EVALUATION OF ADHERENCE TO ANTIRETROVIRAL THERAPY USING EFAVIRENZ AS A MARKER.

By

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DECLARATION

I, Tambe Lisa Arrah Mbang, declare that this dissertation for the award of a Master of Science degree in Microbiology at the University of Venda is my own work and design. I further affirm that all references have been duly acknowledged and this material has not been previously submitted to this or any other university for degree or examination purposes.

Student Signature……. Date…….

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DEDICATION

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ABSTRACT

Background: Patients on antiretroviral (ART) are expected to be at least 95% adherent to their treatment, as this will increase their chances of achieving treatment success (maximum and durable suppression of HIV-1 viral load); non-adherence may lead to the development of HIV drug resistance, which may lead to virologic failure and treatment failure. Therapeutic drug monitoring (TDM) has been reported to be the most efficient method to assess treatment adherence in HIV individuals, since it quantifies the concentration of ARTs in biological matrices. This is very effective when using a robust technique such as liquid chromatography tandem mass spectrometry (LCMS/MS), which has played a significant role in the evaluation and interpretation of bioavailability, bioequivalence and pharmacokinetic data. Even with patient adherence, various intra-individual factors have an influence on the expression and function of the genes responsible for the transport (MDR1) and metabolism (CYP2B6) of Efavirenz (EFV). This may lead to single nucleotide polymorphisms (SNPs) in these genes, and this may affect the way antiretrovirals (ARVs) are metabolized. The aim of this study was to evaluate the EFV concentration in plasma to assess patient adherence to treatment and correlate this with genomic occurrences in human and viral genes.

Hypothesis: The concentration of ARVs in patient plasma can be used to estimate adherence to treatment; while ARVs' transport and metabolism can affect bioavailability in a patient's system.

Research Question: Can EFV concentration in plasma be used to estimate patient adherence to treatment? Can transport and metabolism of EFV affect their bioavailability in the patient's system?

Objectives: To determine EFV concentration in plasma to assess patient adherence to treatment and correlate this with genomic occurrences in human genes and viral genes.

Methodology: Twenty blood samples were collected from HIV positive individuals before treatment initiation (baseline) and between six to twelve months following treatment initiation (follow-up). The concentration of EFV in patient plasma was measured by LC-MS/MS technique. To infer other factors influencing patient pharmacokinetics output, drug resistance and human genetic characteristics were analyzed. A 1.65kb fragment of the HIV-1 *Pol* gene was amplified and sequenced to determine drug resistant mutations; while 363bp and 289bp of the MDR-1 and CYP2B6 human genes respectively, were also amplified and sequenced to determine polymorphisms in the transport and metabolism genes. Obtained sequences were manually edited

and analyzed using Geneious Version 11.1.5 software. The Stanford HIV Drug Resistance database was used for drug resistant mutation (DRMs) analysis and MDR1 and CYP2B6 test sequences were compared with variant reference sequences to detect the presence of any SNPs.

Results: The plasma EFV concentration at baseline and follow-up range was as follows: 0 – 1183ng/ml and below limits of quantification (BLQ) to 15,670ng/ml, respectively. At baseline, 0ng/ml is the expected plasma EFV concentration for patients about to commence treatment; however, two out of twenty patients had 769.9 and 1,183ng/ml drug levels in their system. Post treatment, plasma EFV levels in patients are expected to range from 1,000 – 4,000ng/ml, however, of the twenty patients, two had <1,000ng/ml, and three patients had >4,000ng/ml in their plasma. For *Pol* amplification, 35% (7/20) were positively amplified at baseline and 25% (5/20) were positively amplified from the follow-ups; 100% (20/20) samples were amplified for both CYP2B6 and MDR1 genes. Detection of drug resistance in the baseline *Pol* sequences revealed the absence of major mutations in both NRTI and NNRTI drug classes. The G516T polymorphism was present in 15% of the study participants while the homozygous GG and heterozygous GT genotype was present in 25% and 40% of the study participants, respectively. Allele determination was impossible in 20% of the samples, due to the poor nature of the sequence. The homozygous TT variant polymorphism at position 3435 was absent in the entire population, however, the CC and CT genotype was present in 15% and 85% of the study participants respectively. Analysis of EFV concentration in close proximity with the human genetic characteristics reveals that the presence of a Single Nucleotide Polymorphism affects the pharmacokinetic output observed.

Discussion and Conclusion: Post treatment, 90% of the study participants indicate adherence to treatment, with only 10% of them having lower than expected EFV concentrations, implying they were non-adherent to their treatment. However, because plasma drug concentrations only reflect a patient's adherence pattern for a few hours to at most two days, the adherence patterns of these individuals cannot be concluded with certainty. Using plasma EFV as a biomarker to evaluate adherence to treatment in HIV seropositive individuals is a feasible technique, however, its application in non-research settings is still a drawback due to the cost of the method. Characterizing patient inter-individual differences should be taken into consideration, especially since any polymorphism in their transporter and metabolizing genes may influence their overall treatment success.

Key Words: HIV treatment adherence, EFV concentration, CYP2B6, MDR1

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LIST OF ABBREVIATIONS

ADR:	Adverse Drug Reaction
AIDS:	Acquired Immune Deficiency Syndrome
ART:	Antiretroviral Treatment
BLQ:	Below limits of quantification
cARV:	Combination Antiretroviral
CYP450:	Cyptochrome P450
CYP2B6:	Cytochrome P450 sub-family 2B isoenzyme6
DRMs:	Drug resistance mutations
EDTA:	Ethylene diamine tetra-acetic acid
EFV:	Efavirenz
EMS:	Electronic Monitoring System
FTC:	Emtricitabine
HAART:	Highly active antiretroviral therapy
IN:	Integrase
ISTD:	Internal standard
Kb:	Kilobases
LC-MS/MS:	Liquid Chromatography Tandem Mass Spectrometry
MDR-1:	Multi Drug Resistant gene 1
MEMS:	Medication Events Monitoring System
mRNA:	Messenger RNA

MS:	Mass Spectrometry
m/z:	Mass-to-charge ratio
NNRTI:	Non-Nucleoside Reverse Transcriptase Inhibitors
NRTI:	Nucleoside Reverse Transcriptase Inhibitors
P-gp:	P glycoprotein
PI:	Protease Inhibitors
Pol:	Polymerase protein
PR:	Protease
PV:	Pharmacovigilance
RNA:	Ribonucleic Acid
RT:	Reverse Transcriptase
Spp.:	Species
STDs:	Standards
TDF:	Tenofovir
TDM:	Therapeutic Drug Monitoring
QCs:	Quality controls
3TC:	Lamivudine

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1. INTRODUCTION

In 1983, Barre-Sinoussi and colleagues established that the causative agent of Acquired Immune Deficiency Syndrome (AIDS) is the Human Immunodeficiency Virus (HIV) (Barre-Sinoussi et. al, 1983). At the beginning of the AIDS epidemic, more than 70 million people were reported to have been infected with the virus, with approximately 35 million deaths (Global Health Observatory (GHO) data, 2016). By the end of 2016, approximately 36.7 million people were living with HIV, and 0.8% (293, 600) of these were adults and only 53% (>19.5 million) of them have access to treatment (WHO, 2016).

Sub-Saharan Africa is reported to be the region which accounts for the highest burden of disease, as it accounts for 70% of those infected globally. According to the WHO statistics of July 2016, close to 7 million people in South Africa are living with HIV, with 19.2% prevalence among adults and about 380,000 reported new infections. Of the 7 million, approximately 48% of those living with HIV have access to antiretroviral treatment (WHO, 2017).

Antiretroviral therapy (ART) is a potent, convenient and well tolerated form of treatment which is administered to HIV positive patients in order to reduce the viral replication in the blood. This form of treatment decreases the morbidity and mortality rate, thereby leading to a prolonged life span among those living with the disease (Palmisano and Vella, 2011). There are over 25 ARTs which fall into six different classes, which have been clinically approved for use in treatment of AIDS (Andrade et al., 2011). Therapy administration in people living with HIV has evolved from monotherapy to the administration of a combination of antiretroviral agents, also known as Highly Active Antiretroviral Therapy (HAART). In South Africa, the treatment guidelines suggest that ARTs should consist of a cocktail of two classes of drugs: two Nucleoside Reverse Transcriptase Inhibitors and one Non-Nucleoside Reverse Transcriptase Inhibitor (Meintjes et al., 2012).

Since a greater part of treatment success relies on how adherent a patient is, adherence to treatment regimen has been continually emphasized. Adherence can be defined as the extent to which individuals take medications as prescribed (Nachenga et al., 2011). Measuring ART drug exposure is important for monitoring the response of patients to treatment. This is because it is through a patient's adherence to treatment that successful outcomes can be determined, although partially so (Huang et al., 2008).

Various methods have been put forward in an attempt to evaluate antiretroviral treatment adherence in patients. They include studies on self-reported adherence, pharmacy pill refill, electronic monitoring (Medication Events Monitoring System – MEMS) and measurement of biological or laboratory markers (Turner B.J., 2002; Simoni et al., 2006; San Lio et al., 2008; Gachara et al., 2017). However, methods such as self-reported adherence, pharmacy pill refill and MEMS, are subjective, in the sense that an aspect of their approach is influenced or affected by a particular entity, which makes them to over-estimate a patients adherence. Therapeutic drug monitoring (TDM) measures the concentration of Anti-HIV drugs in the plasma or urine of a patient. That is, it shows exactly what is present in a patient's system (Turner B.J., 2002, Huang et al., 2008). This could be done using specific techniques such as liquid chromatography tandem mass spectrometry (LC-MS/MS).

However, even with the utilization of a combination of drugs to minimize the replication capacity of the virus, as well as an individual adhering to their treatment, drug resistance may still occur due to the lack of proof reading made by the error-prone reverse transcriptase during viral replication (Campo et al., 2012). The errors made during replication may generate drug resistant mutations and these drug resistant variants may play a role in treatment failure. Hence, it is important to evaluate the role that is played by drug resistant mutations in treatment adherence or potential treatment failure.

Measuring the concentration of biological markers in patient samples is very effective in confirming patient adherence to treatment. However, the levels of drugs shown are influenced by inter-individual variability in drug bioavailability and metabolism. Drug transporters and metabolizers, such as P-glcoprotein (P-gp) and cytochrome P450 (CYP450) respectively, determine the bioavailability of the drug in the system. But, there are some factors as well as proteins which are responsible for the alteration in the metabolism process. Their combined activity may result in a low

bioavailability of drugs in the system (Masebe et al., 2012) and this may lead to patients' poor or no adherence to treatment (Nachega et al., 2011).

EFV is primarily metabolized by CYP2B6, which is a member of the CYP450 family of enzymes (Turpeinen and Zanger, 2012) and it is transported by the Multidrug resistance 1 (MDR1) gene (Dong et al., 2009). Reported polymorphisms in both the CYP2B6 and MDR1 genes are said to affect the pharmacokinetics observed in a patient, hence, inferring the effect of these human genes may answer questions of pharmacogenetic influence on treatment adherence.

1.2 LITERATURE REVIEW

1.1 Origin and Classification of HIV

In 1973, Barre-Sinoussi and colleagues reported a new virus – the Human Immunodeficiency Virus, which was prevalent in homosexual males with multiple partners, and this caused acquired immune deficiency syndrome (AIDS) (Barre-Sinoussi et al., 1983). HIV belongs to the family *Retroviridae*and the *Lentivirus* genus. It has been further classified into two types: HIV-1 and HIV-2. HIV type 1 is suspected to originate from the Simian immunodeficiency virus (SIV) which affected the chimpanzee (SIVcpz) (Tebit and Arts, 2011).

HIV-1 comprises of four distinct lineages, consisting of various groups. These include; group(s) M (Main) – the first to be discovered, and the pandemic form of HIV-1 is responsible for millions of infections worldwide (Sharp and Hahn, 2011), O (Outlier) – was discovered next, and its distribution was reported mostly in Western African countries such as Cameroon, Gabon and their neighboring countries (Peeters et al., 1997), N (non-M, non-O) – was identified in 1998, with a much less prevalence than that of group O (Vallari et al., 2010), and P, which was the last to be discovered in a Cameroonian woman living in France, and was designated as group P (Plantier et al., 2009). It is further classified into nine subtypes (A-D, F-H, J and K), several sub-subtypes and 48 circulating recombinant forms (CRFs).

HIV-2 is said to have originated from SIV in the sooty mangabeys (SIVsmm), and is classified into eight main groups (A-H), with group A and B being the most prominent (Sharp and Hahn, 2011; Tebit and Arts, 2011). It is predominantly distributed in West Africa with the center being Guinea-Bissau and Senegal (de Silva et al., 2008).

1.2 Epidemiology of HIV

Since the beginning of the AIDS epidemic, more than 70 million people have been infected with HIV, with about 35 million of them dying from the disease. Globally, approximately 36.7 million people were reported to be living with HIV at the end of 2016. Of this total number, 0.8% of them (293, 600) are adults aged 15–49 years. The global burden of this epidemic varies from one country to the next and one region to the next. Sub-Saharan Africa remains the most severely affected region, with nearly 1 in every 25 adults (4.2%) living with HIV and accounting for nearly two-thirds of the people living with HIV worldwide (Global Health Observatory (GHO) data).

According to the WHO statistics of July 2017, close to 7 million people are living with HIV in South Africa, with 19.2% prevalence in adults and close to 380,000 new infections. Of this total number, approximately 48% of those living with HIV have access to antiretroviral treatment (WHO, 2017).

1.3 Replication Stages of HIV

Replication of HIV-1 occurs in two stages, the early phase and the late phase. In the early phase, the mature virion is attached to the target cell and a cascade of processes leading to and including integration of the genomic DNA into the chromosome of the host cell follows. Late phase replication involves all processes up to and including virus budding and maturation (Turner and Summers, 1999).

Entry into the host is achieved when the virus adsorbs its glycoproteins (gp120 and gp41) into the $CD4^+$ T-cells and macrophages of the host. The $CD4^+$ binding domain on viral gp120 fuses to the receptors on the host $CD4^+$ molecule, and this interaction causes a conformational change in the gp120. This change allows the chemokine Co-Receptor 5 (CCR5) to attach to the host cell. The viral envelope then fuses with the cell membrane and the viral capsid is injected into the cell, while the envelope remains at the cell surface.

Uncoating of the viral particle occurs in the cell, wherein the viral capsid and matrix proteins are cleaved and three viral enzymes: Reverse Transcriptase (RT), Integrase (IN) and Protease (PR) as well as the viral RNA genomes, are released. With the use of host nucleotides, RT converts viral

RNA into single stranded DNA (ssDNA), in a process called reverse transcription. A major characteristic of RT is its poor proof reading ability, so it makes random errors during the replication process. Double stranded DNA (dsDNA) is then formed by the addition of a complimentary strand to the already formed ssDNA (Sherman and Greene, 2002). The dsDNA is then transported to the nucleus, and integrated into the host chromosome with the help of the viral IN.

Viral IN attaches to the dsDNA and carries it through the nucleopore and into the nucleus of the cell. Within the cell nucleus, the viral IN makes a nick (cut) in the host DNA and allows the virus to insert itself into the host chromosome, which establishes lifelong infection. The integrated provirus is then transcribed to RNA and spliced to produce mature messenger ribonucleic acid (mRNA). These mRNAs are then shuttled to the cytoplasm where they are translated into viral regulatory proteins (*Tat* and *Rev*). The Rev protein binds to copies of the full-length viral RNA which is unspliced and facilitates its exit from the nucleus. The RNAs produced have different functions: some function as new copies of the viral genome, while others are translated and code for the Gag and Env structural proteins. New viral particles are produced when the *Gag* protein binds to genomic RNA and packages them into new particles (Freed, 2015).

Assembly and release of these viral components occurs at the cell surface. The viral proteins and RNA transcript, which had been previously shuttled out of the cell, reconstruct and form an immature virion. The viral PR functions in cutting the polypeptide chain and releasing the viral enzymes and other proteins. This then constitutes a mature infective viral particle. The mature virion buds out of the cell and infects other cells. **Figure 1** illustrates the various steps occurring during replication and the processes and enzymes involved.

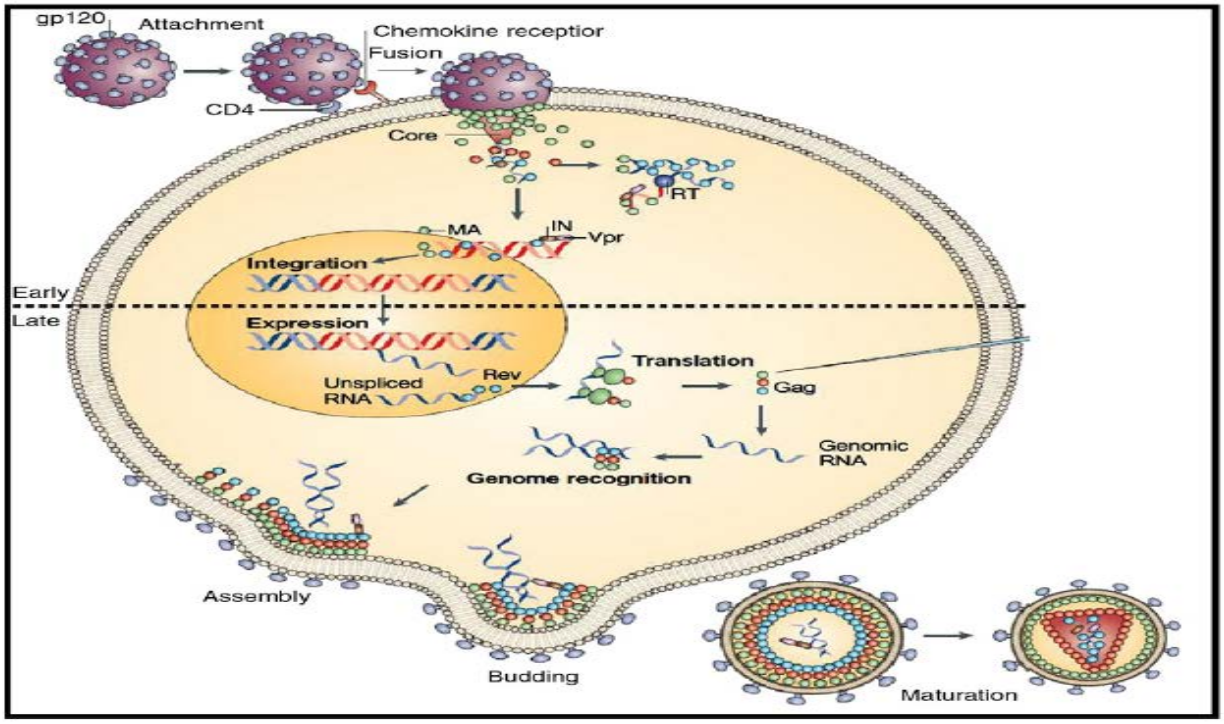

Figure 1: Showing replication stages of HIV and the development of each particle. Adapted from an article by Rajarapu (2014), entitled “Genes and Genomes of HIV-1”, and accessed in March 2016.

1.4 Treatment of HIV

There has been success in the discovery of Anti-HIV drugs (Antiretroviral therapy), which have been used to prolong the lives of those living with the disease. In the early 1990’s, monotherapy was administered; however this has evolved over time to a combination of antiretroviral therapy (cART), also known as HAART (Andrade et al., 2011; Palmisano and Vella, 2011; Arts and Hazuda, 2012). A combination of ART results in a dramatic suppression of viral replication, and this reduces the viral load in the plasma to undetectable levels e.g. even the most sensitive clinical assays, with an increase in circulating CD4 T-lymphocytes (Arts and Hazuda, 2012). There are about 25 Anti-HIV drugs, which belong to six different classes which include: Entry Inhibitors; Fusion Inhibitors; Nucleoside Reverse Transcriptase Inhibitors (NRTIs); Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs); Protease Inhibitors (PIs) and Integrase Strand Inhibitors (INSTIs) (Arts and Hazuda, 2012). These compounds fall into different categories, depending on their target within the replicative cycle of the virus. (Andrade et al., 2011; Palmisano and Vella, 2011).

The combination ART (cART) usually consists of a combination of at least two different drug classes. In other words, for each country, the combination ART varies, depending on individual patient accessibility of this treatment. **Figure 2** below depicts HIV replication stages and drug classes used for inhibition of the various stages.

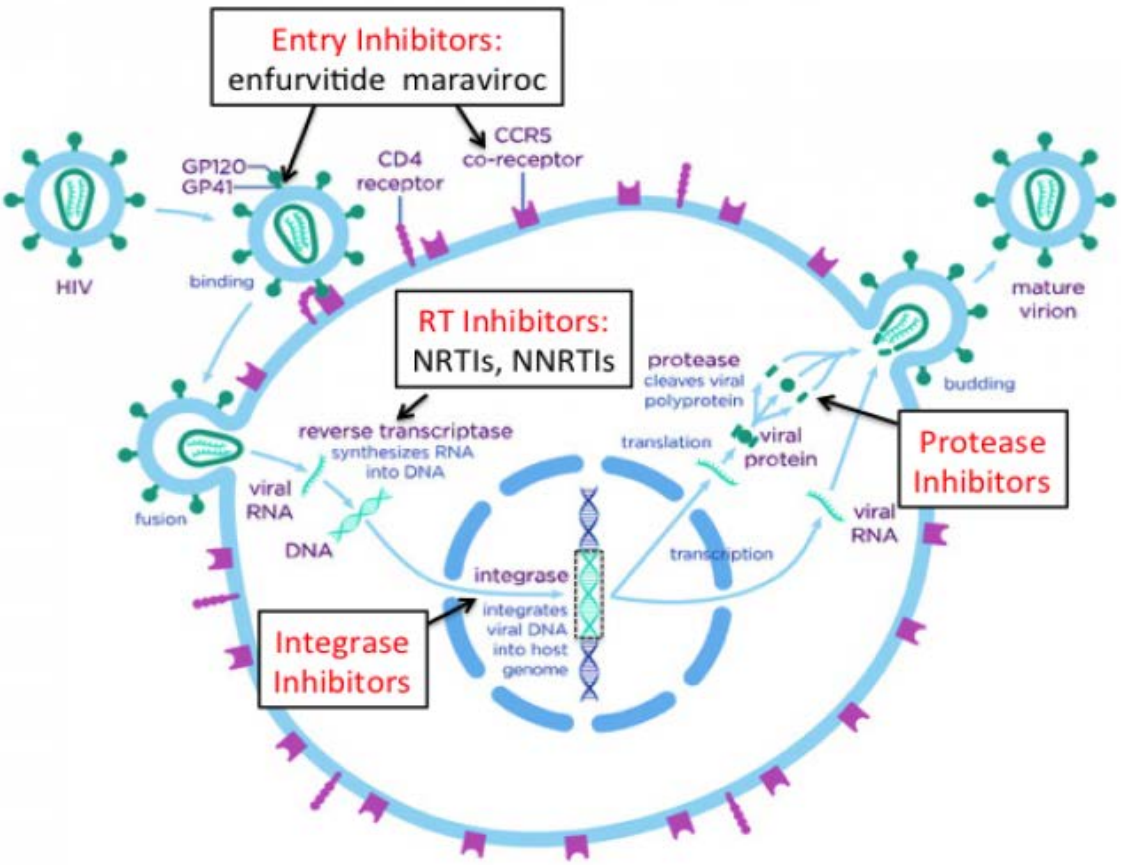

Figure 2: HIV replication stages in the host cell and anti-HIV drug targets used for inhibition of the various replication stages. Adapted from http://tmedweb.tulane.edu/pharmwiki/doku.php/hiv_haart and accessed in July 2019.

1.5 South African Treatment Guideline

The Southern African treatment guideline was written to address issues relevant to Southern Africa (Meintjes et al., 2012). Hence key principles were taken into consideration during the writing process. Some of them include the affordability of the countries in this region, as they range from middle income to low income countries, treatment and diagnosis options available in Southern Africa, as well as the need to bridge the gap between the private and public sector treatment programmes (Meintjes et al., 2017). From the time South Africa first received ART to date, the regimen has been constructed from single dosage to a cocktail of drugs (HAART) and this is in an effort to tailor the needs of those living with HIV.

Today, the South African treatment guideline recommends a combination of two classes of drugs for those initiating treatment (1 NNRTI + 2 NRTI). The most preferred combination includes a cocktail of Tenofovir (TDF), Emtricitabine (FTC), or Lamivudine (3TC) and Efavirez (EFV). For patients failing the first line treatment, a second line treatment consisting of two NRTIs and one PI is administered. This treatment is a combination of Zidovudine (AZT), 3TC and a boosted Lopinavir (LPV/r) (Meintjes et al., 2017).

1.6 Treatment Adherence

Treatment adherence can be defined as the extent to which individuals take medications as prescribed (Nachenga et al., 2011). The importance of patients adhering to their medication regimen has been stressed over the years, since non-adherence can lead to virologic failure, and eventually treatment failure. As a result of this, several methods have been put forth, in an attempt to assess patient adherence to treatment. They include: patient self-report, health care provider estimates, pill counts, pharmacy pill refill, electronic monitoring systems (EMS) and therapeutic drug monitoring (TDM) (Nachenga et al., 2011; Turner, 2002; Chesney, 2000), and these have their advantages and limitations.

Patient self-report is the most common method used by investigators to infer treatment adherence, since its design is relatively flexible, of low cost and data is easily collected. The limitation is that it

reflects only short-term or average adherence and may, in most cases overestimate the adherence rate, since patients may not accurately recall their behaviour and may not provide honest answers. Adherence estimates given by health care providers such as nurses and physicians have been shown to be highly inaccurate; in one study their predictions were found to be slightly better than that of a coin toss (Turner, 2002). Pill counting refers to counting the number of pills remaining in a patients' medication bottle by a health care provider. The advantage of this method is that, if excess pills are returned, there is tangible evidence of non-adherence. The drawback of this method is that patients may want to appear socially desirable (in that they are taking all their medications correctly) and engage in "pill dumping", which tends to inflate the adherence results. Pharmacy measures examine the rate at which a patient refills their medication over a period of two months. This method assumes that patients use the same pharmacy to collect their medications and that all bills are sent to the same source for payment. This method, is strengthened by the fact that bias cannot be reported and population based information is offered. However, the patients may receive their pills from their doctor and not all paid claims are a reflection of patients collecting their medication or not. Electronic monitoring systems, used to measure adherence use the approach of inserting a chip into the medication bottle caps, so that the date and time are recorded each time the bottle is opened. A good example is the medication events monitoring system (MEMS), which is thought to be more objective than the other methods. Some drawbacks of MEMS are: the cost of the devices which makes it impractical to use in a non-research setting, the caps could get lost or malfunction, and since it is assumed that a single dose is taken each time the bottle is opened, patients may take more than what is prescribed to appear more adherent, and this may give inaccurate results (Turner, 2002, Chesney, 2000).

Therapeutic drug monitoring (TDM) or measurement of biological or laboratory markers measures the concentration of drugs present in a patient's system using their plasma or urine samples, accompanied by specific techniques such as liquid chromatography tandem mass spectrometry (LC-MS/MS). These samples demonstrate the concentration of drugs present; and are able to represent the dose taken for the last two days. The drawback of using matrices like plasma and urine to measure drug concentration is that they show only a short-term drug report. Drugs present in an individual's system have also been measured using one's hair sample, since it is able to estimate long-term exposure to ARTs. Preliminary studies have reported the meaningfulness of this method, since it can predict treatment outcomes, as well as how efficient a patient's ART is (Hickey et al., 2014; Baxi et al., 2015; Yan et al., 2016). Other techniques optimized for measuring drug

concentrations include infrared matrix-assisted laser desorption electrospray ionization (IR-MALDESI), which was recently used by Rosen and colleagues (Rosen et al., 2016). Using these techniques is often seen as impractical because of their expense and lack of general availability, especially in resource-limited settings.

Although there is no “gold standard” for measuring treatment adherence because of each method’s advantages and limitations, such as bias, cost, complexity, accuracy, precision and intrusiveness (Nachenga et al., 2011), these approaches enable physicians to estimate how adherent patients are, and may predict a patient’s treatment outcome.

1.7 Association between Treatment Adherence and HIV Drug Resistance

Patients on ART are expected to attain almost perfect adherence, in order to achieve optimal adherence. Optimal adherence is defined as a patient attaining adherence above 95% and is said to be achieved when the virologic, immunologic and clinical outcome of a patient shows treatment success (Nachenga et al., 2011). Moreover, effective adherence reduces the risk of developing drug resistance as well as limiting the probability of transmitting drug resistant viruses to newly infected individuals which results in reducing the cost of having to use the second and third line treatment regimens as these are especially expensive and sometimes unavailable in some resource-limited countries.

Non-adherence to treatment has been proven to be associated with the development of HIV drug resistance, which may lead to virologic failure, treatment failure, disease progression and eventually death (von Wyl et al., 2013; Nachenga et al., 2011; Gardner et al., 2009; Sethi et al., 2003). It has been proven that drug resistance arises in all antiretroviral drugs, hence there is the development of drug resistance to ARVs within the same class, and this is defined as class-specific drug resistance (von Wyl et al., 2013).

Class-specific resistance is usually seen to develop more in the NNRTI class when compared to the NRTIs and the PIs. Factors influencing the development of NNRTI resistance include; the long half-life of NNRTIs which leads to resistance in the case of treatment interruption lasting more than

48hrs. The genetic barrier for NNRTI allows resistant viruses to accumulate rapidly. There is also little impact on the viral fitness since NNRTI resistance mutations have a minor effect on reverse transcriptase's overall function. Also, the short half-lives of NRTI during treatment interruption allow a prolonged monotherapy of the NNRTIs. As a result, resistant mutations in NNRTIs tend to be highest when the adherence levels are low. The most common mutation which emerges in patients failing EFV-based ART is the K103N mutation, and it confers cross-resistance to other NNRTIs such as Nevirapine and Delavirdine. If the K103N mutation develops, secondary mutations such as L100I, V108I and P225H can develop, which will increase EFV resistance to K103N (Koval et al., 2006).

The PIs and NRTIs, on the other hand, have less class-specific drug resistance since they have a rapid clearance rate (short half-life), and require multiple mutations which alter the enzyme's function to make the virus less fit (Gardner et al., 2009; Nachenga et al., 2011; von Wyl et al., 2013).

1.8 Cytochrome P (CYP450) and MDR1 in Metabolism

CYP450 are a superfamily of intracellular hemeproteins that are responsible for the activation of molecular oxygen for the oxidative metabolism of a great variety of lipophilic organic chemicals (Hasleret al., 1999). This enzyme family plays an important role in the Phase-1 metabolism of many drugs. The broad range of drugs that undergo CYP-mediated oxidative biotransformation is responsible for the large number of clinically significant drug interactions during multiple drug therapy (Badyal et al., 2001). The polymorphic forms of CYP450 are usually responsible for the development of adverse drug reactions (ADR). Mutations in these genes can produce enzyme products with abolished, reduced, altered or increased enzyme activity. This leads to the formation of four major phenotypes: poor metabolizers – who lack functional enzymes, intermediate metabolizers – who are heterozygous for a defect allele, efficient metabolizers – who carry two functional gene copies and ultrarapid metabolizers – who carry more than two functional copies (Ingelman-Sundberg and Rodriguez-Antona, 2005).

CYP2B6 belongs to the CYP450 family of enzymes, and it is highly variable due to the existence of strong inhibitors and inducers, as well as its extensive genetic polymorphism; this high variability is the result of both genetic and non-genetic factors. It is primarily expressed in the liver as well as in the skin, kidney, brain and different parts of the gastrointestinal tract (G.I tract). CYP2B6 participates in the metabolism of a wide range of drug classes, including antiretrovirals, antidepressants, anesthetics, and anticancer as well as smoking agents. It is a major component responsible for the elimination of Efavirenz (EFV), which is subject to extensive hepatic oxidative metabolism which leads to the several inactive hydroxylated metabolites (Turpeinen and Zanger, 2012).

P glycoprotein (P-gp) is a 170-kDA plasma membrane protein encoded by the multi-drug resistant gene 1 (MDR-1) is a member of the large ATP-binding cassette (ABC) functions as an ATP-dependent drug efflux pump. It transports a wide range of compounds that include: hydrophobic amphipathic drugs; calcium channel blockers; antihistamines peptides and steroids (Sankatsing et al., 2004). MDR1 comprises of 28 exons, and is located on the long arm of chromosome 7, at position 21 (chromosome 7q21); it is expressed in the liver, pancreas, small intestine, colon, kidney, adrenal gland, lymphocytes, testes, macrophages, brain and hematopoietic progenitor cells. Anti-HIV drugs such as Indinavir, Nelfinavir, Efavirenz, are substrates transported by the MDR1 gene (Dong et al., 2009). Identification and characterization of single nucleotide polymorphisms (SNPs) in the MDR1 gene has been of specific interest, since it is associated with drug response, clinical outcome and susceptibility to certain diseases (Tang et al., 2004).

1.8.1 Pharmacogenetics of Antiretroviral Drugs

The first pharmacogenetic study conducted in 2002 by Fellay and colleagues reported evidence supporting the idea that a patient's initial response to ART could be influenced by allelic variants of a gene (Cressey and Lallemant, 2007). The NNRTIs and PIs are primarily metabolized by CYP450 enzymatic pathways and are substrates for the P-gp drug transporter, while the NRTIs are substrates for MDR-1 transporters such as the multi-drug resistant protein 4 (MRP4) and the breast cancer resistant protein (BCRP/ABCG2), but are not metabolized by the CYP450 enzymes.

It has been reported that there is an association between the polymorphisms present in the MDR-1 gene and the level of NNRTI and PI antiretroviral drug shown in the plasma. These studies report a better virologic response as well as an improvement in the CD4+ cell count in patients with a polymorphism in the C3435T region of the MDR-1 gene. However, other studies have contradicted these results, by reporting no association between a patient's response to therapy and a polymorphism in the MDR-1 C3435T gene (Cresseyand Lallemant, 2007). Dong and colleagues also reported that the C3435T polymorphism in exon 26 is correlated with the efficacy of drug delivery and disposition (Dong et al., 2009). This SNP is located at a wobble position in exon 26, and does not result in an amino acid change.

Polymorphisms which occur in the CYP450 enzymes have shown to have an association with the level of NNRTI and PI drugs present in the plasma. The presence of a G516T polymorphism in exon 4 of the CYP2B6 gene results in an amino acid change from Glutamine to Histidine (Q to H). For patients on EFV based ART, this polymorphism is responsible for the lower hepatic EFV clearance and higher amounts of EFV found in patient plasma (Sankatsing et al., 2004, Cressey and Lallemant, 2007). This means that the G>T SNP at position 516 leads to decreased catalytic CYP2B6 activity which results in increased plasma EFV concentrations and a greater probability of neuropsychological toxicity in patients with this SNP (Gounden et al., 2010). Across different populations, the variant allele has a 15 – 50% frequency, with the highest frequency occurring in African populations and the lowest in the Asian populations (Turpeinen and Zanger, 2012).

1.9 Metabolism of Efavirenz (EFV)

Efavirenz (EFV) belongs to the NNRTI class, and its main function is to bind and induce the formation of a hydrophobic pocket which changes the spatial conformation of the substrate-binding site and decreases polymerase activity. As a result, they are termed non-competitive inhibitors (Arts and Hazuda, 2012). EFV is found in almost all treatment regimens recommended for the first line treatment, according to the South African treatment guidelines.

Efavirenz (EFV) is extensively metabolized with its major isolated metabolite being O-glucuronide conjugate of the metabolite 8-hydroxy-efavirenz. It also undergoes glucuronidation to form an N-

glucuronide conjugate. Except for humans, sulfation of 8-hydroxy-efavirenz occurs in all other species. Other reactions such as the hydroxylation of the cyclopropane ring (C-14) of 8-hydroxy-efavirenz, to produce 8, 14-dihydroxy-efavirenz occurs. Using human liver microsomes, both *In vivo* and *In vitro*, it was observed that 8-hydroxy-efavirenz was obtained as a major metabolite. It was also shown that CYP2B6 is a major isoform responsible for the production of 8-hydroxy-efavirenz and 8, 14-dihydroxy-efavirenz, while other isoforms such as CYPs 1A2, 3A4 and 3A5 participate at a minority level. Preliminary studies conducted have reported that a CYP2B6 genetic polymorphism contributes to the variations in EFV plasma concentrations. This proves its involvement in EFV metabolism (Andrade et al., 2011).

1.10 STUDY RATIONALE

Antiretroviral therapy (ART) is used to reduce the replication and proliferation of the virus in HIV seropositive individuals, and this has led to a decrease in morbidity and mortality among seropositive individuals. As a result, the lives of people living with HIV are being prolonged. However, it is imperative for patients to adhere to their treatment regimen, as this reduces the viral load in patient plasma and prevents transmission as well as reducing the probability of treatment failure.

Several methods have been proposed to infer patient adherence to treatment (Chesney, 2000, Turner 2002). However, most of the methods put forth are subjective and may overestimate a patients' adherence, hence therapeutic drug monitoring methods are more preferred since they reflect the concentration of drugs in a patient's plasma, urine or hair sample. This can be done using sensitive and specific methods such as LC-MS/MS. Measuring the concentration of drugs in a patient's plasma sample can be used to infer how adherent a patient is to their treatment (Chesney, 2000, Turner 2002).

Efavirenz is a Non-Nucleoside Reverse Transcriptase Inhibitor (NNRTI) used in combination with Emtricitabine (FTC) and Tenofovir (TDF), as recommended by the South African treatment guideline for patients initiating treatment (Meintjes et al., 2012). Adherence to treatment is the extent to which individuals take medications as prescribed; patients on ART are expected to be at least 95% adherent to their treatment, as this will increase their chances of treatment success. A patient who is non-adherence to treatment will likely develop HIV drug resistance, which may lead to virologic failure, treatment failure, disease progression and eventually death (Nachenga et al., 2011).

Several intra-individual factors exist which influence the pharmacokinetic variability in patients on EFV-based ART. However, analysis of genetic variations has become the method of choice since the presence of a SNP in either the transporter or metabolizing gene may alter the protein expression, and explain in part the pharmacokinetic outcome observed (Sanchez et al., 2011). These polymorphisms may predict the probability of achieving treatment success or treatment failure in HIV patients. EFV is transported by MDR1 and extensively metabolized by the CYP2B6 genes. It has been reported that a single nucleotide polymorphism (SNP) at position 516G>T in the

CYP2B6 gene is associated with decreased activity of the enzyme which leads to increased concentrations of EFV in the plasma of patients possibly resulting intoxication and increased neuronal toxicity (Gounden et al., 2010). A polymorphism at position 3435C>T in exon 26 of the MDR1 gene is associated with better immune recovery upon treatment initiation (Swart et al., 2012).

Preliminary studies in South Africa have investigated patient adherence to treatment using non-therapeutic drug monitoring methods such as patient self-report, pharmacy pill count or pill refills and MEMS (Gachara et al., 2017; Hornschuh et al., 2017; Evans et al., 2016). A few studies have reported on treatment adherence utilizing therapeutic drug monitoring methods (Van Zyl et al., 2011). The purpose of this study was to use Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS) to measure the concentration of EFV in patient plasma, in order to evaluate their adherence to treatment, as well as infer the influence of patient genetic variability on the pharmacokinetic outcome observed.

1.10.1 RESEARCH QUESTION

- Can the concentration of EFV in patient plasma be used to estimate adherence to treatment?
- Can the transport and metabolism of EFV affect their bioavailability in the patient's system?

1.10.2 HYPOTHESIS

- The concentration of ARVs in patient plasma can be used to estimate adherence to treatment.
- The transport and metabolism of ARVs can affect its bioavailability.

1.10.3 OBJECTIVES OF THE STUDY

Main Objective:

- To determine EFV concentration in plasma to assess patient adherence to treatment and correlate this with genomic occurrences in human genes and viral genes

Specific Objectives:

1) To determine the concentration of efavirenz in plasma samples using LC-MS/MS technique.
2) To infer the presence of drug resistance mutations.
3) To infer the effect of CYP2B6 and MDR-1 genes in drug metabolism.
4) To determine the extent of correlation of treatment adherence, the development of drug resistant mutations and the impact of CYP2B6 and MDR-1 genes in ARV drug metabolism.

CHAPTER TWO: MATERIALS AND METHODS

2.1 ETHICAL CLEARANCE

This study is a subset of a main study which aims to determine the adherence of patients to treatment by measuring the concentration of ARV present in each patient's hair and blood samples. Ethical clearance was obtained from the University of Venda's Research Ethics Committee with the project number: SMN/17/MBY/05.

2.2 BIOLOGICAL SAMPLES USED IN THE STUDY

The study samples were obtained from Rethabile and Seshego health clinics in the Capricorn District of the Limpopo Province, South Africa. The purpose of the study was explained to each participant, and their consent was obtained before collection of samples, and completion of demographic data and clinical histories through questionnaires. Specific research codes were assigned to each sample collected to ensure patient confidentiality.

In order to ascertain the minimum concentration of EFV in the plasma of each patient, blood samples were collected from each patient before treatment commencement (Baseline) and between six to twelve months after treatment commencement (Follow-up). For the main study, a total of 79 paired blood and hair baseline samples were collected from participants. Of this total number, only 20 study participants returned for follow-up for the subset analysis, hence just 20 blood samples were selected and utilized for this project. A summary of the demographic data, CD4 count measurement and viral load information collected are documented in **Section 3.1** in the results section.

2.3 SAMPLE COLLECTION

For CD4+ cell count and viral load measurements, five milliliters (5ml) of venous blood was collected into purple cap and white cap EDTA tubes; the blood in the purple cap tube was to be used for genomic testing and determination of patient EFV concentration, while the blood in the white cap tube was used for viral load testing. The samples were transported to the University of Venda's HIV/AIDS and Global Health Research Program laboratory and Lancet Laboratories, respectively for further processing.

2.4 SAMPLE PROCESSING

Upon arrival of samples at the HIV/AIDS and Global Health Research Program laboratory, CD4+ cell measurements were done for all the blood samples using the BD FACSPRESTO machine. It measures CD4+ cell cells per microliter, the percentage of CD4 cells, as well as the amount of Haemoglobin (g/dL) present. After CD4+ cell counting, the samples are further processed for downstream experiments.

2.4.1 Plasma Isolation

Plasma was prepared by centrifugation of the whole blood specimen at 4000rpm for five minutes. Two hundred microliters of plasma was then aliquoted into labeled sterile cryotubes and stored at -80^{o}C for subsequent experiments. This was done in strict sterile conditions.

2.4.2 PBMC Isolation and DNA Extraction

After the plasma was aliquoted into the cryotubes, peripheral blood mononuclear cells (PBMCs) were extracted from the total cells using the Histopaque-1077 extraction protocol from SIGMA-ALDRICH. Briefly, three milliliters (ml) of Histopaque-1077 was aliquoted into a 15ml conical centrifuge tube and brought to room temperature; 3ml of whole blood was carefully added onto the

Histopaque-1077. This product was centrifuged at 2600rpm for exactly 30 minutes at room temperature after which the opaque interface which appeared (containing the mononuclear cells) was transferred into a clean conical 15ml centrifuge tube using a Pasteur pipette. The cells were washed by adding 10ml of isotonic phosphate buffered saline (PBS); the mixture was thoroughly mixed to homogeneity by gently drawing it in and out of a Pasteur pipette, after which it was centrifuged at 1300rpm for 10 minutes. The resulting supernatant was aspirated and discarded; the cell pellet was re-suspended with 5ml of isotonic PBS and mixed as described previously, after which it was centrifuged at 1300rpm for 10 minutes. This last step was repeated in order to get a concentrated product.

DNA was extracted from the isolated PBMC using the QIAamp DNA mini extraction kit according to the manufacturer's protocol. This extracted DNA was used for the amplification of the HIV-1 *Pol*, MDR-1 and CYP2B6 genes.

2.5 LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY (LC-MS/MS) ANALYSIS OF PLASMA SAMPLES

The LC-MS/MS procedure for the quantification EFV concentration in plasma was conducted at FARMOVS Bioanalytical Services Division (FARMOVS BASD) laboratories in the University of the Free State (UFS).

2.5.1 Principle of LC-MS/MS

This is a technique which incorporates liquid chromatography with mass spectrometry (MS) for quantitative and qualitative analysis of drug substances, products and biological samples (Begumet al., 2015). The mass spectrometer is an instrument designed to separate gas phase ions according to their mass to charge ratio value (m/z). MS involves the separation of charged species that could be produced by various ionization methods (Example: Atmospheric Pressure Chemical Ionisation – APCI, Electrospray Ionisation – ESI) in LC-MS/MS. The charged species (spp) are produced as gas phase ions under atmospheric pressure conditions; these gas phase ions are achieved within the

mass spectrometer through electrical and / or magnetic fields which are used to differentiate the ions. In addition to the analyzer, the mass spectrometer also includes an atmospheric ionisation chamber, a vacuum system and a detector.

There are several discrete stages in LC-MS/MS analysis. First, the compounds are separated using an HPLC column where the analytes are differentially partitioned between the mobile phase (eluent) and the stationary phase (coated unto a support material and packed into the column). The mechanism of retention and separation usually depends on the mode of chromatography. The separated sample spp. are then sprayed into an atmospheric pressure ion (API) source where they are converted into ions in the gas phase, and the majority of the eluent is pumped to waste. The mass analyzer is used to sort the ions according to their mass to charge ratio (m/z); examples of analyzers used include: Quadrupole, Time of Flight (TOF), Ion Trap, and Magnetic Sector. The detector is used to “count” the ions emerging from the mass analyzer and may also amplify the signal generated from each ion; some commonly used detectors are the Electron Multiplier, Dynode, Photodiode and the Multi-channel plate. All mass analysis and detection is carried out under high vacuum pressure that is established using a foreline and turbomolecular pumps (www.chromacademy.com).

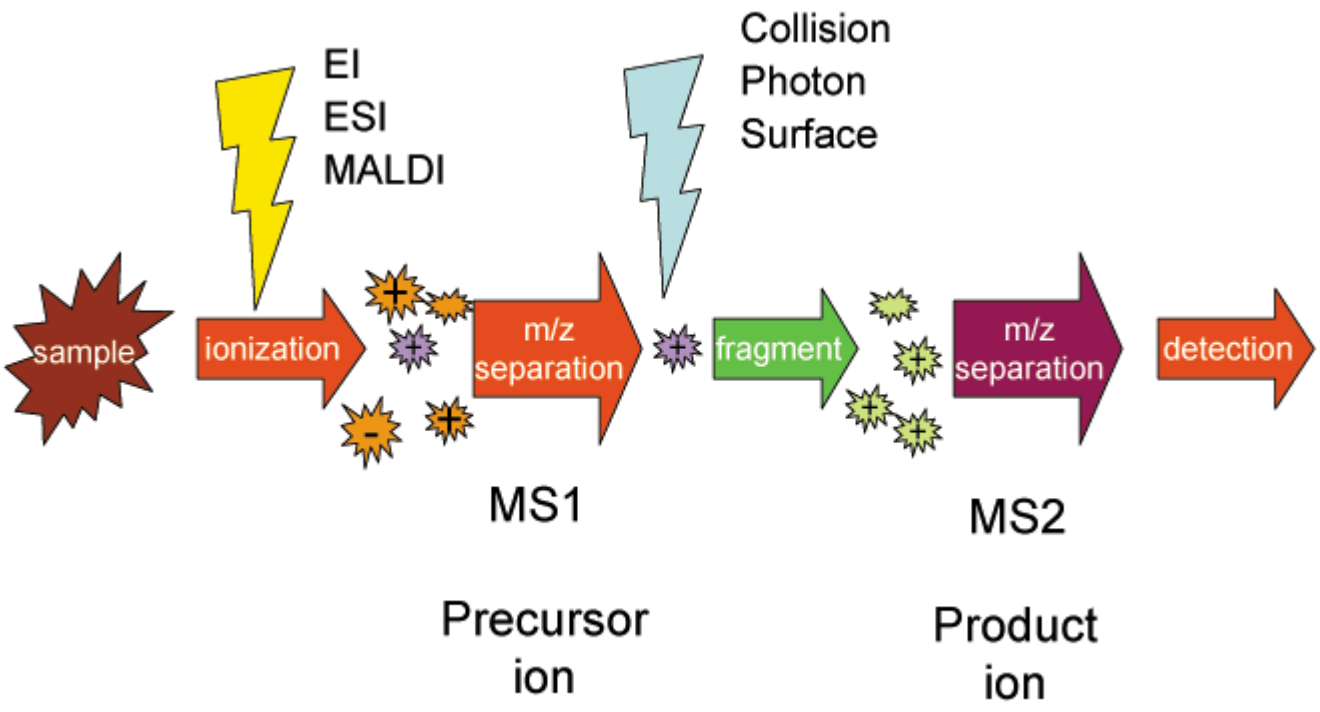

Figure 3: A schematic representation of the tandem mass spectrometry system. Adapted from https://en.wikipedia.org/wiki/Tandem_mass_spectrometry and accessed in March 2019

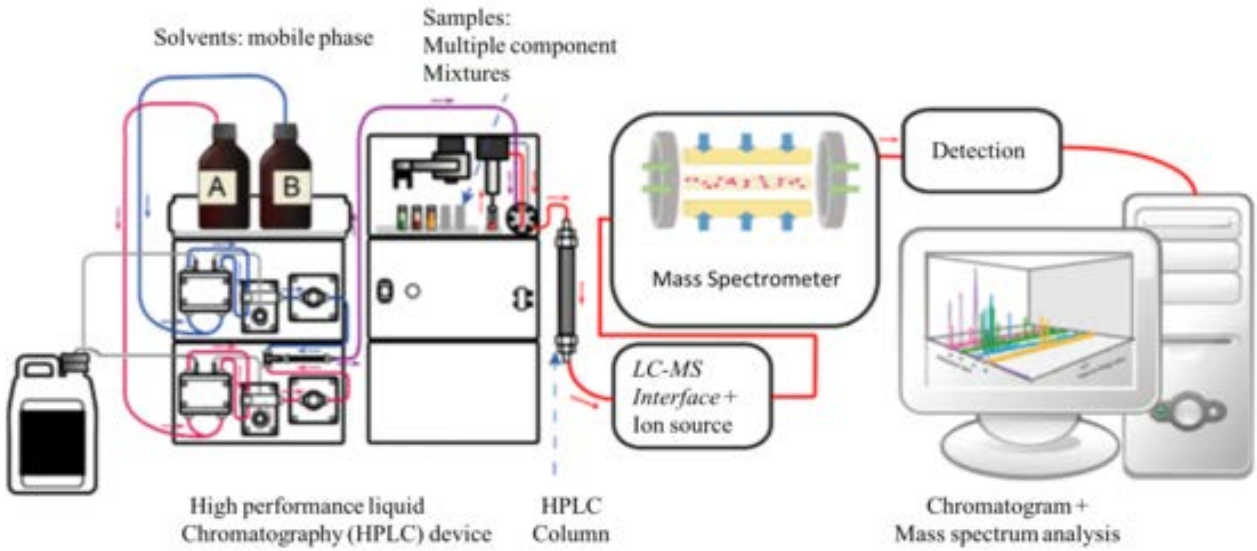

Figure 4: Diagram of LC-MS/MS system Adapted from https://en.wikipedia.org/wiki/Liquid_chromatography-mass_spectrometry and accessed in March 2019

2.5.2 Standards and Internal Standard Solutions

The analytical standard used in this experiment was racEfavirenz (R937a – BASD reference number) from Toronto Research chemicals, having a purity of 98.0%. Deuterated Efavirenz (Efavirenz-d5) was used as the internal standard (ISTD) during extraction, and had a molecular weight of 320.71.

2.5.3 Preparation of the Internal Standard Working Solution

The internal standards were spiked into the precipitation solution (consisting of 100% methanol) to a concentration of ~1000ng/ml efavirenz-d5, prepared in a polypropylene container from stock solutions in methanol. The internal standard working solution was then added to each sample (excluding blank samples).

2.5.4 Preparation of Calibration Standards and Quality Control Samples

Calibration standards (STDs) and quality control samples (QCs) were prepared gravimetrically in human K_2EDTA plasma. A stock solution of each analyte was prepared and used to spike a pool of normal blank plasma for the preparation of the STDs. For the preparation of the QCs, a second stock solution of each analyte was prepared and used to spike a pool of normal blank plasma. Each pool was then serially diluted with normal blank plasma to attain the desired concentrations.

It is a requirement at the laboratory that the prepared concentrations of STDs and QCs be within 1% of the target concentrations. The target concentrations are assigned as the nominal concentrations.

The STDs and QCs were aliquoted into individual polypropylene tubes and stored at approximately -70ºC until required for the analysis of samples.

2.5.5 Drug Extraction Conditions for Plasma Samples

The plasma samples were thawed in a water bath at approximately 22ºC. They were then vortexed briefly and centrifuged for 5 minutes at 1300 x g. One hundred and fifty microliters (150µl) of the plasma was aliquot into a microcentrifuge tube and a precipitation solution with approximately 1,000ng/ml of Efavirenz-d5 was added to the samples. Four hundred microliters of a ten percent formic acid solution was added to the microcentrifuge tube and vortexed for 30 seconds and centrifuged for 3 minutes at 8000 x g. The analyte of interest was extracted manually via the solid phase extraction (SPE) procedure, which used PhenomenexStrata™-X 33µm Polymeric Reverse Phase (200mg/3mL) columns and a SPEEDISK® 48-plate positive pressure system. The procedure was as follows: 1ml of Methanol and 2ml of 1% Formic acid was used to condition the SPE column. A total of 800µl of the sample was transferred onto the SPE columns and washed with 2ml of 15mM Ammonium Acetate solution. The columns were dried for 2 minutes by applying high pressure through the SPEEDISK® 48-plate positive pressure system. The analyte was then eluted by adding 1000µl of Methanol to the column. The solution was then evaporated to dryness under a gentle stream of Nitrogen at approximately 50°C, and later reconstituted with 1:1 Methanol-Water (50:50, v/v) solution. The solution was vortexed for 30 seconds and later transferred to a 96-well plate. Five microliters (5µl) of the extracted solution was injected into the HPLC column.

2.5.6 Instrumental and Chromatographic Conditions

The following instrument assembly was used for the assay of the analyte: Autosampler (Agilent – G1367B), Binary Pump (Agilent – G1312B), Sample Cooler (G1330B). The column compartment was manufactured by Hewlett Packard (G1316A), while the Mass Spectrometer used was manufactured by Sciex (API4000). The analytical column used was a 5µm Phenomenex®Gemini C18, 150 × 2.00 mm, fitted with a PhenomenexSecurityGuard® system containing a C18 (4 x 3 mm) pre-column and kept at 25°C in a column compartment. Mobile phase A consisted of 100% Methanol solution while mobile phase B was made up of 0.1% formic acid.

Table 1 indicates the flow-rate at which the pump delivered both mobile phase A and B at different time points. The autosampler equipped with a 96-well tray, was used to inject 5µl of each sample onto the column; it is fitted with a cooling device to keep the samples at 5°C. The settings on the

mass spectrometer using Electro Spray Ionization (ESI) were optimized and the values of the settings are indicated in **Table 2** and **3**.

Table 1: Indicates the flow-rate and the percentage obtained in both mobile phase A and B, when the time is gradually increased.

Step	Total Time (min)	Flow Rate (µl/min)	A (%)	B (%)
0	0.00	200	50.0	50.0
1	1.50	200	50.0	50.0
2	1.60	200	85.0	15.0
3	11.0	200	85.0	15.0
4	11.10	200	50.0	50.0
5	19.00	200	50.0	50.0

Table 2: Indicates the ESI settings used in the detection of the analyte of interest.

Electro Spray Ionization Settings	Value
Nebulizer gas (Gas 1) (arbitrary unit)	30
Turbo gas (Gas 2) (arbitrary unit)	40
CUR (curtain gas) (arbitrary unit)	12
CAD (collision gas) (arbitrary unit)	12
TEM (source temperature) °C	550
IS (Ion Spray Voltage) (V)	5000

Table 3: Depicts the MS/MS settings with the mass to charge ratio (m/z) of the precursor ion before fragmentation and the product ion after fragmentation for analyte of interest and the internal standard (ISTD) in a positive ionization mode.

MS/MS Settings	Efavirenz	ISTD
Monoisotopic Mass	315.7	320.7
Protonated Precursor Ion Molecular Mass (m/z)	316.1	321.3
Product Ion Molecular Mass (m/z)	244.0	172.4
Dwell Time (ms)	150	150
DP (Declustering Potential) (V)	60	60
EP (Entrance Potential) (V)	11	11
CE (Collision Energy) (eV)	20	20
CXP (Collision Cell Exit Potential) (V)	14	14

2.5.7 Method Validation

A. Calibration Standards

The standard curve was generated by preparing a set of calibration standards. This was done by spiking the solutions with 100µl of the prepared standards and serially diluting them with blank plasma from the highest to the lowest (2, 560 – 20 ng/ml).

B. Accuracy and Precision

Accuracy is expressed as the percentage difference between the true nominal concentration and the measured concentration (expressed as % Bias) while the precision is expressed as the percentage coefficient of variation (expressed as % CV).

C. Linearity

A set of quality control samples was prepared to confirm the linearity of the standard curve. This was done by serial diluting the samples from the highest (4096ng/ml) to the lowest limit of quantification – LLOQ (20ng/ml).

D. Specificity

Specificity was determined by injecting samples 3 times and then determining the fragment pattern in comparison with standard to see if instrument picks up the analyte each time.

The qualification run was required to meet the following criteria:

- The calculated regression line should have a coefficient of determination (r^2) not less than 0.990.
- The calibration curve must consist of at least six STDs levels including the ULOQ and LLOQ.
- At least 75 % of the total number of back-calculated STD concentrations must be within 15 % of the nominal concentrations (except at LLOQ where it should be within 20 % of the nominal concentration). One of each of the replicates per concentration level should also meet this criterion.
- In case a STD does not meet the above criterion, this STD must be rejected, and the calibration curve without this STD must be re-evaluated. This includes re-evaluation of the regression model.
- In the event that both the replicates of the LLOQ or the ULOQ are rejected then the run should be rejected, the possible source of the failure must be determined and the method should be revised (if necessary).
- At least four of the six QC samples of each level included in a qualification run must be within 15% of their respective nominal concentrations (within 20% of the nominal concentration at LLOQ)

  And the mean within-run accuracy of each QC level included in a qualification run must be within 15% (20 % at the LLOQ)

And the within-run precision of each QC level included in a qualification run must be equal to, or less than 15% (20% at LLOQ). This criterion is not applicable to runs with only two QC samples per level.

- If only two QCs per level were included in a run, 67% of the total number of QCs must be within 15% (within 20% at LLOQ) of their respective nominal concentrations and at least one QC sample per level must be within its respective nominal concentration.

2.6 POLYMERASE CHAIN REACTION

2.6.1 Primer Reconstitution

The primers used to target a partial fragment of the HIV-1 Polymerase (*Pol*) gene – approximately 1.65 kilo-bases (kb), for drug resistance analysis are in-house primers used in the HIV/AIDS and Global Health Research laboratory. The primers used are shown in **Table 4**. Since the protocol for amplification was a nested reaction, the first round primer sets were 1353 and 1395; primers 1389 and 1396 were used for the nested reaction.

For drug transport and metabolism, primers used to target the MDR1 and CYP2B6 genes were obtained from Masebe et al., 2012, and they targeted a 363bp and 289bp product, respectively. . The primers used for both genes are shown in **Table 5**

Table 4: Primers used to amplify a 1.65 kb product of the HIV-1 *Pol* gene.

Amplification of HIV-1 Partial Pol Fragment		
Primer Name	**Sequence**	**Position on HXB2**
1395 (Forward)	5'-TGGCAAGGAAGGGCACATAGCCAAAAAATTG-3'	1974 – 2003
1353 (Reverse)	5'-TTGGAGTCTTTCCCCATATTACTATGCTTT-3'	3711 – 3682
1389 (Forward)	5'- AAATTGCAGGGCCCCTAGG-3'	1998 – 2016
1396 (Reverse)	5'-CTCTGTTAACTGTTTTACATCATTAGTGTGGG-3'	3659 – 3628

Table 5: Primers used for amplification of a 363bp and 289bp product of the MDR1 and CYP2B6 genes respectively.

Amplification of MDR1 and CYP2B6 Genes		
Primer Name	**Sequence**	**Primer Position**
MDR1-F	5'-GGCAGTTTCAGTGTAAGAAATAATG-3'	86,750,557 – 86,750,916
MDR1-R	5'-TCAAACTATAGGCCAGAGAGGC-3'	
CYP2B6-F	5'- AGGTGACAGCCTGATGTTCC -3'	46,204,586 – 46,204,874
CYP2B6-R	5'-TTTCTCGTGTGTTCTGGGTG-3'	

2.6.2 Partial Pol Amplification

Amplification of a 1.65kb fragment of the HIV-1 Pol gene was done by a nested conventional PCR. The final concentration of the reaction mix used for amplification consisted of: 2mM of 10X buffer containing 20mM $MgCl_2$, 200µM of 10mM dNTP solution, 0.5µM of each forward and reverse primer from a 10µM working solution and 2U of 5U/µl FastStartTaq enzyme. The cycling conditions are illustrated in **Table 6**. After amplification, gel electrophoresis was performed using a 1% agarose gel stained with Ethidium bromide to visualize the outcome of the PCR and confirm the presence of the desired band of interest.

Table 6: Cycling conditions for the amplification of a 1.65 kb fragment of the HIV-1 *Pol* gene.

First Round Cycling Condition				**Nested Reaction Cycling Conditions**			
Step	**Temperature**	**Time**	**Cycles**	**Step**	**Temperature**	**Time**	**Cycles**
Pre-Denaturation	95ºC	2 mins	1 Cycle	Pre-Denaturation	95ºC	2 mins	1 Cycle
Denaturation	95ºC	1 min	30 Cycles	Denaturation	95ºC	1 min	30 Cycles
Annealing	55ºC	1 min		Annealing	53ºC	1 min	
Extension	72ºC	1 min		Extension	72ºC	2 mins	
Final Extension	72ºC	10 mins	1 Cycle	Final Extension	72ºC	10 mins	1 Cycle
Holding Temperature	4ºC	Infinite	∞	Holding Temperature	4ºC	Infinite	∞

2.6.3 Amplification of the MDR1 and CYP2B6 Genes

The final concentration of the reaction mix for the amplification of both CYP2B6 and MDR1 genes consisted of: 2µM of forward and reverse primers from a 20µM working solution, 200µM dNTPs, 2U FastStartTaq polymerase, 10X buffer (with 20mM $MgCl_2$), 1µM of DNA template and nuclease free water in a 50µl total reaction volume. The cycling conditions used for both genes are shown in **Table 7**.After amplification, gel electrophoresis was performed using a 1% agarose gel stained with

Ethidium bromide to visualize the outcome of the PCR and confirm the presence of the desired band of interest.

Table 7: Cycling conditions for the amplification of the MDR1 and CYP2B6 genes.

CYCLING CONDITIONS FOR MDR1 AMPLIFICATION				**CYCLYING CONDITIONS FOR CYP2B6 AMPLIFICATION**			
Step	**Temperature**	**Minutes**	**Cycles**	**Step**	**Temperature**	**Minutes**	**Cycles**
Pre-denaturation	95ºC	3min	1 cycle	Pre-denaturation	95ºC	3min	1 cycle
Denaturation	95ºC	2 min	35 cycles	Denaturation	95ºC	2 min	35 cycles
Annealing	52ºC	1 min		Annealing	55ºC	1 min	
Extension	72ºC	2 min		Extension	72ºC	2 min	
Final Extension	72ºC	10 min	1 cycle	Final Extension	72ºC	10 min	1 cycle
Holding temperature	4ºC	Infinite		Holding temperature	4ºC	Infinite	

2.7 PURIFICATION OF PCR PRODUCTS

Positively amplified PCR products for all three genes were purified using the QIAGEN purification kit according to the manufacturer's instructions. The final elution step was performed twice in order to concentrate the purified product, and was visualized on a 2% agarose gel. This cleaned up product was sequenced for further genotypic analysis.

2.8 GENOTYPING

2.8.1 Sequencing of HIV-1 *Pol*, MDR1 and CYP2B6 Purified Products

Sequencing of all three genes was done by population based methods (Sanger sequencing). The primers used for sequencing all three genes are shown in **Table 8**.

Table 8: Primers used for sequencing the HIV-1 *Pol*, MDR1 and CYP2B6 genes.

Primers used for sequencing the HIV-1 *Pol* Gene	
Primer Name	**Sequence**
Pol 1F (forward primer)	5' TTT TCC ATT AGT CCT ATT GAA CCT GT 3'
Pol 1R (reverse primer)	5' CAT GCT ACT CTG GAA TAT TGC TGG TG 3'
Pol 2F (forward primer)	5' CTG CAT TCA CCA TAC CTA GTA TAA AC 3'
Pol 2R (reverse primer)	5' TGA TGG GTC ATA ATA TAC TCC ATG 3'
Primers used for sequencing the MDR1 and CYP2B6 Genes	
MDR1-F	5'-GGCAGTTTCAGTGTAAGAAATAATG-3'
MDR1-R	5'-TCAAACTATAGGCCAGAGAGGC-3'
CYP2B6-F	5'- AGGTGACAGCCTGATGTTCC -3'
CYP2B6-R	5'-TTTCTCGTGTGTTCTGGGTG-3'

2.8.2 HIV-1 *Pol* Sequence Analysis

The Geneious version 11.1.5 software package was used for sequence analysis. For each sample, both the forward and reverse strand was aligned first before bases called on the ambiguous sites on the electrophoregram. After base-calling, the consensus was then mapped to a reference sequence obtained from GenBank; this is done to confirm the position of the test sequence. To corroborate the results shown after mapping to a reference sequence, the consensus nucleotide sequence was extracted and submitted to the NCBI Nucleotide BLAST software. The results obtained showed the description of the sequences which match that of the query sequence, the query coverage, percentage identification as well as the accession number. This consensus sequence is saved for further drug resistance analysis.

2.8.3 HIV-1 Drug Resistance Genotyping

The consensus sequence obtained for each baseline sample was then submitted to the Genotypic Resistance Interpretation Algorithm of the HIVdb Program (https://hivdb.stanford.edu/hivdb/by-sequences/) to infer the presence of drug resistant mutations in a patient sequence, which could potentially lead to treatment failure. This site shows results for any mutations present in various ARV drug classes – NRTI, NNRTI, INSTI, and PI.

2.8.4 MDR1 and CYP2B6 Sequence Analysis and SNP Identification

After sequencing completion, the sequences obtained were manually edited and analyzed using the Geneious version 11.1.5 software package. After aligning the forward and reverse strand, bases were called on the ambiguous sites on the electrophoregram. The consensus was then mapped to a reference sequence obtained from GenBank for each gene; accession number NM_000927.4 and NG_007929.1 were used as reference sequences for the MDR1 and CYP2B6 test samples, respectively. This consensus nucleotide sequence was extracted and submitted to the NCBI Nucleotide BLAST software. BLAST results for the MDR1 sequences showed the description of the sequences which match that of the query sequence, the query coverage, percentage identification as well as the accession number.

To infer for the presence of any single nucleotide polymorphisms (SNPs), with keen interest in the C3435T SNP – which is implicated in altering drug uptake and transport mechanisms, edited patient sequences were compared to exon 26 of the variant reference sequence of the MDR1 gene. Similarly, a comparison was done between the CYP2B6 reference sequence and the edited test sequences; the G516T SNP, which is implicated in altering plasma EFV exposure and increased central nervous system (CNS) side effects, was of specific interest.

2.9 STATISTICAL ANALYSIS

The Hardy-Weinberg equilibrium (HWE) was used for allele and frequency determination. The HWE states that the amount of genetic variation in a population will remain constant from one generation

to the next in the absence of disturbing factors. Allelic frequencies were calculated based on the observed number of different alleles present after sequence analysis. This was done using the Michael H. Court lab HW-5 calculator.

CHAPTER THREE: RESULTS

3.1 DEMOGRAPHIC DATA

A summary of the demographic data collected for all 20 participants is shown in **Table 9** and **10**.Briefly, the population was 85% female with an age range of 30 – 56 years old and many of them reporting 2015 – 2016 as the probable year of infection. In this cohort, all the participants were administered the first line combination of Tenofovir + Emtricitabine + Efavirenz to be taken once every night (1TFE; Nocte). A detailed breakdown of the demographic data for all 20 participants is represented in **Appendix 1**.

Table 9: Representation of a summary of the demographic information collected from the study participants.

Demographic Parameter	
Sex	Female: 17 (85%)
	Male: 1 (5%)
	No Data: 2 (10%)
Age	Age Range: 30 – 56 years' old
	Mean Age: 36.3
	No Data: 2 (10%)
Marital Status	Single: 10 (50%)
	Married: 7 (35%)
	Widowed: 1 (5%)
	No Data: 2 (10%)
WHO Stage of AIDS	Stage 1: 18 (90%)
	Stage 2: 0 (0%)
	No Data: 2 (10%)

Table 10: A summary of the Complementary Medicine used by the study participants, alongside the CD4 count and Viral Load measurements before and after treatment

Parameter		**Baseline**	**After Treatment**
CD4 Count (Cells/µl)	Range	28 – 1068	160 – 1148
	No Data	2 (10%)	8 (40%)
	Too Low To Detect	0 (0%)	3 (15%)
Viral Load (Copies/mL)	Range	207 – 2,746,469	31 – 144
	<20 Copies/mL	0 (0%)	3 (15%)
	Not Detected	0 (0%)	8 (40%)
	No Data	3 (15%)	5 (25%)
Use of Complementary Medicine	Not Taking	19 (95%)	6 (30%)
	Taking	1 (5%)	9 (45%)
	No Data	0 (0%)	5 (15%)

3.2 PHARMACOKINETIC ANALYSIS OF EFAVIRENZ (EFV)

The mass spectrum of Efavirenz (standard) and Efavirenz-d5 (internal standard – ISTD) after collision induced dissociation in the fragmentation cell is shown in **Figure 5** and **6**, respectively. It represents the m/z ratios observed, the protonated precursor ions as well as the product ions in each molecule. The calibration standards were run in pairs of 8 (B – I), with B being the lowest concentration, and I being the highest concentration. The theoretical concentrations ranged from 20 ng/ml – 2, 560 ng/ml, whereas the mean concentration achieved were 19.43 – 2, 667 ng/ml (lowest to highest). The quality control samples were run in sets of six (QC A – QC H), with QC A being the lowest limit of quantification and QC H being the highest concentration. The theoretical concentration ranged from 20 – 4, 096 ng/ml, and the mean concentrations achieved ranged from 18.87 – 4, 316 ng/ml. The calibrated curve obtained is depicted in **Figure 7** and it has a linearity of 0.998859.

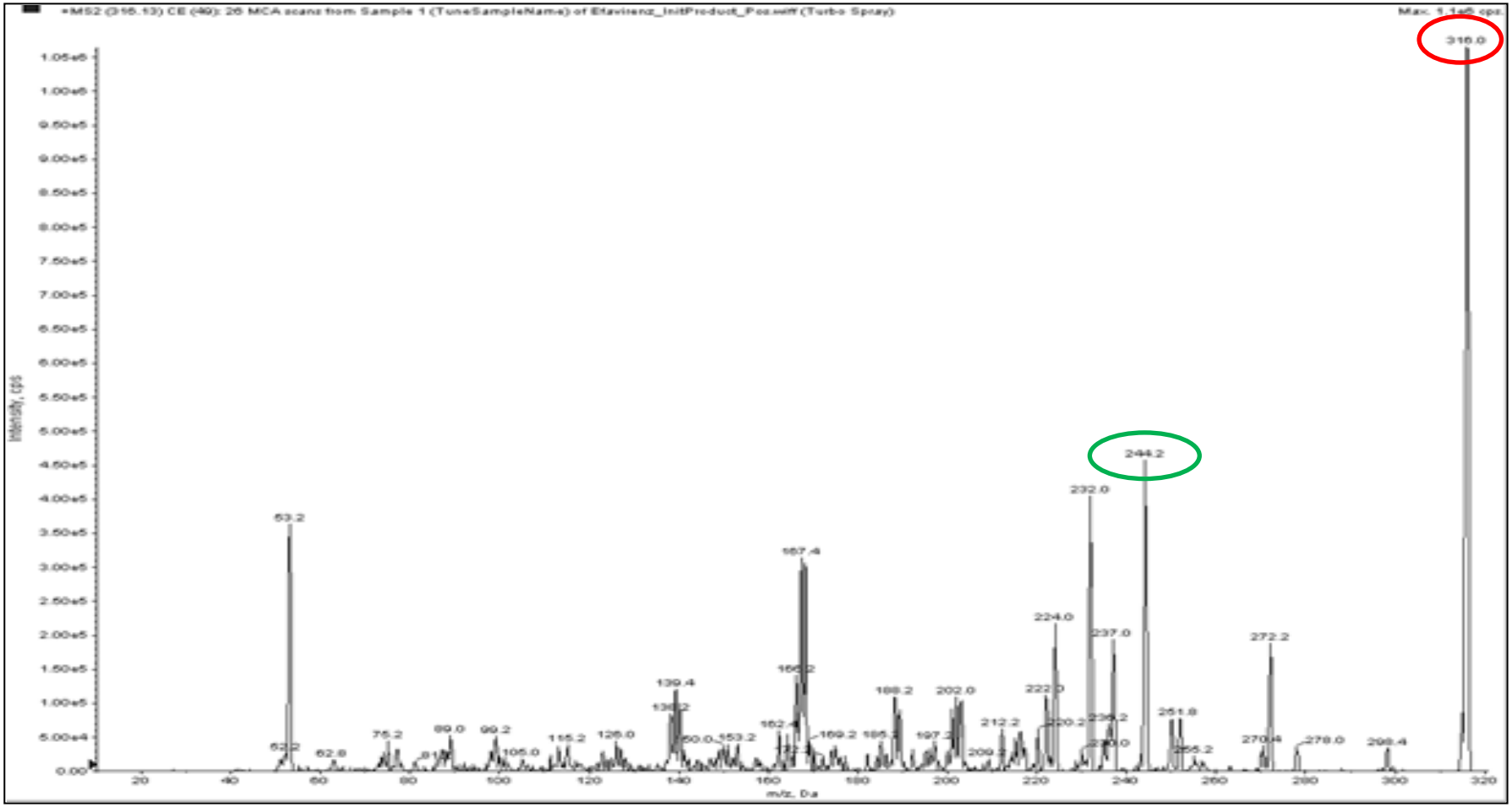

Figure 5: Representation of EFV after collision induced dissociation in the fragmentation cell with the protonated precursor ion at *m/z*316.1 (circled in red), and the product ion at *m/z* 244.2 (circled in green).

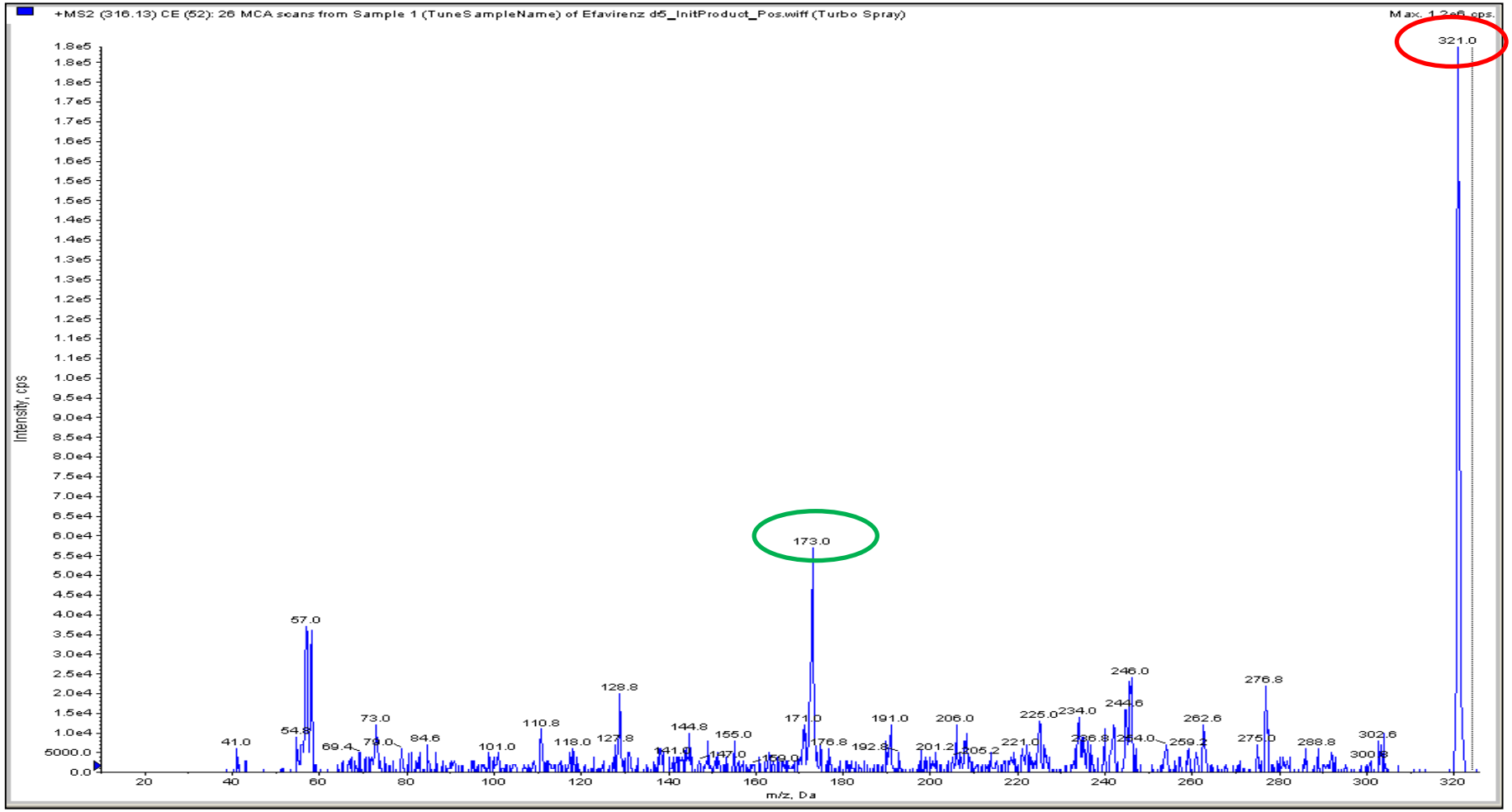

Figure 6: Representation of d-efavirenz after collision induced dissociation in the fragmentation cell with the protonated precursor ion at *m/z* 321.3 (circled in red), and the product ion *m/z* 173.0 (circled in green)**.**

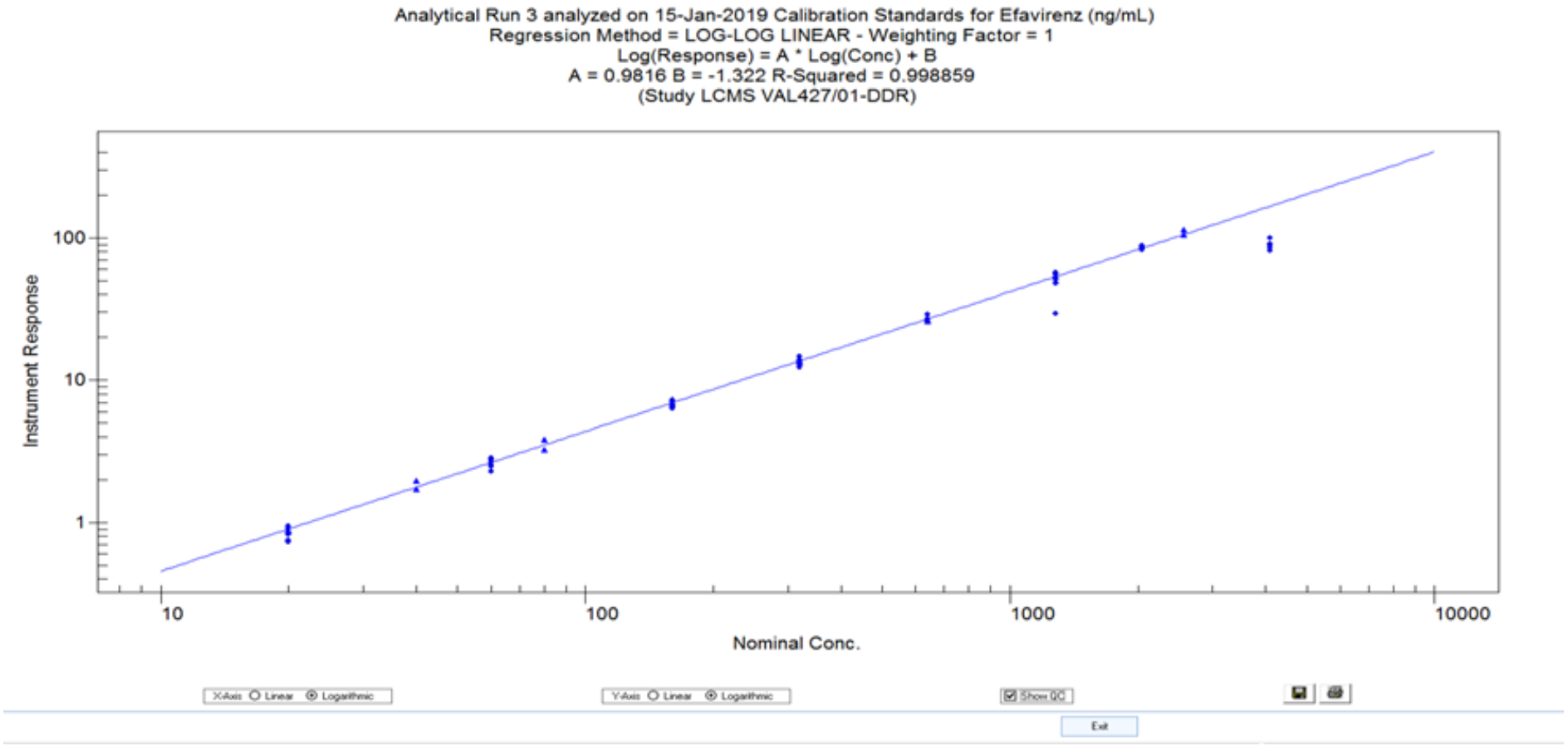

Figure 7: Representation of the calibration curve obtained after running the standard solutions. The Y-axis indicates the instrument response, while the X-axis shows the nominal concentrations obtained.

It has been suggested that, individuals on EFV-based ART should have plasma EFV levels ranging from 1, 000 – 4, 000 ng/ml (Puthanakit et al., 2009). Patients with less than 1, 000 ng/ml of EFV are liable to develop class specific drug resistance mutations, while those with concentrations higher than 4, 000 ng/ml will likely develop central nervous system (CNS) toxicity. The plasma EFV concentrations in the study subjects ranged from below limits of quantification (BLQ) to 15, 670 ng/ml. Representations of these results are depicted in **Table 11**.

At baseline (t=0), no drug (0ng/ml) is expected to be seen in patient plasma; this is because the patient is still to be initiated on ART. However, patients 1 and 12 (AHDR-R295 and AHDR-S94) are seen to have plasma EFV levels ranging from 1, 183 and 769.9 ng/ml, respectively. This suggests that both patients may have already been on treatment at the time of sample collection. This can be attributed to the fact that some patients may have defaulted treatment or relocated, hence they are re-initiated at a new treatment site.

In this study, the plasma EFV levels range from below limits of quantification (BLQ) to 15, 670 ng/ml at follow-up (6 – 12 months post treatment). In the follow-up, fifteen (75%) samples had EFV plasma concentrations within the expected range, three (15%) samples – patients 10, 19 and 20 (AHDR-S88, AHDR-S117,and AHDR-S120), had above range concentrations (supra-therapeutic) after 6 months of treatment each, and two samples (10%) i.e. patient 12 and 16 (AHDR-S101 and AHDR-S110) had less than expected (sub-therapeutic) plasma EFV concentrations even after 7 and 10 months of treatment respectively.

As already discussed in **Section 3.1**, all patients are expected to take their ARV once every night, hence it can be said that, patients in whom sub-therapeutic plasma levels are seen, are not adhering to their treatment, which explains the low levels observed. For patients with higher plasma EFV concentrations, their genetic characteristics were evaluated in order to understand if they may have influenced the supra-therapeutic concentrations observed. This is further discussed in **Section 3.7**. Symptoms of intoxication range from mild e.g. dizziness, irritability, euphoria, which is usually well tolerated, to more severe neurological symptoms such as delusion, paranoia, depression, which occur in roughly 2% of individuals on EFV based ART (Apostolova et al., 2015). In this study, the questionnaire was not designed to inquire about the effect of the ART in the newly initiated patients, but to find out if adherence of patients can be

determined using their blood sample, hence, there was no report about how patients are reacting to their drugs.

Table 11: Drug concentrations obtained after LC-MS/MS analysis for both baseline and follow-up samples. Patient plasma EFV concentrations ranged from below limit of quantification (BLQ) levels to 15, 670 ng/ml.

EFV Drug Concentration (ng/ml)			
Patient No.	**Sample Code**	**Baseline Concentration (t = 0)**	**Follow-Up Concentration (t = 6 – 12 months)**
1	AHDR-R295	1, 183	2, 873
2	AHDR-R298	0, 000	2, 071
3	AHDR-R305	0, 000	3, 738
4	AHDR-R311	0, 000	3, 330
5	AHDR-R315	0, 000	2, 419
6	AHDR-R318	0, 000	3, 562
7	AHDR-R328	0, 000	3, 450
8	AHDR-R331	0, 000	1, 283
9	AHDR-R340	0, 000	1, 688
10	AHDR-S88	0, 000	6, 685
11	AHDR-S92	0, 000	2, 671
12	AHDR-S94	769.9	1, 944
13	AHDR-S101	0, 000	506.8
14	AHDR-S102	0, 000	3, 661
15	AHDR-S106	0, 000	2, 999
16	AHDR-S110	0, 000	BLQ
17	AHDR-S111	0, 000	1, 180
18	AHDR-S113	0, 000	2, 781
19	AHDR-S117	0, 000	4, 358
20	AHDR-S120	0, 000	15, 670

Study participants who show unexpected levels of EFV in their plasma are represented in **Table 12**. Patient 1 and 12 are seen to have EFV concentrations in their baseline sample, suggesting that these patients may have already been on treatment. Three patients i.e. patients 10, 19 and 20 show **HIGH** (Supra-therapeutic) EFV concentration post treatment with only six months post treatment; patients 13 and 16 have **LOW** (Sub-therapeutic) concentrations of EFV in their system, even after being on treatment for over six months.

Table 12: Drug concentrations, the time point at which the follow-up sample was collected and the interpretation, for patients with unexpected drug concentration

EFV Drug Concentration (ng/ml)				**Time Point**	**Comments**
Patient No.	**Sample Code**	**Baseline Concentration**	**Follow-Up Concentration**	**Follow-Up Collection**	
1	AHDR-R295	1, 183	2, 873	9 Months Post Treatment	Plasma EFV concentration in baseline sample
12	AHDR-S94	769.9	1, 944	14 Months Post Treatment	
13	AHDR-S101	0, 000	506.8	7 Months Post Treatment	Low (Sub-therapeutic) plasma EFV concentration
16	AHDR-S110	0, 000	BLQ	10 Months Post Treatment	
10	AHDR-S88	0, 000	6, 685	6 Months Post Treatment	High (Supra-therapeutic) plasma EFV concentration
19	AHDR-S117	0, 000	4, 358	6 Months Post Treatment	
20	AHDR-S120	0, 000	15, 670	6 Months Post Treatment	

3.3 AMPLIFICATION OF A PARTIAL FRAGMENT OF THE HIV-1 *POL* GENE

Of the 20 baseline samples amplified using the optimized *Pol* protocol, 8 samples were positively amplified while the other 12 samples were negative after an amplification attempt. For the follow-up samples, of the 20 samples attempted for amplification, 5 of them were positively amplified while the rest were negative. **Figure 8** is a representation of the first 10 baseline samples amplified after optimization of the protocol.

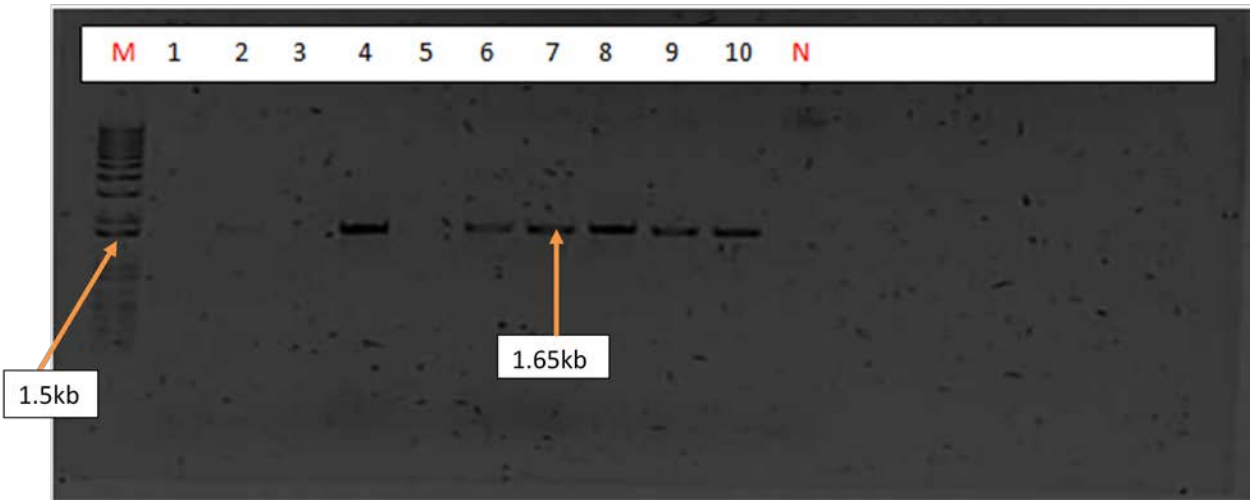

Figure 8: A representative gel picture depicting amplification of the targeted 1.65kb fragment of the HIV-1 *Pol* gene. Lane M shows the 1kb molecular weight marker used, Lanes 1 – 10 are the test samples and Lane N is the negative control. Of the 10 samples ran 7 of them were positive.

3.4 AMPLIFICATION OF MDR1 AND CYP2B6 GENES

All 20 samples were positively amplified for both the MDR1 and CYP2B6 genes. The target for the MDR1 gene was located on exon 26 and the primers were set to amplify a 363bp fragment; a 289bp fragment was targeted by the primers used to amplify the CYP2B6 gene located on the long q-arm of chromosome 19 of exon 4. **Figures 9** and **10** illustrate gel pictures for MDR1 and CYP2B6 amplifications respectively.

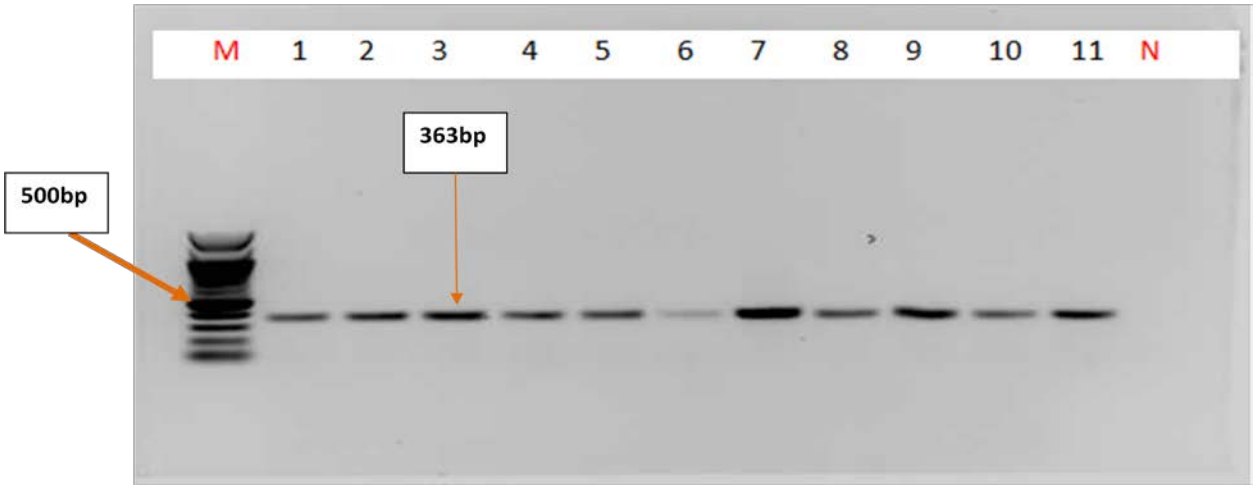

Figure 9: Represents the gel electrophoresed amplicons of MDR1 PCRs, loaded on a 1% ethidium bromide agarose-stained gel. The first lane, Lane M, is the 100bp molecular marker; the test samples follow, and Lane N is the negative control (nuclease free water).

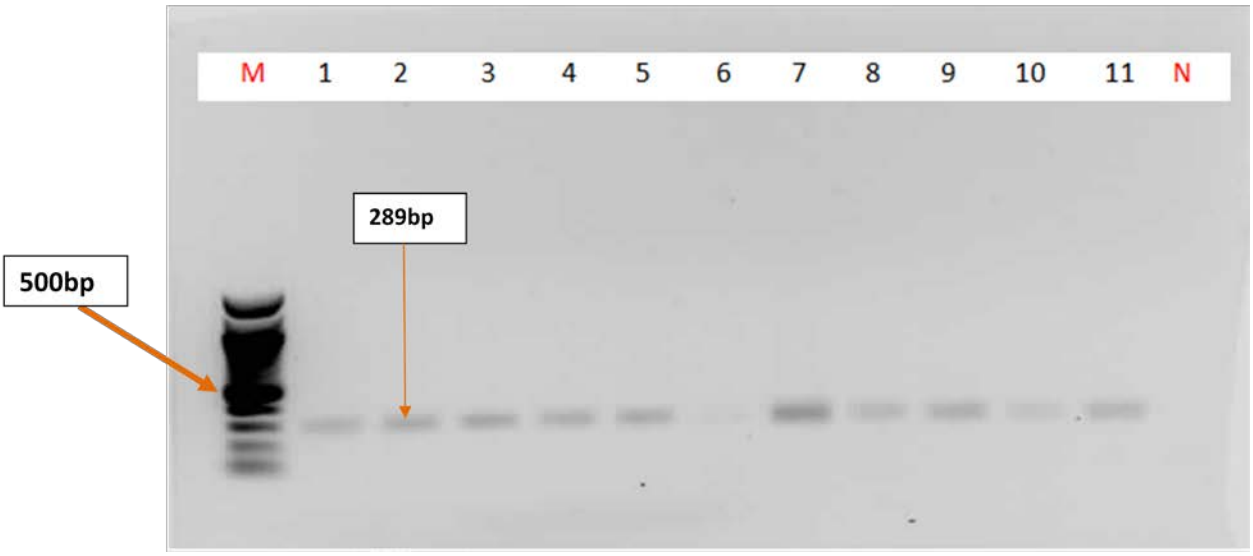

Figure 10: Represents the gel electrophoresed amplicons of CYP2B6 loaded on a 1% ethidium bromide agarose-stained gel. The first lane, Lane M, is a100bp molecular marker; the test samples follow, and Lane N is the negative control (nuclease free water).

3.4 PURIFICATION OF *POL*, MDR1 AND CYP2B6 PCR AMPLICONS

All positively amplified samples for the *Pol*, MDR1 and CYP2B6 genes were purified using the QIAGEN QIAquick PCR purification kit, as per manufacturer's instructions, in preparation for genomic sequencing. **Figures 11**, **12** and **13** below show a few samples after purification using the above-mentioned purification kit.

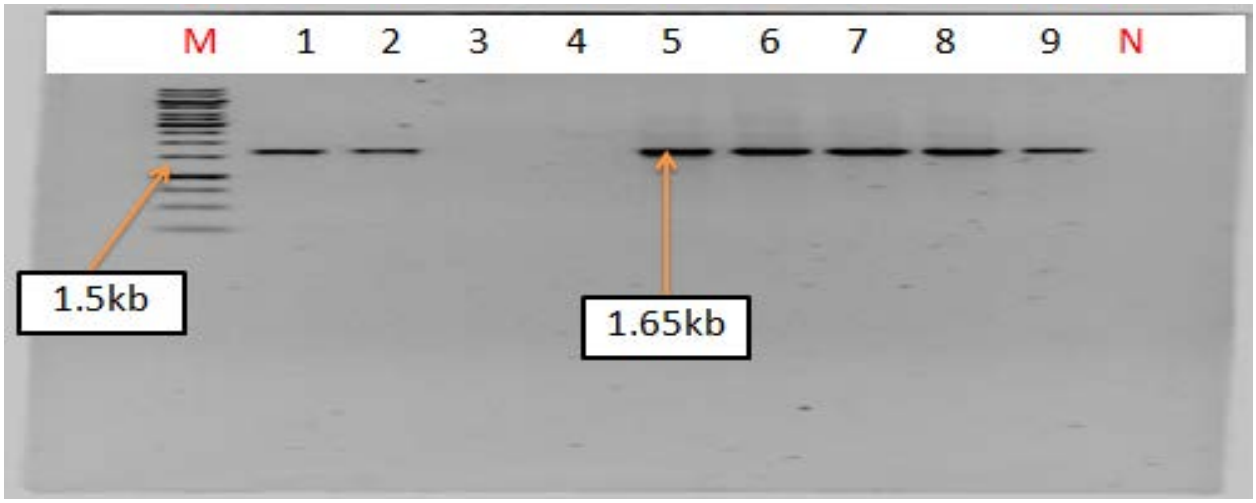

Figure 11: Gel representation of *Pol* amplicons which were purified using the QIAGEN QIAquick PCR purification kit. Lane M represents a 1kb molecular weight marker, Lanes 1-9 represent the test samples loaded in each well. Test samples were loaded in Lane 3 and 4, but because of their minute DNA concentrations after amplification, they are not visible after purification.

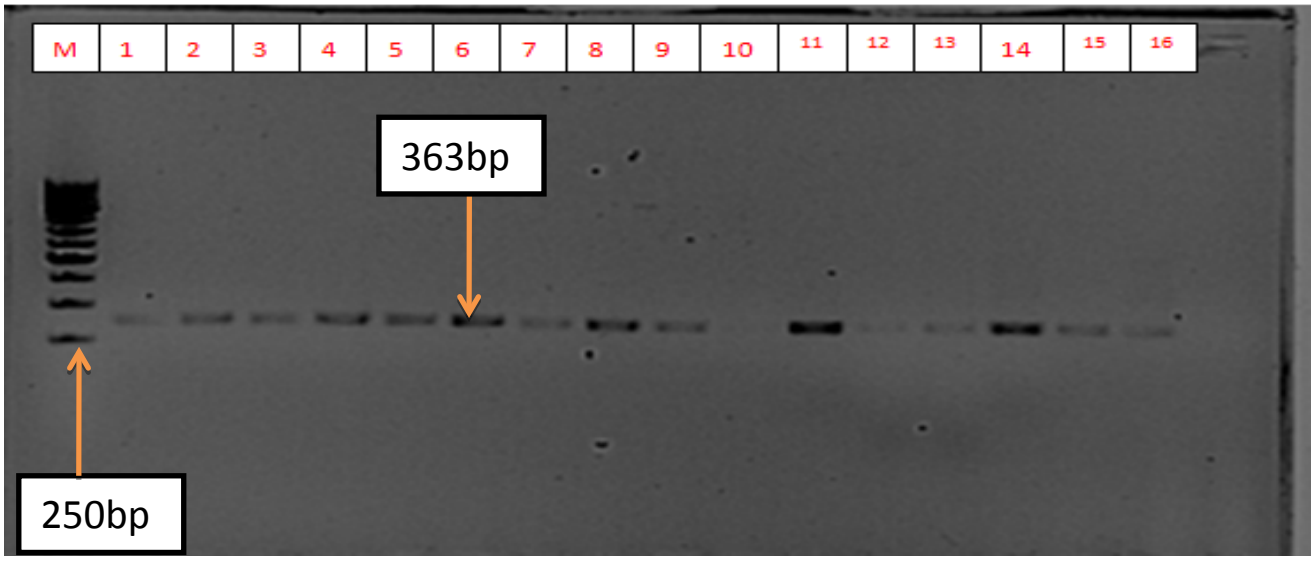

Figure 12: Gel representation of some (16) MDR1 amplicons which were purified using the QIAGEN QIAquick PCR purification kit. Lane M represents a 250bp molecular weight marker, Lanes 1-16 represent the test samples loaded in each well.

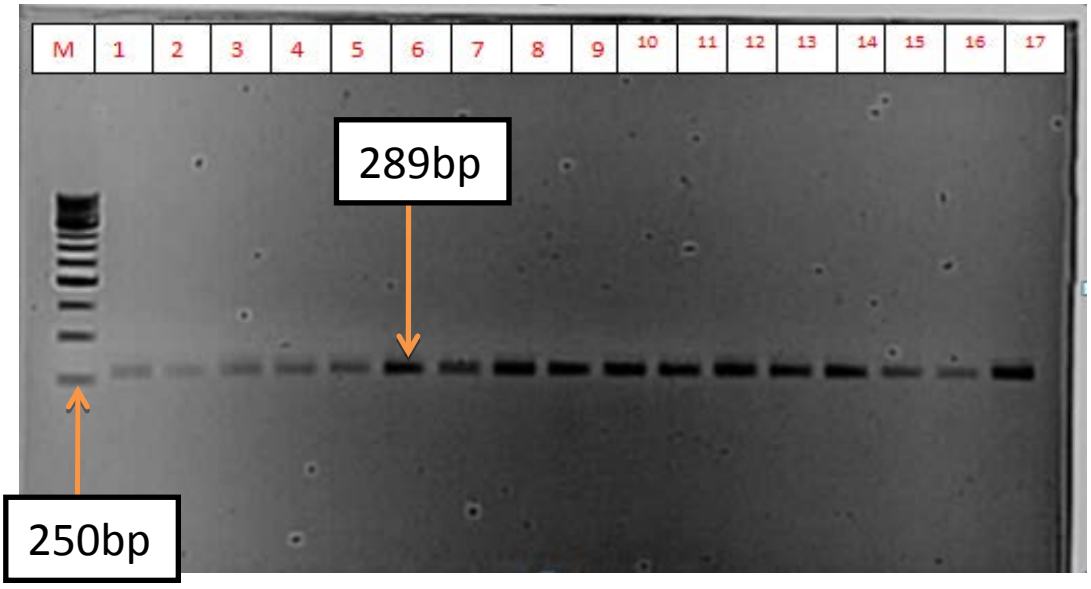

Figure 13: Gel representation of 17 CYP2B6 amplicons which were purified using the QIAGEN QIAquick PCR purification kit. Lane M represents a 250bp molecular weight marker, Lanes 1-17 represent the test samples loaded in each well.

3.5 GENOTYPIC ANALYSIS OF *POL*, MDR1 AND CYP2B6

3.5.1 Sequencing Results of the Pol Gene

Eight sequenced samples for the Pol gene were submitted for drug resistance analysis. No major NRTI and / or NNRTI mutations were present in any of the samples; however, minor mutations and polymorphisms were present. This is depicted in **Appendix 2**. The interpretation is that, in the absence of major mutations, the sample will be susceptible to all NRTI and NNRTI drugs. **Figure 14** is a representation of the results obtained after drug resistance analysis of patient 12 (AHDR-S94). However, due to time constraints, positively amplified follow-up samples were not sequenced.

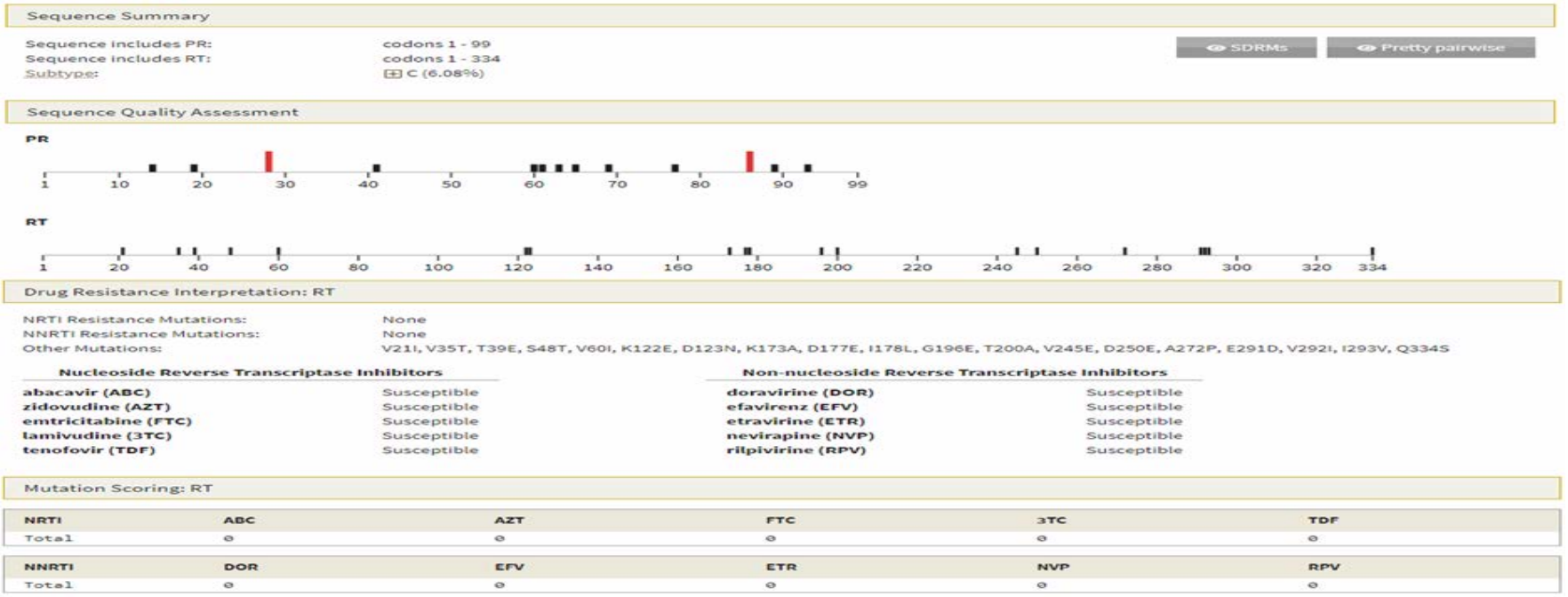

Figure 14: Representative sample (AHDR-S94) submitted to the Stanford HIV DR database indicating the subtype present, as well as the absence of major NRTI or NNRTI resistance mutations. The red bars indicate the presence of two unusual mutations: A28T and APOBEC G86E, which are present in the PR and APOBEC genes respectively.

3.5.2 Sequencing Results of the MDR1 Gene

Of the 20 samples sequenced for the MDR1 samples, 17 samples (85%) coded for the homozygous CC wildtype genotype, 3 samples (15%) possessed the heterozygous CT genotype, and no sample had the homozygous TT polymorphism. The C3435T SNP is located on exon 26, at a wobble position, hence it does not code for an amino acid change. **Figure 15** and **16** represent the contig view after mapping the forward and reverse sequence to a variant reference sequence (NM_000927.4) which was obtained from GenBank.

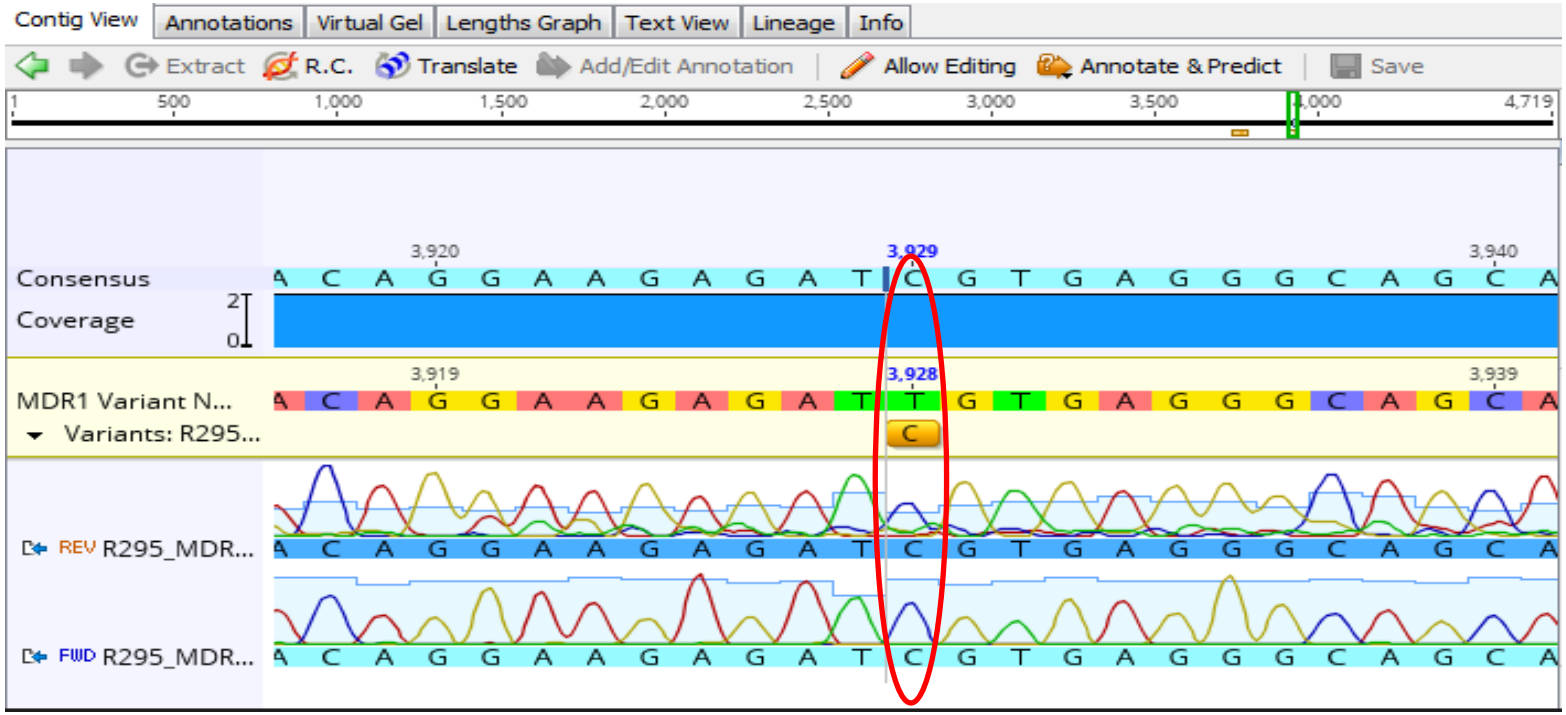

Figure 15: Representation of a contig view of the MDR1 sample for patient 1 (AHDR-R295) mapped to a variant reference sequence NM_000927.4. At position 3435 the test sequence possesses a homozygous CC genotype, and this differs from the variant sequence which has a T allele.

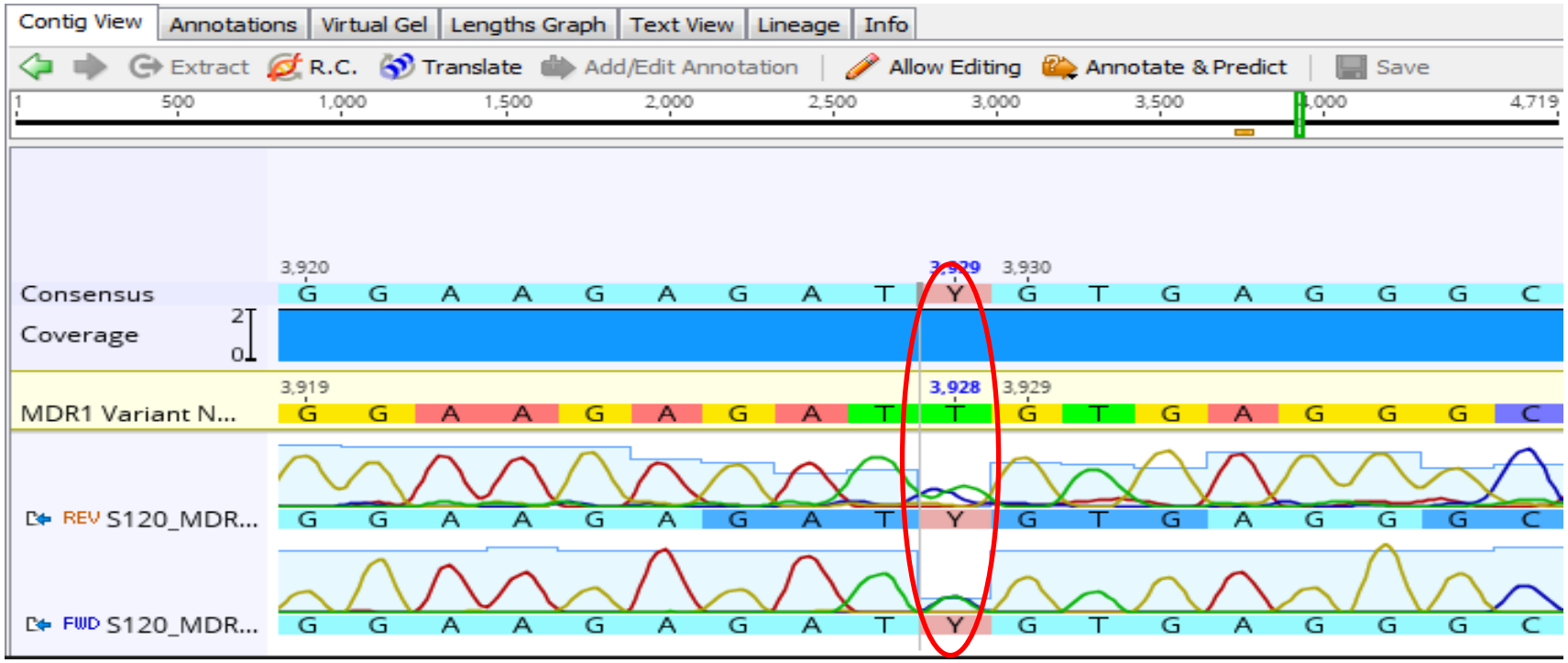

Figure 16: Representation of a contig view of the MDR1 sample for patient 20 (AHDR-S120) mapped to a variant reference sequence NM_000927.4. At position 3435 the test sequence possesses a heterozygous CT genotype.

3.5.3 Sequencing Results of the CYP2B6 Gene

Of the 20 samples sequenced for the CYP2B6 gene, 5 samples (25%) possessed the homozygous GG genotype; 8 samples (40%) had the heterozygous GT polymorphism, while 3 samples (15%) had the TT polymorphism. The G>T SNP is located at position 516 on the CYP2B6 gene, on exon 4; it is a transversion mutation and codes for an amino acid change from Q (Glutamine) to H (Histidine). Genotype determination was not possible for 4 sequences (20%) due to the poor nature of the sequence. **Figure 17** represents the contig view after mapping the forward and reverse sequence to a variant reference sequence (NG_007929.1) which was obtained from GenBank.

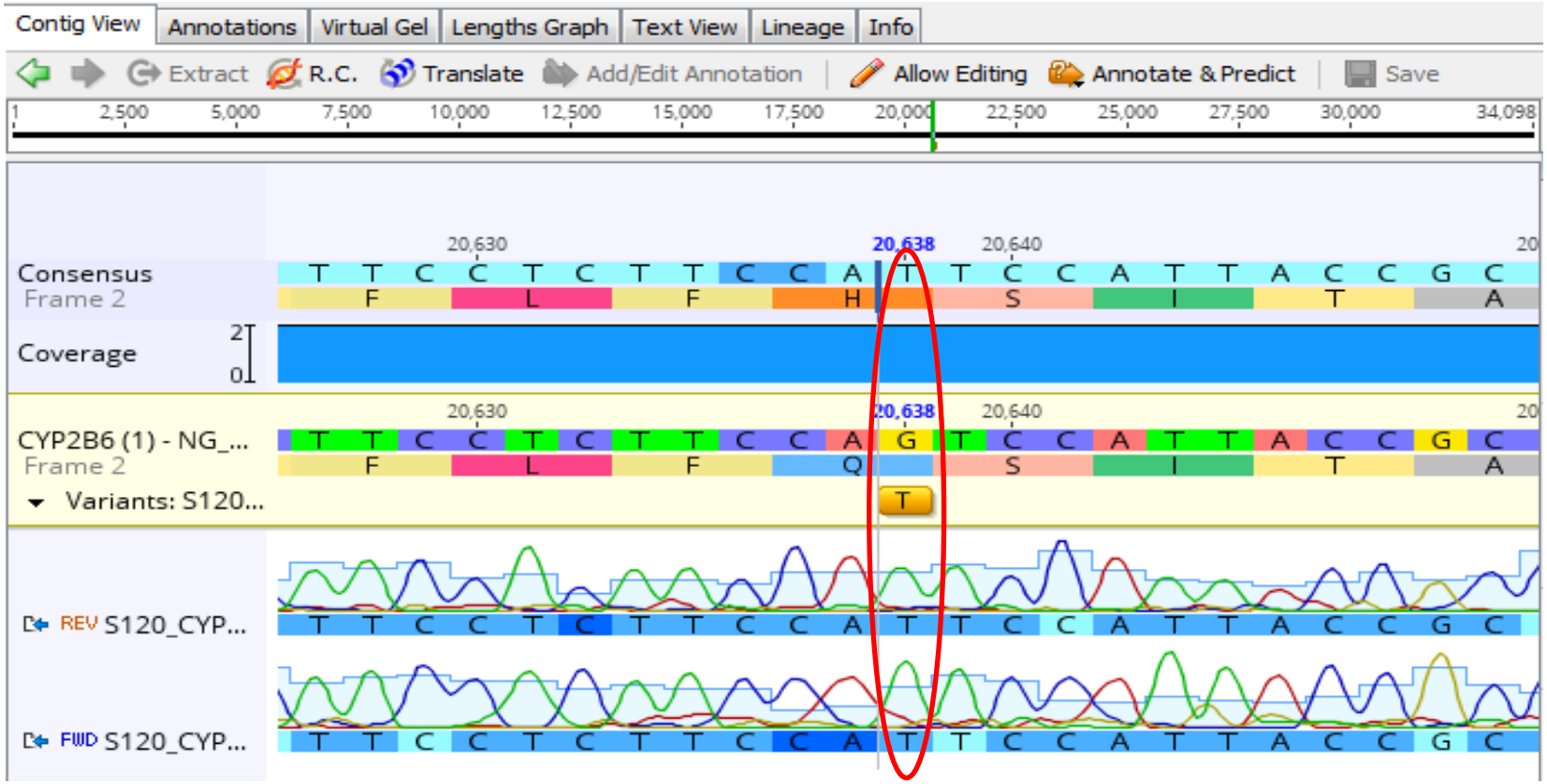

Figure 17: Representation of a contig view of the CYP2B6 sample for patient 20 (AHDR-S120) mapped to a variant reference sequence NG_007929.1. At position 516 the test sequence possesses a homozygous TT variant, and this differs from the reference sequence which has a G allele. This allele change results in a transversion amino acid change from a Q (Glutamine) to H (Histidine).

3.6 HARDY-WEINBERG EQUILIBRIUM

The Michael H. Court lab HW-5 calculator was used to determine whether the observed genotype frequencies are consistent with the Hardy-Weinberg Equilibrium (HWE). The variant allele frequency for position 3435C>T and 516G>T was 0.08 and 0.44, respectively. The P-value observed for the 3435C>T polymorphism in the MDR1 gene and the 516G>T polymorphism in the CYP2B6 gene indicates that these results are not statistically significant. However, confirming these results for a bigger population is needed.

Table 13: Observed and expected genotype frequencies of the MDR1 and CYP2B6 alleles in the surveyed population.

Genotype	**Observed # (Frequency)**	**Expected #**	**Variant Allele Frequency**	**X^2**	**X^2 P-value**
MDR1 3435C>T			0.08	0.131482834	0.716
CC	17	17.1			
CT	03	2.1			
TT	00	0.1			
CYP2B6 516G>T			0.44	0.004031242	0.949
GG	05	5.1			
GT	08	7.9			
TT	03	3.1			

Key: **X^2** – Chi Square; If P<0.05 – Not consistent with HWE; # - Number

3.7 CORRELATION BETWEEN PLASMA EFV CONCENTRATION, DRUG RESISTANT MUTATIONS and HUMAN GENETIC CHARACTERISTICS

To determine the correlation between treatment adherence, the development of drug resistant mutations and the impact of CYP2B6 and MDR-1 genes in ARV drug metabolism, the results obtained from all three aspects were jointly analyzed; see **Table 14**.Further analysis was done for patients who showed unexpected EFV concentrations in their plasma, as mentioned in **Section 3.2**.The analysis gave some insight notions why these samples may have had higher or lower than expected drug concentrations. This was explained by the presence of a SNP in either the transporter or metabolizing genes of the patient, with some patients having a SNP in both their transporter and metabolizing genes – see **Table 15**. Drug resistance analysis revealed that no study participant had any major drug NRTI or NNRTI associated resistance mutations; this study was unable to corroborate the relationship between drug resistance mutations and treatment adherence.

Table 14: Plasma EFV concentrations, human genetic characteristic (CYP2B6 and MDR1 genes) and baseline drug resistant mutations characteristics for all study participants.

EFV Drug Concentration (ng/ml)				Human Genetic Characteristics		Drug Resistant Mutations
Patient No.	**Sample Code**	**Baseline Concentration**	**Follow-Up Concentration**	**CYP2B6**	**MDR1**	**Major NRTI or NNRTI Mutations**
1	AHDR-R295	1, 183	2, 873	GG	CC	Negative Amplification
2	AHDR-R298	0, 000	2, 071	GT	CC	Negative Amplification
3	AHDR-R305	0, 000	3, 738	GG	CC	No Major Mutations
4	AHDR-R311	0, 000	3, 330	Not determined (poor sequence)	CC	Negative Amplification
5	AHDR-R315	0, 000	2, 419	GG	CC	Negative Amplification
6	AHDR-R318	0, 000	3, 562	GT	CC	No Major Mutations
7	AHDR-R328	0, 000	3, 450	GT	CC	Negative Amplification
8	AHDR-R331	0, 000	1, 283	GG	CC	No Major Mutations
9	AHDR-R340	0, 000	1, 688	GT	CC	No Major Mutations
10	AHDR-S88	0, 000	6, 685	TT	CC	Negative Amplification
11	AHDR-S92	0, 000	2, 671	GT	CC	Negative Amplification
12	AHDR-S94	769.9	1, 944	GT	CC	No Major Mutations
13	AHDR-S101	0, 000	506.8	GT	CT	Negative Amplification
14	AHDR-S102	0, 000	3, 661	GG	CC	No Major Mutations
15	AHDR-S106	0, 000	2, 999	Not determined (poor sequence)	CC	Negative Amplification
16	AHDR-S110	0, 000	BLQ	TT	CC	Negative Amplification
17	AHDR-S111	0, 000	1, 180	Not determined (poor sequence)	CC	No Major Mutations
18	AHDR-S113	0, 000	2, 781	GT	CC	Negative Amplification
19	AHDR-S117	0, 000	4, 358	Not determined (poor sequence)	CT	No Major Mutations
20	AHDR-S120	0, 000	15, 670	TT	CT	Negative Amplification

In patients who had unexpected drug concentration (shown in **Table 12**), patient 12 and 13, have the GT genotype, while patients 10, 16 and 20 have the TT SNP in their CYP2B6 gene – this change is associated with an increased plasma EFV concentration; patient 1 has the GG genotype, and genotype determination was not possible for patient 17 due to the poor nature of the sequence. Two of the three patients with the TT SNP (patient 10 and 20) have HIGH (Supra-therapeutic) EFV concentration, while the third person (patient 16) has sub-therapeutic concentrations (BLQ), even with the TT polymorphism present. Patient 19 also showed supra-therapeutic plasma EFV concentration, although the genotype was not determined. This is demonstrated in **Table 15**.

Analysis of the MDR1 genotypes in the aforementioned samples is as follows: three patients had the CT polymorphism (patient 13, 19 and 20), while four of them (Patient 1, 10, 12 and 16) had the CC genotype at position 3435. Patient 19 and 20, who have the CT genotype have HIGH plasma EFV concentrations, while patient 13 had plasma EFV concentrations below the expected range. Similarly, of the four samples with the CC genotype, two of them had concentrations within the expected drug range (patient 1 and 12), while one patient (patient 10) had high plasma EFV, and the other patient (patient 16) had below limits of quantification (BLQ) plasma EFV concentration. See **Table 15**.

Table 15: Plasma EFV concentrations and human genetic characteristic (CYP2B6 and MDR1 genes) for study participants with unexpected drug levels.

EFV Drug Concentration (ng/ml)				**Human Genetic Characteristics**	
Patient No.	**Sample Code**	**Baseline Concentration**	**Follow-Up Concentration**	**CYP2B6**	**MDR1**
1	AHDR-R295	1, 183	2, 873	GG	CC
12	AHDR-S94	769.9	1, 944	GT	CC
13	AHDR-S101	0, 000	506.8	GT	CT
16	AHDR-S110	0, 000	BLQ	TT	CC
10	AHDR-S88	0, 000	6, 685	TT	CC
19	AHDR-S117	0, 000	4, 358	Not determined (poor sequence)	CT
20	AHDR-S120	0, 000	15, 670	TT	CT

CHAPTER FOUR: DISCUSSION, RECOMMENDATIONS AND CONCLUSION

Being at least 95% adherent to treatment is required for HIV positive individuals on ART in order to achieve treatment success and prevent development of HIV DRMs which would likely yield virologic failure and eventually treatment failure (Nachenga et al., 2011). Various techniques can be used to measure treatment adherence (Gachara et al., 2017; Hornschuh et al., 2017; Evans et al., 2016). However, due to the subjectivity and bias of these techniques therapeutic drug monitoring methods are the methods of choice. This is because they have the ability to optimally measure the exact amount of ARV in a patient's hair, plasma or urine sample, with the use of robust techniques such as LC-MS/MS. A matrix like plasma is able to demonstrate the concentration of drugs present in a patient's sample for the last few hours to at most two days. However, individual variability has an impact on the way administered ARVs are transported and metabolized. This may be as a result of genetic polymorphisms, which likely affect the plasma concentration of drugs observed in a patient (Swart et al., 2012, Soeria-Atmadja et al., 2017). The aim of this study was to assess patient adherence to treatment by measuring EFV plasma concentration and using genomic markers of transporters and resistance to correlate discrepancies observed in the pharmacokinetic output.

Interestingly, two study participants (patient 1 and 12) showed drug concentrations levels ranging from 769.9 to 1,183 ng/ml at baseline. This could be attributed to the fact that, some patients are previously initiated on ART at another treatment facility, however, after relocation, they present themselves as new incoming patients to the health care provider and so are re-initiated. Post treatment, these patients are seen to have plasma EFV concentrations within the expected range. Several studies which have used TDM methods to measure patient adherence have rarely reported measuring drug concentrations at t=0 (Gounden et al., 2010; Ngaimisi et al., 2013; Ribaudo et al., 2010).This shows the importance of better history taking and access to digital patient records before a patient is initiated on treatment. This will enable the healthcare provider to have a full history of the patient, since inconsistencies in ART adherence such as these may lead to the development of drug resistance.

Although patient 13 and 16 have a synonymous change at position 516 of theCYP2B6 gene (demonstrated in **Table 15**) they still have plasma EFV concentrations below 1, 000 ng/ml. The presence of a G516T polymorphism in exon 4 of the CYP2B6 gene which results in an amino acid change from Glutamine to Histidine (Q to H) is responsible for the lower hepatic EFV clearance and higher amounts of EFV found in patient plasma (Sankatsing et al., 2004, Cressey and Lallemant, 2007). This means that the G>T SNP at position 516 leads to decreased catalytic CYP2B6 activity which results in increased plasma EFV concentrations and a greater probability of neuropsychological toxicity in patients with this SNP (Gounden et al., 2010).This means both patients were not adhering to their treatment. Marzolini and colleagues (2001) stated that, one major cause of low plasma levels is non-compliance, which suggests the omission of several consecutive doses. Also, in looking at the MDR1 genes of these patients, it can be seen that patient 13 has the CT genotype at position 3435, while patient 16 has the homozygous CC wildtype. The CT genotype is associated with better immune recovery while the CC genotype implies a lower concentration of substrate in plasma. However, the concentration of EFV in patient 13 still does not reflect the impact of the SNPs in their transporter and metabolizing genes. All this indicates that these patients were not adhering to treatment. After drug resistance analysis, no drug resistance mutations were observed in these patients.

The patients who had supra-therapeutic levels of EFV in their plasma showed the presence of either a GT or TT genotype in their CYP2B6 gene; except for the patient whose genotype could not be determined. This finding is consistent with other studies which have shown that the presence of the G516T polymorphism in the CYP2B6 gene leads to higher plasma EFV concentrations (Puthanakit et al., 2009; Gounden et al., 2010) and may lead to toxicity in the CNS of the patient. Symptoms of intoxication range from mild e.g. dizziness, irritability, euphoria, which is usually well tolerated, to more severe neurological symptoms such as delusion, paranoia, depression, which occur in roughly 2% of individuals on EFV based ART (Apostolova et al., 2015). Symptoms of toxicity were not reported by any patient; the main aim of this study was to determine EFV concentration in plasma to investigate patient adherence patterns. Hence the questionnaire was not designed to inquire about the effect of the ART in the newly initiated patients. Therefore, there was no report about how patients are reacting to their drugs. However, it is important for patients to be correctly monitored, so that in the case of adverse drug reactions (ADR) occurrence, this can be reported immediately. Van Graan and colleagues in their retrospective study reported that 98.8% of ADRs were reported by medical doctors, compared to the 0.9% reported by professional registered nurses (Van Gran et

al., 2018). The benefits of long-term reporting of ADRs when correct ADR reporting is coupled with closer interaction between healthcare professionals and pharmacovigilance centers was highlighted by Gupta and Udupa in their 2011 study (Gupta and Udupa, 2011). Hence, the importance of pharmacovigilance (PV) should be emphasized among healthcare professionals. Soeria-Atmadja (2017) stated that developing CNS toxicity is dependent on the concentration of EFV, and this is mostly evident during the first week of treatment, since tolerance is developed by the patient later, and the effects become more subtle. Patient 19 and 20 carry the heterozygous CT SNP, implying that EFV drug levels are maintained in the intestinal epithelial cells and in the plasma, hence there will be a better drug efficacy and minimal chances of drug resistance accumulation. Patient 10 on the other hand has a homozygous CC allele at position 3435, meaning there is more of EFV exported out of the intestinal epithelial cells, hence there will be less of the drug in the patient's plasma sample (Dong et al., 2009); however, this patient has >4000ng/ml of EFV. Mixed views have been expressed with regards to the effects of the C3435T SNP on observed plasma concentrations (Cressey and Lallemant, 2007, Dandara et al., 2011, Swart et al., 2012). Hence, it cannot be concluded whether this C3435T SNP affects EFV plasma concentration or not. From the observation in this study, it can be said that this genotype impacted the amount of drug metabolized by the CYP2B6 gene, implying that if less of the drug is transported, then less of the drug is metabolized. As a result, the G516T SNP was only able to over-express what the drug transporter made available.

Interestingly, there were some patients who had the CYP2B6 heterozygous GT genotype, but did not present with any above limit quantifications of EFV in their plasma. This could be attributed to the role of inter-patient variability such as drug-drug interactions, especially for ARV drugs taken which are simultaneously metabolized by the CYP450 enzyme family (Cressey and Lallemant, 2007). Also, the bioavailability of ARVs can be reduced considerably by food, disease state, pregnancy, as well as the sex of a patient (Pavlos and Phillips, 2012).

This study is in agreement with other studies which have shown that patient adherence to treatment can be determined using TDM methods (Marzolini et al., 2001; Van Zyl et al., 2011). In addition, this study has confirmed that the homozygous CC wildtype is more prevalent in people of African descent, rather than CT or TT genotypes of the C3435T polymorphism (Dandara et al., 2011, Swart et al., 2012).As indicated by these studies, a clear conclusion cannot be made about the impact of the C3435T polymorphism on the pharmacokinetic output observed, since some studies have found

an association between the CT and TT polymorphism with better immunological output, whereas others have not. Similarly, the CYP2B6 G516T polymorphism has been proven to be prevalent in the black population, when compared to people of other ethnicities (Barco and Rodriguez-Novoa , 2013); this is corroborated in this study with a prevalence of 40% and 15% of the GT and TT genotype, respectively. Several studies have also reported an association between the CYP2B6 516G>T polymorphism and increased plasma EFV levels, since the heterozygous GT and homozygous TT genotypes are known to be intermediate and poor metabolizers, respectively (Mukonzo et al., 2009; Swart et al., 2016). All but one patient (patient 16) in this study who carried the homozygous variant allele (TT) are seen to possess supra-therapeutic plasma EFV concentrations, which is in agreement with what is observed in other studies. However, the effect of the heterozygous GT polymorphism on plasma EFV concentrations is not seen in this study population; in this study, all but one patient (patient 13) having the heterozygous GT allele and are seen to have plasma EFV concentrations within the expected range of 1, 000 – 4, 000ng/ml.

From the results obtained in this project, it can be recommended that more TDM studies should be conducted in the Northern part of South Africa, so as to have better insight about the adherence pattern of individuals on antiretroviral therapy. This should be done in parallel with characterization of single nucleotide polymorphisms present in the transporter and metabolizing genes of the patients, since inter-individual differences are an important consideration to assess drug efficacy, safety, as well as the overall treatment outcome.

One major limitation in this study was the sample size; having a bigger sample size would have given a better picture about treatment adherence in this population, as well as what influence patient inter-individual differences have on the pharmacokinetic output observed. Also, due to time constraints the positively amplified follow-up samples for the Pol gene were not sequenced; this additional data would have given more understanding, about what influence DRMs have on adherence, should there have been any identified.

Future studies should investigate the mechanism by which the C3435T polymorphism impacts P-gp expression in the MDR1 gene. Also, it would be interesting to know the extent to which they impact what is observed at the CYP2B6 level, as well as the pharmacokinetic outcomes. Furthermore, estimating patient adherence to treatment using patient hair samples should be considered. This information will add knowledge to what is known about the South African population in terms of

adherence to treatment using TDM methods, especially since it does not have the intra-individual variability present in single plasma levels. More genetic characterization studies should also be conducted in the various ethnic groups in South Africa, in order to know what transport and metabolism genotypes are prevalent in the population. This knowledge will be helpful to healthcare providers and direct ART dose recommendations especially in those presenting symptoms of adverse drug reactions when initiated to treatment.

It can be concluded that the study participants were adhering to treatment. This is evident in the 90% of study participants who were adherent to treatment; 10% of them showed lower than expected EFV concentrations, implying they were non-adherent to their treatment. However, because plasma drug concentrations only reflect a patient's adherence pattern for a few hours to at most two days, the adherence patterns of these individuals cannot be concluded with certainty. It can be concluded with certainty that the concentration of ARVs in patient plasma can be used as a biomarker to estimate adherence to treatment; this is evident in the patients who possess sub-therapeutic plasma EFV concentrations. In addition, the presence of a SNP in a patient's transporter or metabolizing gene influences the drug availability in their system. The only drawback of using TDM methods is its cost, since it cannot be applied in non-research settings.

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APPENDICES

Appendix 1: Patient demographic data collected before blood collection from all 20 study participants

Sample Code	Sex	Age	Marital Status	Probable Year of Infection	Risk Factor	Current ARV Regimen	WHO Stage of AIDS	Use of Complimentary Medicine	CD4 Count (Cells/ µl)	Viral Load (counts/ ml)	Use of Complimentary Medicine (Follow-up)	CD4 Cells (Cells/µl) Follow-Up	Viral Load (counts/ml) Follow-Up
AHDR-R295	F	49	M	2015	Sexual	TFE	1	None	547	207	None	570	ND
AHDR-R298	No Info	No Info	No Info	No Info	No Info	TFE	No Info	No Info	497	6, 950	None	No Info	ND
AHDR-R305	F	32	S	2016	Sexual	TFE	1	None	No Info	31, 100	None	No Info	<20
AHDR-R311	No Info	No Info	No Info	No Info	No Info	TFE	No Info	No Info	No Info	137, 000	None	No Info	68
AHDR-R315	F	56	M	2016	Sexual	TFE	1	None	416	919, 000	None	TLTD	No Info
AHDR-R318	F	40	S	2016	Sexual	TFE	1	None	113	2, 600, 000	None	TLTD	No Info
AHDR-R328	F	37	S	2017	Sexual	TFE	1	None	484	No Info	INH	No Info	45
AHDR-R331	F	36	M	2017	Sexual	TFE	1	None	354	No Info	None	No Info	ND
AHDR-R340	F	37	S	2010	Sexual	TFE	1	None	278	No Info	None	No Info	90
AHDR-S88	F	32	M	2014	Sexual	TFE	1	None	191	16, 900	INH	160	ND
AHDR-S92	F	47	M	2015	Sexual	TFE	1	None	28	254, 000	INH	167	ND
AHDR-S94	F	54	M	2013	Sexual	TFE	1	None	101	100, 000	None	No Info	<20
AHDR-S101	M	31	S	2015	Sexual	TFE	1	None	500	9, 120	INH	No Info	ND
AHDR-S102	F	44	W	2015	Sexual	TFE	1	None	567	2, 746, 469	None	TLTD	50
AHDR-S106	F	34	S	2017	Sexual	TFE	1	None	334	68, 941	INH	TLTD	31
AHDR-S110	F	30	S	2016	Sexual	TFE	1	None	174	17, 600	None	No Info	<20
AHDR-S111	F	36	S	2016	Sexual	TFE	1	None	1068	6, 400	None	TLTD	ND
AHDR-S113	F	55	M	2014	Sexual	TFE	1	None	88	5, 490	INH	1148	ND
AHDR-S117	F	42	S	2015	Sexual	TFE	1	Bactrim	85	84, 600	INH	261	ND
AHDR-S120	F	38	S	2009	Sexual	TFE	1	None	186	3, 490	INH	415	<20

The table displays the sample code given to each patient, depending on the clinic the sample was collected for example AHDR-R denotes a sample collected from Rethabile clinic, while AHDR-S indicates a sample collected from the Seshego clinic.

Key: Male – M, Female – F; Married – M, Single – S, Widowed – W; Not detected – ND; Too Low to Detect – TLTD; Tenofovir + Emtricitabine + Efavirenz – TFE

Appendix 2: Results obtained after submission of baseline sequences to the Stanford HIV Drug Resistance Algorithm.

No.	Sample Code	Subtype	Major NRTI Mutations	Major NNRTI Mutations	Other Mutations	Interpretation
1	AHDR-R305	C	None	None	K173T, Q174K, D177E, T200A, Q207E, R211K, V245Q, K281R, T286A, E291D, V292I, I293V, D320E	Susceptible to all NRTI and NNRTI drugs
2	AHDR-R318	C	None	None	K173A, D177E, I178L, I195L, T200A, E204K, Q207E, R211K, V245Q, A272S, K281R, T286A, T290S, E291D, V292I, I293V, D320E, S322I	Susceptible to all NRTI and NNRTI drugs
3	AHDR-R331	C	None	None	K173A, Q174K, D177E, T200A, Q207E, R211K, V245Q, A272P	Susceptible to all NRTI and NNRTI drugs
4	AHDR-R340	C	None	None	K173A, Q174K, D177E, T200A, Q207A, R211K, V245Q, K277R, A288S, I293V, D320E, S322I	Susceptible to all NRTI and NNRTI drugs
5	AHDR-S94	C	None	None	V21I, V35T, T39E, S48T, V60I, K122E, D123N, K173A, D177E, I178L, G196E, T200A, V245E, D250E, A272P, E291D, V292I, I293V, Q334S	Susceptible to all NRTI and NNRTI drugs
6	AHDR-S102	C	None	None	T131P, S162C, P170S, K173A, D177E, T200A, Q207E, R211K, V245Q, A272P, T286A, E291D, V292I	Susceptible to all NRTI and NNRTI drugs
7	AHDR-S111	C	None	None	S162A, K173T, T200A, Q207E, R211K, V245K, D250E, A272P, E291D, V292I, I293V, D320E, S322I	Susceptible to all NRTI and NNRTI drugs
8	AHDR-S117	C	None	None	K173A, Q174A, D177E, G196K, T200A, Q207A, V245Q, A272P, K277R, T286A, E291D, V292I, I293V	Susceptible to all NRTI and NNRTI drug