kb (lanes 2-4 and 8 respectively). Lane 6 represents a failed amplification, and Lane 7 had nonspecific amplification which was also considered a failure

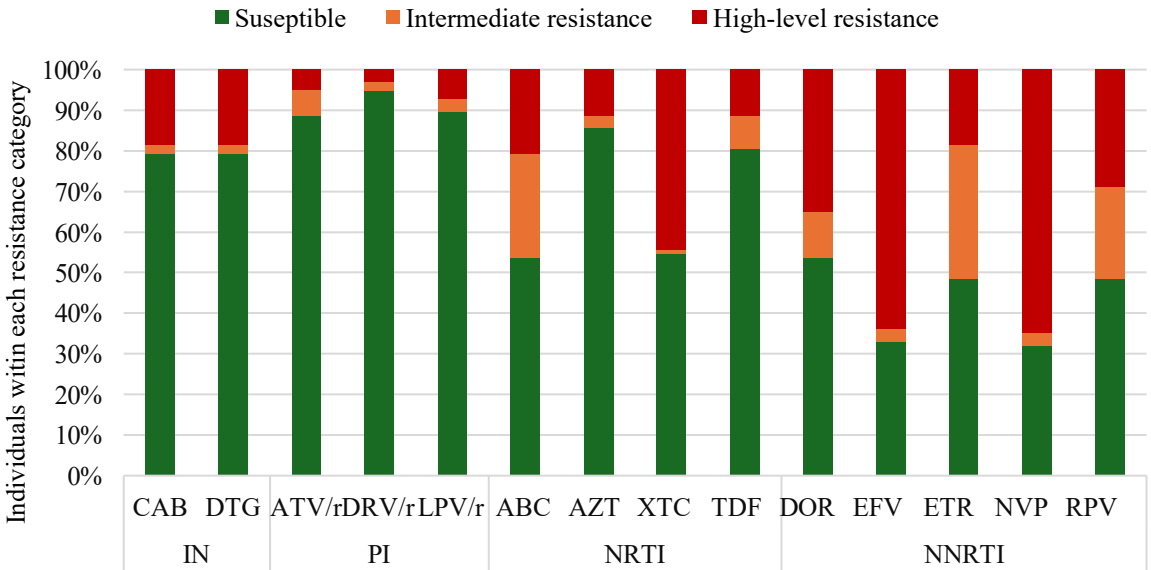


Figure 3.3: Sanger drug resistance profile summary for relevant ARVs in South Africa; ABC: Abacavir; ATV/r: Atazanavir/ ritonavir; AZT: Zidovudine; CAB: Cabotegravir; DOR: Doravirine; DRV/r: Darunavir/ ritonavir; DTG: Dolutegravir; EFV: Efavirenz; ETR: Etravirine; LPV/r: Lopinavir/ ritonavir; NVP: Nevirapine; RPV-Rilpivirine; TDF: Tenofovir; XTC: lamivudine or emtricitabine

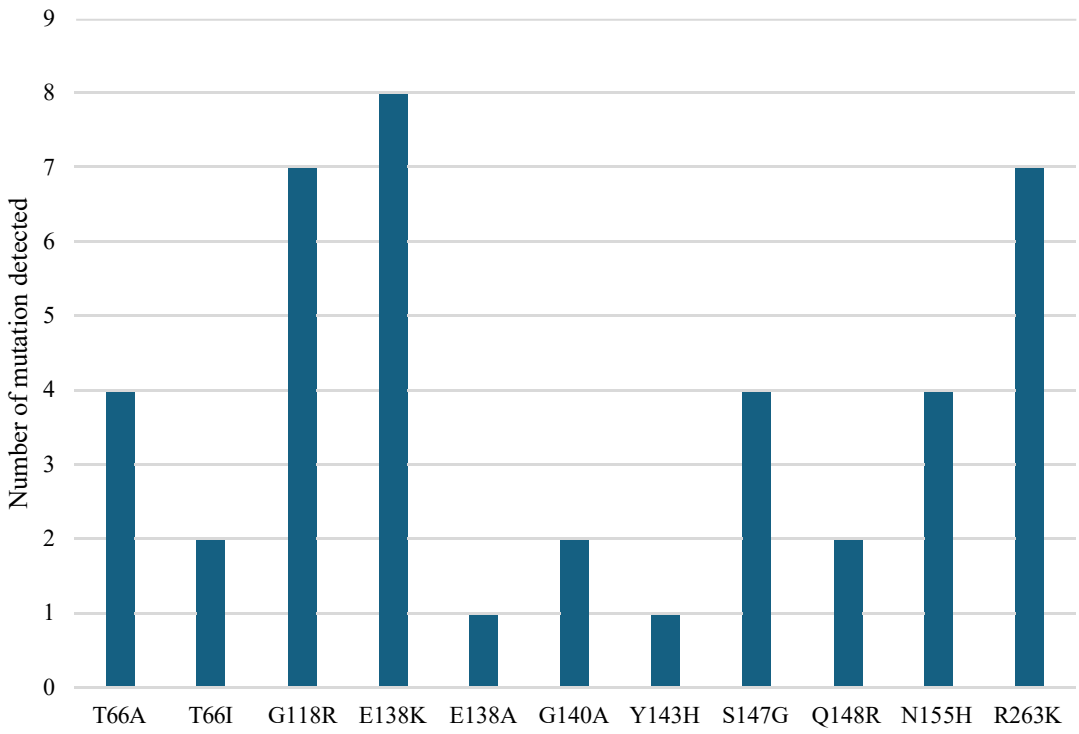


Figure 3.4: Bar chart of major InSTI mutations detected in the Integrase region of the pol gene; InSTI: Integrase-strand Transferase Inhibitor

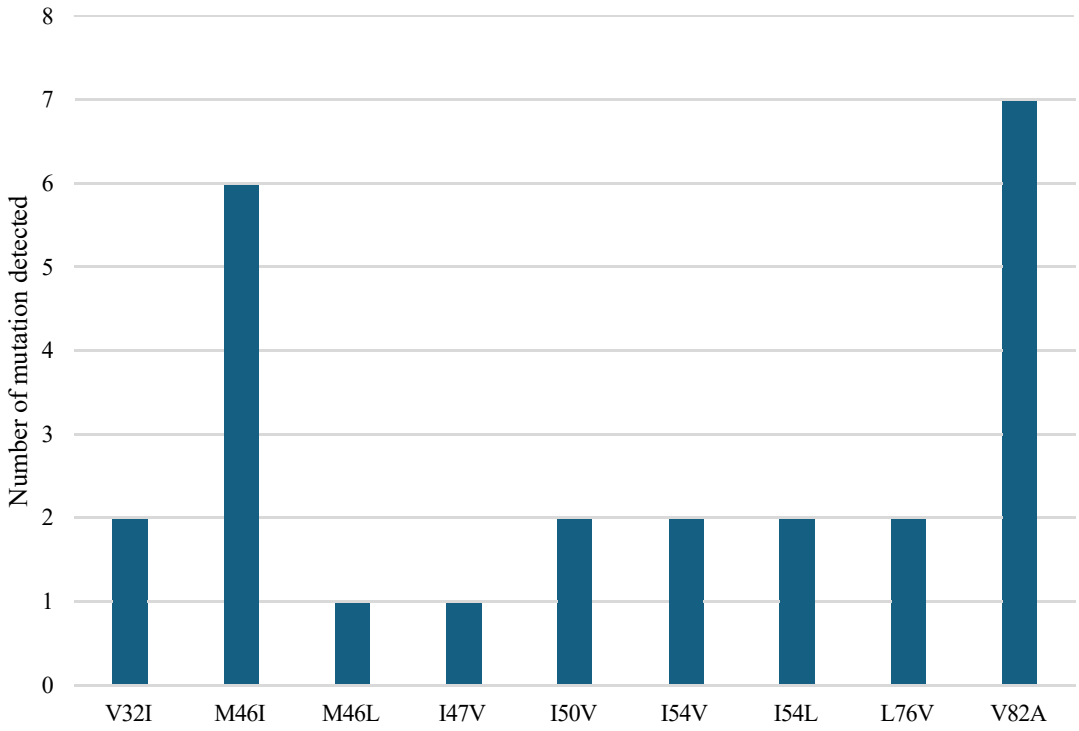


Figure 3.5: Bar chart of major PI mutations detected in the protease region of the pol gene; PI: Protease inhibitor

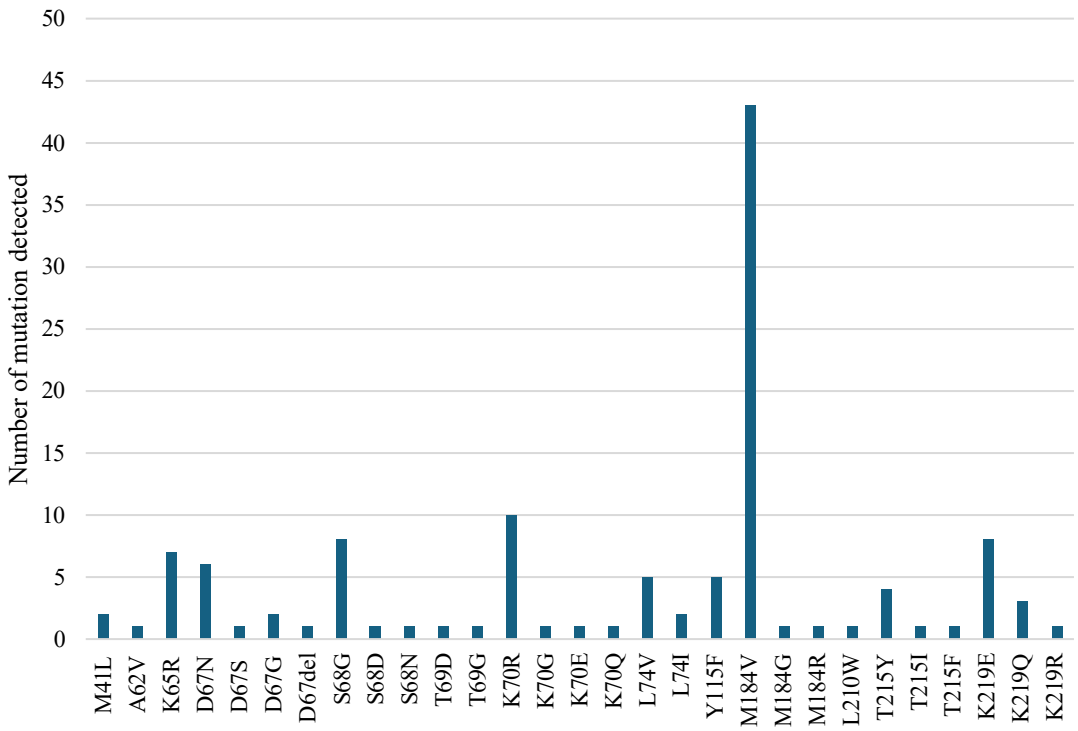


Figure 3.6: Bar chart of major NRTI mutations detected in the reverse transcriptase region of the pol gene; NRTI: Nucleoside Reverse Transcriptase Inhibitor

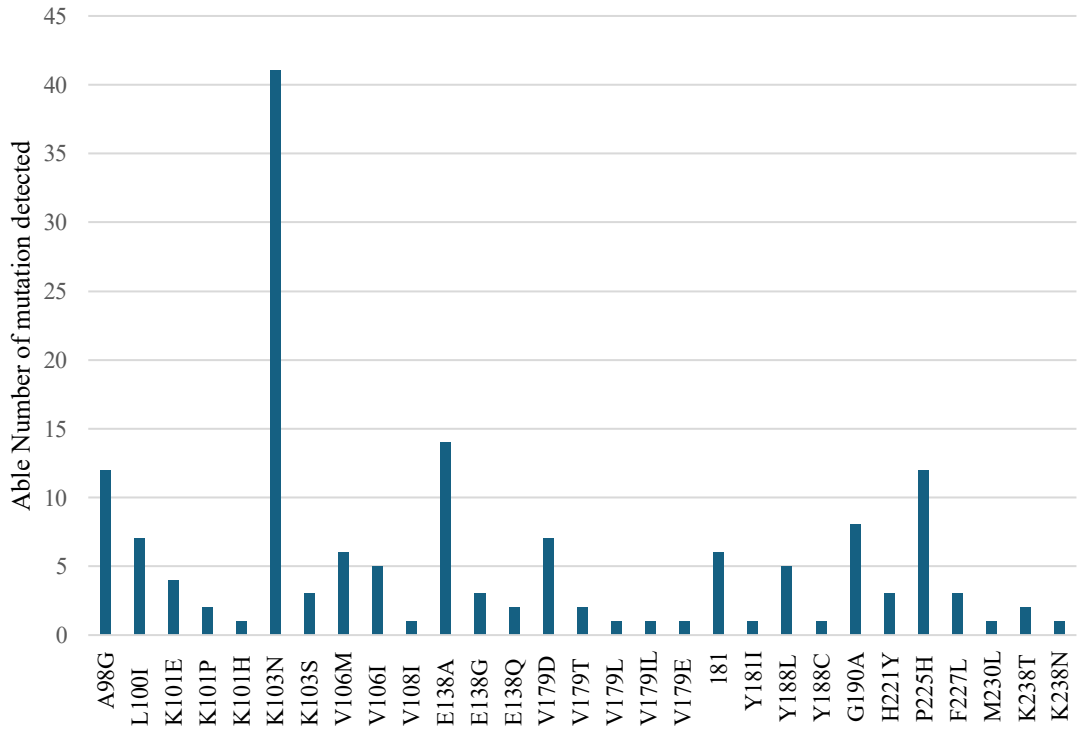


Figure 3.7: Bar chart of major NNRTI mutations detected in the reverse transcriptase region of the pol gene; NNRTI: Non-nucleoside Reverse Transcriptase Inhibitor

3.2.2. DTG- drug resistance and drug exposure analyses

Drug exposure was qualitatively assessed by considering a concentration of ≥ 0.02 µg/mL as a detectable plasma DTG drug exposure (pDTG DE), 45.3% (n=44) had detectable DTG levels. Individuals with undetectable pDTG were more likely to be younger (p=0.023). Individuals with at least one NRTI mutation were significantly more common in the positive pDTG group than the negative pDTG group (59.1% vs 37.7%, p=0.036) (Table 3.5).

Table 3.5: Analysis of individuals with positive and negative pDTG

Parameter	Positive pDTG (n=44)	Negative pDTG (n=53)	P value
Age (years)	37 [30-45]	26 [17-40]	**0.023**
ART duration (months)	68.9 [43.4-113.3] [a]	73.4 [32.8-136.7] [b]	0.750
DTG duration (months)	13.5 [4.5-24] [a]	19.3 [6.2-28] [b]	0.229
Latest Viral load (Log copies/mL)	4.6 [3.4-5.3]	4.8 [4.3-5.1]	0.503
Regimen at time of failure			0.751
ALD % (n)	11.4 (5)	7.5 (4)	0.727
TLD % (n)	50 (22)	50.9 (27)	1.000
ZLD % (n)	34.1 (15)	32.1 (17)	1.000
DTG + DRV/r % (n) *	4.5 (2)	9.5 (5)	0.451
Sex			0.089
Males % (n)	38.6 (17)	22.6 (12)	
Females % (n)	61.4 (27)	77.4 (41)	
Prior ART exposure			0.438
True % (n)	79.5 (35)	79.3 (42)	
False % (n)	9.1 (4)	15.1 (8)	
Unknown % (n)	11.4 (5)	5.6 (3)	
Prior PI exposure			0.887
True % (n)	54.5 (24)	56.6 (30)	
False % (n)	34.1 (15)	37.8 (20)	
Unknown % (n)	11.4 (5)	5.6 (3)	
Prior NNRTI exposure			0.363
True n (%)	54.5 (24)	69.8 (37)	
False n (%)	29.5 (13)	24.5 (13)	
Unknown % (n)	16 (7)	5.6 (3)	
Mutations per sequence			
≥1 PI mutation	9.1 (4)	11.3 (6)	0.719
≥1 NRTI mutation	59.1 (26)	37.7 (20)	**0.036**
≥1 NNRTI mutation	75 (33)	67.9 (36)	0.444

* Specific 3rd line regimens: TLD + DRV/r = 3, TLD + DRV/r + ETR = 2, TLD + ATV/r = 1, ZLD + DRV/r = 1; Missing data [a]n = 11, [b]n = 15; ALD: Abacavir-Lamivudine-Dolutegravir ;ART: Antiretroviral Therapy; ATV/r: Atazanavir/ritonavir; ETR: Etravirine; DRV/r: Darunavir/ritonavir; NNRTI: Non-nucleoside Reverse Transcriptase Inhibitor; p(DTG): Plasma (Dolutegravir); PI: Protease Inhibitor; TLD: Tenofovir-Lamivudine-Dolutegravir; ZLD: Zidovudine-Lamivudine-Dolutegravir

Clinically relevant resistance to DTG was detected in 19.5% (95% CI: 12.8-28.7) (n=19) of the study population. Individuals who were susceptible to DTG were significantly younger (p=0.033) (Table 3.6). Overall, individuals had a median ART duration of 81.2 months with a median time of 18.2 months on DTG-based regimens. However, treatment duration was unknown for four individuals. Although the duration of DTG treatment was longer in the group with resistance; this difference was not statistically significant (p=0.087. A significant association between individuals with susceptible DTG profiles and undetectable pDTG was observed ($p < 0.001$). Individuals with detectable pDTG levels were more likely to have DTG resistance (OR: 9.54; 95% CI 2.49 – 36.53, p=0.001). Undetectable pDTG predicted the absence of DTG resistance with high accuracy (negative predictive value: 94.2%, 95% CI: 85.1-97.9), a sensitivity of 82.4% (95% CI 56.7% – 96.2%), and specificity of 67.1% (95% CI 54.9% - 77.9%). All individuals with DTG resistance had NRTI mutations which was significantly higher compared to the DTG susceptible group (100% vs 34.6%, $p < 0.001$), they were also more likely to have NNRTI mutations (89.5% vs 66.7%, $p = 0.049$).

All observed mutation patterns and resistance profiles to DTG are summarised in Table 3.7, with the G118R and the R263K mutations being the most prevalent, both present in seven individuals respectively. Moreover, five of these individuals with DTG resistance did not initially have an InSTI HIVDR test requested by their clinician, as per clinical guideline recommendations. Duration of DTG-based treatment in these five individuals ranged from 8.4 months to 18.2 months.

Table 3.6: Analysis of individuals with clinically relevant DTG drug resistance

Parameter	DTG susceptible (n=78)	DTG resistant (n=19)	P value
Age (years)	32 [19-42]	39 [32-48]	**0.033**
ART duration (months)	70.0 [36.7-139.1] [a]	81.2 [45-112] [b]	0.938
DTG duration (months)	11.6 [4.9-25.9] [a]	18.2 [14.5-29.3] [b]	0.087
Latest Viral load (Log copies/mL)	4.7 [3.9-5.2]	4.5 [3.6-5.1]	0.510
Regimen at time of failure			0.599
ALD % (n)	9 (7)	10.5 (2)	1.000
TLD % (n)	50 (39)	52.6 (10)	1.000
ZLD % (n)	32 (25)	36.8 (7)	0.690
DTG + DRV/r % (n) *	9 (7)	0	0.339
Sex			0.467
Males % (n)	28.2 (22)	36.8 (7)	
Females % (n)	71.8 (56)	63.2 (12)	
Prior ART exposure			0.898
True % (n)	79.6 (62)	79 (15)	
False % (n)	12.8 (10)	10.5 (2)	
Unknown % (n)	2.6 (6)	10.5 (2)	
Prior PI exposure			0.909
True % (n)	56.5 (44)	47.4 (9)	
False % (n)	35.9 (28)	42.1 (8)	
Unknown % (n)	2.6 (6)	10.5 (2)	
Prior NNRTI exposure			0.816
True % (n)	61.5 (48)	68.4 (13)	
False % (n)	28.2 (22)	21.1 (4)	
Unknown % (n)	10.3 (8)	10.5 (2)	
Detectable pDTG			**< 0.001**
True % (n)	35.9 (28)	84.2 (16)	
False % (n)	64.1 (50)	15.8 (3)	
Mutation per sequence			
≥1 PI mutation	8.9 (7)	15.8 (3)	0.381
≥1 NRTI mutation	34.6 (27)	100 (19)	**< 0.001**
≥1 NNRTI mutation	66.7 (52)	89.5 (17)	**0.049**

* Specific 3rd line regimens: TLD + DRV/r = 3, TLD + DRV/r + ETR = 2, TLD + ATV/r = 1, ZLD + DRV/r = 1; Results are presented as median values [lower quartile; upper quartile] for skewed data and % (n) for categorical variables; Missing data: [a]n = 22, [b]n = 4; ALD: Abacavir-Lamivudine-Dolutegravir ;ART: Antiretroviral Therapy; ATV/r: Atazanavir/ritonavir; ETR: Etravirine; DRV/r: Darunavir/ritonavir; NNRTI: Non-nucleoside Reverse Transcriptase Inhibitor; p(DTG): Plasma (Dolutegravir); PI: Protease Inhibitor; TLD: Tenofovir-Lamivudine-Dolutegravir; ZLD: Zidovudine-Lamivudine-Dolutegravir

Table 3.7: Mutation profiles of patients with clinically relevant resistance to dolutegravir

Number of individuals with specific resistant profiles (n=19)*	**DTG resistance level**
R263K (6) [a]	Intermediate
E157Q + S230R (1)	Low level
N115H + S147G (2)	Intermediate
R263K +Y143H (1)	Intermediate
G118R + E138AKT (1)	High level
G118R + T66I + E138K (2) [b]	High level
G118R + T66A + E138A (1)	High level
G118R + T66A +E138K (3)	High level
E138K + G140A + S147G + Q148R (1)	High level
E138K + G140A + S147G + Q148R + N155H (1)	High level

* One additional individual had the N155H DRM and was susceptible to DTG; [a] Four individuals had DTG exposure for < 24 months and no routine InSTI HIVDR test was requested. [b] One individual had DTG exposure for < 24 months and no routine InSTI HIVDR test was requested; DRM: Drug Resistance Mutation; DTG: Dolutegravir; HIVDR: HIV Drug Resistance; InSTI: Integrase Strand Transferase Inhibitor

3.2.3. Nanopore sequencing data

The ONT platform together with the native barcoding kit for amplicons (SQK-NBD114.96) allows the user to barcode and multiplex sequence up to 96 individual samples in one run. All samples (n=100) (before exclusions based on sequence similarity as described in section 3.2) were sequenced in four separate batches consisting of 5, 10, 39, and 51 samples on a flongle flow cell (Table 3.8) — the last run consisting of 51 samples contained five previously failed samples. The size of each batch was arbitrarily selected based on comfort level with the library preparation workflow. Samples were considered failed for this study if both regions of the *pol* gene had coverage below 250x. We noticed there was a skewed representation of sequencing data favouring the PR-RT region/amplicon during the first two runs. It was thought that there was competition binding between the two amplicons during the barcode ligation step. Therefore, we decided to end-prep and ligate the barcodes for each amplicon separately before pooling the sample set together for the downstream steps of the procedure. Theoretically, this would yield a more uniform coverage across the two regions. This is partly true for the batch consisting of five samples, however as the batch size increases to 51, the coverage for the IN region decreased again.

Table 3.8: Nanopore data output summary

Batch order	Number of samples	Barcoding	IN concentration (fmol)	PR-RT concentration (fmol)	PR coverage	RT coverage	IN coverage	Failure % (n)
1	10	Together	55.1 [63.6-66.9]	37.2 [40.5-44.1]	609 [714-846]	773 [996-1327]	643 [797-891]	20 (2)
2	39	Together	138 [112-150.2]	60.4 [51.0-77.4]	1065 [813-1233]	1307 [927-1498]	1069 [864-1356]	10.3 (4)
3	5	Separate	28.7 [58.4-130.4]	33.0 [40.2-87.4]	1255 [1265-1443]	1319 [1549-1983]	1334 [1355-1371]	0
4	51	Separate	35.1 [14.1-111.7]	53.4 [34.2-71.1]	1184 [811-1226]	1265 [772-1531]	270 [194-374]	52.9 (27)

Results are presented as median values [lower quartile; upper quartile] for skewed data, and % (n) for categorical variables; fmol: Femtomoles; IN: Integrase; PR: Protease; RT: Reverse Transcriptase

MEGA analysis was only performed on the samples that had a sequencing coverage of ≥ 250X per region which produced a matching consensus sequence. A close association between one pair of samples was observed, but these samples were from the same patient and only one sample was included as previously described. Another pair had a phylogenetic distance of 0.019 for the integrase region, however, this pair was still included in the analysis as the phylogenetic distance was close to the 0.02 cut-off and the association was not recorded in the PR-RT region and thus cross-contamination was ruled out.

3.2.4. Correlating Sanger- and nanopore sequencing data

We conducted four analyses correlating Sanger- and nanopore sequencing data (Figure *3.8*). Some fasta files failed to produce a resistance profile for either region of the *pol* gene at specific mixture thresholds analysed by NanoRecall, these samples were subsequently excluded from the relevant analysis. The NanoRecall software suggests a minimum coverage of 400X per *pol* gene region at a 25% mixture threshold to accurately compare nanopore sequencing data (n=54) with the gold standard (Sanger), this is supported by Delaney et al. [105,106]

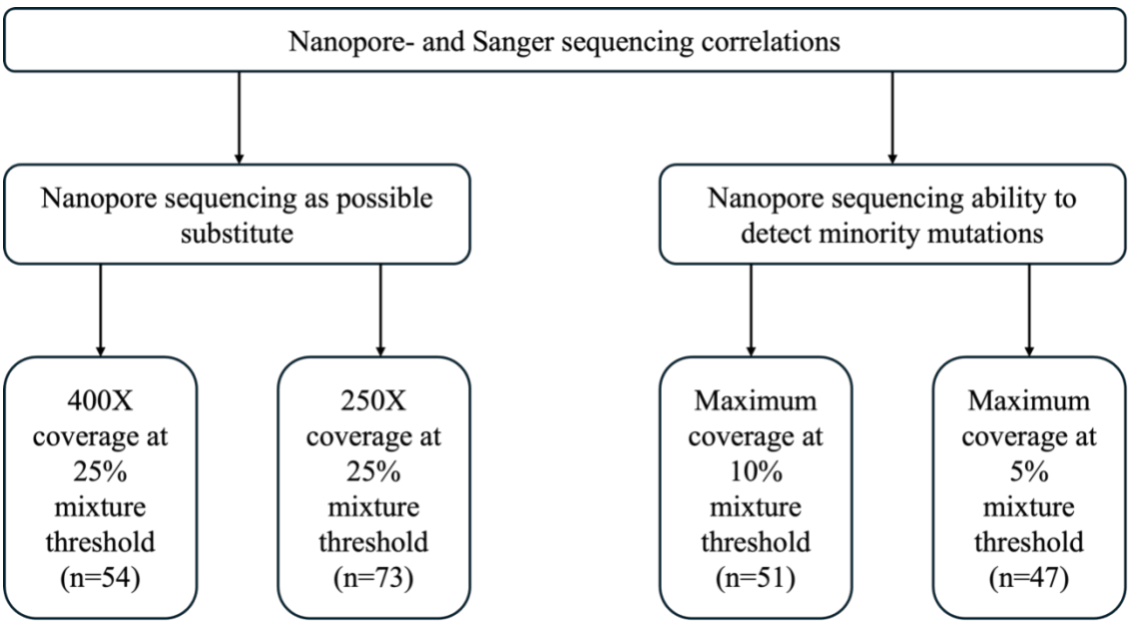


Figure 3.8: Flow diagram illustrating correlation tests between Nanopore- and Sanger sequencing data.

For the first analysis, NanoRecall was set to a minimum coverage of 400X and a mixture threshold of 25%. Resistance mutations detected by Sanger and nanopore had a 99.6% (range: 88.9-100) and 97.9% (range: 75.6-100) pairwise identity for integrase and protease-reverse transcriptase respectively (Table *3.9* and Figure *3.9*). For the IN region, two samples did not

meet the 93% similarity threshold, both scoring 88.9%. In one sample (P031), the R263K mutation was detected as a pure mutant by Sanger sequencing but detected as a mixture of wild type and mutant by nanopore. The resistance profile of DTG changed from intermediate resistance by Sanger to susceptible by nanopore. In the second sample (P041), Sanger detected an E138AK mixture but nanopore sequencing only reported E138A which did not alter the resistance profile. Six samples (P002, P006, P016, P031, P64, and P100) failed to meet the similarity threshold of 97% for the PR-RT region, mostly due to the mixture picked up by nanopore sequencing. The DRM differences and their impact on resistance level interpretation are listed in Table 3.12 and 3.13). However, in one of these samples (P031) three major PI mutations were not detected by Nanopore sequencing which resulted in a resistance profile change from high-level resistance to susceptible for ATV/r and LPV/r.

The software settings were then changed to a minimum coverage of 250X and a mixture threshold of 25% to determine the correlation between Sanger- and nanopore sequencing data (n=72) at a lower coverage than recommended. The pairwise identity for this analysis was 99.7% (range: 88.9-100) for IN and 97.8% (range: 73.1-100) for the PR-RT region. The same two samples (P031 and P041) from the IN 400X analysis were flagged for not meeting the similarity threshold. The same five samples (P002, P006, P016, P031, and P100) from the PR-RT 400X analysis were also flagged while one sample (P064) from the same analysis failed to produce nanopore consensus sequences. Two additional sequences (P036 and P061) were flagged where nanopore detected major PI mutations (M46I/I50L/V82V/L90M for P036 and I50L for P061) resulting in ATV/r and LPV/r high-level resistance which was initially susceptible.

To determine the presence of low abundance mutations we ran each sample (n=54) at its maximum coverage at both 10% and 5% mixture thresholds. The software failed to produce a sequence for three and seven specimens at mixture thresholds of 10% and 5% respectively. At a 10% level, the IN region had a pairwise identity of 91.9% (66.7-100) vs 84.6% (range: 44.4-100) at a 5% level, and similarly the PR-RT region 96.3% (range: 63.4-100) vs 92.1% (range 58.5-100) at a 10% and 5% level respectively. As more low-abundance mutations (<25%) were expected to be picked up by Nanopore. the similarity acceptance criteria were not applied in this analysis. Figure *3.9* visually represents these results.

Table 3.9: Correlating Sanger- and nanopore sequencing data

Mixture threshold	PR-RT: Coverage	PR-RT: % Pairwise identity (Range)	IN: Coverage	IN: % Pairwise identity (Range)
25%	≥ 400X	97.9 (75.6-100)	≥ 400X	99.6 (88.9-100)
25%	≥ 250X	97.8 (97.1-100)	≥ 250X	99.7 (88.9-100)
10%	1113 [804-1369]	96.3 (63.4-100)	926 [709-1203]	91.9 (66.7-100)
5%	1185 [809-1382]	92.1 (58.5-100)	954 [727-1229]	84.6 (44.4-100)

Results are presented as median values [lower quartile; upper quartile] for skewed data. CI: Confidence Interval; IN: Integrase; PR: Protease; RT: Reverse Transcriptase

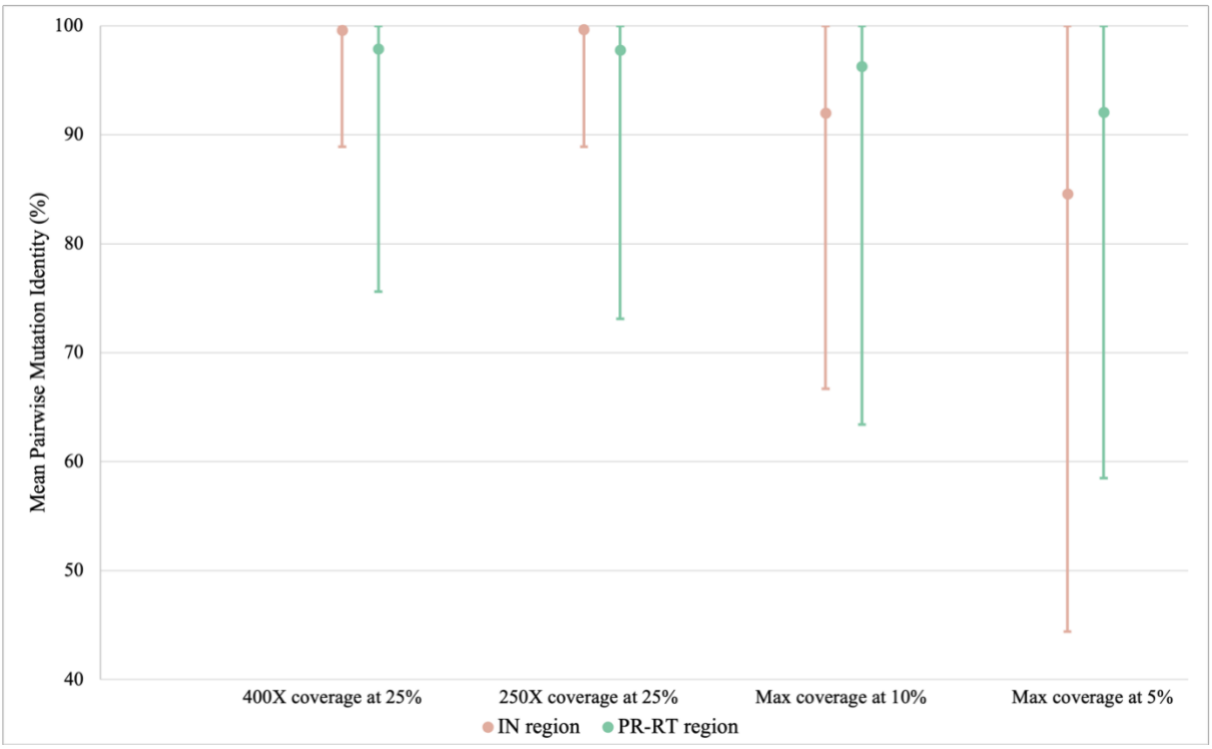


Figure 3.9: Mean pairwise identity of mutation detected by Nanopore sequencing compared to Sanger sequencing; IN: Integrase; PR-RT: Protease – Reverse Transcriptase

We examined the detection rates of low-abundance mutations and the potential changes in predicted resistance profiles. Discrepancies in InSTI DRM detection between Sanger sequencing and low-abundance mixture thresholds (5%) were observed in 41 individuals at a nanopore minimum coverage of 400x. The detection of low-abundance DRMs influenced the resistance profile to DTG in 28 individuals with the G140A being detected in 23 individuals. However, the changes in resistance profiles were only clinically relevant in three of these

individuals. Two individuals had a DTG-resistance level change from susceptible to high-level resistance with the detection of the G140AR and R263K mutations in two cases (P013) and the E138K, G140IS and Y143S* mutations in another (P033) (Table *3.10*). Patients with unchanged InSTI resistance profiles are listed in Table *3.11*. Of note, none of the InSTI DRMs that were only detected by Nanopore exceeded 25% prevalence, indicating these could not have been picked up by Sanger sequencing.

The detection of different low-abundance DRMs at 5% was observed in 7 individuals when considering the PI resistance profiles, with five resulting in different resistance profiles (P031, P035, P049, P061, and P121, Table 3.12). Nanopore sequencing failed to detect three major mutations (M46I, I54V, and V82A) in one individual (P031) resulting in a susceptible resistance profile to the three available PIs (Table *3.12*). The PI mutation profiles detected in individuals whose predicted resistance profile did not change are listed in Table *3.13*.

Table 3.10: Full InSTI mutation profiles of patients with changed DTG resistance levels using Nanopore sequencing

ID	Viral load	Sequence	InSTI DRM position							DTG level
			138	140	143	151	155	157	263	
P070	587 000	Sanger		-						1
		Nanopore		G140R(10%)						2
P058	110 000	Sanger		-						1
		Nanopore		G140R(10%)						2
P028	1 800	Sanger		-						1
		Nanopore		G140R(10%)						2
P068	205 000	Sanger		-						1
		Nanopore		G140A(11%) E(5%)						2
P007	228 000	Sanger		-						1
		Nanopore		G140A(5%)						2
P008	3 470	Sanger		-						1
		Nanopore		G140A(8%)						2
P050	29 700	Sanger		-						1
		Nanopore		G140A(9%)						2
P035	40 700	Sanger		-						1
		Nanopore		G140A(11%)						2
P057	46 200	Sanger		-						1
		Nanopore		G140A(11%)						2
P054	307 000	Sanger		-						1
		Nanopore		G140A(11%)						2
P037	270 000	Sanger		-		-				1
		Nanopore		G140A(11%)		V151A(5%)				2
P034	114 995	Sanger		-		-				1
		Nanopore		G140A(11%)		V151A(6%)				2
P067	557	Sanger		-				E157Q		1
		Nanopore		G140A(7%)				E157Q(90%)		2

Please refer to Appendix C: Table *5.4* for amino acid codes. The minimum mixture threshold for detection of specific mutation is given in brackets; DTG level 1: Susceptible, 2: Potential low-level resistance, 3: Low-level resistance, 4: Intermediate resistance; DRM: Drug Resistant Mutation; DTG: Dolutegravir.

Table 3.10: Continued

ID	Viral load	Sequence	InSTI DRM position							DTG level
			138	140	143	151	155	157	263	
P043	1 500	Sanger		-						1
		Nanopore		G140A(14%)						2
P071	87 800	Sanger		-		-				1
		Nanopore		G140A(11%) R(5%)		V151A(5%)				2
P101	58 600	Sanger		-		-				1
		Nanopore		G140A(9%) R(5%)		V151A(5%)				2
P097	464 000	Sanger		-						1
		Nanopore		G140A(14%) R(5%)						2
P002	85 700	Sanger		-		-				1
		Nanopore		G140 A(7%) R(6%)		V151A(5%)				2
P121	42 800	Sanger		-						1
		Nanopore		G140 A(12%) R(6%)						2
P055	97 700	Sanger		-		-				1
		Nanopore		G140 A(12%) R(5%)		V151A(5%)				2
P092	263 000	Sanger		-	-					1
		Nanopore		G140A(10%)	Y143D(5%)					2
P061	1 870	Sanger		-	-	-				1
		Nanopore		G140A(11%)	Y143D(5%)	V151A(5%)				2

Please refer to Appendix C: Table *5.4* for amino acid codes. The minimum mixture threshold for detection of specific mutation is given in brackets; DTG level 1: Susceptible, 2: Potential low-level resistance, 3: Low-level resistance, 4: Intermediate resistance; DRM: Drug Resistant Mutation; DTG: Dolutegravir.

Table 3.10: Continued

ID	Viral load	Sequence	InSTI DRM position							DTG level
			138	140	143	151	155	157	263	
P004	109 448	Sanger Nanopore	-	- G140A(13%) R(9%)	- Y143D(6%)					1 2
P016	16 300	Sanger Nanopore	-	- G140A(9%) R(5%)	- Y143D(5%)					1 2
P013	65 500	Sanger Nanopore	-	- G140A(10%) R(7%)	-	-	-	-	- R263K(10%)	1 4
P100	60 800	Sanger Nanopore	-	- G140 A(9%) R(5%)	-	- V151A(6%)	N155NH N155H(68%)	E157Q E157Q(82%)	-	2 3
P005	3 190	Sanger Nanopore	- E138K(5%)	-	- Y143*(7%)	- V151A(7%)	-	-	-	1 2
P033	929 000	Sanger Nanopore	- E138K(14%)	- G140I(6%) S(6%)	- Y143S(7%) *(5%)	-	-	-	-	1 4

Please refer to Appendix C: Table *5.4* for amino acid codes. The minimum mixture threshold for detection of specific mutation is given in brackets; DTG level 1: Susceptible, 2: Potential low-level resistance, 3: Low-level resistance, 4: Intermediate resistance; DRM: Drug Resistant Mutation; DTG: Dolutegravir.

Table 3.11: InSTI mutation profiles of patients with unchanged DTG resistance levels

ID	Viral load	Sequence	InSTI DRM amino acid position									DTG level
			66	118	138	140	143	147	148	155	263	
P022	9 260	Sanger					-					1
		Nanopore					Y143*$_{(5\%)}$					1
P024	1 190	Sanger					-					1
		Nanopore					Y143H$_{(5\%)}$					1
P032	3 210	Sanger					-					1
		Nanopore					Y143H$_{(5\%)}$					1
P038	51 200	Sanger					-					1
		Nanopore					Y143H$_{(5\%)}$					1
P025	659 000	Sanger			E138K	G140A		S147G	Q148R	N155H		5
		Nanopore			E138K$_{(87\%)}$	G140A$_{(80\%)}$		S147G$_{(90\%)}$	Q148R$_{(79\%)}$	N155H$_{(89\%)}$		5
P041	11 100	Sanger	-	G118R	E138EAKT							5
		Nanopore	T66M$_{(5\%)}$	G118R$_{(84\%)}$	E138A$_{(77\%)}$ K$_{(9\%)}$							5
P042	52 700	Sanger	T66I	G118R	E138K	-	-					5
		Nanopore	T66I$_{(79\%)}$	G118R$_{(70\%)}$	E138K$_{(79\%)}$	G140A$_{(7\%)}$	Y143D$_{(5\%)}$					5
P044	582	Sanger				-	-	S147G		N155H		4
		Nanopore				G140A$_{(10\%)}$ R$_{(5\%)}$	Y143D$_{(9\%)}$	S147G$_{(92\%)}$		N155H$_{(91\%)}$		4
P045	414 000	Sanger					-					1
		Nanopore					Y143H$_{(5\%)}$					1
P047	2 100	Sanger	T66I	G118R	E138EK	-						5
		Nanopore	T66I$_{(76\%)}$ T$_{(11\%)}$	G118R$_{(76\%)}$ G$_{(11\%)}$	E138K$_{(65\%)}$	G140A$_{(9\%)}$						5

Please refer to Appendix C: Table *5.4* for amino acid codes. The minimum mixture threshold for detection of specific mutation is given in brackets; DTG level 1: Susceptible, 4: Intermediate resistance, 5: High-level resistance; DRM: Drug Resistant Mutation; DTG: Dolutegravir.

Table 3.11: Continued

ID	Viral load	Sequence	InSTI DRM amino acid position									DTG Level
			66	118	138	140	143	147	148	155	263	
P051	2 600	Sanger	T66A	G118R	E138A	-						5
		Nanopore	T66A(76%)	G118R(78%)	E138A(90%)	G140A(12%)						5
P056	154 000	Sanger				-					R263K	4
		Nanopore				G140A(8%) R(5%)					R263K(82%)	4
P082	2 460	Sanger				-	-				R263K	4
		Nanopore				G140A(11%) R(6%)	Y143D(7%)				R263K(77%)	4

Please refer to Appendix C: Table *5.4* for amino acid codes. The minimum mixture threshold for detection of specific mutation is given in brackets; DTG level 1: Susceptible, 4: Intermediate resistance, 5: High-level resistance; DRM: Drug Resistant Mutation; DTG: Dolutegravir.

Table 3.12: Resistance profiles of patients with change PI resistance levels

ID	Viral load	Sequence	PI DRM amino acid position						ATV/r Level	DRV/r Level	LPV/r level
			32	46	47	50	54	82			
P031	879	Sanger		M46I			I54V	V82A	5	1	5
		Nanopore		-			-	-	1	1	1
P035	40 700	Sanger	V32I	M46I	-		I54L	V82A	5	4	5
		Nanopore	$V32I_{(95\%)}$	$M46I_{(95\%)}$	$I47V_{(87\%)}$		$I54L_{(88\%)}$	$V82A_{(95\%)}$	5	5	5
P049	568	Sanger						-	1	1	1
		Nanopore						$V82L_{(5\%)}$	3	1	3
P061	1 870	Sanger				-			1	1	1
		Nanopore				$I50L_{(37\%)}$			5	1	1
P121	42 800	Sanger					-		1	1	1
		Nanopore					$I54M_{(6\%)}$		3	3	3

Please refer to Appendix C: Table *5.4* for mutational codes. The minimum mixture threshold for detection of specific mutation is given in brackets; Resistance level 1: Susceptible, 3: Low-level resistance, 4: Intermediate resistance, 5: High-level resistance; ATV/r: Atazanavir/ ritonavir; DRM: Drug Resistant Mutation; DRV/r: Darunavir/ ritonavir; LPV/r: Lopinavir/ ritonavir.

Table 3.13: Resistance profiles of patients with unchanged PI resistance levels

ID	Viral load	Sequence	PI DRM amino acid position						ATV/r Level	DRV/r Level	LPV/r level
			30	46	47	54	76	82			
P004	109 448	Sanger	-			I54V	L76V	V82A	4	3	5
		Nanopore	$D30N_{(15\%)}$			$I54V_{(92\%)}$	$L76V_{(94\%)}$	$V82A_{(38\%)}$ $T_{(10\%)}$	4	3	5
P032	3 210	Sanger	-						1	1	1
		Nanopore	$D30N_{(5\%)}$						1	1	1

Please refer to Appendix C: Table *5.4* for amino acid codes. The minimum mixture threshold for detection of specific mutation is given in brackets; Resistance level 1: Susceptible, 3: Low-level resistance, 4: Intermediate resistance, 5: High-level resistance; ATV/r: Atazanavir/ ritonavir; DRM: Drug Resistant Mutation; DRV/r: Darunavir/ ritonavir; LPV/r: Lopinavir/ ritonavir.

Chapter 4

Discussion

Herein we undertook to describe and evaluate HIV-1 ARV drug resistance in individuals in the South African public sector who are failing treatment in two different cohorts; one cohort consisted of individuals failing PI-based treatment, whereas the other cohort included individuals failing DTG-based treatment. In the latter cohort, we also evaluated the use of drug exposure testing to triage individuals for an HIVDR test and compared a relatively novel sequencing technology to the gold standard in HIVDR testing for its suitability in the public sector.

In the current DTG era, the use of PIs to treat PLWH will decline as guidelines promote the switch to DTG-based regimens. However, drugs like DRV/r, a second-generation PI, are still vital in personalised third-line ART regimens highlighting the need to track resistance to this class. [21] As expected, the V82A mutation was most abundant in the cohort of PLWH who failed PI-based treatment. This mutation is frequently reported with LPV/r and to a lesser extent ATV/r resistance. [107] In our cohort, most individuals failed a LPV/r-based regimen (76.4%). Moreover, 62.1% of those on a failing ATV/r-based regimen had previously recorded LPV/r exposure. Before 2023, LPV/r was the preferred PI for second-line ART, however, individuals on LPV/r were switched to ATV/r if dyslipidaemia occurred or if diarrhoea associated with LPV/r was diagnosed. When the 2023 guidelines suggested that ATV/r is to be used as the preferred PI, clinicians switched patients from LPV/r to ATV/r during the transition period to DTG. [26,27] In our cohort, the LPV/r group had significantly more PI mutations per sequence than the ATV/r group ($p=0.002$). This significant accumulation of mutations in the LPV/r group can likely be attributed to the longer treatment duration in this group as exposure to a certain drug leaves room for the acquisition of more mutations. [108] Unfortunately, we did not have access to treatment duration in this cohort to corroborate this finding. Gastrointestinal toxicity associated with LPV/r may also contribute to suboptimal plasma levels, increasing the risk for DRM acquisition. [34,58]

Although the proportion of individuals with PI resistance within each group was quite similar (p=0.538), DRV/r cross-resistance was significantly more prevalent in the LPV/r group (p=0.013). This is likely linked to a higher proportion of individuals with ≥ 3 PI mutations in the LPV/r group. Furthermore, two of the four individuals with high-level cross-resistance to DRV/r within the ATV/r group expressed LPV/r-specific DRMs; it is therefore likely that DRV/r resistance had already been acquired before the ATV/r regimen switch. All of these justify the preferred use of ATV/r as outlined by previous studies. Menshawy et al. [34] determined that ATV/r is non-inferior to LPV/r with much better gastrointestinal tolerability. Moreover, Tigabu et al. [58] also deemed ATV/r fit for better viral suppression at a lower risk for dyslipidaemia. Findings such as these shaped the South African ART guidelines to include ATV/r as the preferred PI. [21,26]

Individuals in the DTG cohort had a median age of 35 years and consisted of 70.1% females who also had a longer duration on a DTG-based regimen (p=0.051). While females are more inclined to seek healthcare, in South Africa they were previously shown to be eight times more likely to be infected with HIV. [109,110] The UNAIDS initiative has brought the HIV infection rate down, however HIV prevalence is still higher in females compared to males in South Africa (22.6% vs 11.5%). [17,111] Moreover, UNAIDS reported that more females (81%) in South Africa are receiving ART as opposed to males (71%) in 2023. [111]. Men on the other hand delay treatment initiation and do not always adhere to follow up visits after they have been enrolled in HIV care. [112] The more favourable healthcare-seeking behaviour by females could explain the longer duration of DTG treatment.

Most individuals in the DTG cohort were on a ZLD regimen (33%) followed by TLD (29.9%). For a long time, the ART dogma suggested that at least two drugs from the first-line regimen were to be replaced by two active drugs in the second-line regimen, e.g. TEE (TDF-FTC-EFV) to AZT-3TC-LPV/r. Therefore, many countries, including South Africa followed the conservative approach, which led to most patients initially being switched from TEE to ZLD, despite ZLD not being available in a FDC. However, the NADIA trial found tenofovir and lamivudine to be non-inferior to zidovudine and lamivudine as the NRTI backbone in combination with DTG. [78] Moreover, they compared these two DTG combinations to DRV/r which are both second-generation drugs in their respective classes, offering the most robust genetic barriers. The study showed that the use of DTG was non-inferior to DRV/r, however, DTG is preferred over DRV/r due to the availability of FDCs with an NRTI backbone, which

improves adherence and reduces treatment costs. [78] Although viral suppression rates in the NADIA trial were similar between the ZLD and TLD arm, in those patients with virological failure, DTG resistance was more common in patients who switched to a ZLD regimen, indicating a preference towards TLD. [78] This combination is also supported by the VISEND trial, which showed that tenofovir and XTC recycled in second-line therapy are more effective than DTG with zidovudine. [113] The ARTIST trial conducted in Cape Town, South Africa, also supported switching patients from a failing TEE regimen to TLD due to the high genetic barrier of DTG. [73,114]

Overall 19.5% of the cohort presented with DTG resistance which is in line with estimates from Mozambique for patients failing DTG-based ART and having failed a prior regimen. [33] We conducted 59 additional standard InSTI HIVDR tests which were not requested by the clinician as outlined by the guidelines as all these individuals had DTG exposure for < 24 months. [21] Intermediate to high-level DTG resistance was observed in 8.5% (n=5) of these individuals which is concerning as adequate patient care could not be provided to these individuals due to a lack of resistance information. We also observed that PLWH in our cohort were more likely to present with DTG resistance if NRTI mutations were present. Resistance to NRTIs in treatment-experienced patients is common and has been identified as a risk factor to developing InSTI mutations. [82] This highlights the need to identify those at the highest risk for DTG resistance, especially if they have been exposed to ART before switching to DTG-based regimens.

Younger individuals were more likely to present with susceptible resistance profiles for DTG (p=0.033) and to test negative for pDTG DE (p=0.023), indicating poor adherence. It is not unexpected that adolescents struggle with ART adherence owing to possible negative family dynamics and conflict between school and clinic times. [115,116] However, due to our small sample size and the limited number of children and adolescents included in this cohort, these conclusions need to be confirmed in a larger cohort including individuals with a wider age range

Poor treatment adherence is a main contributor to virological failure for PLWH on ART. [89,117] Kangee et al. explained that adherence is not always the patient's fault, in poverty-stricken communities, attending clinic visits can be difficult, and public health services are sometimes inadequate. [37] This is supported by another study, which reported a virological non-suppression prevalence of 4.2% where non-adherent individuals said that transport to a health

facility is a major hurdle. [118] Virological non-suppression can also arise due to drug-drug interactions, e.g. Rifampicin prescribed to co-infected TB patients lowers the bioavailability of DTG. [21] Although DTG has less gastrointestinal toxic side effects, PLWH have reported discontinuation of ART due to DTG intolerability. [119]

It is therefore important to identify the root cause of suboptimal treatment adherence when managing PLWH and viral non-suppression. However, the objective assessment of treatment adherence is a challenge, limiting the proposed objective adherence measures to pharmacy refills- and clinic visit records. [21] Although patient clinic attendance is used as a proxy for adherence, this metric does not always reflect pill taking in the setting of HIV. [120] Moreover, given varying patterns of tracking clinic attendance by paper charts, electronic medical records, or patient-reported data sources in South Africa, comprehensive data sources reflecting retention in HIV care remain elusive without a centralised medical records system. [121,122] For the same reason pharmacy refill data may be difficult to comprehensively collect and are not always predictive of HIV treatment outcomes. [123] Without centralised data sources, both clinic visit attendance and pharmacy refill data can be difficult to accurately capture.

There is evidence that the detection of ARVs in biological specimens could be used to predict virological failure and/or resistance. Undetectable urine tenofovir, measured by a point-of-care immunoassay, predicted viral rebound with 66% sensitivity and 100% specificity. [90] In the same analysis, individuals with detectable urine tenofovir were 12.8 times more likely to have NRTI resistance. In comparison, urine-tenofovir predicted ART adherence with a sensitivity of 94% and excellent specificity of 99%. [91] Another study found that undetectable urine LPV/r, measured via LC-MS/MS, predicted PI susceptibility with a negative predictive value of 94%. [88] Our findings, which suggest the ability to triage individuals for an HIVDR test based on the presence or absence of DTG in their blood adds to this growing body of evidence. We demonstrated that individuals without detectable pDTG DE have a 94.2% likelihood of presenting without any clinically relevant DTG DRMs, which is in line with that of Hermans et al. [88] This means that 50 (51.5%) of the HIVDR tests in our cohort could have been avoided by using pDTG DE testing as a screening tool for HIVDR testing. On a national level, this would benefit the monitoring and evaluation of the HIV programme since more objective adherence data would become available. Additionally, the financial burden associated with resistance testing on the public health system would be reduced. However, these implications would need further investigation.

These findings suggest that ARV drug exposure testing can be divided into two categories. A screening method using immunoassays and urine as a substrate which are available as point-of-care testing, and laboratory-based tests suitable for either urine or plasma samples. [124] Both urine and plasma testing approaches could have a distinct role in the management of virological failure, each with its advantages and limitations. While a urine lateral flow test can provide immediate results in the presence of the patient, which can aid in time-efficient adherence counselling, these tests still require extensive validation before clinical use as they tend to be less sensitive and specific. [90,91,124] Plasma testing using LC-MS/MS would be preferred as it can provide more accurate results but with a longer turnaround time compared to a point-of-care test. [88,124] Plasma DTG drug exposure testing can be considered an adequate screening method to identify patients who are unlikely to benefit from HIVDR testing, decreasing the financial burden associated with these tests.

Resistance testing is crucial for patient management as outlined by Korn et al. [39] however it remains expensive in a public health setting. [41] Although drug exposure testing is being considered as a triaging tool for resistance testing, other avenues to make HIVDR testing more accessible in LMICs should be explored. Next-generation sequencing could be an alternative sequencing method, should it be proven to be cost-efficient. In addition, NGS technologies can detect low-abundance mutations at levels < 25%, which is the current detection limit for Sanger Sequencing. The value of these low-abundance mutations is still under investigation. The WHO HIVResNet working group systematically reviewed 25 studies focused on this topic. They reported that only 11 (44%) of these studies that included individuals on first-line NNRTI-based regimens reported a significant association between the presence of low-abundance mutations and virologic failure. [125] Although not assessed in this study, Maruapula et al. [126] also reported that individuals with NNRTI-associated low-abundance DRMs were 2.68 more likely to experience virologic failure. Chimukangara et al. [127] found that the prevalence of pretreatment drug resistance in HIV-1/TB-co-infected individuals increased from 4.7% (at a ≥ 20% threshold) to 18.2% if low-abundance mutations are taken into account at a ≥ 2% threshold. They observed that individuals with low-abundance pretreatment drug resistance had 1.6 times higher odds of experiencing virologic failure. [127] Data describing low-abundance InSTI mutations and their clinical impact is currently limited. Although the value of the clinical impact of these low-abundance mutations is still controversial, it is worth exploring these technologies for their feasibility.

The Nanopore technology was selected to be used in this study because it has the potential to be mobile, require less complex laboratory equipment, provide more simplified protocols and has the potential to be more cost-effective compared to other NGS platforms. [55] The value of nanopore sequencing for drug resistance testing has been endorsed by recent studies. [55–57,106] They all deemed nanopore sequencing a suitable alternative for HIVDR testing with great resistance profile similarity to Sanger sequencing. Our results are in line with those of Delaney et al. [106] , showing excellent concordance between Nanopore- and Sanger sequencing data for both the IN and PR-RT region at a minimum coverage of 400X for nanopore sequencing. Gonzalez et al. [56] report nanopore sequencing data obtained from a minION flowcell, and although the Flongle flow cell used in our study is less powerful in terms of data output, we can conclude that the Flongle fits the purpose intended for HIVDR testing.

Although we obtained excellent concordance between Sanger and nanopore sequencing, we had one sample in which high-abundance PI mutations were not detected by nanopore sequencing. A possible explanation could be a sample swap while remnant samples were anonymised or a processing error during the barcoding procedure. However, the reason for this discrepancy remains unclear, as reprocessing the sample was not possible due to insufficient sample volume. The phylogenetic analysis did not show any association with another sample in the cohort, which reduces the chances of cross-contamination. While this type of sequencing and analysis is in its infancy, we could not relate the result to a systematic error during sample processing. There were no discrepancies in InSTI mutations between Sanger and nanopore data at a mixture level above 25%. However, aside from the previously mentioned sample, two other samples had a high-abundance PI mutation (31% and 87% respectively) detected by nanopore sequencing but missed by Sanger sequencing. Unfortunately, no comparison between nanopore sequencing and other NGS platforms was done for these samples to compare the results. Discordant results like these require further investigation and validation before implementing the nanopore system in our clinical setting.

As previously stated, the true impact of low-abundance mutations is yet to be elucidated, and correctly distinguished from PCR artefacts. In this study, the clinical decisions for five individuals (10.6%) — excluding the one sample with three high-abundance PI mutations — would have been different if the low-abundance DRM results had been available. Two individuals were categorised as susceptible to DTG based on Sanger sequencing; whereas they would have been classified as intermediate resistance with the detection of G140AR/R263K

and E138K/G140IS/Y143S, respectively by nanopore. One individual with potential low-level resistance predicted by Sanger would have been identified as low-level resistance to DTG with the addition of G140AR and V151A, detected by nanopore. Regarding PI mutations, the additional detection of V82L changed one individual's susceptibility to ATV/r and LPV/r from susceptible to low-level resistance, while the detection of I54M in another individual, changed the resistance interpretation to all three available PIs in South Africa from susceptible to low-level resistance. These DRMs were reported at a mixture level of 5% to 14% for InSTI and PI resistance. One possible way to determine if this was sequencing background noise or DRM accumulation is through longitudinal studies to determine if the prevalence of the mutation increased within the individuals. This was however not possible during our retrospective analysis. The clinical impact of the detection of low-abundance InSTI DRMs needs to be investigated further as one does not want to recommend unnecessary treatment switches, especially in LMICs where access to different ARVs is limited.

Although this study has shown the added value of drug exposure testing and the feasibility of using nanopore sequencing for drug resistance testing, we acknowledge some limitations. Because of the retrospective nature of the study, accurate treatment history and viral load testing data for patients were not always available as we could only access information captured on laboratory request forms. The number of samples to accurately estimate the prevalence of HIVDR within the South African public sector was limited. We needed a statistical estimate of 674 samples, however, only 126 samples were available that met the inclusion criteria. Moreover, these were remnant samples that were exposed to prolonged storage which may have affected sample quality and negatively impacted downstream applications. This resulted in some samples that failed to amplify or produce sequencing data, which further decreased the availability of data. Additionally, the application of the ONT platform is in its infancy and we were restricted by financial resources and time constraints to fully optimise the protocol. This led to several samples that had to be excluded from the final analysis due to poor sequence coverage. We did however find that a more uniform coverage across the regions can be obtained in smaller batch sizes. Moreover, the NanoRecall software is still in its developmental phase and is only used for research purposes. [105] Unfortunately, we were unable to complete a cost analysis between Nanopore and Sanger Sequencing as running samples on a sub-optimised platform and multiple sample failures restricted this analysis. A follow-up study to optimise the nanopore protocol, conduct an extensive cost analysis, and determine the impact of turn-around time would answer most of these questions.

Nonetheless, this study provides a useful starting point for future research and possible policy changes. Despite the initial hope that the acquisition of DTG resistance would be rare, resistance is more common than expected, especially in patients with previous ART exposure. Therefore, resistance profiling is essential for PLWH on DTG-based regimens and should be continuously monitored through surveillance. From an individual patient management point of view, our results indicate that pDTG DE testing can be used to triage individuals failing DTG-based regimens for resistance testing and decrease the financial pressure on the public health system. Our findings have contributed to the National Department of Health's decision to embark on a pilot study introducing drug exposure testing before resistance testing in three provinces. The results from this pilot will be crucial to determine whether this approach should be included in the treatment guidelines (personal communication). The nanopore sequencing platform holds potential, once fully optimised, to replace Sanger sequencing. However, a detailed cost and time analysis, along with thorough validation to address discrepancies, is needed before implementation.

In summary, we have shown that ATV/r is the most appropriate drug for individuals who cannot be switched to a DTG-based regimen, preserving the activity of DRV/r in salvage treatment. Second, we showed the applicability to triage individuals with a detectable drug exposure test for resistance testing, potentially reducing the number of unnecessary drug resistance testing. Third, DTG resistance can be detected in PLWH prior to being treated with DTG for at least 24 months, indicating that resistance testing should be performed earlier in those at highest risk of resistance. Finally, nanopore sequencing can be an alternative to Sanger sequencing in the South African public sector. Our results uncover new strategies to provide a more individualised approach to HIV care. These contributions can ultimately aid in the joint UNAIDS effort to eradicate HIV/AIDS as a public health threat by 2030.

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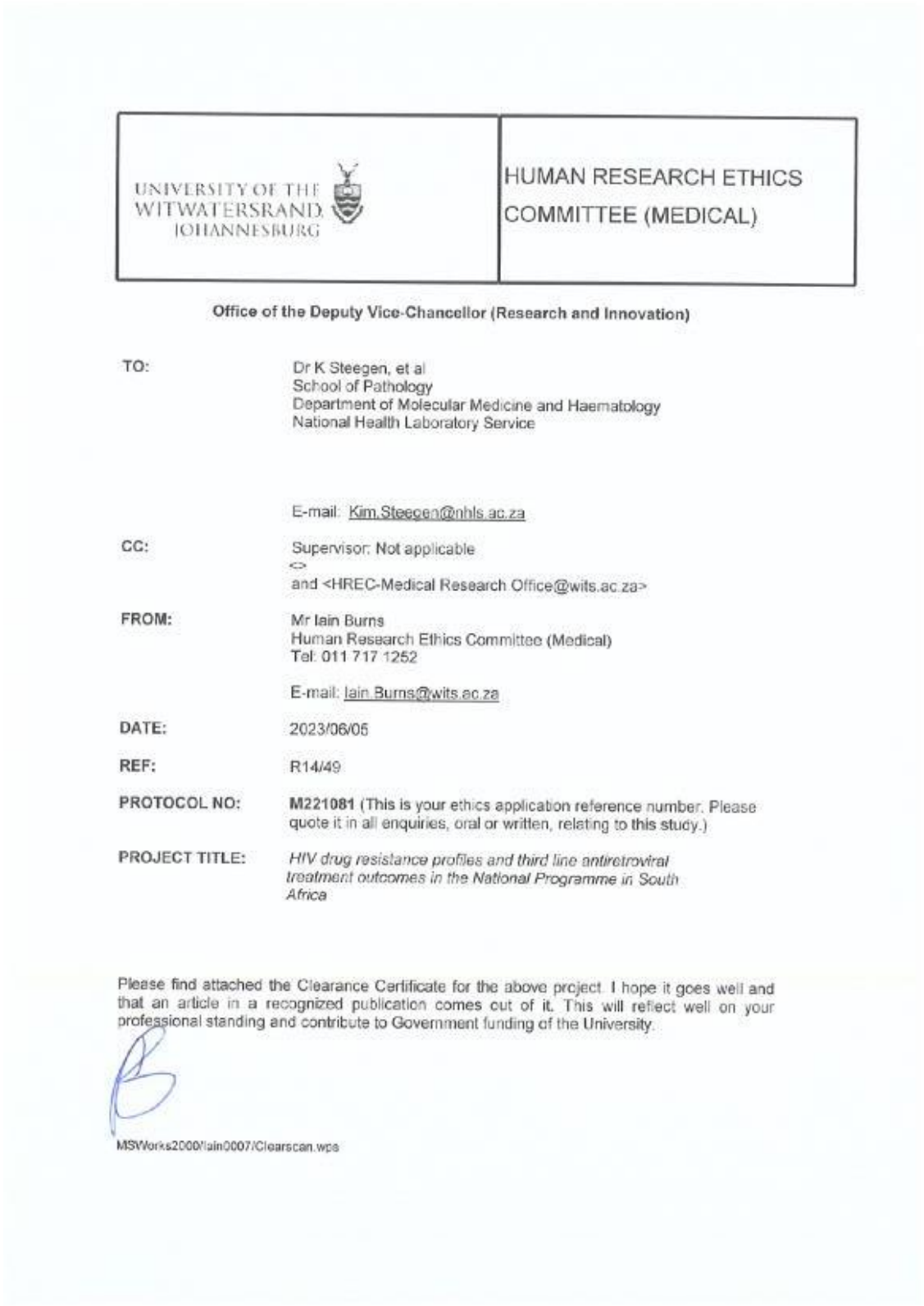
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Appendix A: Approval letters



UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research and Innovation)

TO: Dr K Steegen, et al
School of Pathology
Department of Molecular Medicine and Haematology
National Health Laboratory Service

E-mail: Kim.Steegen@nhls.ac.za

CC: Supervisor: Not applicable
<>
and <HREC-Medical Research Office@wits.ac.za>

FROM: Mr Iain Burns
Human Research Ethics Committee (Medical)
Tel: 011 717 1252

E-mail: Iain.Burns@wits.ac.za

DATE: 2023/06/05

REF: R14/49

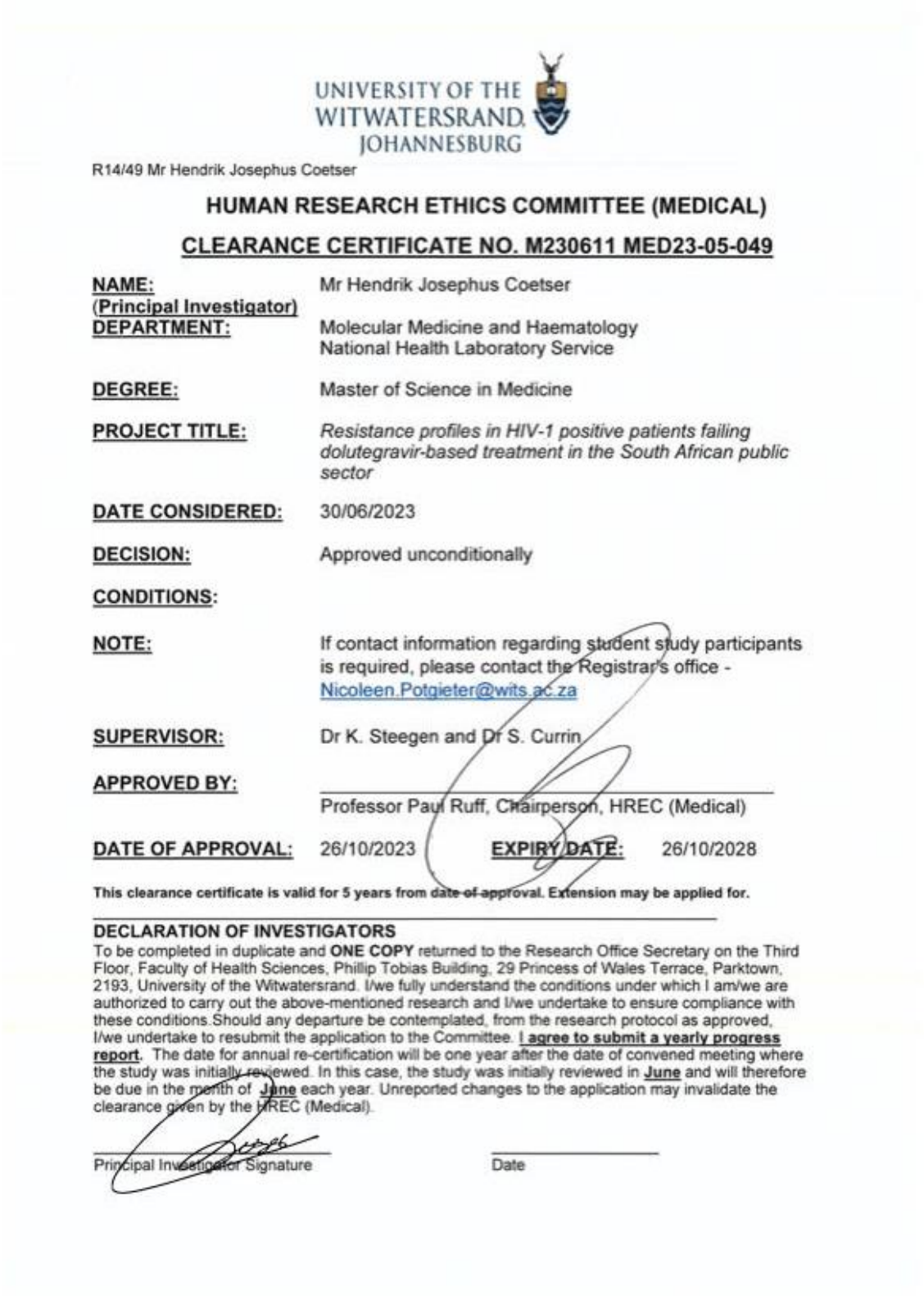
PROTOCOL NO: **M221081** (This is your ethics application reference number. Please quote it in all enquiries, oral or written, relating to this study.)

PROJECT TITLE: *HIV drug resistance profiles and third line antiretroviral treatment outcomes in the National Programme in South Africa*

Please find attached the Clearance Certificate for the above project. I hope it goes well and that an article in a recognized publication comes out of it. This will reflect well on your professional standing and contribute to Government funding of the University.

MSWorks2000/Iain0007/Clearscan.wps

Figure 5.1: Wits University medical HREC clearance certificate: PI-based regimen cohort



UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

R14/49 Mr Hendrik Josephus Coetser

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M230611 MED23-05-049

NAME: (Principal Investigator)	Mr Hendrik Josephus Coetser
DEPARTMENT:	Molecular Medicine and Haematology National Health Laboratory Service
DEGREE:	Master of Science in Medicine
PROJECT TITLE:	*Resistance profiles in HIV-1 positive patients failing dolutegravir-based treatment in the South African public sector*
DATE CONSIDERED:	30/06/2023
DECISION:	Approved unconditionally
CONDITIONS:	
NOTE:	If contact information regarding student study participants is required, please contact the Registrar's office - Nicoleen.Potgieter@wits.ac.za
SUPERVISOR:	Dr K. Steegen and Dr S. Currin
APPROVED BY:	Professor Paul Ruff, Chairperson, HREC (Medical)
DATE OF APPROVAL:	26/10/2023 **EXPIRY DATE:** 26/10/2028

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on the Third Floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions.Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report.** The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in **June** and will therefore be due in the month of **June** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature Date

Figure 5.2: Wits University medical HREC clearance certificate: DTG-based regimen cohort

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01 October 2023

Applicant: Hendrik Coetser
Institution: University of the Witwatersrand
E-mail Address: josephus.coetser@gmail.com
Cell: 082 443 1749

Project Title: RESISTANCE PROFILES IN PATIENTS FAILING DOLUTEGRAVIR-BASED TREATMENT IN THE SOUTH AFRICAN PUBLIC SECTOR.
Reference Number: PR2346132

Research Application Type(s):
1. Request for Samples
2. Request for Samples
3. Permission for use of NHLS Facilities

RE: APPROVAL LETTER: REQUEST TO ACCESS NHLS RESOURCES FOR RESEARCH PURPOSES

This letter serves to advise that the application requesting permission to conduct the above-mentioned research using the listed NHLS resources has been reviewed and **"Approved"**. Please note that the approval is granted on the condition that you comply with the NHLS Research Material and Data Access Policy and requirements stated below.

1. All material and data requested shall be used as per the research protocol submitted to the NHLS and as approved by the relevant Health Research Ethics Committee (HREC) in South Africa.
2. Access to the NHLS material and/or data shall be limited to the minimum required for successful completion of the approved study and shall be made available ***without patient names and other patient identifiers (including, but not limited to, national identity numbers, hospital/clinic file numbers, addresses and telephone numbers)***.
3. Confidentiality shall be maintained at the participant and institutional level and there shall be no disclosure of personal information or confidential information.
4. Data and/or material shall not be shared with other parties unless approved by the NHLS
5. The material and/or data obtained from the NHLS shall be anonymised and not, for any reason, be used to track or recruit patients as no pre-approval/consent is obtained from patients.
6. Processes shall be discussed with the relevant NHLS departments (i.e. Corporate Data Warehouse (CDW), NHLS Laboratory Management, Operations Office, etc.) and agreed upon.
7. Any amendments to the study requirements, including the use of the material and/or data for purposes not initially disclosed to the NHLS) shall be cleared by an approved HREC and submitted to the NHLS for approval via the AARMS system – https://aarms.nhls.ac.za.
8. The NHLS shall be acknowledged as a source of material and/or data in any output, such as abstracts and journal articles, emanating from the project.
9. A final report of the research study and any published output resulting from this study shall be submitted to the NHLS via AARMS

Please note that this letter constitutes approval by the NHLS Academic Affairs and Research Office. The NHLS entities tasked with providing the material and/data may have additional requirements for access. Data related queries may be directed to NHLS CDW, email: zarina.sabat@nhls.ac.za; contact number: 011 386 6074 and sample related queries (if applicable) shall be directed to the relevant business manager.

Dr Babatyi Malope-Kgokong
National Manager: Academic Affairs and Research

Figure 5.3: NHLS AARMS approval: DTG-based regimen cohort

Appendix B: Methodology

Table 5.1: In-house integrase genotyping assay primers

Primer name	Primer direction	Primer position	Sequence 5' – 3'
RT-PCR 1ST round			
KVL068	Sense	3854-3880	AGGAGCAGAAACTTWCTATGTAGATGG
KVL069	Antisense	5955-5981	TTCTTCCTGCCATAGGARATGCCTAAG
Nested PCR 2nd round			
KVL070	Sense	4013-4042	TTCRGGATYAGAAGTAAAYATAGTAACAG
KVL084	Antisense	5243-5266	TCCTGTATGCARACCCCAATATG
Cycle sequencing			
KVL084	Antisense	5243-5266	TCCTGTATGCARACCCCAATATG
KVL076	Sense	4161-4188	GCACAYAAAGGRATTGGAGGAAATGAAC
KVL082	Sense	4647-4669	GGVATTCCCTACAATCCCCAAAG
KVL083	Antisense	4750-4772	GAATACTGCCATTTGTACTGCTG

B.1: 1X TAE Buffer preparation

1960 mL dH2O

40 mL 50X TAE buffer (manufacturer, country)

Appendix C: Additional results and statistics

Table 5.2: Regimen within the study population

Regimen at time of failure	Number of patients on regimen
TLD	29
TLD + DRV/r	3
TLD + DRV/r + ETR	2
TLD + ATV/r	1
ZLD	32
ZLD + DRV/r	1
ALD	9
DTG	1
DTG+ RAL	1
Unknown	18

ALD = Abacavir-Lamivudine-Dolutegravir; TLD = Tenofovir-Lamivudine-Dolutegravir; ZLD = Zidovudine-Lamivudine-Dolutegravir

Table 5.3: Resistance profiles for patients with suspected cross-contamination

	IN region	NNRTI	NRTI
HDK026	R263RK	V106M, V179D, G190A	L74I, M184V,
HDK049		K103N, E138A, P225H	M184V

Table 5.4: Amino acid one letter codes

Code	Amino Acid
*	Stop Codon
A	Alanine
C	Cysteine
D	Aspartic acid
E	Glutamic acid
G	Glycine
H	Histidine
I	Isoleucine
K	Lysine
L	Leucine
M	Methionine
N	Asparagine
Q	Glutamine
R	Arginine
S	Serine
T	Threonine
V	Valine
W	Tryptophan
X	Any amino acid
Y	Tyrosine

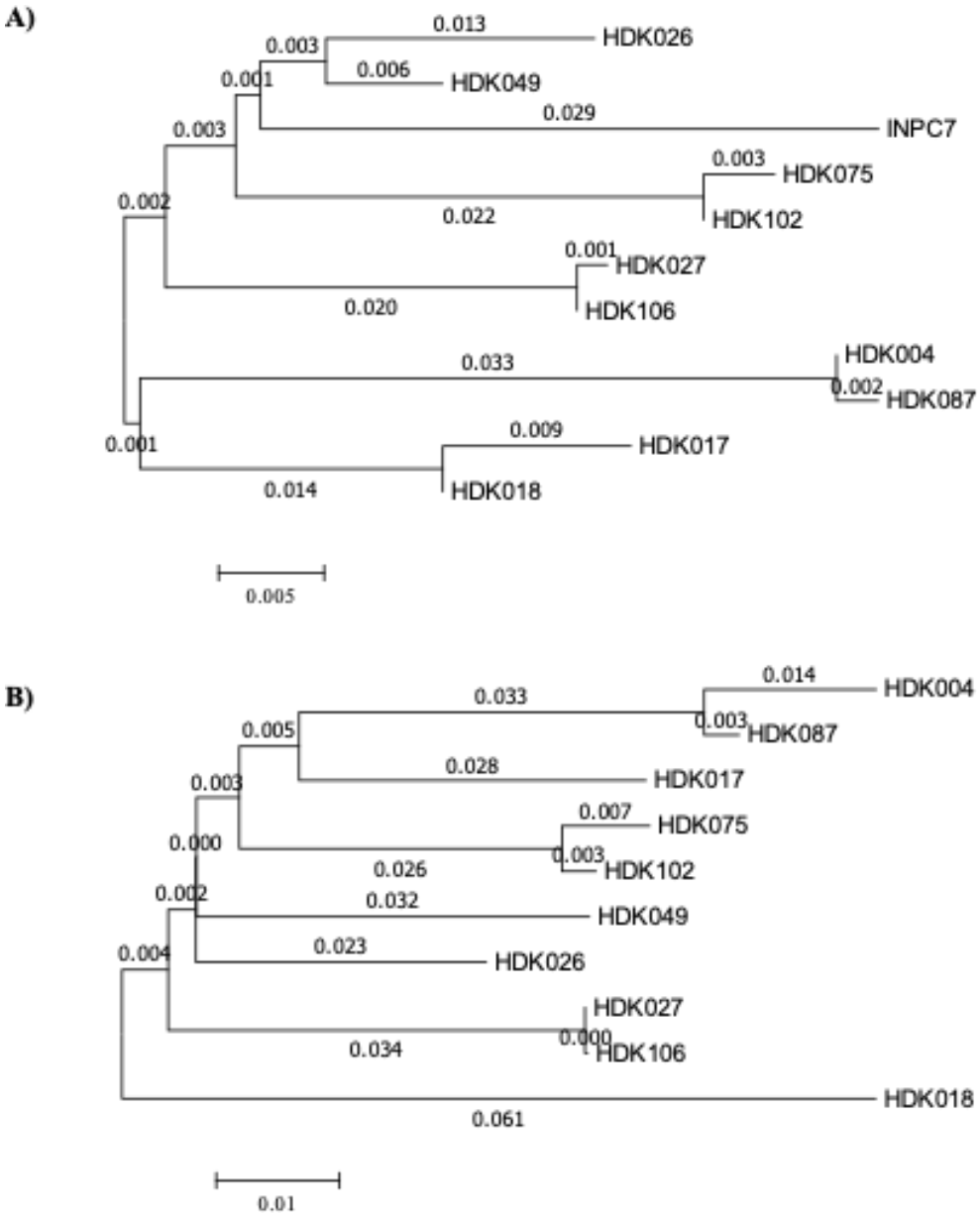


Figure 5.4: Phylogenetic representation of clustered amplicons. **A)** Integrase amplicons, **B)** Protease-reverse transcriptase amplicons. HDK026 and HDK049 are not from the same patients but were included for further analysis. HDK075 and HDK102 are from the same patient. The same is true for pairs HDK027/HDK106 and HDK004/HDK087. Cross-contamination was observed in HDK017 and HDK018, these samples were excluded from the study. *Phylogenetic tree generated by MEGA software. (100)*