Pharmacogenetics of efavirenz response in Bantu-speaking South African HIV/AIDS patients

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Abbreviations

°C	degrees Celsius
µg	microgram
3’	three prime end
5’	five prime end
A	adenine
ABCB1	ATP-binding cassette subfamily B1
ADMET	absorption, distribution, metabolism, excretion and transport
ADR	adverse drug reaction
AHR	aryl hydrocarbon receptor
AIDS	acquired immune deficiency syndrome
ALT	alanine transaminase
ART	antiretroviral treatment
ARV	antiretroviral drug
AST	aspartate transaminase
BMI	body mass index
bp	base pair
C	cytosine

CAR	constitutive androstane receptor
CD-4	cluster of differentiation 4
CNS	central nervous system
Conc.	concentration
CYP	cytochrome P450
dNTP	deoxynucleotide triphosphate
ddNTP	dideoxynucleotide triphosphate
dF	degrees of freedom
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EFV	efavirenz
EM	extensive metaboliser
et al.	*et alii*
F	forward
FDA	Food and Drug Administration
g	g-force
G	guanine
HAART	highly active antiretroviral therapy

HCl	hydrogen chloride/hydrochloric acid
HDACs	histone deacetylase complexes
HIV	human immunodeficiency virus
IM	intermediate metaboliser
IU	international units
l	litre
LC	liquid chromatography
LD	linkage disequilibrium
MDR1	multidrug resistance gene 1
mg	milligram
$MgCl_2$	magnesium chloride
miRNAs	microRNAs
ml	millilitre
mM	millimolar
mmol	millimole
MS	mass spectrometry
MS/MS	tandem mass spectrometry
ng	nanogram

N-gln	N-glucuronide
nm	nanometre
NNRTI	non-nucleoside reverse transcriptase inhibitor
NRTI	nucleoside/nucleotide reverse transcriptase inhibitor
NRs	nuclear receptors
OH	hydroxy/hydroxyl
PCR	polymerase chain reaction
P-gp	P-glycoprotein
PI	protease inhibitor
PM	poor metaboliser
pM	picomolar
PXR	pregnane X receptor
R	reverse
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RPM	revolutions per minute
RXR	retinoid X receptor
SD	standard deviation

sdH_2O	sterile distilled water
SNP	single nucleotide polymorphism
T	thymine
T_a	annealing temperature
Taq	*Thermus aquaticus*
TBE	Tris/Borate/EDTA
TE	Tris EDTA
T_m	melting temperature
Tris	tris(hydroxymethyl)aminomethane
U	unit
UCT	University of Cape Town
UM	ultrarapid metaboliser
UV	ultraviolet
V	volts
v/v	volume per volume
w/v	weight per volume
WHO	World Health Organisation

Abstract

Introduction: Efavirenz (EFV) is used in first-line antiretroviral (ARV) therapy of HIV-infected patients, and is principally metabolised by CYP1A2, CYP2A6, CYP2B6, CYP3A4/5 and the drug transporter ABCB1. Genetic variability in the above genes may contribute to differences in EFV plasma concentrations which affect the levels of viral suppression as well as development of side effects in patients. The aim of this project was to evaluate the effects of the different genetic polymorphisms on EFV plasma concentration levels in HIV/AIDS patients receiving first-line antiretroviral treatment (ART) containing EFV.

Methods: Patients were recruited from Themba Lethu Clinic in Gauteng, South Africa. PCR/RFLP and SNaPshot were used to genotype for SNPs in *CYP1A2, CYP2A6, CYP2B6, CYP3A4/5* and *ABCB1* among 464 Bantu-speaking South Africans (301 HIV/AIDS patients and 163 controls). EFV drug plasma concentration levels were measured using LC-MS/MS.

Results: We report baseline frequencies of the variant alleles of genetic polymorphisms in these genes in the Bantu-speaking South African population and their correlation, alone or in combination, with EFV plasma concentration. *CYP2B6 516T, 785G* and *983C* alleles were associated with significantly high EFV plasma levels; while the *CYP1A2 -163A* allele showed a trend towards low EFV plasma levels in smokers. Combining *CYP2B6 516T, 785G* and *983C* and *CYP1A2 -163A* alleles resulted in better prediction of EFV plasma levels.

Conclusion: *CYP1A2* and *CYP2B6* are predictors of EFV plasma concentration in the Bantu-speaking South African population, The *CYP2B6 516T/T, 785G/G* and *983C/C* and *CYP1A2 -163A/A* genotypes may be useful in deciding on the starting dosages or HAART regimens to use.

Chapter 1: Introduction

1.1. HIV/AIDS epidemiology

The global burden of disease due to HIV-infection is extensive and over 33 million individuals [31.4-35.3 million] were living with HIV at the end of 2009. The HIV/AIDS prevalence in Africa varies substantially from as low as <1% in North Africa, to as high as 28% in Southern Africa (Figure 1.1). In Botswana and South Africa approximately 300 000 and 5.6 million individuals were HIV-infected at the end of 2009, respectively. AIDS-related deaths in 2009 were estimated at about 6000 and 300 000 in Botswana and South Africa, respectively. In South African adults of 15 - 49 years, the incidence of new HIV infections in 2009 was 1.49[1]. This evidently illustrates the need for effective management of HIV/AIDS in South Africa.

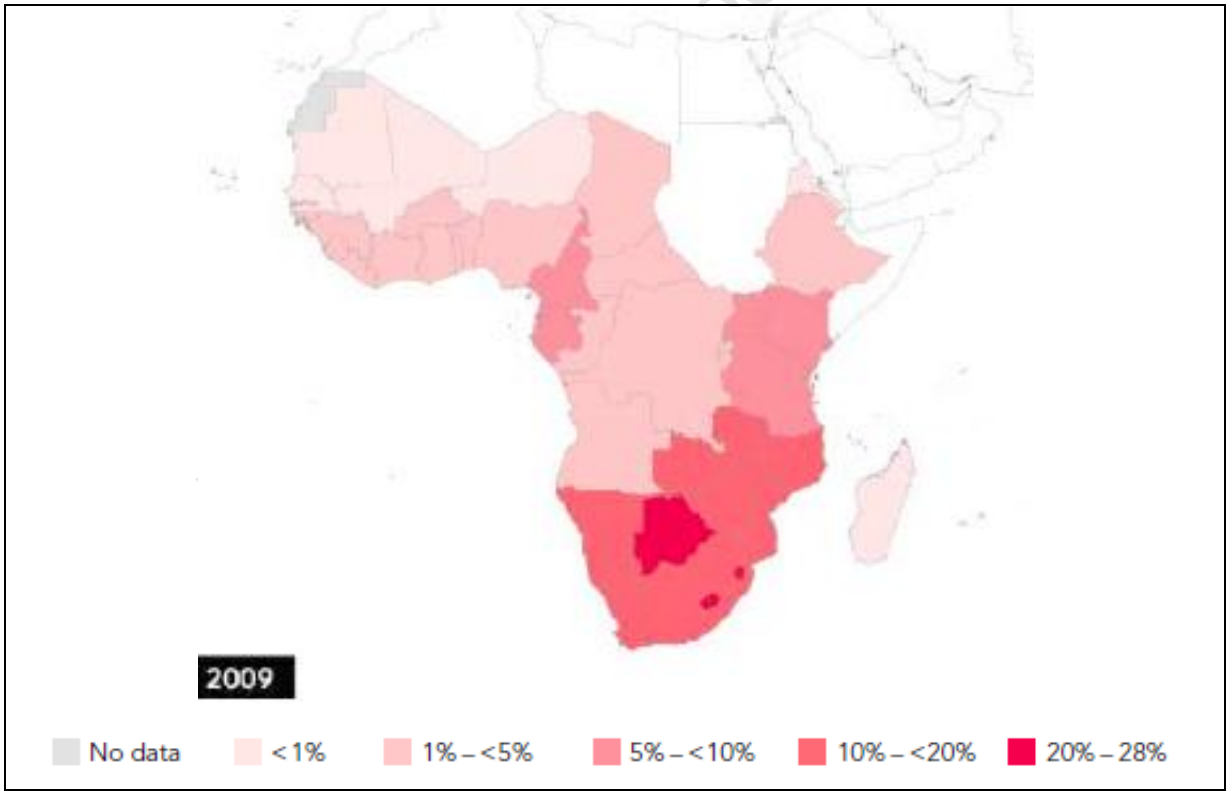

Figure1.1: Map showing the HIV prevalence in sub-Saharan Africa at the end of 2009[1].

Susceptibility to HIV-infection and progression into acquired immune deficiency syndrome (AIDS) is dependent on host genetic factors. The human leukocyte antigen (HLA) B*5701 class I allele is an example of a genetic factor that has been associated with slow progressing to HIV/AIDS in patients with stable CD-4 cell counts and HIV RNA levels < 50 copies/ml after two years of untreated infection[2]. With the high HIV prevalence in South Africa, it is priority to develop HIV/AIDS prevention programmes.

1.2. Prevention of HIV/AIDS

A number of HIV prevention interventions for the South African youth, including school and community-based programs (e.g. the Tshwane peer education and support programme) have a noteworthy impact on the improvement of HIV-related risk behaviours including condom use, HIV testing and alcohol use[3]. Large scale national AIDS awareness campaigns have been implemented, but the effect of these campaigns have not been systematically reviewed.

Another approach to prevention of HIV/AIDS is the prevention of mother-to-child transmission (PMTCT). Guidelines proposed in 2010 include recommendations such as initiation of prophylaxis at 14 weeks of pregnancy for all HIV positive pregnant women[4]. However, besides the prevention of mother-to-child transmission, South Africa has an extensive antiretroviral therapy (ART) program involving more than a million HIV/AIDS patients[1].

1.2.1. Antiretroviral therapy (ART)

Major progress in treatment of HIV/AIDS has been made since the discovery of the HI virus. Currently there are over 20 ARV drugs that function by targeting different steps in the HIV replication cycle. Table 1 lists all the ARV drugs that have been approved by the Food and Drug Administration (FDA) as of 31 July 2011. Approved ARV drugs are divided into six classes based on their mechanism of action[5]. Figure 1.2 is a diagrammatical presentation of the sites of action of the various ARV classes[6].

Table 1: FDA approved ARV drugs (as of July 31, 2011).

Class	ARV	Abbreviation	Mechanism of action
Entry inhibitors (CCR5 antagonists, Fusion inhibitors)	enfuvirtide	T-20	Prevent virus attachment to the membrane receptors and interfere with the membrane insertion processes.
	maraviroc	MVC	
Integrase inhibitors	raltegravir	RAL	Inhibit HIV integrase to prevent insertion into the host genome.
Protease inhibitors (PIs)	amprenavir	APV	Inhibit HIV protease from assembling infectious virions.
	fosamprenavir	FOS	
	atazanavir	ATV	
	darunavir	DRV	
	indinavir	IDV	
	lopinavir	LPV	
	ritonavir	RTV	
	nelfinavir	NFV	
	saquinavir	SQV	
	tipranavir	TPV	
Nucleoside/Nucleotide reverse transcriptase inhibitors (NRTIs)	lamivudine	3TC	Mimic nucleotides and is incorporated into viral DNA leading to chain termination.
	abacavir	ABC	
	zidovudine	AZT or ZDV	
	stavudine	d4T	
	didanosine	ddI	
	emtricitabine	FTC	
	tenofovir	TDF	
Non-nucleoside reverse transcriptase inhibitors (NNRTIs)	delavirdine	DLV	Inhibit HIV reverse transcriptase by disrupting the shape of the catalytic pocket and deter substrate attachment.
	efavirenz	EFV	
	etravirine	ETR	
	nevirapine	NVP	
	rilpivirine	RPV	
Recommended ARV Combinations:			
South Africa 1st line	TDF+3TC/FTC+EFV/NVP, d4T+3TC+EFV or AZT+3TC+EFV		
South Africa 2nd line	TDF+3TC/FTC+LPV/r or AZT+3TC+LPV/r		
Preferred WHO recommendations	TDF+3TC/FTC+EFV/NVP or AZT+3TC+EFV/NVP for 1st line and TDF+3TC/FTC+LPV/r or AZT+3TC+LPV/r for 2nd line.		

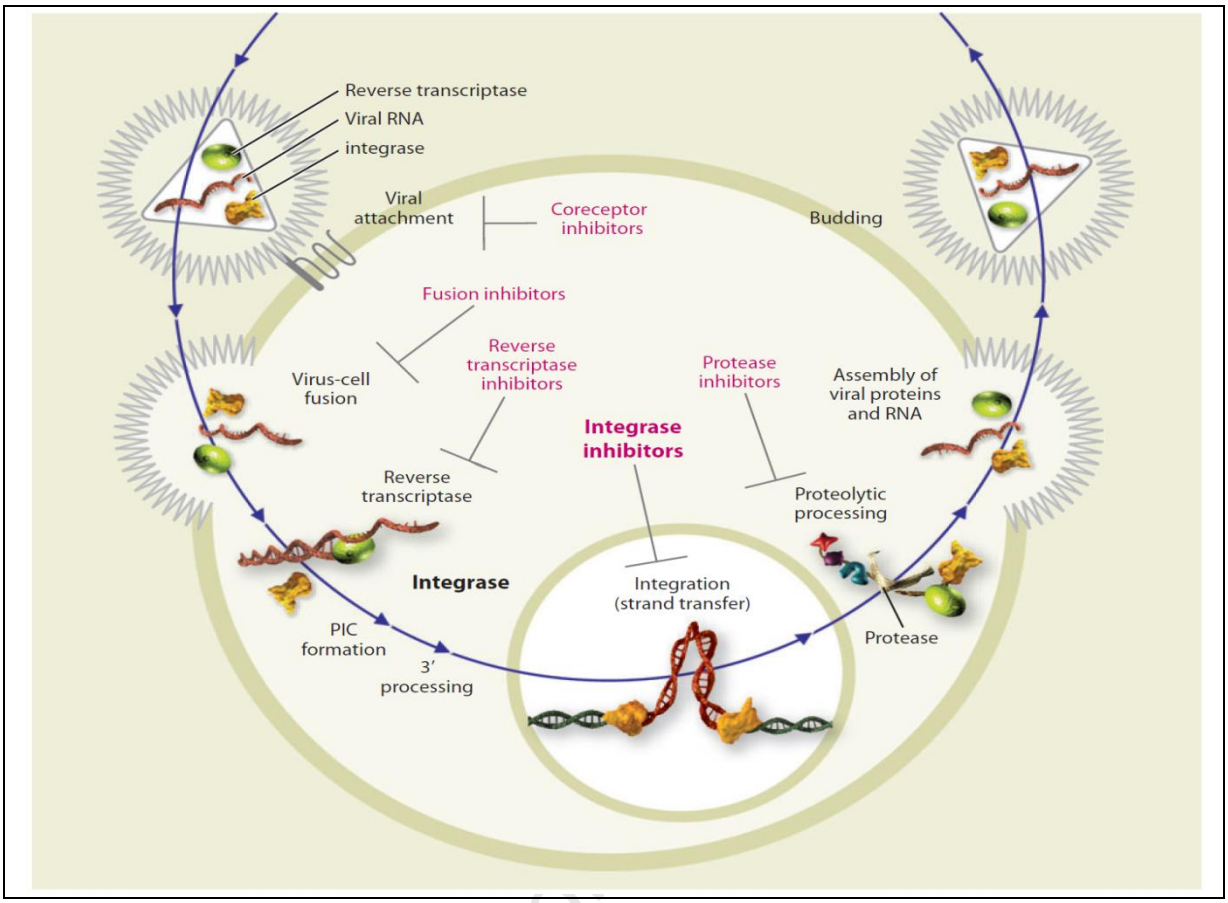

Figure 1.2: Diagrammatical presentation of the sites of action of the various ARV classes (Adapted from Hazuda *et al.* [6]).

As shown in Figure 1.2, fusion inhibitors prevent viral entry by binding to the viral envelope, while the integration of viral DNA into host DNA is avoided by integrase inhibitors. PIs bind to viral protease and prevent the cleavage of HIV proteins into mature viral particles[7, 8]. NNRTIs (e.g. EFV) function by binding non-competitively to HIV reverse transcriptase and lead to a change in its structure and function. The reverse transcriptase enzyme is then incapable of converting viral RNA into DNA preventing HIV viral replication[9].

A combination of the drugs used in primary treatment is more effective in HIV/AIDS management and is commonly referred to as highly active antiretroviral therapy (HAART). HAART consists of three or more antiretroviral drugs from two different classes[10]. ARV regimens combine two

NRTIs with a NNRTI, one or two PIs, or a third NRTI depending on the ARV treatment recommendations of the country[11].

1.2.2. ARV treatment recommendations

The World Health Organisation (WHO) recommends ART initiation for all HIV/AIDS individuals with a CD-4 cell count $\leq$ 350 cells/mm[3]. First-line therapy is recommended as a combination of a NNRTI and two NRTIs (e.g. lamivudine). Second-line ART consists of a ritonavir-boosted PI (atazanavir or lopinavir) and two NRTIs (zidovudine or tenofovir as used in first-line therapy)[12].

In South Africa approximately 1.2 million people are currently undergoing ARV therapy at state hospitals[1]. All HIV/AIDS adults are started on a combination of 300 mg tenofovir (TDF), 150 mg lamivudine (3TC) or 200 mg emtricitabine (FTC) every 12 hours and 600 mg efavirenz (EFV) at night. Nevirapine (NVP) is used in cases where women cannot guarantee proper contraception or in cases of pregnancy during ARV therapy, because of the teratogenicity of EFV[13]. NVP is given in first-line treatment at a lead-in dose of 200 mg daily for two weeks followed by 200 mg every 12 hours. The use of stavudine (d4T) is gradually being decreased due to its toxicity, thus it has been replaced by TDF, but is being continued in patients tolerating it. Patients presenting with renal disease are put on regimens containing AZT instead of TDF. In the South African setting, patients who do not respond to first-line treatment are switched to a combination of TDF, 3TC/FTC and LPV/r or AZT, 3TC and LPV/r, depending on the reason for treatment failure[14]. Although roughly 700 000 life years among adults have been gained due to ART between 1996 and 2009, several complications such as the development of cardiovascular disorders and adverse effects were encountered[1].

1.3. Complications of ARV treatment

1.3.1. Treatment failure and viral resistance

A considerable number of patients experience viral rebound and clinical progression to AIDS. Treatment failure, often defined as two consecutive viral load values of more than 5000 copies/ml, can be explained by the emergence of drug-resistant HIV and difficulties in maintaining long-term adherence to treatment[15]. Prolonged auto-induction of the enzymes involved in ARV metabolism can also lead to sub-therapeutic plasma concentration levels[16]. Repeated exposure to sub-therapeutic ARV (e.g. EFV) drug plasma concentration levels can lead to the appearance of EFV-resistant mutants in patients with low trough concentration levels. A single mutation (K103N) in the HIV reverse transcriptase gene has been reported to induce a high level of phenotypic resistance[17]. The combination of ARV drugs as well as concomitant treatment with other drugs can lead to significant drug-drug interactions. These interactions may also result in either sub-therapeutic or toxic drug concentration levels with a high risk for treatment failure or adverse drug reactions[9]. Treatment failure necessitates the change to different drug combinations or four drug regimens including both a NNRTI and a PI[11], which is expensive. Lifelong therapy is thus necessary to delay the development of drug-resistant viral strains, suppress HIV replication and reduce viral load by maintaining high drug levels in plasma and tissue[9]. Failure of ARV treatment to permanently eliminate HIV-infection after years of treatment and the development of ADRs are major limitations[8].

1.3.2. Adverse drug reactions (ADRs)

The most common reason for poor adherence is the development of ADRs after therapy initiation[18]. In the United States approximately 100 000 deaths per year are caused by ADRs[19]. Up to 40% of patients receiving EFV treatment experience central nervous system (CNS) adverse events during the first month of treatment. The CNS disturbances are usually mild to moderate in

severity and range from dizziness, insomnia, skin rash, bloating, nausea, diarrhoea and fatigue to hallucinations and dreams. However, the CNS adverse effects disappear in most of the HIV/AIDS patients and persist only in a proportion of individuals. EFV side effects occur more frequently and more severely in patients with high plasma concentrations of EFV. EFV plasma concentrations can thus be used to predict the efficacy of treatment and the occurrence of CNS ADRs[20, 21]. Discontinuation of EFV treatment occurs in approximately 4% of patients if adverse events are severe and persistent. The therapeutic range of EFV has been reported to be between 1 µg/ml and 4 µg/ml and patients with sub-therapeutic plasma concentrations (below 1 µg/ml) have a higher risk of treatment failure and the development of drug resistance. An increased risk of CNS side effects resulted from plasma concentrations above 4 µg/ml[17]. Considerable inter-patient variability in response to EFV therapy has been observed, despite administration of the same EFV dosage. The inter-patient variability complicates the treatment of HIV/AIDS individuals notably. Other ARV drugs are associated with different ADRs but this project focussed on treatment with EFV.

In the standard dose-response paradigm (excluding variability caused by non-adherence), pharmacokinetics (PK) describes the study of drug absorption, distribution, metabolism, elimination and toxicity (ADMET), while the relationship between drug plasma concentration and response to treatment is described by pharmacodynamics (PD). Inter-individual variability in drug and xenobiotic metabolism is extensive, resulting in ineffective drug treatment. ARV exposure in the human body is affected by the patient's physiological parameters (e.g. age, body weight, gender, ethnicity, pregnancy and renal function), secondary diseases, drugs treating secondary diseases, diet and genetic polymorphisms in DMEs and transporters[22, 23]. Genetic variation accounts for a considerable amount of inter-individual variability and leads to impaired drug metabolism and transport (reviewed by Ingelman-sundberg [24]). For example, Autar *et al.* [25] reported on inter-individual variability in drug plasma concentration for ritonavir boosted saquinavir in Thai and UK patiens. This inter-individual variation has serious implications in drug development[26].

As mentioned before, HAART, whose backbone is the concomitant administration of a combination of drugs, has the potential to result in adverse drug-drug interactions. These interactions can influence ADMET and complicate treatment[11, 27]. HIV/ AIDS patients are more prone to infection with *Mycobacterium tuberculosis* (TB) thus inevitably end up requiring treatment for both HIV/AIDS and TB. The co-administration of ART and anti-tubercular therapy is complicated by the resulting drug-drug interactions. The drugs used for TB treatment, rifampicin, pyrazinamide, isoniazid, and ethambutol, are substrates, inducers or inhibitors of enzymes involved in the metabolism of ART. Rifampicin is an effective inducer of the CYP2B6 and CYP3A4/5 enzymes which are involved in the metabolism of both EFV and NVP[28, 29]. Rifampicin-based anti-tubercular therapy has been reported to reduce EFV and NVP plasma concentrations by up to 30%[30].

1.4. Metabolism of ARVs

ARVs from the different classes are metabolized by diverse enzymes in the small intestine and liver. Cytochrome P450 (CYP) enzymes are responsible for the bulk of all drug metabolism of clinically, physiologically, and toxicologically important compounds[31, 32]. Table 2 shows some of the compounds and drugs that are metabolized by enzymes that have been reported to play a role in the metabolism of efavirenz and which form the basis of this project[33].

Table 2: Examples of drugs that are metabolized by enzymes that participate in efavirenz metabolism (ARV drugs are highlighted).

DMEs	Substrates	Inhibitors	Inducers
CYP1A2	caffeine	amiodarone	modafinil
	clozapine	fluvoxamine	omeprazole
	verapamil	methoxsalen	tobacco
	phenytoin	mibefradil	insulin
	warfarin	ticlpidine	methylcholanthrene
	acetaminophen	ciprofloxacin	nafcillin
	efavirenz	cimetidine	beta-naphthoflavone
	tipranavir	interferon	
	ritonavir		
	tamoxifen		
	rifampicin		
CYP2A6	coumarin	ketoconazole	phenobarbital
	tegafur	methoxsalen	rifampicin
	nicotine	pilocarpine	pyrazole
	5-fluorouracil	cannabidiol	dexamethazone
	efavirenz		
CYP2B6	bupropion	clopidogrel	phenobarbital
	diazepam	thiotepa	phenytoin
	efavirenz	ticlopidine	rifampicin
	nevirapine		
	nicotine		
	ibuprofen		
	cyclophosphamide		
	ifosfamide		
	methadone		
CYP3A4/5	alprazolam	**delaviridine**	rifabutin
	midazolam	**indinavir**	**efavirenz**
	indinavir	**ritonavir**	**nevirapine**
	nelfinavir	**saquinavir**	phenobarbital
	ritonavir	**nelfinavir**	rifampicin
	simvastatin	clarithromycin	barbiturates
	amlodipine	erythromycin	phenytoin
P-glycoprotein (ABCB1)	**amprenavir**	midazolam	cyclosporine
	indinavir	tamoxifen	**efavirenz**
	nelfinavir	amiodarone	cisplatin
	rifampicin	erythromycin	doxorubicin
	ritonavir	simvastatin	verampil
	saquinavir	quinidine	tacrolimus

RTV, a PIs that is often co-administered with IDV and SQV, is metabolised by CYP3A4/5[34, 35]. Figure 1.3 shows the metabolic pathways of ritonavir. Ritonavir is principally metabolised through catalysis by CYP3A4/5, thus, RTV potently inhibits the CYP-mediated metabolism of other drugs that are also substrates of CYP3A4/5[35]. In addition, indinavir is also under the predominant metabolism by CYP3A4/5. Careful titration of the amount of RTV is used in boosting the plasma

concentrations of other PIs (e.g. LPV/r) and ensures the achievement of therapeutic concentration levels[36].

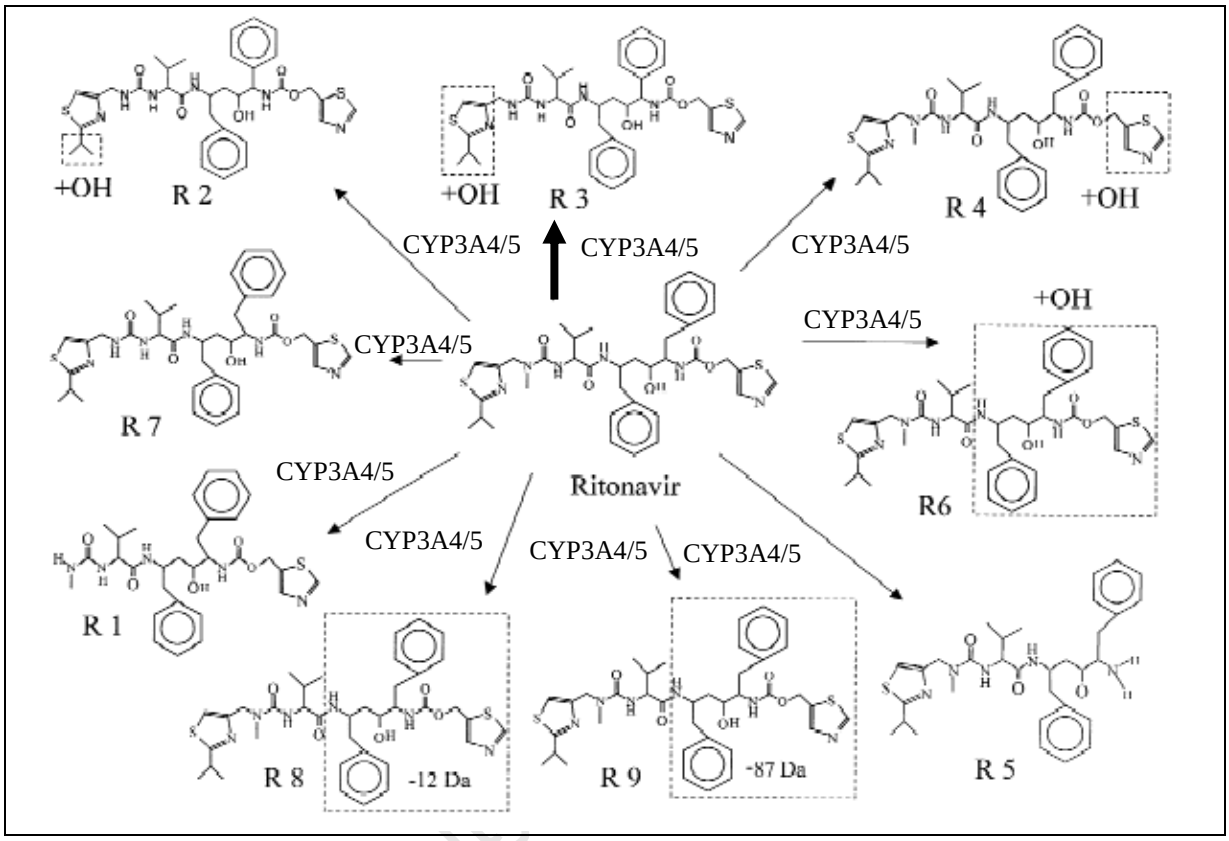

Figure 1.3: RTV metabolism by CYP3A4/5 with the most dominant route of metabolism producing metabolite R3 (Adapted from Gangl *et al.* [37]).

NVP is principally metabolized by CYP3A4/5 and other enzymes such as CYP2B6 have a minor contribution. The four main metabolites of NVP: 2-, 3-, 8-, and 12-hydroxyNVP result from metabolism mediated by CYP2B6, and CYP3A4/5 enzymes. Figure 1.4 shows the metabolic pathway for NVP and the enzymes that are involved in the production of specific metabolites. Given that the same enzymes involved in NVP metabolism are also involved in the metabolism of drugs used for TB treatment, there are vast implications of drug interactions[38]. Genetic polymorphisms in the DMEs differ between populations and could result in various responses to co-administered drugs[39].

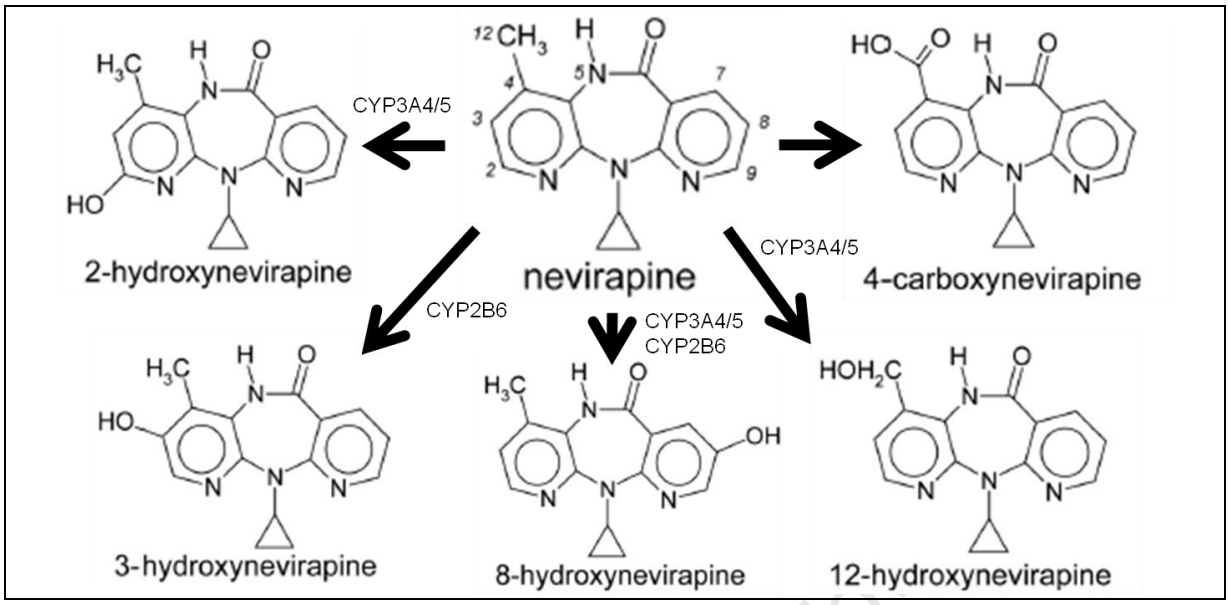

Figure 1.4: NVP metabolism by CYP2B6 and CYP3A4/5 (Adapted from Erickson *et al.* [40]; Cammett *et al.* [41]).

EFV, which forms the major part of this study, goes through rapid absorption after oral administration and reaches maximum plasma concentrations in 3-6 hours. Therapeutic concentration levels are reached within a few days after treatment initiation. Steady-state EFV plasma concentrations is reached in 6-10 days after multiple dosing has been initiated in healthy individuals[42]. The prolonged half-life of EFV (approximately 40-55 hours after repeated dosing) allows for efficient repression of HIV replication and is good in treatment adherence[43, 44]. The narrow therapeutic range of EFV and correlation of high plasma concentration levels with severe CNS side effects are however a major problem in treatment of HIV/AIDS.

Similar to NVP, EFV is principally metabolised by CYP2B6 to 8-hydroxy-EFV (8-OH-EFV), and to the minor metabolite 7-OH-EFV by CYP2A6, CYP2B6, CYP1A2, CYP3A5 and CYP3A4, in order of importance[43, 45]. EFV is also metabolised to the secondary metabolite N-glucuronide-EFV by UGT2B7[43, 45]. Figure 1.5 shows the EFV metabolic pathway. Due to the variation in the distribution in *CYP2B6* genetic polymorphisms, Pfister *et al.* [46] showed a 30% higher hepatic clearance of EFV in Caucasians when compared to African Americans and Hispanics. In contrast with the well characterised Caucasian populations, information on the African populations,

particularly, South African Bantu-speaking population is very limited. A PK study by Cohen *et al.* [47] in HIV/AIDS patients, investigated the effect of only one SNP *CYP2B6 G516T* and showed that this SNP is associated with high EFV concentrations and present in 32% Xhosa South Africans. This project therefore aims to investigate the combined effects of SNPs in the genes that have been associated with EFV metabolism.

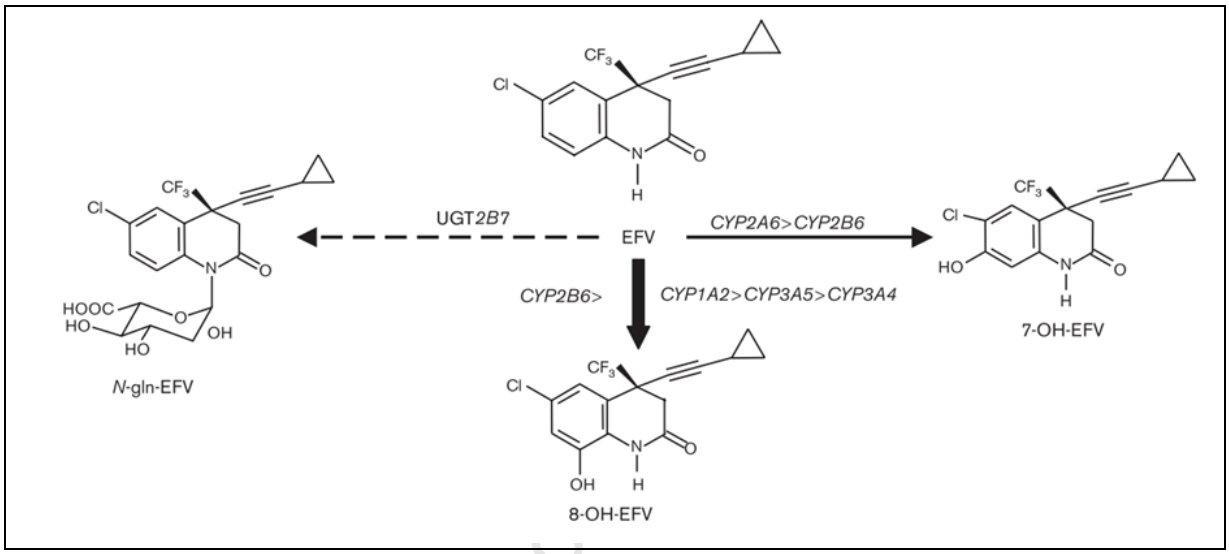

Figure 1.5: EFV metabolism by CYP2B6, with minor influence from CYP1A2, CYP2A6 and CYP3A4/5 (Adapted from di Iulio *et al.* [45]).

However, it has been shown that not all patients experience the same effects to treatment and this is thought to be due to variability in the genes responsible for ARV metabolism and transport. A brief overview of each of the genes that forms part of this study will be given below.

1.5. Variation in the enzymes associated with EFV ADMET

CYP1A2 is one of the most abundant CYP enzymes expressed in the human liver, and contributes to about 13% of the total hepatic CYP content[48]. CYP1A2 metabolizes many drugs among them, phenytoin for treatment of epilepsy. CYP1A2 is not only responsible for the metabolism of EFV, but also endogenous compounds for example steroids as shown in Table 2. Smoking has been shown to induce CYP1A2 activity and alter drug response in treatment of Schizophrenia[49]. CYP1A2 is responsible for the primary metabolism of caffeine, and caffeine is therefore used as probe for

CYP1A2 activity. Inter-individual variability in caffeine metabolism of as high as 60% has been reported previously[50-52]. Up to 70-fold variation in CYP1A2 activity and 15-fold variation in mRNA expression levels has also been observed among individuals[53, 54].

The CYP1A2 enzyme is encoded by the *CYP1A2* gene and located in a cluster with *CYP1A1* and *CYP1B1* on chromosome 15q24.1[55]. The *CYP1A2* gene, which contains seven exons, is well-conserved across humans and mice[56]. Polymorphisms in the *CYP1A2* regulatory region have been reported (www.cypalleles.ki.se) of which the following polymorphisms have been documented in African populations: *CYP1A2 C-163A, CYP1A2 T-739G* and *CYP1A2 C-729T*[52, 57-60]. The *CYP1A2 C-729T* polymorphism is associated with decreased CYP1A2 expression, because of the elimination of an E-twenty six (Ets) nuclear factor binding site. The *C-729T* SNP has however only been observed at a frequency of about 3% in African populations together with the *C-163A* and *T-739G* polymorphisms. The presence of the *C-163A* change in intron 1 has been largely associated with increased enzyme inducibility and activity[58, 61]. The *C-163A* SNP is the most widely studied *CYP1A2* SNP and forms part of this investigation[58]. Several studies have described an influence of the *C-163A* polymorphism on response to antipsychotic drugs; with the *-163A* allele associated with lower drug concentrations and a higher risk of treatment failure and the *-163C* allele with higher drug concentrations and greater risk of ADRs[62]. There is nonetheless a need for studies with well-defined phenotype groups of adequate sample size to determine the effect of genetic polymorphisms on enzyme activity and response to treatment.

CYP2A6 enzyme is predominantly expressed in the liver and inter-individual variability of up to 300-fold in activity has been reported by Xu *et al.* [63]. CYP2A6 is involved in the primary metabolism of nicotine, coumarin and also EFV (see Table 2)[64, 65]. Variability in CYP2A6 activity has been reported to alter the risk of becoming nicotine dependent and the development of tobacco-related cancer[66]. The variability in CYP2A6 enzyme expression shows ethnic differences

with the CYP2A6 poor metabolizers being more frequent among Asians as compared to Caucasian individuals[67].

The *CYP2A6* gene, located on chromosome 19q13.2, is highly polymorphic with more than 30 known alleles (www.cypalleles.ki.se). The polymorphisms are associated with abolished activity, reduced enzyme activity or increased gene expression and enzyme activity[67]. Among the polymorphisms identified, is a 58 bp gene conversion in the 3'UTR which is present at an allele frequency of about 30% in Caucasians, 40% in Asians, 55% in Canadian Native Americans and 10% in Africans[68]. The genetic polymorphism namely *CYP2A6 G1093A* was investigated in the present study and is located in a substrate recognition site in exon 7 of *CYP2A6*[64, 69]. The allele frequency for the *G1093A* polymorphism was below 10% in Caucasians and African Americans, with no information for the South African population[70, 71].

CYP2B6 makes up 2%-10% of the hepatic CYP content and is expressed in the brain[17, 72-74]. CYP2B6 is responsible for the metabolism of approximately 4% of the FDA-approved drugs including EFV and NVP. Inducers of CYP2B6 expression include the TB drug rifampin, phenobarbital and phenytoin (see Table 2)[38, 43]. Inter-individual (20-250 folds) as well as ethnical variation in CYP2B6 expression has been reported by Code *et al.* [32]. Differential transcriptional regulation or genetic variation has been shown to result in altered enzyme functionality[75, 76].

CYP2B6 (gDNA of 6968 bp) is located on chromosome 19q13.2, contains nine exons and encodes the CYP2B6 enzyme of 491 amino acids[77, 78]. *CYP2B6* is highly polymorphic, with more than 70 identified SNPs in intronic, exonic, and promoter regions[75, 76, 79]. Five mRNA splice variants have been identified for this gene and the most common splice variant (SV1) lacking exons 4 to 6, was linked to the *G516T* SNP in exon 4. The *G516T* SNP is responsible for aberrant splicing of pre-mRNA by disruption of an exonic splice enhancer and leads to reduced functional mRNA and enzyme activity[76]. The occurrence of the *G516T* (Q172H) SNP together with the non-synonymous

A785G (K262R) SNP have been shown to exhibit 4-fold less protein expression in Caucasian individuals compared to individuals without these SNPs[18, 80]. The frequency of the *CYP2B6 G516T* SNP ranged from 14% in Koreans[81] to over 40% in Africans[79, 82] and 62% in Papua New Guineans[83]. *CYP2B6* SNPs show inter-ethnic variability and this may have serious implications for treatment of HIV/AIDS taking into account that CYP2B6 principally metabolises EFV and NVP. *CYP2B6 516T* and *983C* variants diminish CYP2B6 enzyme activity resulting in lower EFV and NVP clearance (50-60%), increased steady-state plasma concentrations and a high frequency of CNS adverse effects[84-89]. The clinical relevance of *CYP2B6* polymorphisms in EFV metabolism has been studied to great extent in other populations but very little or no information is available on South African patients. Even in situations where the polymorphisms have been studied, very few have looked at the effects of combinations of SNPs on metabolism. Thus, this study evaluated the role of the following SNPs: *G516T*, *A136G*, *A785G*, *T983C* and *C1459T*.

The human CYP3A subfamily encompasses CYP3A4, CYP3A5, CYP3A7 and CYP3A43. CYP3A43 is believed to most likely not translate into active enzyme, while foetal hepatic CYP3A activity is thought to be due to CYP3A7 and CYP3A5[90, 91]. CYP3A4 and CYP3A5 are both expressed in the adult liver and intestine, while CYP3A5 is predominantly expressed in extra-hepatic tissues. CYP3A4/5 account for approximately 30% of hepatic CYP expression and are responsible for metabolism of a range of different xenobiotics including approximately 50% of currently prescribed drugs as indicated in Table 2[31]. The substrate specificity of CYP3A4 and CYP3A5 overlap and include antiretroviral drugs such as ritonavir and saquinavir[35]. Knowledge of the inducibility of the CYP3A4 enzyme is also important in combination therapy of HIV and TB, given that the TB drug rifampicin is an effective inducer of CYP3A4[92]. Extensive variability in CYP3A4/5 protein expression was previously detected in the livers of Caucasian individuals, where only 10% of individuals expressed high levels of CYP3A5 and the remaining showed on average

nine times less expression[93]. In contrast, CYP3A5 is thought to be highly expressed in African and African-American individuals and this is of importance in the current study[94].

The *CYP3A* gene family is located on chromosome 7, and *CYP3A4* along with *CYP3A5* was mapped to 7q22.1 by Inoue *et al.* [95] in 1992. *CYP3A4* is about 27 kb in length and contains 13 exons, while *CYP3A5* consists of nine exons and encodes a 502 amino acid protein[96, 97]. *CYP3A* expression is polymorphic with more than 40 allelic variants that have been identified in the *CYP3A4/5* genes[97]. *CYP3A4 A-392G* polymorphism is located in the nifedipine-specific response element of the promoter region and in vitro expression analysis of this SNP produced conflicting results[98, 99]. The *CYP3A4 -392G/G* genotype has been associated with deterred enzyme activity and therefore a decrease in the rate of clearance of the anticonvulsant midazolam and immunosuppressant cyclosporine[100], while the same genotype was correlated to increased transcriptional activity[99]. Chu *et al.* [101] found that a small number of individuals with the *-392G/G* genotype had an elevated risk of developing endometrial cancer, but this study needs to be confirmed. The frequencies of the *A-392G* SNP ranged from being absent in Asians to 9% in Caucasians, 55% in African-Americans and 77% in Xhosa South Africans[102-104].

Inter-ethnic variability in *CYP3A5* expression is the reason for the absence of CYP3A5 in 70% of Caucasians and was linked to the *CYP3A5 A6986G* polymorphism. The *6986G* allele results in alternative splicing of the transcripts and absence of CYP3A5 protein. The frequency of the *6986G* allele were 95% in Caucasians, 73% in Chinese, 71% in Japanese, 70% in Koreans to 27% in African-Americans and 14% in Black Africans[97, 105]. *CYP3A5 G14690A* is a less common polymorphism that is present mainly in the African-American population. The *14690A* allele causes a loss of protein activity by skipping exon 7 (alternative splicing) and leads to protein truncation[97]. Another polymorphism, the *CYP3A5 27131-2insT* leads to premature termination of the open reading frame by resulting in a shift in the reading frame. This polymorphism occurs at a frequency of 10% in African-Americans, but has not been observed in Caucasians[93, 97, 106]. The genetic

polymorphisms of *CYP3A5* have been studied in the South African population in the context of oesophageal cancer by Dandara *et al.* [105], but not with respect to HIV/AIDS therapy. The distribution of these polymorphisms in South Africa differed from that of other ethnic groups and individuals with abolished CYP3A5 enzyme activity had a reduced risk of developing oesophageal cancer[105]. A weak association between the *CYP3A5 6986G/G* genotype and increased risk of hepatotoxicity in patients receiving NVP treatment as well as higher EFV plasma concentration levels has been observed previously in other populations[84, 107]. Elucidation of the factors affecting an individual's CYP3A4/5 enzyme activity could allow for individualized dose adjustments in therapies with drugs that are CYP3A4/5 substrates[106].

The P-glycoprotein (P-gp) encoded by *ABCB1*, is a 1280 amino acid protein expressed in the liver, adrenal gland, kidney, small and large intestines and brain. P-gp forms a dimer that results in a transmembrane pore on the epithelial cells lining the above mentioned tissue. This ATP-dependent efflux pump is responsible for the elimination of anticancer drugs, immunosuppressants, steroids, statins, calcium channel blockers, beta-blockers, antihistamines, anticonvulsants, antidepressants and the ARV drugs EFV, NFV and NVP (see Table 2)[108, 109]. P-gp transports substrates out of the blood brain barrier, excretes substrates into urine and limits absorption in the bowel. Expression and function of P-gp is influenced by environmental factors such as induction by other drugs. Interactions between compounds are substrate-specific, tissue-specific and concentration-dependent[108].

ABCB1 forms part of the ATP-binding cassette gene family (49 members) that encodes transporter (e.g. P-gp) and channel proteins. The *ABCB1* gene is located close to *ABCB4* on chromosome 7q21.12, spans 209.6 kb and contains 29 exons[110]. The two exons closest to the *ABCB1* promoter are untranslated and the mRNA is 4872 bp with the 5'UTR included[110, 111]. More than 1200 SNPs have been identified in the *ABCB1* gene, with about 20 synonymous and 40 non-synonymous polymorphisms. Genetic variation in the *ABCB1* gene, excluding the 3'UTR, is known to alter the

mRNA stability or splicing activity[112]. The three most common SNPs in the protein coding region are *ABCB1 C1236T, T2677G/A* and *C3435T/A* as shown on Figure 1.6. The *C1236T* polymorphism occurring in exon 13 does not result in any change in the amino acid sequence at residue 412. The frequency of the *1236T* allele ranged from as low as 30% among Africans (this study) to 90% among Asians[112]. The *T2677G/A* SNP result in a change from serine to alanine or threonine at residue 893. The *C3435T/A* SNP is one of three coding SNPs in *ABCB1* and has been associated with reduced expression of P-gp in the intestines. The *ABCB1 3435T* allele does not result in any change in the amino acid sequence at residue 1145 in a cytoplasmic loop of P-gp[113]. Large inter-ethnic variability was observed for the *C3435T/A* polymorphism with the *ABCB1 3435C* allele present at a frequency of 83% in individuals from Ghana and Kenya[114].

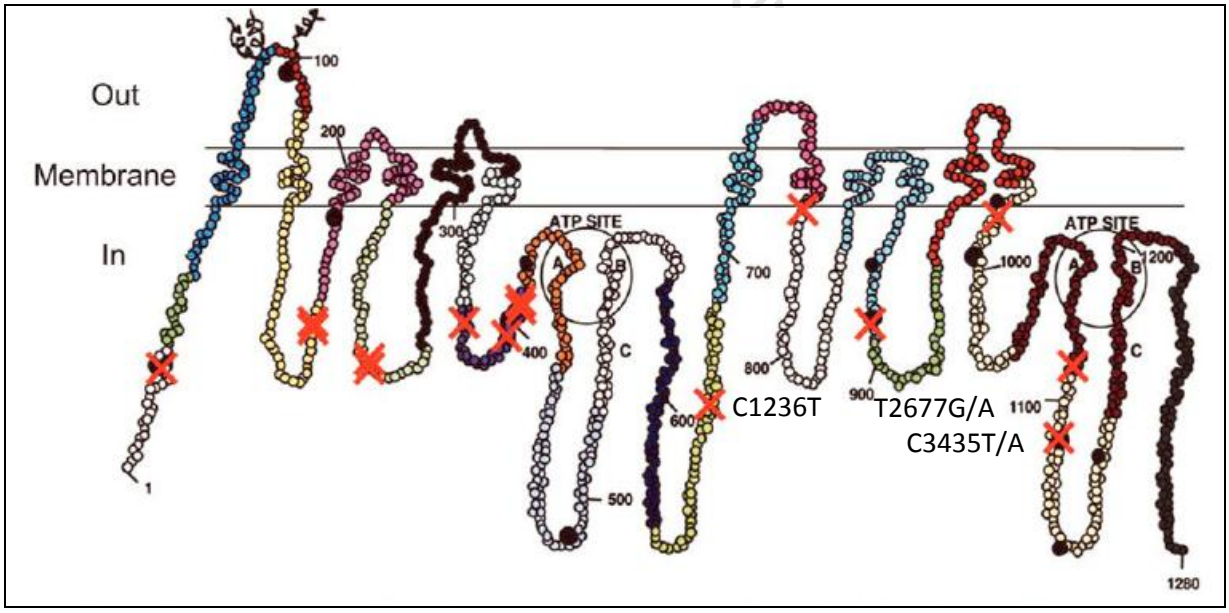

Figure 1.6: The position of common genetic polymorphisms in P-gp (encoded by the ABCB1 gene) is shown with red "X" marks (Adapted from Kimchi-Sarfaty *et al.*[115]).

The *ABCB1 3435T* variant has been linked to good immune recovery in HIV/AIDS individuals. The presence of the *ABCB1 2677T* and *CYP2B6 516T* variants has been strongly associated with virological failure[85, 116]. Conflicting reports have discussed the effect of these SNPs on treatment

response[116-118]. Replication studies are thus necessary to understand the influence of *ABCB1* genetic variation on disease susceptibility, multi-drug resistance and drug-drug interactions[108].

However, not all the inter-individual variability in response to ARV treatment can be explained by genetic variation in the *CYP* and *ABCB1* genes alone. The expression of these genes is controlled by nuclear receptors (NRs) for example the pregnane X receptor (PXR). Although they are not a major component of this project, nuclear receptors will be briefly reviewed in order to give a clear picture of the factors related to DME associated drug responses.

1.6. Regulation of DMEs and drug transporters by nuclear receptors (NRs)

Members of the nuclear receptor superfamily (NRs) are found in the cytoplasm and play a vital role in regulation of phase I and II DME expression[119]. These receptors are responsible for recognition of xenobiotics and then trigger their elimination through inducing DMEs and transporters. In their inactive form, NRs are bound to histone deacetylase complexes (HDACs). Upon ligand binding (xenobiotic or drug e.g. EFV) the HDACs are dissociated and the drug-NR complex is translocated into the nucleus. This complex then heterodimerises with retinoid X receptor (RXR) and binds to nuclear receptor response elements located in the promoter region of the genes responsible for drug metabolism. In addition to regulation by NRs, DMEs are also regulated by microRNA (miRNA). For example, *CYP3A4*, *CYP2B6* and *CYP2E1* have been shown to be regulated by microRNAs miR-148a, miR-378 and miR-27a/b. Thus, any genetic variation at the level of the NRs and miRNA is bound to affect drug metabolism[120, 121]. It will be worthwhile to evaluate the effects of genetic variation at the level of miRNA and NRs in combination to that of DMEs on the response to commonly used therapy, in this case ART. Although the genetic variation in DMEs, NRs and miRNA is known for Caucasian and Asian populations, little (with respect to DMEs), almost nothing (with respect to NRs) and presently nothing (with respect to miRNAs) is known in African populations especially the South African population.

1.7. Linguistic and genetic characterization of South African population

There are little genetic data on African populations with respect to pharmacogenetics. The few studies that have been done have looked at single SNPs, thus failing to get the complete picture on the functional effects of genetic variation in genes of pharmacogenetics relevance. This study makes a valuable contribution by investigating the distribution of several polymorphic variants of pharmacogenomics relevance, in the South African Bantu-speaking population. South Africa is made up of four major population groups, Bantu-speaking Africans (80%), Mixed Ancestry (8%), Caucasians (9%) and Asians, particularly Indians (2%) (www.statssa.gov.za). Data related to the Mixed Ancestry, Caucasians and Asians can be inferred from previous studies that focussed largely on Caucasians and Asians, but are not relevant to the Bantu-speaking South Africans. The Bantu-speaking population group is hypothesized to have originated in West Africa between the present-day Cameroon and Nigeria, near the Benue river, approximately 3000-5000 years ago (Figure 1.7). These populations are thought to have subsequently spread to east, west, central and southern Africa, through one of the most studied human movements, the Bantu-migration[122].

In Africa, the Bantu languages are part of the Niger-Congo language family which together with Nilo-Saharan, Afro-Asiatic and Khoisan, form the major language groups[123]. In South Africa, the Xhosa, Southern Sotho, Zulu, Tsonga, Venda, Pedi and Tswana groups belong to what is loosely referred to as the Southern branch of the Eastern Bantu languages. Although these groups originated from a common ancestral population and were not isolated for long, their languages have diverged in the past 2000 years[124].

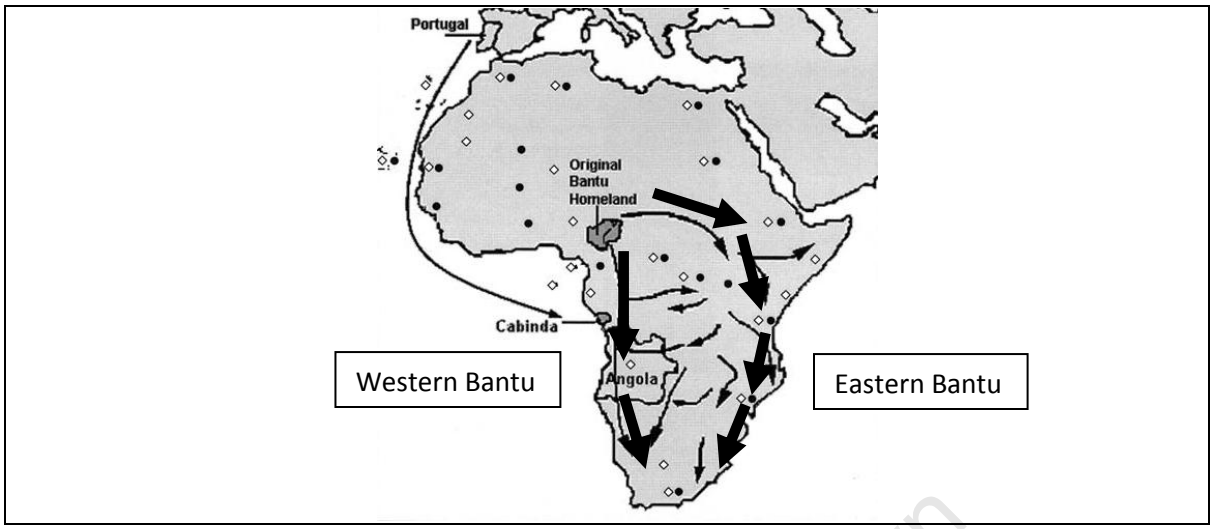

Figure 1.7: Map of Africa showing the origin of the Bantu-speaking populations and migration southward in different times and manners: first along the western coast and second along the eastern coast as shown in thick arrows (Adapted from Beleza *et al.*[125]).

The genetic relatedness of the South African Bantu-speaking groups has been extensively characterised at the level of Y-chromosome DNA and mtDNA[126-128]. In general it has been observed that they share about 99% sequence similarity at the level of Y-chromosome DNA[124]. In addition, analysis at nine other STR autosomal DNA loci also revealed more than 99% similarity[124]. Besides genetic characterisation at the Y-chromosome and other loci, some work has also looked at mitochondrial DNA and the haplogroups frequently found in south-eastern Bantu-speakers include L2a1b, L3e1 and L3e3[127].

When genetic variation associated with response to treatment is taken into account, isolated and limited studies have documented the baseline frequencies of gene polymorphisms of pharmacogenomic importance in Bantu-speaking populations[129-135]. However, very little studies have been done using well characterized phenotypes to investigate the effects of such variation. This project therefore focuses on the aspect of pharmacogenetics of ARVs with an emphasis on the response to EFV treatment.

1.8. Aim and objectives

The aim of the project was to investigate the role of genetic variation in *CYP1A2, CYP2A6, CYP2B6, CYP3A4/5* and *ABCB1* genes in response to treatment with EFV-based ART in a Bantu-speaking South African population.

Objectives:

- Baseline frequencies of previously characterized genetic polymorphisms in pharmacogenes were determined in a sample of healthy, Bantu-speaking South African individuals and the frequencies were compared to other populations.
- The effect of identified genetic variations on the response to treatment with EFV-containing ARV regimens was investigated by genotyping HIV/AIDS patients.

Chapter 2: Materials and Methods

2.1. Recruitment of research participants

Four hundred and sixty-four (n=464) unrelated individuals from South Africa were recruited to participate in this study and informed consent was obtained from all subjects (Appendix A). These South African subjects were either healthy research participants (n=163) or HIV/AIDS patients (n=301). All subjects were black South African Bantu-speakers of different ethnic groups, including Zulu and Xhosa speakers. This study was approved by the University of Cape Town Health Sciences Faculty Research Ethics Committee, Cape Town, South Africa (REC REF: 103/2009) as well as by the University of Witwatersrand Human Research Ethics Committee, Gauteng, South Africa (M080124) as indicated in Appendix B.

The healthy research participants were included as general population controls with no phenotypic data, and were not tested to determine HIV infection. The criteria for their inclusion were based on availability of adult Bantu-speakers from mainly Xhosa and Zulu groups, who did not present with disease. The HIV/AIDS patients were recruited prospectively from Themba Lethu clinic, Helen Joseph Hospital in Gauteng, South Africa and were on the following EFV-based regimens for at least six months; 3TC_TDF_EFV (n=25), AZT_3TC_EFV (n=62) and d4T_3TC_EFV (n=214). HIV/AIDS individuals were not newly diagnosed and included in the study based on the availability of the following information from a questionnaire: age, gender, ethnicity, and disease stage, body mass index (BMI) at baseline, level of education, employment, smoking and alcohol use. The CD-4 cell count, viral load, haemoglobin levels, aspartate transaminase (AST) and alanine transaminase (ALT) levels were available from the clinic patient records. The CD-4 cell count and viral load was available at baseline as well as six months after treatment initiation. Detailed recordings on the ART regimens (start and end date), change in regimens as well as treatment adherence and the use of other concomitant medication were also provided. Only patients who were on treatment for at least six months were included in the study.

2.2. Sample collection

2.2.1. EFV plasma sample preparation

A 5 ml whole blood sample (in EDTA-containing Vacutainer tube) was collected from each patient between 14 and 18 hours post EFV dose. Plasma was obtained from the blood samples by centrifugation at 700 x g for 5mins, and stored immediately at -80^{0}C until pharmacological analysis.

EFV plasma concentrations were determined by liquid chromatography (LC) and triple-quadrupole tandem mass spectrometry (MS/MS) (API 4000 triple quadrupole MS/MS Applied Biosystems, South Africa) using a modification of the method by Chi *et al.* [136], in the Division of Clinical Pharmacology, University of Cape Town. LC-MS/MS is a technique used to separate compounds based on their physical properties such as hydrophobicity, and their mass. LC-MS/MS allows for the detection and concentration measurement of a compound (e.g. EFV and EFV metabolites) within a complex mixture.

An aliquot of 500 µl of precipitation reagent (80% methanol: 20% 0.2M $ZnSO_4$ v/v) (methanol from Merck KGaA, Darmstadt, Germany; $ZnSO_4$ from Sigma Chemical Co.) with reserpine (Sigma Chemical Co.) at 0.4 µg/ml as internal standard was added to a 50 µl aliquot of the plasma sample and mixed at high speed for 15s. The suspension was then sonicated for 10mins and centrifuged at 4000 x g for 10mins to precipitate and remove the proteins. The extracts were transferred to vial inserts and placed in the autosampler tray for injection onto LC/MS/MS column (Phenomenex Gemini C18, 50× 2.00 mm, with 5 micron particle size packing).

2.2.2. EFV plasma concentration assay by LC/MS/MS

Mobile phase A was 10% methanol and mobile phase B was 90% methanol: 10% 10mM of ammonium acetate in 0.1% acetic acid v/v. The flow gradient was initially 100% of phase A for

0.20mins, switched to 100% phase B for 0.21mins, held at 100% phase B for 2.79mins. Then returned to 100% phase A for 0.10mins and held for a further 3.90mins prior to the injection of another sample. The total run time for each sample was 7.00mins. The flow rate of mobile phase was kept constant at 500 µl/min. The volume of injection was 5 µl.

The plasma calibration curves of EFV (Merck Research Laboratories, Rahway N.J., USA) were linear over the range of 0.20-20.00 mg/l. Plasma calibration curves were prepared by spiking EFV in drug-free plasma at each calibration concentration. Three quality controls were prepared in the same manner (0.3 mg/l, 4 mg/l and 11 mg/l) for EFV. The lower limit of quantification (LLOQ) for EFV was 0.10 mg/l. A sample with plasma concentrations below the LLOQ was treated as 40% of LLOQ concentration in the data analysis. Samples with plasma concentrations higher than the upper limits of quantification were diluted with drug-free plasma and re-analysed.

Accuracy of the EFV plasma concentration assay was above 90%. The intra-day and inter-day precisions ranged from 1.34% to 9.61% and from 2.23% to 7.04%, respectively (Appendix C).

2.2.3. DNA isolation and integrity check

DNA was isolated from the healthy participants in the Division of Human Genetics, University of Cape Town. DNA isolation from the 5 ml whole blood was completed according to the method adapted from Gustafson *et al.* [137]. The salting-out method involves lymphoblast cell lyses using Sucrose Triton-X (Sigma-Aldrich, St Louis, MO, USA) and T20E5 (Sigma-Aldrich, St Louis, MO, USA and Promega Corporation, Madison, WI, USA) lysis buffers, the use of high salt concentrations for the removal of proteins, followed by ethanol precipitation of the DNA (Merck, Darmstadt, Germany). DNA samples were stored at 4^0C during genetic analysis.

Buffy coat and plasma samples were obtained from the whole blood sample collected from each HIV/AIDS patient, by centrifugation at 4 000 x g for 10mins. 400 µl plasma and 200 µl buffy coat samples were used to extract DNA using the QIAmp Blood Midi Kit (QIAGEN, Hilden, Germany)

and GENelute (Sigma-Aldrich, St Louis, MO, USA) extraction methods, respectively. DNA was bound to a silica membrane (as a result of the lower pH) within the spin column and contaminants were removed by washing of the spin column. DNA was then eluted from the silica membrane using elution buffer. The QIAmp spin protocol for 0.3 – 1 ml whole blood was used with the following deviations; centrifugation steps were performed at 2000 x g and extended to double the time described in the protocol (in order to efficiently remove impurities), DNA elution with 150 µl elution buffer was extended to overnight and repeated to yield maximum DNA concentrations. The GENelute spin protocol (using up to 0.2 ml whole blood) was adapted by repeating the DNA elution step twice overnight, using 200 µl elution solution. The integrity of the DNA samples was checked after extraction, using a UV-spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) as well as agarose gel electrophoresis (Appendix D).

2.3. Gene annotation and primer design

DNA sequences were attained from the ENSEMBL website (URL: http://www.ENSEMBL.org) and the NCBI Gene website (URL: http:// ncbi.nih.gov). These DNA sequences were annotated using the program Perl version five, created by Dr. Rebello (Division of Human Genetics, UCT, Personal communication) to show the gene structure and previously identified genetic variation.

The following free web-based programs were used to determine the specificity of the primers to the human genome (including the region of interest), to calculate product length, the melting temperature, and the GC-content of the designed primers; NCBI Primer-BLAST[138] which can be found at the following URL: http://www.ncbi.nlm.nih.gov/tools/primer-blast/, Primer 3 version 2004 which was developed by Rosen and Skaletsky [139] in 2000 (URL: http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and the IDT Oligo Analyzer from Integrated DNA Technologies (URL; http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/). Primers were 18 bp-28 bp in

length and had a 50%-60% GC-content. Primer pairs had a difference in melting temperature of less than 5°C and were ordered from Integrated DNA Technologies.

2.4. Genotyping of genetic polymorphisms

The genetic variants investigated in this study as well as the genotyping method used is indicated in Table 3 below. The number of individuals with genotypic data available was also reported. The allele frequencies of the healthy volunteers where compared to allele frequencies previously reported for the Yoruba, African-American and Caucasian populations (National Centre for Biotechnology Information (NCBI) dbSNP database (http://www.ncbi.nlm.nih.gov/) and frequencies for Xhosa, Luhya and Maasai individuals were obtained from Ikediobi *et al.* [104].

Table 3: List of genes and SNPs genotyped in this study.

Gene	ENSEMBL Accession	SNP ID	Position & Alleles	R	O	N	Genotyping method
CYP1A2	ENSG00000140505	rs762551	-163C>A	C	A	242	PCR/RFLP
CYP2A6	ENSG00000255974	rs28399454	1093G>A	G	A	458	PCR/RFLP
CYP2B6	ENSG00000197408	rs35303484	136A>G	A	G	460	SNaPshot
CYP2B6		rs3745274	516G>T	G	T	460	PCR/RFLP
CYP2B6		rs2279343	785A>G	A	G	458	PCR/RFLP
CYP2B6		rs28399499	983T>C	T	C	451	SNaPshot
CYP2B6		rs3211371	1459C>T	C	T	461	PCR/RFLP
CYP3A4	ENSG00000160868	rs2740574	-392A>G	G	A	187	PCR/RFLP
CYP3A5	ENSG00000106258	rs55817950	3699C>T	C	T	460	SNaPshot
CYP3A5		rs776746	6986A>G	G	A	455	PCR/RFLP
CYP3A5		rs10264272	14690G>A	G	A	457	PCR/RFLP
CYP3A5		rs41303343	27131-2insT	-	T	460	PCR/RFLP
CYP3A5		rs28383479	19386G>A	G	A	460	SNaPshot
CYP3A5		rs15524	31611C>T	C	T	457	SNaPshot
ABCB1	ENSG00000085563	rs9282564	61A>G	A	G	460	SNaPshot
ABCB1		rs1128503	1236C>T	T	C	460	SNaPshot
ABCB1		rs1045642	3435C>T/A	T	G/A	460	SNaPshot
ABCB1		rs2032582	2677T>G/A	T	G/A	456	SNaPshot
ABCB1		rs3842	4036A>G	A	G	460	SNaPshot

N = number of samples with genotyping results, R = reference allele and O = other allele.

2.4.1. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) genotyping

Genetic polymorphisms of pharmacogenetics relevance in *CYP1A2 (C-163A), CYP2A6 (G1093A), CYP2B6 (G516T, A785G* and *C1459T), CYP3A4 (A-392G)* and *CYP3A5 (A6986G, G14690A* and *27131-2insT)* were genotyped using PCR-RFLP (see Table 4) according to the methods of Pavanello *et al.* [140], Fukami *et al.* [141], Lang *et al.* [75], van Schaik *et al.* [142] and van Schaik *et al.* [93], respectively.

The primer annealing temperature (T_a) was estimated from the melting temperature (T_m) of a primer set, as T_a (°C) = T_m – 5^0C. The T_m was calculated based on the nucleotide composition of the primer sequence, using the formula: T_m (°C) = 2(G+C) + (A+T). A temperature gradient PCR was used to establish the optimal T_a for each primer set (Table 4). PCR amplification was performed using the "MyCycler™ Thermal Cycler" from Bio-Rad (Bio-Rad Laboratiories, Hercules, CA, USA). PCR cycling conditions was set up at 94^0C for 3mins, and then repeated 40 cycles with 94^0C for 30s, the optimal T_a for each primer set (Table 4) for 30s and 72^0C for the specific extension time (Table 4), followed by 72^0C for 10mins.

The PCR reaction contained the following reagents at final concentrations: 50-100 ng DNA, 1X GoTaq poly flexi reaction buffer (Promega Corporation, Madison, WI, USA), 0.4 mM of each dNTPs (Bioline, London, UK), 1.5 mM $MgCl_2$ for all SNPs except *CYP1A2 C-163A* which required 1.25 mM (Promega Corporation, Madison, WI, USA), 0.4 µM of each forward and reverse primer specific to the genetic variation (Integrated DNA Technologies, Inc., Coralville, IA, USA), 0.1 µl GoTaq flexi DNA polymerase (Promega Corporation, Madison, WI, USA) and the volume was made up to a total of 25 µl using sterile distilled water (sdH_2O). The presence of the expected PCR products were determined on a 1.5% (w/v) agarose gel at 160 V for 30mins and the gel visualised

using EtBr staining (Sigma-Aldrich, St Louis, MO, USA). Tables 4 and 5 show the PCR conditions and either the RFLP or SNaPshot conditions that were used in the genotyping of the 19 SNPs.

In the case of RFLP, PCR amplification was followed by digestion of each specific PCR product using the appropriate restriction endonucleases as specified in Table 4. The digestion reaction contained the following reagents: 10 µl of PCR product, 1X specific restriction buffer (Fermentas Life Sciences, Burlington, Canada), 3 U restriction enzyme (Fermentas Life Sciences, Burlington, Canada), and made up to a total volume of 30 µl with sdH_2O. The digestion reaction was incubated for at least two hours at the temperature mentioned in Table 4. Digestion products were separated on a 2.5% or 4.5% (w/v) agarose gel at 160 V for 45mins and visualised using EtBr staining. 4.5% (w/v) agarose gels were prepared for the separation of small DNA fragments, using NuSieve®3:1 and SeaKem agarose (Roche, Mannheim, Germany) in a 2:1 ratio. The restriction patterns (Table 4) indicating the presence or absence of the genetic variation and were used to determine the genotype of each individual.

Table 4: Parameters and conditions used in the PCR-RFLP genotyping protocols (All PCR reactions required 1.5 mM $MgCl_2$ except the *CYP1A2 C-163A* which required 1.25 mM).

SNP	Primer sequence (5'-3')	T_a, ext	PCR product	Digestion (bp)	Ref
CYP1A2 C-163A	F:CAACCCTGCCAATCTCAAGCA R:AGAAGCTCTGTGGCCGAGAAG	65°C, 1min	920	*ApaI* C:709, 211 A:920	1.
CYP2A6 G1093A	F:TTGAAAAACCTGGTGATGAC R:CTGAGATTTCTGTCCCTAT	56°C, 1min	1046	*TaiI* A:491,364,191 G:855,191	2.
CYP2B6 G516T	F:GGTCTGCCCATCTATAAAC R:CTGATTCTTCACATGTCTGCG	54°C, 40s	526	*BsrI* G:268,241,17 T:509,17	3.
CYP2B6 A785G	F:GACAGAAGGATGAGGGAGGAA R:CTCCCTCTGTCTTTCATTCTGT	55°C, 1min	640	*StyI* A:297,171,116,56 G:468,116,56	3.
CYP2B6 C1459T	F:TGAGAATCAGTGGAAGCCATAGA R:TAATTTTCGATAATCTCACTCCTGC	62°C, 1,5mins	1401	*BglII* C:1401 T:1185,216	3.
CYP3A4 A-392G	F:GGACAGCCATAGAGACAACTG* R:CTTTCCTGCCCTGCACAG	58°C, 1min	334	*PstI* A:220,81, 33 G:199,81, 33,21	4.
CYP3A5 A6986G	F:CATCAGTTAGTAGACAGATGA R:GGTCCAAACAGGGAAGA AATA*	51°C, 30s	293	*SspI* A:148,125,20 G:168,125	5.
CYP3A5 G14690A	F:TGGAAGATGATTCAGCAGATA R:GTGGGGTGTTGACAGCTAAAG	58°C, 40s	495	*DdeI* G:230,137,103,25 A:230,137,128	5.
CYP3A5 27131-2insT	F: CTTCAATAGTACTGCATGGAC R: CTGTACCACGGCATCATAGCT	53°C, 30s	107	*DdeI* 61,24,22 T:41,24,22,20	5.

* Mismatches with the *CYP3A4* and *CYP3A5* reference sequences are underlined. References; 1= Pavanello *et al.* [140], 2= Fukami *et al.* [141], 3= Lang *et al.* [75], 4= van Schaik *et al.* [142], 5= van Schaik *et al.* [93].

2.4.2. SNaPshot genotyping

The ABI prism SNaPshot multiplex method was used to genotype two polymorphisms in *CYP2B6 (A136G* and *T983C*), three polymorphisms in *CYP3A5 (C3699T, G19386A* and *C31611T*) and five polymorphisms in *ABCB1 (A61G, C1236T, C3435T/A, T2677G/A* and *A4036G*). The SNaPshot method follows PCR amplification of the DNA sequence region of interest, and involves the annealing of an internal SNaPshot primer directly adjacent to the genetic polymorphism. Single base extension is facilitated by Ampli Taq DNA polymerase and fluorescently labelled ddNTPs are

incorporated, resulting in sequence termination. The ddNTPs are labelled as follows: ddATP – dR6G (green), ddCTP – dTAMRA™ (black), ddGTP – dR110 (blue) and ddTTP – dROX™ (red). The SNaPshot™ multiplex kit allows for the genotyping of up to ten genetic polymorphisms simultaneously. The dye-labelled GeneScan™-120LIZ™ was used as size-standard and the ABI 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA) was used for genetic analysis.

PCR amplification of the DNA regions surrounding the genetic polymorphisms was performed in combination as shown in Table 5. The "MyCycler™ Thermal Cycler" from Bio-Rad (Bio-Rad Laboratiories, Hercules, CA, USA) was used with the PCR cycling programme as 94°C for 3mins, and then repeated 40 cycles at 94°C for 30s, the optimal T_a for each primer set (Table 5) for 30s and 72°C for the specific extension time (Table 5), followed by 72°C for 10mins.

Table 5: PCR and SNaPshot amplification conditions for 10 SNPs (all required 1.5 mM $MgCl_2$ except *CYP2B6 T983C* which required 2.5 mM).

SNP	Primer sequence (5'-3')	T_a, ext	PCR product	SNaPshot cocktail	Ref
CYP2B6 A136G	F: ACATTCACTTGCTCACCT R: GTAAATACCACTTGACCA SNaPshot primer: CTTTTGGGAAACCTTCTGCAG	51^0C, 50s	726	A	1.
CYP2B6 T983C	F:GTGATTATTCATTAATTGGGTTC R: TGCAATGGTTGATTGATGCTC SNaPshot primer: GCGCGACACAGAGAGAGTCTACAG GGAGA	50^0C, 2mins	2108	A	1.
CYP3A5 G19386A	F: AGGATCATTCAAGGCACACAC R: CACCTTATTGGGCAAAACTG SNaPshot primer: GTTCTGCCGCTGTTTCACTTTGTAG ATATGGGACC	54^0C, 40s	495	B	2.
CYP3A5 C3699T	F: CCCTCTCTTGACCATTCCAG R: GGACTACAGGCATGGGCTAC SNaPshot primer:: ATGTAACTATGAAAATGAAACAAG CTAGTTACCTTTTAT	51^0C, 50s	467	A	5.
CYP3A5 C31611T	F: TCCATATGCTTGTTTAACTATTG R: TCACTTACCTGGTCACCTCCT SNaPshot primer: AGAGACATCATCAAGTGGAGAGAA ATC	54^0C, 40s	335	B	3.
ABCB1 A4036G	F:CCTCAGTCAAGTTCAGAGTCTTCA R: TCACAGGCAGTTGGACAAG SNaPshot primer: TCTTGGCAGAAACTGCAAAAGGAG ATTGAT	54^0C, 40s	297	B	5.
ABCB1 C3435T/A	F: ACTCTTGTTTTCAGCTGCTTG R:AGAGACTTACATTAGGCAGTGACTC SNaPshot primer: ACTCGTCCTGGTAGATCTTGAAGGG	54^0C, 40s	230	C	4.
ABCB1 T2677G/A	F:ATGGTTGGCAACTAACACTGTTA R:AGCAGTAGGGAGTAACAAAATAACA SNaPshot primer: CTTCGACCTAAGTGGAGAATGAGT TATTCTAAGGA	54^0C, 40s	206	B	4.
ABCB1 C1236T	F:TGTGTCTGTGAATTGCCTTGAAG R: CCTCTGCATCAGCTGGACTGT SNaPshot primer:: TTAATTAATCAATCATATTTAGTTT GACTCACCTTCCCAG	51^0C, 50s	228	B	4.
ABCB1 A61G	F: CTGCGGTTTCTCTTCAGGTC R: GATTCCAAAGGCTAGCTTGC SNaPshot primer: CTCCTTTGCTGCCCTCAC	51^0C, 50s	149	A	5.

References; 1= Lang *et al.* [75], 2= Lee *et al.* [143], 3= Liou *et al.* [144], 4= Rhodes *et al.* [145] and 5= designed.

5 µl of each PCR product was pooled and 10 µl of the combined PCR products were used during the first shrimp alkaline phosphatase (*SAP*) and *ExoI* clean-up. The *SAP* and *ExoI* reaction contained: 1.5 U *SAP* (Fermentas Life Sciences, Burlington, Canada), 2 U *ExoI* (Fermentas Life Sciences, Burlington, Canada) and the volume was made up to a total of 25 µl using sterile distilled water

(sdH_2O). The *SAP* and *ExoI* reaction was incubated at 37^0C for 1 hour and the enzymes were inactivated at 75^0C for 15mins.

SNaPshot single base extension of the genetic polymorphisms was performed in combinations; A, B and C (as shown in Table 5). The "GeneAmp® PCR System 9700 version 3.08" (Applied Biosystems, Carlsbad, CA, USA) was used with the SNaPshot cycling programme as 96°C for 10s, and then repeated for 25 cycles at 50°C for 5s and 60°C for 30s.

The SNaPshot reaction contained the following reagents at final concentrations: 5 µl, 5 µl and 1 µl PCR product from each sample for combinations A, B and C, respectively. 1 µl SNaPshot multiplex ready reaction mix, pooled internal SNaPshot primers were designed and is shown in Table 5 (Integrated DNA Technologies, Inc., Coralville, IA, USA) and the volume was made up to a total of 10 µl using sterile distilled water (sdH_2O). The second shrimp alkaline phosphatase (*SAP*) clean-up was performed by adding 1 U *SAP* (Fermentas Life Sciences, Burlington, Canada) to the total SNaPshot reaction volume of 10 µl. The SNaPshot reaction was incubated at 37^0C for 1 hour, the enzyme was inactivated at 75^0C for 15mins, the SNaPshot products denatured at 94^0C for 5mins and placed on ice until capillary electrophoresis.

0.25 µl of the dye-labelled GeneScan™-120LIZ™ (Applied Biosystems, Carlsbad, CA, USA) and 4 µl HiDi formamide was added to 5.75 µl SNaPshot product and capillary electrophoresis was performed using a ABI 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA). SNaPshot results were analysed on the GeneMapper © Software version 4.1 (Applied Biosystems, Carlsbad, CA, USA).

2.4.3. Cycle sequencing

Direct cycle sequencing was used to identify novel genetic polymorphisms in *CYP2B6*, *CYP3A5* and *ABCB1*, as well as confirm the genotyping results from three individuals of each genotype per SNP. The ABI Prism BigDye Terminator v3.1 kit (Applied Biosystems, Carlsbad, CA, USA) with the

terminator mix and sequencing buffer, was used. The terminator mix contained the ddNTPs, as well as the thermo-stable DNA polymerase. The sequencing reaction (20 µl) included 2 µl PCR product, 0.5 X terminator mix, 10 pM of primer and 1 X sequencing buffer. Sequencing reactions were made up to 20 µl with sdH_2O. The cycling conditions of the sequencing reaction used an initial denaturation step at 98°C for 5mins, 30 cycles consisting of a denaturation step at 96°C for 10s, an annealing step at 50°C for 15s and an extension step at 60°C for 4mins.

Clean-up of the cycle sequencing reaction was required to remove unincorporated ddNTPs and excess primer, also to precipitate the sequencing product. This involved the addition of 44 µl absolute ethanol and 2 µl 5 M sodium oxaloacetate (pH 4.5) to each reaction, followed by storage at -20°C for a minimum of two hours. After storage at -20°C, the supernatant was removed by centrifugation at 10 000 RPM for 5mins. 40 µl 70% (v/v) ethanol was added, to remove the absolute ethanol and samples were centrifuged at 10 000 RPM for another 5mins. The supernatant was discarded and the samples were left to air-dry for at least 30mins. Sequencing samples were resuspended in 10 µl sdH_2O and analysed on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA).

Sequencing analysis was performed using Bioedit Sequence Alignment Editor version 7.0.0 (Tom Hall, Isis Pharmaceuticals, Inc.) and all sequences were aligned to the reference gene DNA sequences database using the ClustalW multiple alignment accessory application.

2.5. Statistical analysis

Statistical analysis was performed using Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA) to calculate Hardy-Weinberg Equilibrium (HWE), while GraphPad Prism version 5 (GraphPad Software, Inc, La Jolla, CA, USA) and Statistica version 10 (StatSoft Ltd, Atterbury, UK) were used for genotype-phenotype correlations. Linkage disequilibrium and haplotype analysis were

performed using SHEsis. SHEsis infers haplotypes using a PL-CSEM (Partition-Ligation Combination-Subdivision Expectation Maximization) algorithm based on the standard Expectation Maximization (EM) algorithm but with the improvement of estimating haplotypes from a large number of individuals and multi-allelic loci. In the first step some alleles are combined randomly to construct an optimum set of candidate haplotypes with frequencies greater than a threshold value (using the EM algorithm). In the subdivision step the alleles are subdivided in two to form the set of candidate haplotypes for the next EM step [146, 147]. A p-value <0.05 was considered statistically significant and a p-value <0.1 was considered a trend.

2.5.1. Sample size calculation

The following equation was used to calculate the sample size required to achieve a 95% confidence-interval: $N=Z^2 (pq) / d^2$. Z is defined as the value for 95% confidence, d is a α-value = 0.05, while p and q are the frequency of the variant allele and 1-p, respectively. A Z-value of 1.96 and allele frequency of 0.1 was used to calculate the sample size.

$N = Z^2 (pq) / d^2$

$N = (1.96)^2 (0.1x0.9) / (0.05)^2$

N = 138 samples

2.5.2. Genotype-Phenotype analysis

Drug plasma concentration levels from time-point A and time-point B were noted for each sample and the change in EFV concentration levels were also calculated. The distribution of mean drug plasma concentrations was established and normality of the data were tested using the Shapiro-Wilk normality test.

The effect of patient demographic and clinical parameters on average EFV plasma concentration was assessed by Mann–Whitney rank sum test and Chi^2-analysis (for gender, alcohol consumption, tobacco smoking, opportunistic infection and change in drug regimen) or by Spearman correlation and Kruskal-Wallis one-way analysis (for age, BMI, CD-4 cell count, viral load, haemoglobin levels, AST levels and ALT levels).

The effect of genetic polymorphisms in *CYP1A2*, *CYP2A6*, *CYP2B6*, *CYP3A4/5* and *ABCB1* on average drug plasma concentration, CD-4 cell count, viral load and opportunistic infection was measured by Kruskal-Wallis one-way analysis or by Mann-Whitney rank sum test. The combined effect of genetic polymorphisms was performed using Kruskal-Wallis one-way analysis and followed by Dunn's post test for multiple comparisons. Chi^2-analysis was carried out to investigate the effect of genetic polymorphisms on change in ARV-regimen.

Chapter 3: Results

3.1. Study subjects and demographic characteristics

The study cohort (n=464) consisted of 163 healthy research participants and 301 HIV/AIDS patients. The clinical and demographic characteristics of the study participants are presented in Table 6. About 67% of the healthy participants were females; less than 20% of all the healthy participants smoked tobacco and about 40% consumed alcohol. The average age (±SD) of the study participants were approximately 38 (±12.28) years.

Efavirenz plasma concentration levels were available for 156 of the 301 patients and information regarding disease stage was available for 286 of the patients. The current study is ongoing and information on disease stage is available from hospital records and therefore updated regularly and also available for all the patients and not only the patients with EFV plasma concentrations. Disease stage 1 is defined by the World Health Organization as asymptomatic HIV-infection with persistent generalized lymphadenopathy, while disease stage 2 is defined by unexplained moderate weight loss, recurrent upper respiratory tract infection and infection with *Herpes zoster* or *Angular cheilitis*. Disease stage 3 is defined by unexplained severe weight loss and disease stage 4 is termed HIV wasting syndrome. Most (63%) of the HIV/AIDS patients were classified as disease stage 1. The average (range) EFV plasma concentration was 4.64 (0.6 – 22) µg/ml, while the average (range) for time-points A and B were 4.78 (0.2 – 34) µg/ml and 4.61 (0.2 – 31.8) µg/ml, respectively. HIV/AIDS patients had an average BMI of 23.23 at baseline. The average CD-4 cell count and viral load of patients initiating ARV treatment was 136.09 (±113.24) cells/µl and 26917.71 (±27133.50) copies/ml, respectively. After 6 months of first-line ARV treatment, the average CD-4 cell count almost doubled, while the average viral load was about 18-times less than at baseline. 21% (n=54) of patients presented with haemoglobin levels below 10 g/dl, 44% (n=112) with AST levels above 35

IU/l and 20% (n=50) with ALT levels above 40 IU/l. Non-ARV medication co-administrated with EFV included mainly bactrim, vitamin B and rifampicin and 42% of HIV/AIDS patients had opportunistic infections. Four patients reported to have missed a day of treatment.

Table 6: **Demographic characteristics of the healthy research participants and HIV/AIDS**

	Healthy research participants (n=163)	**HIV/AIDS patients (n=301)**
Ethnicity	Bantu-speaking	Bantu-speaking
Gender/ sex ratio Male: Female)	1:2.25	1:3.81
Smoking	8 (15.4%)	20 (6.9%)
Alcohol use	22 (42.3%)	28 (9.7%)
Both	8 (15.4%)	11 (3.8%)
Mean age (years)	35.77 (±13.96)	41.29 (±9.3)
Mean BMI (kg/m^2)	N/A	23.23 (±4.74)
Average viral load at baseline (copies/ml)	N/A	26917.71 (±27133.50)
Average CD4 count at baseline (cells/µl)	N/A	136.09 (±113.24)
ARV regimen –	N/A	
3TC_TDF_EFV		25 (1.7%)
AZT_3TV_EFV		62 (20.6%)
d4T_3TC_EFV		214 (71.1%)
Average EFV plasma concentration (µg/ml)	N/A	4.64 (0.6 – 22)
Mean Haemoglobin level (IU/l)	N/A	11.58 (±2.14)
Median AST level (IU/l)	N/A	32 (15 – 228)
Median ALT level (IU/l)	N/A	23.5 (6 – 176)

N/A = Not applicable

3.2. PCR/RFLP genotyping

Genotyping of the *CYP1A2 C-163A, CYP2A6 G1093A, CYP2B6 G516T, A785G* and *C1459T, CYP3A4 A-392G, CYP3A5 A6986G, G14690A* and *27131-2insT* genetic polymorphisms were performed using PCR-RFLP. A typical example of PCR/RFLP genotyping is shown in Figure 3.1 using the genotyping of *CYP2B6 G516T* polymorphism as an example. Presence of the *CYP2B6 516T* variant abolishes a *BsrI* restriction site, thus, digestion of the 526 bp PCR fragment results in two bands (509 and 17 bp) compared to the three bands (268, 241 and 17 bp) obtained when the *516G* variant is present. The PCR/RFLP for the other SNPs are shown in Appendix E.

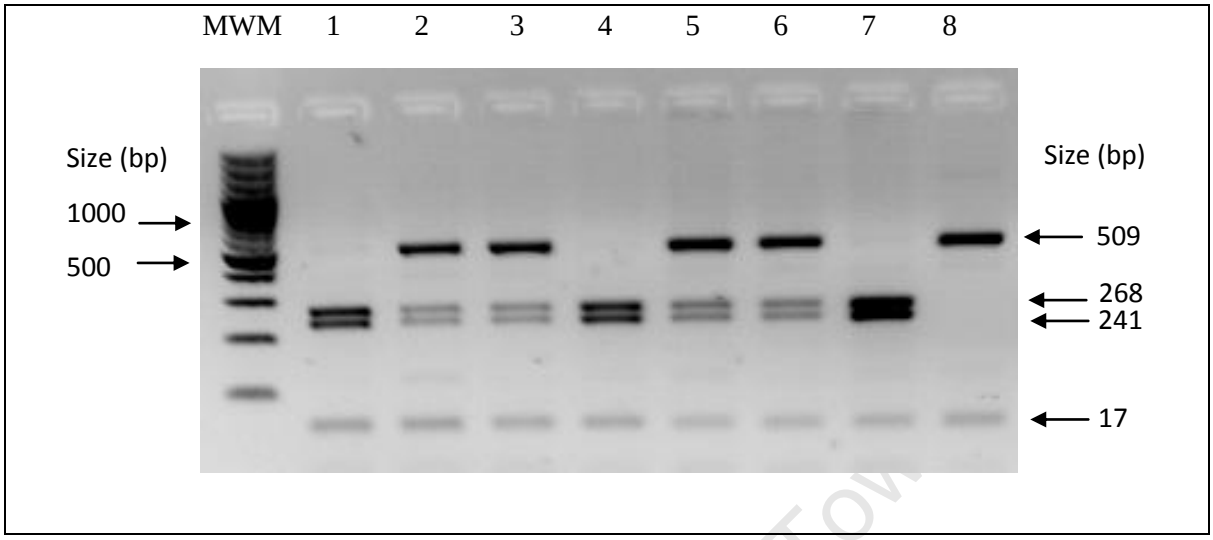

Figure 3.1: Genotyping of *CYP2B6 G516T* is shown on a 2.5% agarose gel. The 100bp plus molecular weight marker (MWM) was used and the genotypes are represented as follows; G/G = lanes 1, 4 and 7, G/T = lanes 2, 3, 5 and 6, with the T/T genotype in lane 8.

3.3. SNaPshot genotyping

Genotyping of the *CYP2B6 A136G* and *T983C, CYP3A5 C3699T, G19386A* and *C31611T, ABCB1 A61G, C1236T, C3435T/A, T2677G/A* and *A4036G* genetic polymorphisms were performed using SNaPshot. Below in Figure 3.2 we show an example of the chromatograms obtained using *ABCB1 A4036G* and *T2677G/A* SNPs as an examples while the other genetic polymorphisms are shown in Appendix F.

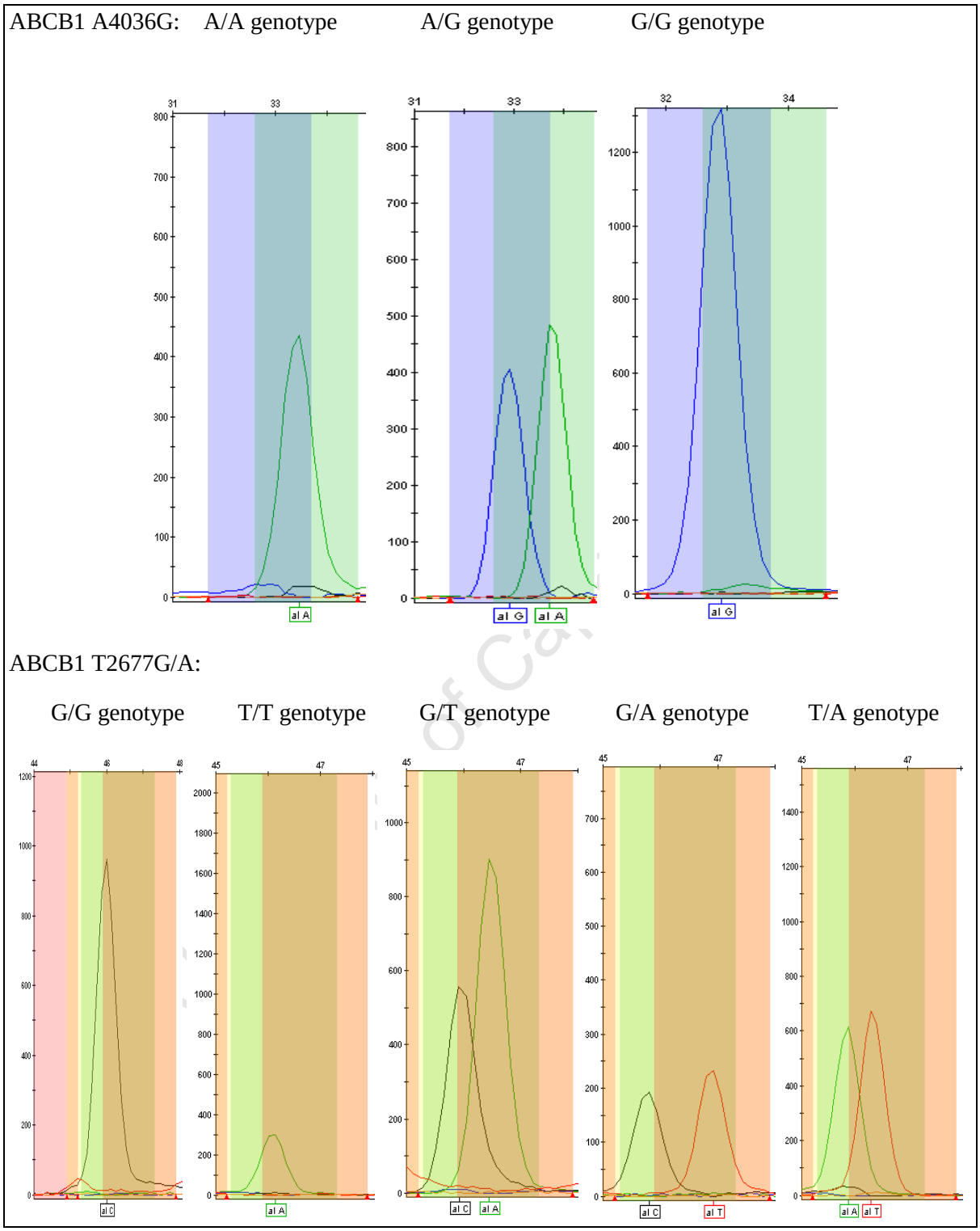

Figure 3.2: SNaPshot genotyping of the *ABCB1 A4036G* and *T2677G/A* genetic polymorphisms using capillary electrophoresis. A reverse internal SNaPshot primer was used in *ABCB1 T2677G/A* genotyping and the *2677T* allele, *2677G* allele and *2677A* allele is therefore represented as a *2677A* allele (green), *2677C* allele (black) and *2677T* allele (red), respectively.

3.4. Allele and genotype frequencies of SNPs

Tables 7 and 8 show the genotype and allele frequencies of the genetic polymorphisms in *CYP1A2*, *CYP2A6, CYP2B6, CYP3A4/5* and *ABCB1* in the healthy participants as well as the HIV/AIDS patients. The allele and genotype frequencies were first established in the healthy participants and then used to target genotyping in the HIV/AIDS patients. Allele and genotype frequencies were expected to be similar between healthy participants and HIV/AIDS patients, however, genotype frequencies differed significantly between these two groups for the *CYP2B6 T983C, CYP3A4 A-392G, CYP3A5 A6986G, CYP3A5 27131-2insT, ABCB1 A61G, ABCB1 C3435T/A* and *ABCB1 T2677G/A* SNPs. Allele and genotype frequencies differ between ethnic groups and the differences observed between the healthy participants and HIV/AIDS patients can be as a result of small differences in the composition of the healthy controls compared to patients with respect to different ethnic groups or possibly undetected genotyping errors. Hardy-Weinberg equilibrium (HWE) was calculated in the healthy research participants and HIV/AIDS patients and genotyping results were confirmed by cycle sequencing. The genotype frequencies of four SNPs deviated from HWE in the healthy participants and patients and could be an indication of genotyping errors in the *CYP2B6 A136G, CYP3A4 A-392G, CYP3A5 A6986G* and *CYP3A5 27131-2insT* SNPs. The variant allele frequencies of all the SNPs were above 0.01, except for the *CYP3A5 C3699T, CYP3A5 G19386A* and *ABCB1 A61G* SNPs that were monomorphic.

Table 7: Genotype frequencies of genetic polymorphisms in *CYP1A2, CYP2A6, CYP2B6, CYP3A4/5* and *ABCB1*.

Genotype	Healthy research participants N (freq)	HIV/AIDS patients N (freq)	P-value	HWE (p-value)	
				Healthy participants	HIV/AIDS patients
CYP1A2: -163C>A	104	138			
C/C	8 (0.077)	18 (0.130)			
C/A	44 (0.423)	70 (0.507)	0.0765	0.755	0.397
A/A	52 (0.500)	50 (0.362)			
CYP2A6: 1093G>A	160	293			
G/G	135 (0.444)	253 (0.863)			
G/A	25 (0.156)	35 (0.119)	0.1470	0.284	0.007
A/A	0 (0.000)	5 (0.017)			
CYP2B6: 136A>G	160	295			
A/A	141 (0.881)	248 (0.841)			
A/G	16 (0.100)	33 (0.112)	0.2693	0.006	0.000
G/G	3 (0.019)	14 (0.047)			
CYP2B6: 516G>T	160	295			
G/G	64 (0.400)	107 (0.363)			
G/T	76 (0.475)	133 (0.451)	0.2356	0.726	0.233
T/T	20 (0.125)	55 (0.186)			
CYP2B6: 785A>G	160	293			
A/A	63 (0.394)	110 (0.375)			
A/G	78 (0.488)	132 (0.451)	0.2945	0.488	0.297
G/G	19 (0.119)	51 (0.174)			
CYP2B6: 983T>C	156	290			
T/T	148 (0.949)	253 (0.872)			
T/C	8 (0.051)	32 (0.110)	0.0261	0.742	0.002
C/C	0 (0.000)	5 (0.017)			
CYP2B6: 1459C>T	163	293			
C/C	158 (0.969)	289 (0.986)	0.2921	0.842	0.906
C/T	5 (0.031)	4 (0.014)			
CYP3A4: -392A>G	101	86			
A/A	22 (0.218)	9 (0.105)			
A/G	30 (0.297)	38 (0.442)	0.0414	0.0002	0.954
G/G	49 (0.485)	39 (0.453)			
CYP3A5: 3699C>T	160	295			
C/C	160 (1.000)	295 (1.000)			
CYP3A5: 6986A>G	155	295			
A/A	100 (0.645)	211 (0.715)			
A/G	41 (0.265)	77 (0.261)	0.0056	0.003	0.994
G/G	14 (0.090)	7 (0.024)			
CYP3A5: 14690G>A	162	290			
G/G	113 (0.698)	194 (0.669)			
G/A	44 (0.272)	91 (0.314)	0.4457	0.777	0.121
A/A	5 (0.031)	5 (0.017)			
CYP3A5:27131-2insT	161	294			
-/-	146 (0.907)	282 (0.959)			
-/T	10 (0.062)	5 (0.017)	0.0313	0.0001	0.000
T/T	5 (0.031)	7 (0.024)			

CYP3A5: 19386G>A	160	295			
G/G	160 (1.000)	295 (1.000)			
CYP3A5: 31611C>T	157	295			
C/C	70 (0.446)	144 (0.488)			
C/T	66 (0.420)	114 (0.386)	0.6912	0.391	0.059
T/T	21 (0.134)	37 (0.125)			
ABCB1: 61A>G	160	295			
A/A	157 (0.981)	295 (1.000)			
A/G	3 (0.019)	0 (0.000)	0.0430	0.905	
ABCB1: 3435C>T/A	160	295			
C/C	102 (0.638)	233 (0.790)			
C/T	50 (0.313)	59 (0.200)	0.0004	0.530	0.730
T/T	8 (0.050)	3 (0.010)			
ABCB1: 4036A>G	160	289			
A/A	98 (0.613)	189 (0.641)			
A/G	56 (0.350)	96 (0.325)	0.8368	0.253	0.605
G/G	6 (0.038)	10 (0.034)			
ABCB1: 1236C>T	160	295			
C/C	112 (0.700)	235 (0.797)			
C/T	45 (0.281)	56 (0.190)	0.0687	0.564	0.750
T/T	3 (0.019)	4 (0.014)			
ABCB1: 2677T>G/A	157	294			
G/G	132 (0.841)	283 (0.963)			
G/T	21 (0.134)	9 (0.031)			
T/T	2 (0.013)	0 (0.000)	<0.0001	0.563	0.789
T/A	1 (0.006)	1 (0.003)			
G/A	1 (0.006)	1 (0.003)			

N=number of individuals

Table 8: Allele frequencies of genetic polymorphisms in *CYP1A2, CYP2A6, CYP2B6, CYP3A4/5* and *ABCB1*.

Allele	Healthy research participants N (freq)	HIV/AIDS patients N (freq)	P-value
CYP1A2: -163C>A	208	276	
C	60 (0.289)	106 (0.384)	0.0333
A	148 (0.712)	170 (0.616)	
CYP2A6: 1093G>A	320	586	
G	295 (0.922)	541 (0.923)	1.0000
A	25 (0.078)	45 (0.077)	
CYP2B6: 136A>G	320	590	
A	298 (0.931)	529 (0.897)	0.0917
G	22 (0.069)	61 (0.103)	
CYP2B6: 516G>T	320	590	
G	204 (0.638)	347 (0.588)	0.1557
T	116 (0.363)	243 (0.412)	
CYP2B6: 785A>G	320	586	
A	204 (0.638)	352 (0.601)	0.2851
G	116 (0.363)	234 (0.399)	
CYP2B6: 983T>C	312	580	
T	304 (0.974)	538 (0.928)	0.0034

C	8 (0.026)	42 (0.072)	
CYP2B6: 1459C>T	326	586	
C	321 (0.986)	582 (0.993)	0.2944
T	5 (0.015)	4 (0.007)	
CYP3A4: -392A>G	202	172	
A	74 (0.366)	56 (0.326)	0.4463
G	128 (0.634)	116 (0.674)	
CYP3A5: 3699C>T	320	590	
C	320 (1.000)	590 (1.000)	
CYP3A5: 6986A>G	310	590	
A	241 (0.777)	499 (0.846)	0.0131
G	69 (0.223)	91 (0.154)	
CYP3A5: 14690G>A	324	580	
G	270 (0.833)	479 (0.826)	0.8540
A	54 (0.167)	101 (0.174)	
CYP3A5:27131-2insT	322	588	
-	302 (0.938)	569 (0.968)	0.0399
T	20 (0.062)	19 (0.032)	
CYP3A5: 19386G>A	320	590	
G	320 (1.000)	590 (1.000)	
CYP3A5: 31611C>T	314	590	
C	206 (0.656)	402 (0.681)	0.4571
T	108 (0.344)	188 (0.317)	
ABCB1: 61A>G	320	590	
A	317 (0.991)	590 (1.000)	0.0432
G	3 (0.009)	0 (0.000)	
ABCB1: 3435C>T/A	320	590	
C	254 (0.794)	525 (0.890)	0.0001
T	66 (0.206)	65 (0.110)	
ABCB1: 4036A>G	320	590	
A	252 (0.788)	474 (0.803)	0.6042
G	68 (0.213)	116 (0.197)	
ABCB1: 1236C>T	320	590	
C	269 (0.841)	526 (0.892)	0.0362
T	51 (0.159)	64 (0.108)	
ABCB1: 2677T>G/A	314	586	
G	286 (0.911)	576 (0.980)	<0.0001
T	26 (0.083)	10 (0.017)	
A	2 (0.006)	2 (0.030)	

N=number of alleles

3.5. Linkage disequilibrium and Haplotype analysis

Linkage disequilibrium (LD) analysis was performed for the five SNPs in the *CYP2B6* gene, the six SNPs in *CYP3A5* and the five SNPs in *ABCB1*. High pairwise linkage disequilibrium coefficients (D´and r^2) indicated that *CYP2B6 G516T* and *A785G* are in tight linkage disequilibrium and are reported in Table 9, while the LD plots are shown in Figure 3.3. No tight LD was observed between the genetic polymorphisms in *CYP3A5* or *ABCB1* (Table 9). D´ and r^2 are both measures of linkage disequilibrium and should therefore be similar; however, these coefficients are not independent of allelic frequencies and the r^2 coefficient is a correlation coefficient which is preferred by the HapMap project and therefore used in this study.

Table 9: Linkage disequilibrium analysis of *CYP2B6, CYP3A5* and *ABCB1*.

SNP pair	South Africans (n=455)
G516T-A785G	
D´	0.967
r^2	0.913
A785G-T983C	
D´	0.999
r^2	0.039
G516T-T983C	
D´	0.906
r^2	0.033
A6986G-C31611T	
D´	0.845
r^2	0.319
G14690A-C31611T	
D´	0.912
r^2	0.085
C3435T/A-C1236T	
D´	0.679
r^2	0.397
C3435T/A-T2677G/A	
D´	0.598
r^2	0.097
C1236T-T2677G/A	
D´	0.641
r^2	0.130

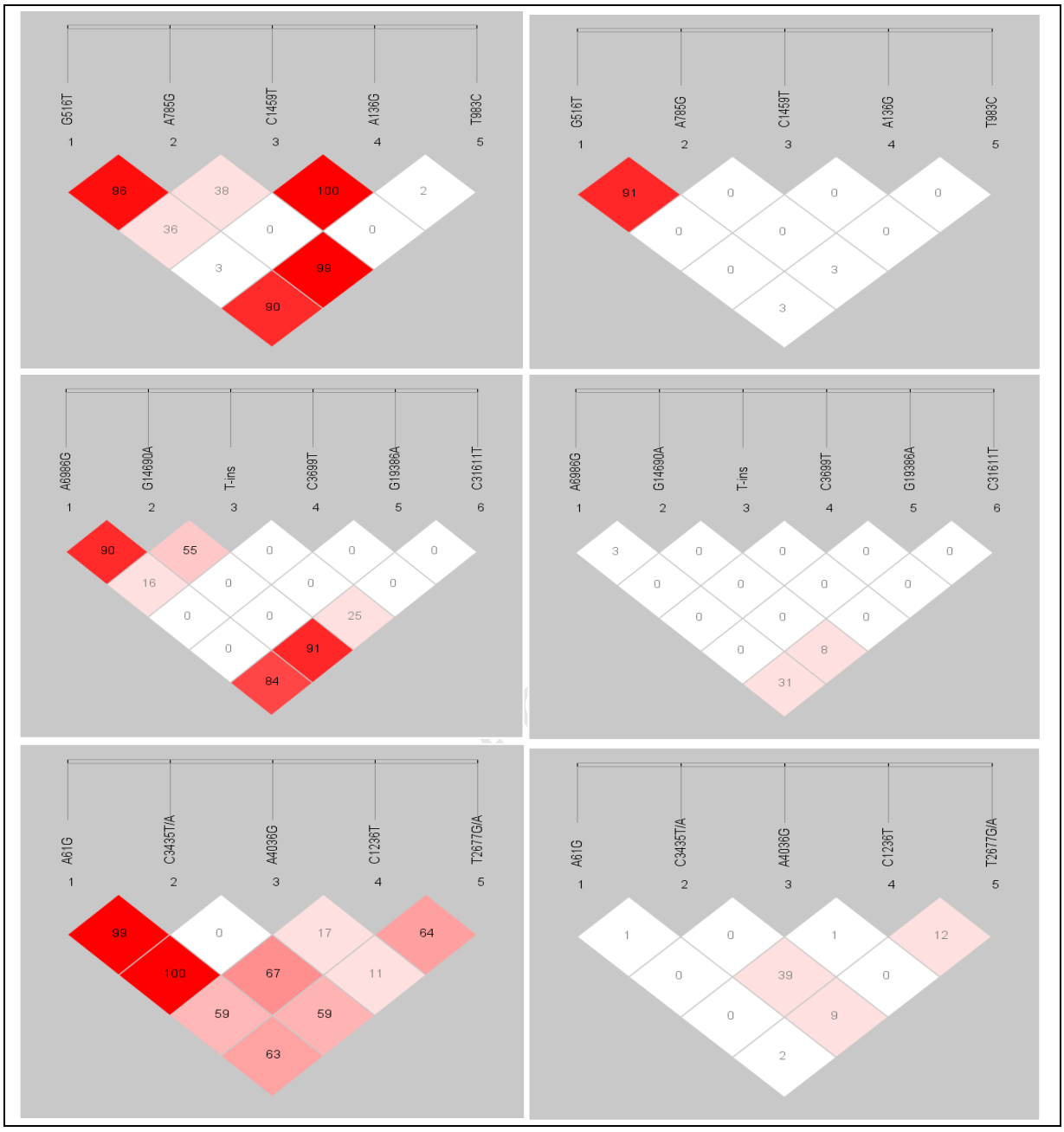

Figure 3.3 Linkage disequilibrium (LD) analysis of genetic polymorphisms in *CYP2B6, CYP3A5* and *ABCB1*. 1and 2) D´ and r^2 linkage disequilibrium coefficients of SNPs in *CYP2B6*. 3 and 4) D´ and r^2 linkage disequilibrium coefficients of SNPs in *CYP3A5*. 5 and 6) D´ and r^2 linkage disequilibrium coefficients of SNPs in *ABCB1*.

Haplotype analysis demonstrated the presence of four, five and seven frequent inferred haplotypes for *CYP2B6*, *CYP3A5* and *ABCB1*, respectively. The inferred haplotypes with a frequency below 5% as well as the inferred haplotypes with a significant difference in frequency between the healthy research participants and HIV/AIDS individuals are reported in Table 10.

Table 10: Haplotype analysis of *CYP2B6, CYP3A5* and *ABCB1* in the healthy research participants (n=153) and HIV/AIDS individuals (n=295).

Haplotype no.	Haplotype						Healthy participants	HIV/AIDS patients
CYP2B6	A136G	G516T	A785G	C1459T	T983C			
1	A	G	A	C	T		0.5441	0.4717
2	A	G	A	C	C		0.0170	0.0624
3	A	T	G	C	T		0.3381	0.3506
4	G	T	G	C	T		0.0095	0.4690
CYP3A5	C3699T	A6986G	G14690A	T-ins	G19386A	C31611T		
5	C	A	G	-	G	C	0.4111	0.4857
6	C	A	G	-	G	T	0.1456	0.1649
7	C	A	A	-	G	C	0.1700	0.1649
8	C	G	G	-	G	T	0.1867	0.1436
9	C	G	G	-	G	C	0.0300	0.0052
ABCB1	A61G	C3435T/A	A4036G	C1236T	T2677G/A			
10	A	C	A	C	G		0.6018	0.6754
11	A	C	G	C	G		0.1457	0.1708
12	A	T	A	T	G		0.0305	0.0573
13	A	T	A	C	G		0.0684	0.0343
14	A	T	A	T	T		0.0455	0.0013
15	A	T	G	T	G		0.0396	0.0077
16	A	C	G	T	A		0.0074	0.0086

3.6. Correlation with EFV plasma concentration

The distribution of EFV plasma concentrations in the HIV/AIDS patients was investigated and a large degree of inter-individual variability (38-fold) was observed, which has implications on ARV treatment response such as the development of ADRs (Figure 3.4).

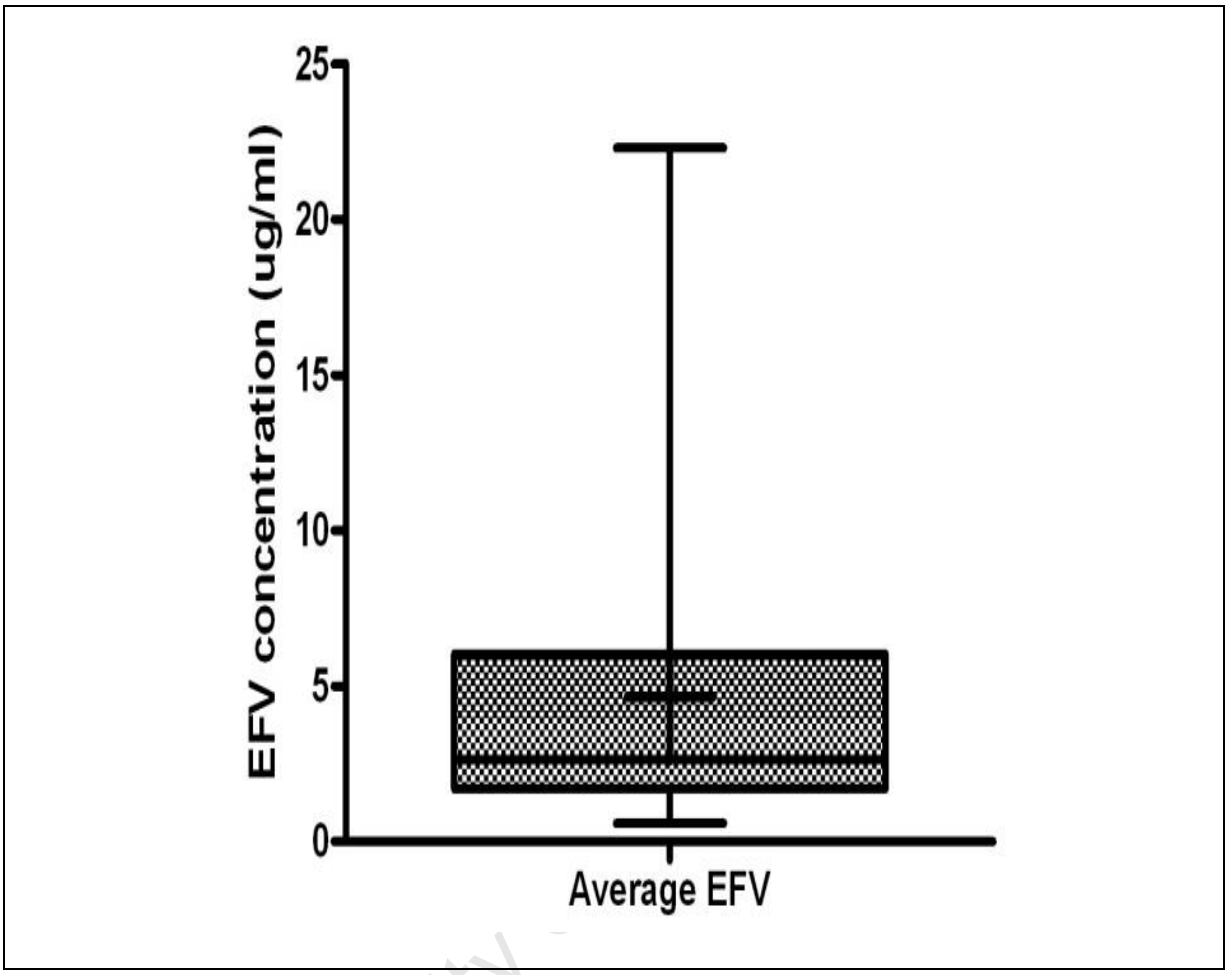

Figure 3.4: The average EFV plasma concentration levels.

The *CYP2B6 516T/T* (P<0.0001), *785G/G* (P<0.0001) and *983C/C* (P=0.027) genotypes were independently associated with high average EFV plasma concentrations. The current assumption that the *CYP1A2 -163A* allele affects enzyme activity only in smokers but not in non-smokers is supported by our results as no significant difference in EFV concentrations are observed in non-smokers (P=0.3523), but a very small number (n=7) of smokers were present in this study and conclusions can therefore not be drawn (Figure 3.5).

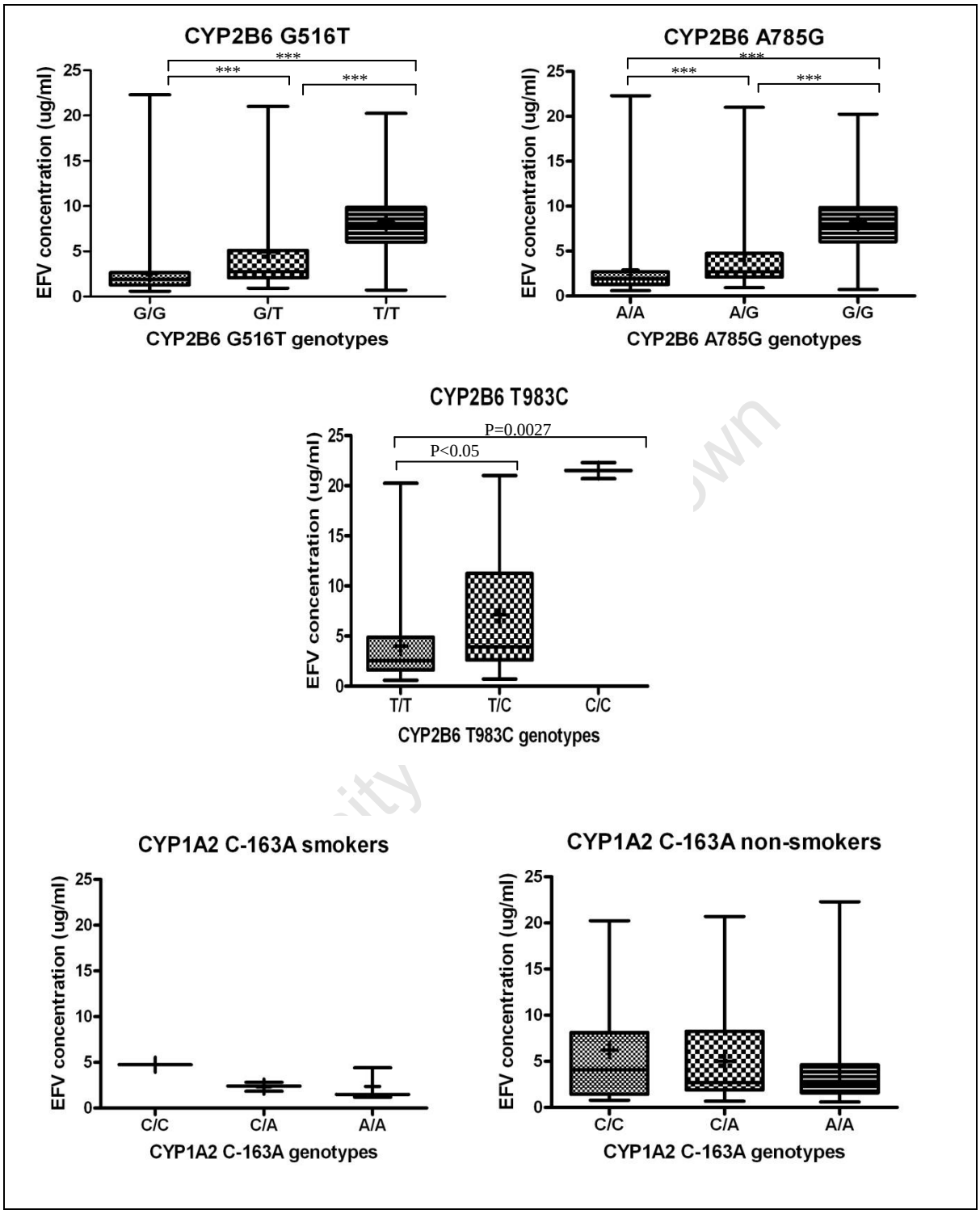

Figure 3.5: The *CYP2B6 G516T, A785G* and *T983C* genetic polymorphisms were significantly associated with high EFV plasma concentrations. *CYP1A2 C-163A* showed a trend towards low EFV concentration levels in the smokers, but not in the non-smokers. ***P<0.0001,

The combined effect of the associated genetic polymorphisms in *CYP2B6* and *CYP1A2* were investigated and is shown in Figure 3.6. The *CYP2B6 516T, 785G, 983C* and *CYP1A2 -163C* alleles are defined as high risk alleles. The individuals with four, five and six high risk alleles have significantly higher EFV plasma concentrations compared to individuals without the *CYP2B6 516T, 785G, 983C* and *CYP1A2 -163C* alleles.

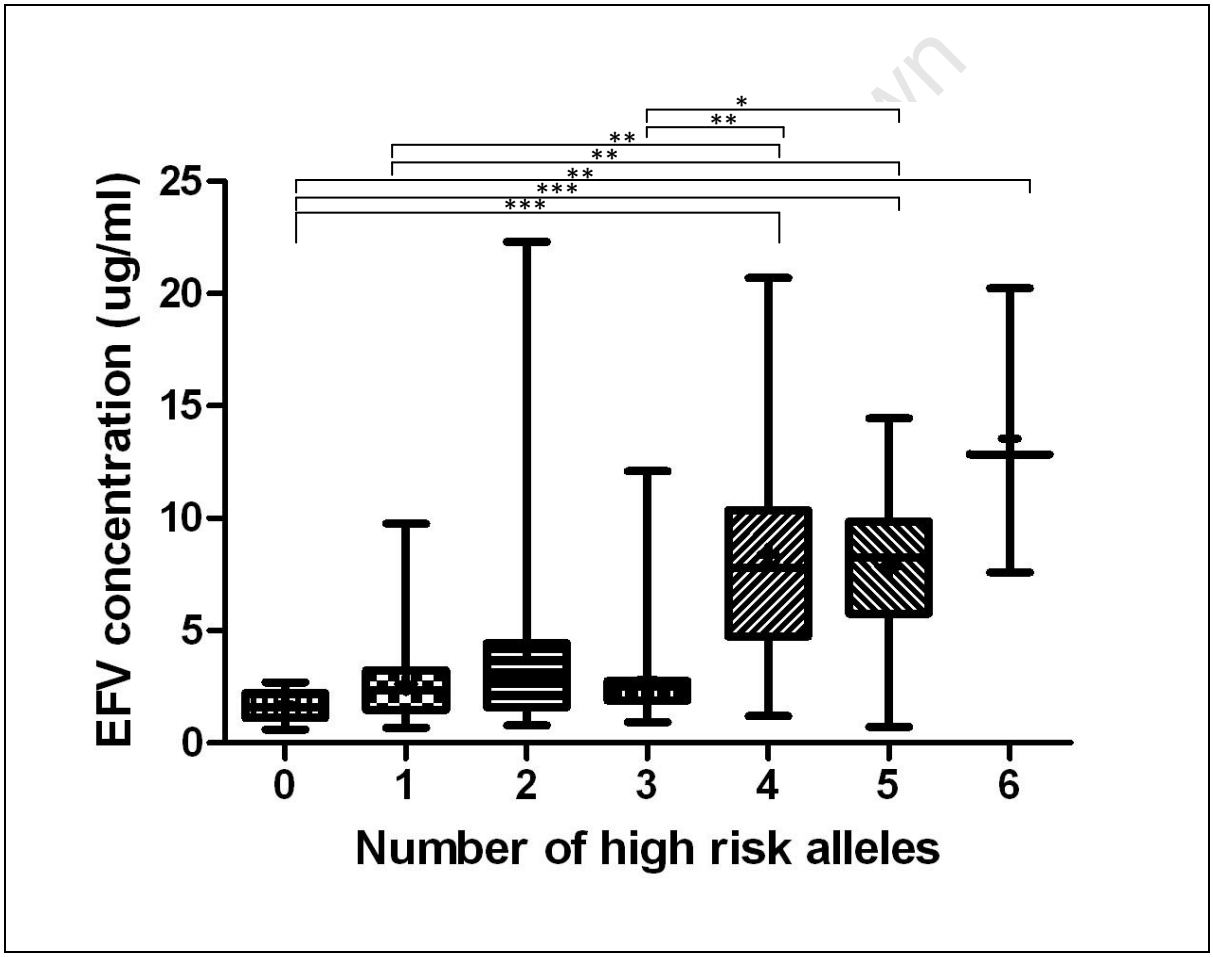

Figure 3.6: The combined effect of *CYP2B6 516T, 785G, 983C* and *CYP1A2 -163C* alleles on the average EFV plasma concentration.***P<0.0001, **P<0.01, *P<0.05.

3.7. Correlation of EFV with disease severity

Comparison of EFV plasma levels in the different disease stages showed no significant difference (P=0.2842), but a trend towards high mean EFV levels in the more advanced stages of disease and disease severity was observed and could be explained by virologic failure as a result of the development of ADRs due to high EFV levels leading to poor adherence to treatment (Figure 3.7).

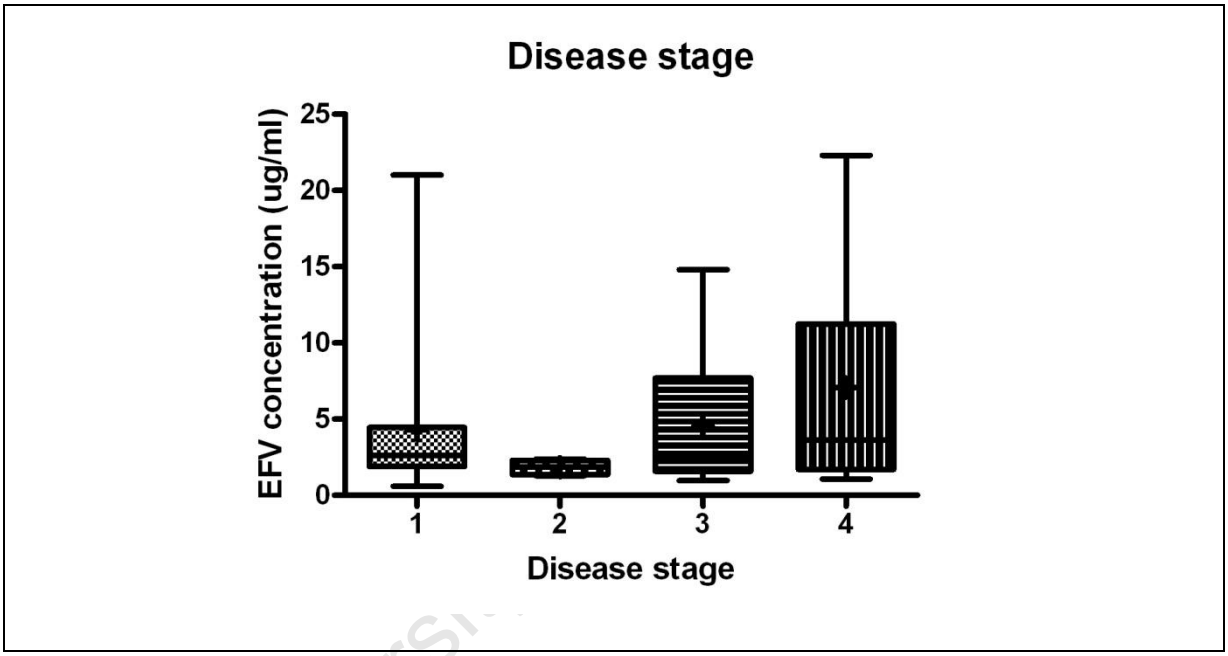

Figure 3.7: Correlation of EFV plasma concentrations with disease severity.

3.8. Correlation of genotypes with change in ART regimen

Information regarding a change in ARV regimen was available and investigated for the *CYP2B6 G516T, A785G, T983C* and *CYP1A2 C-163A* SNPs. A higher percentage of individuals with the *CYP2B6 516T/T* (96%) and *785G/G* (98%) genotypes changed treatment within one year after initiation compared to the *CYP2B6 516G/G* (82%) and *785A/A* (76%) genotypes. The difference can be explained by the high EFV plasma concentrations, resulting in the development of CNS side

effects. The *CYP2B6 983C/C* (40%) and *CYP1A2 -163A/A* (63%) genotypes was shown to result in fewer individuals with a change in treatment compared to the *CYP2B6 983T/T* (89%) and *CYP1A2 -163C/C* (71%) genotypes. HIV/AIDS patients with the *CYP1A2 -163A/A* genotype and low EFV plasma concentrations were less likely to develop ADRs and to change treatment, however very few individuals have the *CYP2B6 983C/C* genotype (Table 11).

Table 11: Change in ARV regimen within three months, six months and one year after treatment initiation

Genotypes	Treatment initiation	Three months	Six months	One year
CYP2B6 516G/G	102	6%	26%	82%
CYP2B6 516G/T	125	12%	39%	90%
CYP2B6 516T/T	53	9%	34%	96%
CYP2B6 785A/A	105	5%	23%	76%
CYP2B6 785A/G	125	13%	42%	93%
CYP2B6 785G/G	49	10%	37%	98%
CYP2B6 983T/T	240	9%	35%	89%
CYP2B6 983T/C	30	10%	33%	93%
CYP2B6 983C/C	5	0%	0%	40%
CYP1A2 -163C/C	17	18%	59%	71%
CYP1A2 -163C/A	64	8%	28%	61%
CYP1A2 -163A/A	49	8%	27%	63%

3.9. Anomalies in EFV plasma concentration

About 5% (n=7) of the total samples (n=138) had drug levels below the EFV therapeutic range of 1.00 µg/ml, while approximately 31% (n=48) of samples had concentrations above the therapeutic range of 4.00 µg/ml (Figure 3.8).

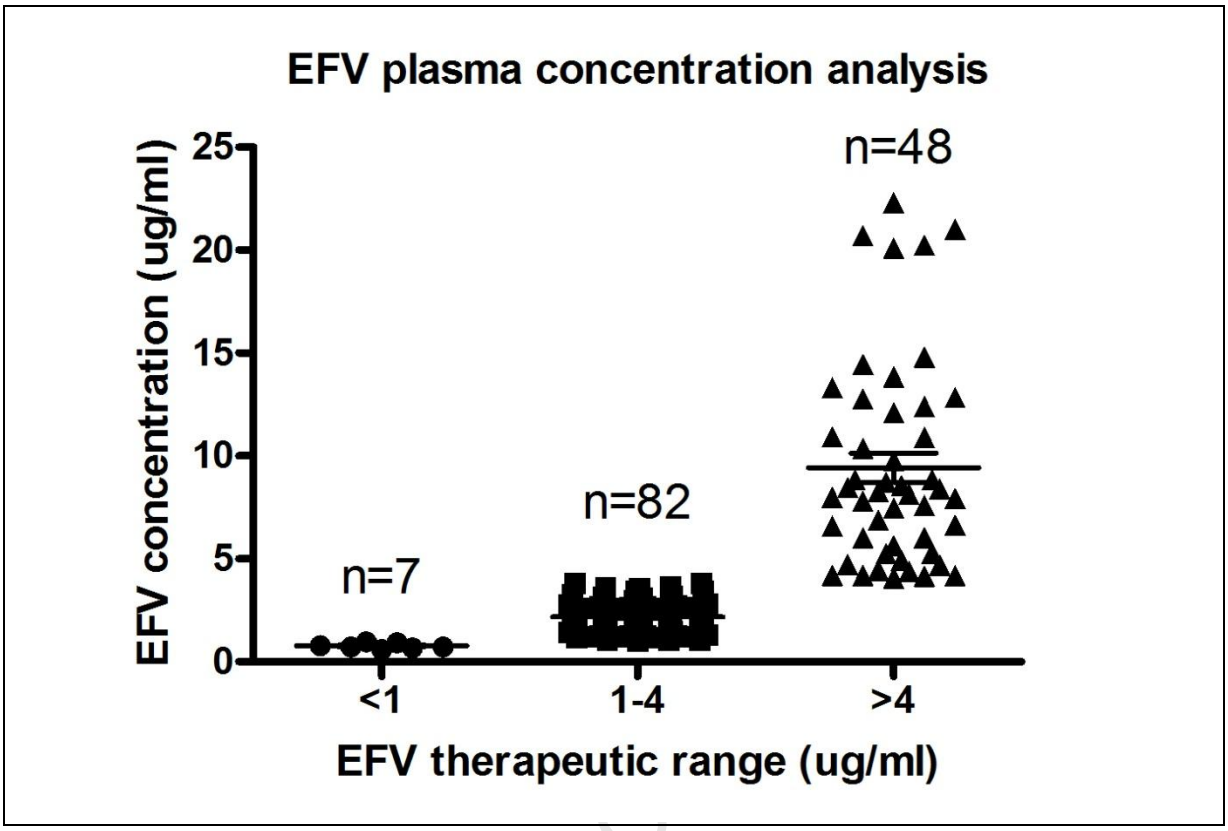

Figure 3.8: The average EFV concentration levels presented according to EFV therapeutic range (1-4 µg/ml).

Two of the four individuals (patients one and four) with the *CYP2B6 516G/G* genotype, but high EFV levels, have the *CYP2B6 983C* allele resulting in decreased enzyme activity. Patient two has decreased CYP3A5 and ABCB1 enzyme activity as a result of the *CYP3A5 6986G* allele and *ABCB1 1236T* and *3435T* alleles and patient 3 has the *CYP3A5 27131-2insT* polymorphism associated with reduced CYP3A5 enzyme activity. All three individuals with low EFV plasma concentrations, but the *CYP2B6 516G/T* or *516T/T* genotypes, have the *CYP1A2 -163A* allele associated with increased enzyme inducibility and therefore increased EFV metabolism (Table 12).

Table 12: Genotypes for patients with EFV plasma concentrations above or below the therapeutic range.

Gene	Variant	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7
EFV levels		>4 µg/ml	>4 µg/ml	>4 µg/ml	>4 µg/ml	<1 µg/ml	<1 µg/ml	<1 µg/ml
CYP1A2	C-163A	A/A	C/A	C/A	C/A	C/A	A/A	C/A
CYP2A6	G1093A	G/A	G/G	G/G	G/G	G/G	G/G	G/G
CYP2B6	A136G	A/A	A/G	A/A	A/A	A/A	G/G	A/A
CYP2B6	G516T	G/G	G/G	G/G	G/G	G/T	G/T	T/T
CYP2B6	A785G	A/A	A/A	A/A	A/A	A/G	A/G	G/G
CYP2B6	T983C	T/C	T/T	T/T	C/C	T/T	T/T	T/T
CYP2B6	C1459T	C/C	C/C	C/C	C/C	C/C	C/C	C/C
CYP3A4	A-392G	N/A	N/A	N/A	N/A	N/A	G/G	N/A
CYP3A5	C3699T	C/C	C/C	C/C	C/C	C/C	C/C	C/C
CYP3A5	A6986G	A/A	A/G	A/A	A/A	A/A	A/A	A/A
CYP3A5	G14690A	G/A	G/G	G/G	G/G	G/G	G/A	G/G
CYP3A5	27131-2insT	-/-	-/-	-/T	-/-	-/-	-/-	-/-
CYP3A5	G19386A	G/G	G/G	G/G	G/G	G/G	G/G	G/G
CYP3A5	C31611T	C/T	C/T	C/C	C/T	C/C	C/C	C/C
ABCB1	A61G	A/A	A/A	A/A	A/A	A/A	A/A	A/A
ABCB1	C1236T	C/C	C/T	C/C	C/T	C/T	C/C	C/C
ABCB1	C3435T/A	C/C	C/T	C/C	C/C	C/C	C/C	C/C
ABCB1	T2677G/A	G/G	G/G	G/G	G/G	G/G	G/G	G/G
ABCB1	A4036G	A/A	A/A	A/A	A/A	A/G	A/A	A/A

Chapter 4: Discussion

4.1. Study subjects and clinical parameters

Genetic polymorphisms with an allele frequency of 0.1 could be detected with 95% confidence given the sample size of 138 samples. The study cohort size (n=464) consisting of 163 healthy research participants and 301 HIV/AIDS individuals was therefore more than adequate. The majority of HIV/AIDS patients were females and this might be the reason for the very low smoking and alcohol use among the study subjects because generally among South African Black people, smoking and alcohol consumption is less common in women than in other racial groups[148]. The current study cohort had few smokers thus, it was impossible to evaluate the effect of tobacco smoking on EFV plasma concentration, although, other studies alluded to the possibility of CYP2A6 induction among smokers and consequently reduced EFV plasma concentration.

Clinical parameters such as CD-4 cell count, viral load, disease stage, haemoglobin, AST and ALT levels were used as an indication of ARV treatment efficacy, underlying liver disease and disease progression in the HIV/AIDS patients. As expected, EFV-containing HAART led to a general (48%) increase in CD-4 cell count (cells/µl) and a 94% decrease in viral load (copies/ml) baseline levels when compared to levels at 6 months. However, a single individual had a 68% increase in viral load between baseline and 6 months after treatment initiation and this individual had to be switched ARV regimens four times since treatment initiation. First from AZT_3TC_NVP to AZT_3TC_LPV/r_TDF; AZT_LPV/r_TDF and back to AZT_3TC_LPV/r_TDF. Interestingly, this individual had the *CYP2B6 516G/T*, *785A/G* and *983T/C* genotypes, associated with high EFV plasma concentrations and development with ADRs. Genotyping this patient for the *CYP2B6 G516T*, *A785G* and *T983C* SNPs before treatment initiation could have been used to reduce the drug dose and avoided the change to more expensive ARV regimens. The AST and ALT levels (119 IU/l and 71 IU/l, respectively) of this patient were above the average AST and ALT levels of 43 IU/l and 30 IU/l of all the study participants, respectively. The average AST levels (43 IU/l) of the study

participants were above the normal AST range of 10-35 IU/l, but the average ALT levels (30 IU/l) were however not above the normal range of 9-40 IU/l. Elevated AST and ALT levels are as a result of underlying liver disease and complicate treatment. Elevated ALT levels were therefore significantly linked to high average plasma drug concentrations.

Failure of reduction in viral load and emergence of opportunistic infections after six months was associated with ARV switching. Sustained viral load after 6 months and the presence of opportunistic infections are indications of possible treatment failure or non-adherence. On the other hand, other studies have shown that high EFV plasma concentrations are associated with development of adverse drug events leading to drug discontinuation[17, 149, 150]. Although non-adherence plays a role in poor drug response, it has to be taken into account that poor metabolism can also result in low drug levels which lead to virologic failure.

4.2. Allele and genotype frequencies of SNPs

To our knowledge, the present study is the first report on the allele and genotype frequencies of the following genetic polymorphisms in the South African population; *CYP2A6 G1093A, CYP2B6 A136G* and *T983C, CYP3A5 G19386A* and *C31611T* as well as *ABCB1 A4036G*. This data contribute to the accumulation of information on genetic variants of pharmacogenetics relevance among Africans.

The allele frequencies of the genetic polymorphisms in the healthy research participants (presented in Tables 7 and 8) were also compared to the allele frequencies in other African, African-American and Caucasian populations (Tables 13 and 14). It is clear that there are significant differences in the allele frequencies between the African populations and the Caucasians for example the allele frequency of the *ABCB1 3435T* allele in African populations ranges from 0.07 to 0.11, but is present at a frequency of almost 0.6 in Caucasian individuals. These differences show that universal treatment of common diseases is not possible. Notably, differences in the South African populations

compared with other African populations also exist. Of particular interest, the allele frequency of the *CYP1A2 -163A* allele was similar in the Yoruba (0.57) and African-American (0.54) individuals. However, the *-163A* allele occurred at a frequency of 0.71 in the present study and at 0.72 among Caucasians (P=0.9092). The *CYP1A2 -163A* allele frequencies are in concordance with what has been reported previously in Africans (0.55), Asians (0.65) and Caucasian (0.7) individuals. The African population can therefore not be treated as a single population and drug dose needs to be adjusted according to ethnic group and ultimately based on the genotypes of an individual. The allele frequencies of the *CYP2A6 1093A* allele were significantly different to the frequency in the Yoruba (P=0.0082) and Caucasian (P<0.0001) individuals. The *CYP2B6 136G* allele was more frequent in the South Africans than in the Caucasians (P=0.0025). Interestingly, the frequency of the *CYP2B6 516T* allele only differed significantly from the frequency reported in the Xhosa individuals (P=0.0046), while the *785G* allele was more frequent in the South Africans than in the Caucasians (P=0.0013). The frequency of the *CYP2B6 983C* allele in the South Africans differed from both the Xhosa and Yoruba individuals (P<0.0001 and P=0.0003). The allele frequencies of *CYP2B6 1459T* allele, *CYP3A5 6986G* allele, *CYP3A5 31611T* allele and *ABCB1 1236T* allele differed from the Caucasians (P=0.0007, P<0.0001, P<0.0001 and P<0.0001, respectively), while the allele frequencies of the *CYP3A5 27131-2insT* and *ABCB1 4036G* allele were different to the frequencies reported in the Yoruba individuals (P<0.0001). The frequency of *CYP3A4 -392G* allele was similar to the African-Americans, but different from the other populations (P=0.0166, P=0.0006 and P<0.0001). The same was observed for the *CYP3A5 14690A* allele compared to the Xhosa, Yoruba, Caucasian, Luhya and Maasai individuals (P=0.0001, P=0.0002, P=0.0009, P<0.0001 and P=0.0088, respectively). The frequency of the *ABCB1 3435T* allele were different among all the populations, while the frequency of the *ABCB1 2677T/A* alleles differed significantly from the Yoruba individuals (P<0.0001) and Caucasians (P<0.0001). The differences in allele frequencies between the African and Caucasian individuals show that therapeutic drugs, including EFV, should not be prescribed at

universal dosages. Similarly, fine scale genetic structure exists within the African population and can therefore not be treated as a single population.

Table 13: Allele frequencies in the healthy research participants, Xhosa individuals, Yoruba individuals, Luhya, Maasai, African-American and Caucasian populations.

Gene	Position and allele	Healthy subjects (n=163)	Xhosa (n=109) [104]	Yoruba (n=226) HapMap	Luhya (n=89) [104]	Maasai (n=143) [104]	African-American (n=46) HapMap	Caucasian (n=226) HapMap
CYP1A2	-163A	0.712	N/A	0.566	N/A	N/A	0.543	0.721
CYP2A6	1093A	0.078	N/A	0.131	N/A	N/A	0.167	0.004
CYP2B6	136G	0.069	N/A	N/A	N/A	N/A	N/A	0.008
CYP2B6	516T	0.363	0.200	0.420	0.310	0.370	0.467	0.270
CYP2B6	785G	0.363	N/A	0.455	N/A	N/A	0.462	0.214
CYP2B6	983C	0.026	0.170	0.124	0.070	0.020	0.038	0.000
CYP2B6	1459T	0.015	N/A	0.042	N/A	N/A	0.000	0.091
CYP3A4	-392G	0.634	0.770	0.792	N/A	N/A	0.679	0.023
CYP3A5	3699T	0.000	N/A	N/A	N/A	N/A	N/A	N/A
CYP3A5	6986G	0.223	N/A	0.155	N/A	N/A	0.367	0.964
CYP3A5	14690A	0.167	0.200	0.168	0.250	0.140	0.115	0.000
CYP3A5	27131-2insT	0.062	N/A	0.000	N/A	N/A	0.214	0.000
CYP3A5	19386A	0.000	N/A	N/A	N/A	N/A	N/A	N/A
CYP3A5	31611T	0.344	N/A	0.274	N/A	N/A	0.370	0.960
ABCB1	61G	0.009	0.000	0.000	N/A	N/A	0.000	0.100
ABCB1	1236T	0.159	0.130	0.124	0.110	0.140	0.136	0.451
ABCB1	3435T	0.206	0.110	0.111	N/A	0.840	0.071	0.571
ABCB1	2677T/A	0.083/0.006	N/A	0.00	N/A	N/A	0.077/0.00	0.34/0.042
ABCB1	4036G	0.213	N/A	0.142	N/A	N/A	0.000	0.142

N/A=Not available

Table 14: Comparison (p-values) of the allele frequencies in the healthy research participants with the Xhosa individuals, Yoruba individuals, Luhya individuals, Maasai individuals, African-American and Caucasian populations.

Gene	SNP ID	Position and allele	Xhosa (n=109)	Yoruba (n=226)	Luhya (n=89)	Maasai (n=143)	African-American (n=46)	Caucasian (n=226)
CYP1A2	rs762551	-163A	N/A	0.0041	N/A	N/A	0.0490	0.9092
CYP2A6	rs28399454	1093A	N/A	0.0082	N/A	N/A	0.0911	<0.0001
CYP2B6	rs35303484	136G	N/A	N/A	N/A	N/A	N/A	0.0025
CYP2B6	rs3745274	516T	0.0046	0.2502	0.4900	0.9057	0.3028	0.0589
CYP2B6	rs2279343	785G	N/A	0.0949	N/A	N/A	0.3028	0.0013
CYP2B6	rs28399499	983C	<0.0001	0.0003	0.1729	1.000	0.6149	0.0302
CYP2B6	rs3211371	1459T	N/A	0.1295	N/A	N/A	1.000	0.0007
CYP3A4	rs2740574	-392G	0.0166	0.0006	N/A	N/A	0.7281	<0.0001
CYP3A5	rs55817950	3699T	N/A	N/A	N/A	N/A	N/A	N/A
CYP3A5	rs776746	6986G	N/A	0.1107	N/A	N/A	0.0542	<0.0001
CYP3A5	rs10264272	14690A	0.0001	0.0002	<0.0001	0.0088	0.1657	0.0009
CYP3A5	rs41303343	27131-2insT	N/A	1.000	N/A	N/A	<0.0001	1.000
CYP3A5	rs28383479	19386A	N/A	N/A	N/A	N/A	N/A	N/A
CYP3A5	rs15524	31611T	N/A	0.1480	N/A	N/A	0.7302	<0.0001
ABCB1	rs9282564	61G	1.000	1.000	N/A	N/A	1.000	0.7786
ABCB1	rs1128503	1236T	0.6006	0.3728	0.1760	0.7488	0.8171	<0.0001
ABCB1	rs1045642	3435T	0.0468	0.0097	N/A	<0.0001	0.0276	<0.0001
ABCB1	rs2032582	2677T/A	N/A	<0.0001	N/A	N/A	0.8678	<0.0001
ABCB1	rs3842	4036G	N/A	0.0764	N/A	N/A	<0.0001	0.0764

N/A=Not available

4.3. Linkage disequilibrium and Haplotype analysis

Tight LD (D´=0.967 and r^2=0.918) was observed only between the *CYP2B6 G516T* and *A785G* genetic polymorphisms in the South African population which is similar to studies done among Caucasians and Asians[84]. This observation has important implications for genetic screening preceding ARV therapy and therefore only screening for the *G516T* polymorphism is necessary in South African HIV/AIDS patients. With respect to the linkage between *G516T* and *T983C*, other studies in Caucasians, reported almost 100% linkage of the three SNPs. The *A785G* SNP is often inherited together with both the *G516T* and *T983C* SNPs and these three polymorphisms were consequently found to be in tight LD[88]. However, in South Africans (our current study), West Africans and African-Americans the LD block between *G516T* and *A785G* does not include the *T983C* polymorphism[151]. Genetic polymorphisms in the *CYP3A5* or *ABCB1* genes were not in LD in the South African population, which is in contrast to the tight LD observed between *ABCB1 C1236T, C3435T/A* and *T2677G/A* among individuals of Caucasian origin[152]. The observation of a greater degree of LD among the Caucasians than in the African populations is explained by a possible bottleneck that occurred with migration from Africa[153]. However, the differences in inferred haplotypes, further confirms the need for consolidated studies among the African Black population to discover relevant haplotype blocks as well as good tag SNPs. This may be accomplished by whole genome sequencing of more samples from Africans.

The *CYP2B6* inferred haplotypes frequently observed in the South African individuals corresponded to the AGACT (*CYP2B6*1*), AGACC (*CYP2B6*18*), ATGCT (*CYP2B6*7*) and GTGCT (*CYP2B6*11*) haplotypes reported previously (www.cypalleles.ki.se). The following inferred haplotypes were identified in *CYP3A5*; CAG-GC (*CYP3A5*1A*), CAG-GT (*CYP3A5*1D*), CAA-GC (*CYP3A5*6*), CGG-GT (*CYP3A5*3A*) and CGG-GC (*CYP3A5*3C*). Previous studies have reported the identification of the *ABCB1* 893Ala-containing CGC haplotype (*ABCB1 1236C, 2677G* and *3435C* alleles) and, as expected, this haplotype was present in the majority of South Africans. The

ABCB1 893Ser-containing haplotype (*ABCB1 1236T, 2677T* and *3435T* alleles) was defined by Kim *et al.* [154] and present at a low frequency in this study. Inferred haplotypes that are different combinations of the three common *ABCB1* SNPs for example *1236T, 2677G* and *3435T* were observed at low frequencies as a result of the lack of LD between these three SNPs in the South Africans.

4.4. Correlation of genotypes with efavirenz plasma concentrations

Efavirenz plasma concentration levels were obtained at two time-points on average two hours apart and the change in plasma concentrations were calculated. The change in EFV concentration can be influenced by a number of factors like the time of dosing, food-intake, underlying liver disease and co-administration of non-ARV medication. The study subjects were grouped according to the therapeutic range of EFV (1 µg/ml - 4 µg/ml). Individuals with sub-therapeutic plasma EFV concentration levels (<1.00 µg/ml) could have been due to any of the following; poor compliance, increased metabolism at another loci that metabolises EFV and concomitant treatment with other drugs. Co-administration of other drugs could induce the enzymes involved in EFV metabolism and this can most likely lead to virologic failure. EFV concentration levels above 4.00 µg/ml were most likely due to poor metaboliser status at the loci that metabolises EFV or concomitant EFV treatment with other drugs either indicated for other diseases or for recreation, which had inhibitory effects on the system that metabolises EFV. The inhibitory effects on EFV metabolism would lead to increased EFV plasma levels, decreased viral load but inadvertently, increased adverse EFV effects such as CNS toxicity as observed by Marzolini *et al.* [17]. The 5% and 31% of individuals with EFV plasma concentrations below and above the therapeutic range is higher than previously reported in Caucasian individuals[155]. The *CYP2B6 G516T* SNP is well characterised as a risk factor of poor EFV metabolism[84]. The average EFV plasma concentrations of the HIV/AIDS patients were therefore grouped according to *CYP2B6 G516T* genotypes to identify other clinical or genetic factors to explain the skewed EFV concentration levels. Four individuals had high EFV concentrations but

with the *CYP2B6 516G/G* genotype, possibly pointing to the existence of other mutations in *CYP2B6* that have not yet been reported and these should be sequenced. On the other hand, the three individuals had low EFV concentrations levels yet they were carrying the *CYP2B6 516T/T* genotype, which points more to the involvement of other genes in the metabolism of EFV if non-compliance can be ruled out. Indeed, the four individuals with high EFV plasma concentrations but carrying the fast *CYP2B6 516G/G* genotype all had other mutations in *CYP2B6* and other genes including *CYP2A6* and *CYP3A5*. The first individual have the *CYP1A2 -163A/A* genotype, the *CYP2A6 1093G/A* genotype, the *CYP2B6 983T/C* genotype, the *CYP3A5 14690G/A* and *31611C/T* genotypes. The second individual have the following genotypes; *CYP1A2 -163C/A, CYP3A5 6986A/G* and *31611C/T, CYP2B6 136A/G, ABCB1 3435C/T* and *1236C/T*. Individual three is heterozygous for the *CYP1A2 -C163A* and the *CYP3A5 27131-2insT* SNPs, while the fourth individual have the following genotypes; *CYP2B6 983C/C, CYP3A5 31611C/T* and *ABCB1 1236C/T*. The combined effects of these additional mutations were probably the reason for slow metabolism of EFV. It is interesting to report that the three individuals with low EFV concentrations yet carrying slow *CYP2B6 516T/T* genotype, carried the *CYP1A2 -163A* variant which has been associated with increased inducibility and activity of CYP1A2[59, 156, 157].

In general, the *CYP2B6 G516T* and *A785G* variants showed significant correlation with EFV concentrations. *CYP1A2 C-163A* SNP showed a trend towards significant correlation with EFV plasma levels. Notably, of the four patients with high EFV concentrations one of them presented with AST and ALT levels above the average of the study cohort (43 IU/l and 30 IU/l) possibly pointing to underlying liver disease.

The influence of genetic polymorphisms on EFV plasma concentrations was further investigated to identify polymorphisms with clinical relevance. The presence of the *CYP2B6 516T, 785G* and *983C* variants were associated with significantly higher EFV plasma concentrations, while the *CYP1A2 -163A* variant was associated with reduced EFV plasma concentrations. The observed effects of

CYP2B6 516T, 785G and *983C* variants are in agreement with other studies involving Caucasians, Africans and Asians[82, 84-89, 158]. Our result further confirm previous findings on *CYP2B6 516T* in the South African population[47, 159] which showed an association of this variant with high EFV plasma levels. However, the effects of the other *CYP2B6* SNPs on EFV levels as well as the inferred haplotypes, has not been reported before in this population. Combining the *CYP2B6 G516T* and *A785G* SNPs did not improve prediction of EFV plasma concentration, because the expected lower EFV plasma concentration in the presence of the *A785G* polymorphism is masked by the presence of the *G516T* polymorphism ($D' = 0.967$ and $r^2 = 0.913$) and this is in agreement to earlier reports[160]. The *CYP2B6 983C* variant was associated with high EFV concentration levels in this study, confirming earlier reports in Caucasians and Asians[20, 88]. Five individuals with the *CYP2B6 983C/C* genotype were identified in this study, of which two whose EFV plasma levels were available, had more than 5-fold higher EFV plasma concentrations with respect to the therapeutic range upper limit. In a recent study by Schipani *et al.* [89], no *CYP2B6 983C/C* homozygotes were reported. The *CYP2B6 983C* variant has been associated with substantial differences in EFV and NVP exposure among individuals of African and Caucasian ancestry[20, 88, 161]. The *G516T* and *T983C* SNPs together had an additive effect and were associated with an almost 2-fold higher EFV concentrations, a finding in agreement to reports by Wyen *et al.* [161] and Schipani *et al.* [89]. These observations suggest that *CYP2B6* polymorphisms can be used as biomarkers of EFV plasma levels further arguing for genotyping-assisted dosing with respect to use of EFV[85, 89, 162]. Already, suggestions based on pharmacogenetics knowledge are being proposed, such as gradual reductions in EFV dose from 600mg/day to 400 mg/day or 200mg/day for intermediate metaboliser and poor metaboliser patient groups, respectively[20, 163]. Nevirapine, which is another substrate of *CYP2B6* has been suggested to be dosed twice-daily (200mg) instead of once-daily (400mg) to avoid sub-therapeutic drug exposure in the fast metaboliser with the *CYP2B6 516G/G* genotype, while also ensuring that EFV levels would not go above the therapeutic range in *CYP2B6 516G/T* and *516T/T* genotype carriers after adjusting for BMI [89]. The same applies to the

CYP2B6 983T/C genotype. The *CYP1A2 C-163A* polymorphism is located in intron 1, modifying a transcription factor binding site and has been associated with increased CYP1A2 inducibility[52, 157]. In the current study, the *CYP1A2 -163A* variant was associated with low EFV plasma concentrations although not statistically significant. The influence of the *CYP1A2 -163A* variant was prominent in carriers of *CYP2B6 516G/T* or *516T/T* genotypes who displayed low EFV levels, contrary to expectations of high levels. Our findings may help to clarify that the *CYP1A2 -163A* variant is most likely associated with increased induction or enzyme activity than the contrary[58, 164].

The only SNP genotyped for in *CYP2A6, 1093G>A*, did not show any correlation with EFV plasma levels, which is contrary to the observation by Kwara *et al.* [165] in a Bantu-speaking Ghanaian population, who reported a nearly 2-fold higher median EFV concentration in *CYP2A6 1093A/A* homozygotes when compared to *1093G/G* homozygotes. To really conclude on the effects or role of *CYP2A6*, more SNPs should be investigated.

We report no association between genetic polymorphisms in *CYP3A4/5* and altered EFV metabolism which supports the results reported previously[116, 166]. This probably suggests a very minor role of this ubiquitous and promiscuous enzyme family.

In addition to drug metabolising enzymes, drug transporters also play a role in drug disposition thus; we investigated 5 SNPs in *ABCB1*. It is surprising that we did not observe a role for any of the *ABCB1* genetic polymorphisms with EFV concentrations, yet, in a different study among Ugandans, the *ABCB1 4036G* variant resulted in higher EFV plasma concentrations[167]. Previous studies investigating the potential phenotypic associations for the *ABCB1 C1236T, T2677G/A* and *C3435T/A* polymorphisms bear no consensus and the *1236C/C* genotype has been linked to increased drug response in treatment of glioblastoma patients[168], yet the *1236T/T* genotype has also been associated with high cyclosporine trough concentrations[169], making it difficult to draw

conclusions. There are conflicting reports as well for the role or effects of *ABCB1 T2677G/A* and *C3435T/A* polymorphisms[170].

The above mentioned studies reported associations based on the presence or absence of a single genetic polymorphism in one of the pharmacogenetically relevant genes. Prediction of response to EFV can however not be made based on genotypic data at a single locus. The combined effect of the *CYP1A2 C-163A* and *CYP2B6 G516T, A785G* and *T983C* polymorphisms produced a more accurate prediction of EFV plasma concentration.

The predictive ability of the *CYP2B6 516T/T, 983C/C* and *CYP1A2 -163A/A* genotypes on EFV plasma concentrations above the therapeutic range were investigated, however because of the strong LD between *CYP2B6 G516T* and *A785G* only screening for the *CYP2B6 G516T* SNP is recommended (Tables 15 and 16). The sensitivity and specificity of the *CYP2B6 516T/T* genotype is high compared to that of the *CYP2B6 516G/G* genotype, but low for the *CYP1A2 -163A/A* genotype compared to the *CYP1A2 -163C/C* genotype. The sensitivity of the *CYP2B6 983C/C* genotype is high, but the specificity low and this is as a result of only two individuals with the *CYP2B6 983C/C* genotype and EFV concentrations above 4 µg/ml. The combined predictive ability of the *CYP2B6 516T/T* and *CYP1A2 -163A/A* genotypes have very high sensitivity and specificity, but low positive and negative predictive values showing the clinical utility of testing for these genotypes. Pharmacogenetic screening for *CYP2B6 516T/T* and *CYP1A2 -163A/A* genotypes with high predictive ability can be useful in the public health sector to predict EFV treatment response.

Table 15: HIV/AIDS patients with EFV concentration above or below 4 µg/ml

Genotypes	HIV/AIDS patients with EFV concentrations >4 µg/ml	HIV/AIDS patients with EFV concentrations <4 µg/ml
CYP2B6 516G/G	4	48
CYP2B6 516G/T	22	37
CYP2B6 516T/T	22	3
CYP2B6 983T/T	37	79
CYP2B6 983T/C	9	9
CYP2B6 983C/C	2	0
CYP1A2 -163C/C	7	5
CYP1A2 -163C/A	20	42
CYP1A2 -163A/A	15	32

Table 16: Predictive ability of *CYP2B6 516T/T, 983C/C* and *CYP1A2 -163A/A* genotypes

Genotype	Predicted enzyme activity	Sensitivity	Specificity	Positive predictive value	Negative predictive value
CYP2B6 516T/T	Decreased	88%	92%	15%	6%
CYP2B6 983C/C	Decreased	100%	68%	95%	0%
CYP1A2 -163A/A	Increased inducibility	32%	42%	32%	86%
CYP2B6 516T/T and CYP1A2 -163A allele	Normal	100%	93%	15%	0%

4.5. Correlation of genotypes with change in ART regimen

The change in ART regimen was used as an indication of treatment failure among South African HIV/AIDS patients. Individuals with the *CYP2B6 516T/T* and *785G/G* genotypes were more likely to change ART regimen. This observation can be explained by the initial development of EFV-related CNS adverse events within the first month after treatment initiation. The development of adverse effects often results in discontinuation of treatment in individuals with high EFV plasma concentrations. Fewer patients with the *CYP2B6 983C/C* and *CYP1A2 -163A/A* genotypes changed treatment and the observation related to *CYP1A2 -163A/A* can be explained by the low EFV plasma concentrations. However, caution should be taken when concluding on the involvement of the *CYP2B6 983C/C* genotype in treatment discontinuation as only five individuals with the *983C/C* genotype were identified.

4.6. Limitations and future directions

In the current study EFV plasma concentrations were only available for 156 of the 301 HIV/AIDS patients. The limitation of using SNaPshot mini-sequencing as genotyping method is the detection of the dR110 labelled ddGTP (blue) is more sensitive than detection of the other ddNTPs. Patients were genetically characterized for a small number of known polymorphisms in the major pharmacogenes involved in EFV metabolism and future studies should firstly investigate the Bantu-speaking South African population for novel genetic variants in these genes, as the African population harbours to greatest degree of genetic diversity, and secondly establish the functional role of these genetic variants and their effect on treatment with a variety of drugs commonly used in treatment of infectious diseases. The effect of genetic variation in genes encoding proteins responsible for the regulation of the genes investigated in the current study should also be focussed on in future studies to completely understand EFV metabolism before pharmacogenetic testing could be utilized in clinical practise.

Chapter 5: Conclusion

The current study showed that *CYP1A2* and *CYP2B6* are the main predictors of response to EFV treatment in the South African population. The *CYP2B6 G516T, A785G* and *T983C* polymorphisms were significantly correlated with high EFV plasma concentration levels, whereas the *CYP1A2 C-163A* polymorphism showed a trend towards low EFV plasma concentrations. The combined effect of the *CYP2B6* and *CYP1A2* polymorphisms, rather than the influence of single polymorphisms are more accurate in predicting the development of ADRs and treatment failure. This strongly suggests that pharmacogenetic testing could assist with deciding of the HAART regimen for HIV/AIDS treatment in a clinical setting as well as the starting ARV dosage. Pharmacogenetic testing could be beneficial in personalization of treatment and avoiding the development of ADRs that are often severe and result in hospitalization of patients. A change to a more expensive treatment regimen and poor adherence to treatment can also be prevented by dose adjustment. Ultimately, pharmacogenetic testing will aid in tailoring currently available treatment for HIV/AIDS and can prevent the development of drug resistance resulting from inefficient suppression of HIV replication. In South Africa, pharmacogenetic testing is currently not indicated in the South African ART guidelines and testing has assisted treatment only in one case with the development of severe CNS ADRs [131, 171, 172].

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Appendix A: Informed consent

INFORMED CONSENT FOR LEGAL GUARDIANS

(Applicable where participants are incapable of giving consent).

Participant number: _____

I, the undersigned, *_____, has fully explained the information leaflet to the patient,

_____

and/or his/her legal guardian_____

The account I have given explained both the possible risks and benefits of the study. The patient and/or his/her legal guardian understand these. The patient and/or his/her legal guardian indicated that he/she understands that the patient will be free to withdraw from the study at any time for any reason and without jeopardizing his/ her subsequent treatment.

I hereby certify that, the patient and/or his/her legal guardian acting on his/her behalf has agreed to participate. I am **willing/not willing** (delete the inappropriate) to be contacted for follow-up studies

PATIENT

_____

Name and Surname (Capital letters) Date

STUDY DOCTOR AND PERSON ADMINISTERING THE CONSENT

_____

Name (Capital letters) Signature Date

LEGAL GUARDIAN

_____

Name (Capital letters) Signature Date

WITNESS

_____

Name (Capital letters) Signature Date

**Name of member of the researcher team who will be administering the informed consent.*

Appendix B: Ethics Approval Letters from the University of Cape Town and the University of Witwatersrand

UNIVERSITY OF CAPE TOWN

Health Sciences Faculty
Research Ethics Committee
Room E52-24 Groote Schuur Hospital Old Main Building
Observatory 7925
Telephone [021] 406 6338 • **Facsimile** [021] 406 6471
e-mail: [illegible]

26 May 2010

REC REF: 103/2009

Dr C Dandara
Human Genetics

Dear Dr Dandara

PROJECT TITLE: THE ROLE OF PHARMACOGENETICS ON THE RESPONSE TO TREATMENT USING ANTIRETROVIRAL DRUGS

Thank you for your letter to the Research Ethics Committee.

Thank you for sending us a copy of the ethics clearance from Malawi (COMREC)

It is a pleasure to inform you that the Ethics Committee has **granted ethical approval** to the following students for their studies titled:

a. Marelize Sauer: Investigation of genes associated with response to efavirenz
b. Elizabeth Kampira: Investigating the pharmacogenetics of drug metabolising enzymes among Malawians and the role of some of the variants in response to Lopinavir, stavudine, nevirapine

Approval is granted to the above students for one year until 28 May 2011.

Please send us an annual progress report if your research continues beyond the approval period. Alternatively, please send us a brief summary of your findings so that we can close the research file.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please quote the REC. REF in all your correspondence.

kmjoll

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS

Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938

This serves to confirm that the University of Cape Town Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Conference on Harmonisation Good Clinical Practice (ICH GCP) and Declaration of Helsinki guidelines.

The Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.

[illegible]

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Dandara

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M080124**
PROJECT	Pharmacogenetics profiling: establishing the baseline frequencies of variants of drug or xenobiotic metabolizing.....
INVESTIGATORS	Dr C Dandara
DEPARTMENT	Molecular & Cellular Biology
DATE CONSIDERED	20080125
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 08.02.05 **CHAIRPERSON** .....
(Professor P E Cleaton Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Not applicable

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10005, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix C: EFV plasma concentration assay by LC/MS/MS

Mobile phase A was 10% methanol and mobile phase B was 90% methanol: 10% 10mM of ammonium acetate in 0.1% acetic acid v/v. The flow gradient was initially 100% of phase A for 0.20mins, switched to 100% phase B for 0.21mins, held at 100% phase B for 2.79mins. Then returned to 100% phase A for 0.10mins and held for a further 3.90mins prior to the injection of another sample. The total run time for each sample was 7.00mins. The flow rate of mobile phase was kept constant at 500 µl/min. The volume of injection was 5 µl.

The plasma calibration curves of EFV (Merck Research Laboratories, Rahway N.J., USA) were linear over the range of 0.20-20.00 mg/l. Plasma calibration curves were prepared by spiking EFV in drug-free plasma at each calibration concentration. Three quality controls were prepared in the same manner (0.3 mg/l, 4 mg/l and 11 mg/l) for EFV. The lower limit of quantification (LLOQ) for EFV was 0.10 mg/l. A sample with plasma concentrations below the LLOQ was treated as 40% of LLOQ concentration in the data analysis. Samples with plasma concentrations higher than the upper limits of quantification were diluted with drug-free plasma and re-analysed.

Accuracy of the EFV plasma concentration assay was above 90%. The intra-day and inter-day precisions ranged from 1.34% to 9.61% and from 2.23% to 7.04%, respectively.

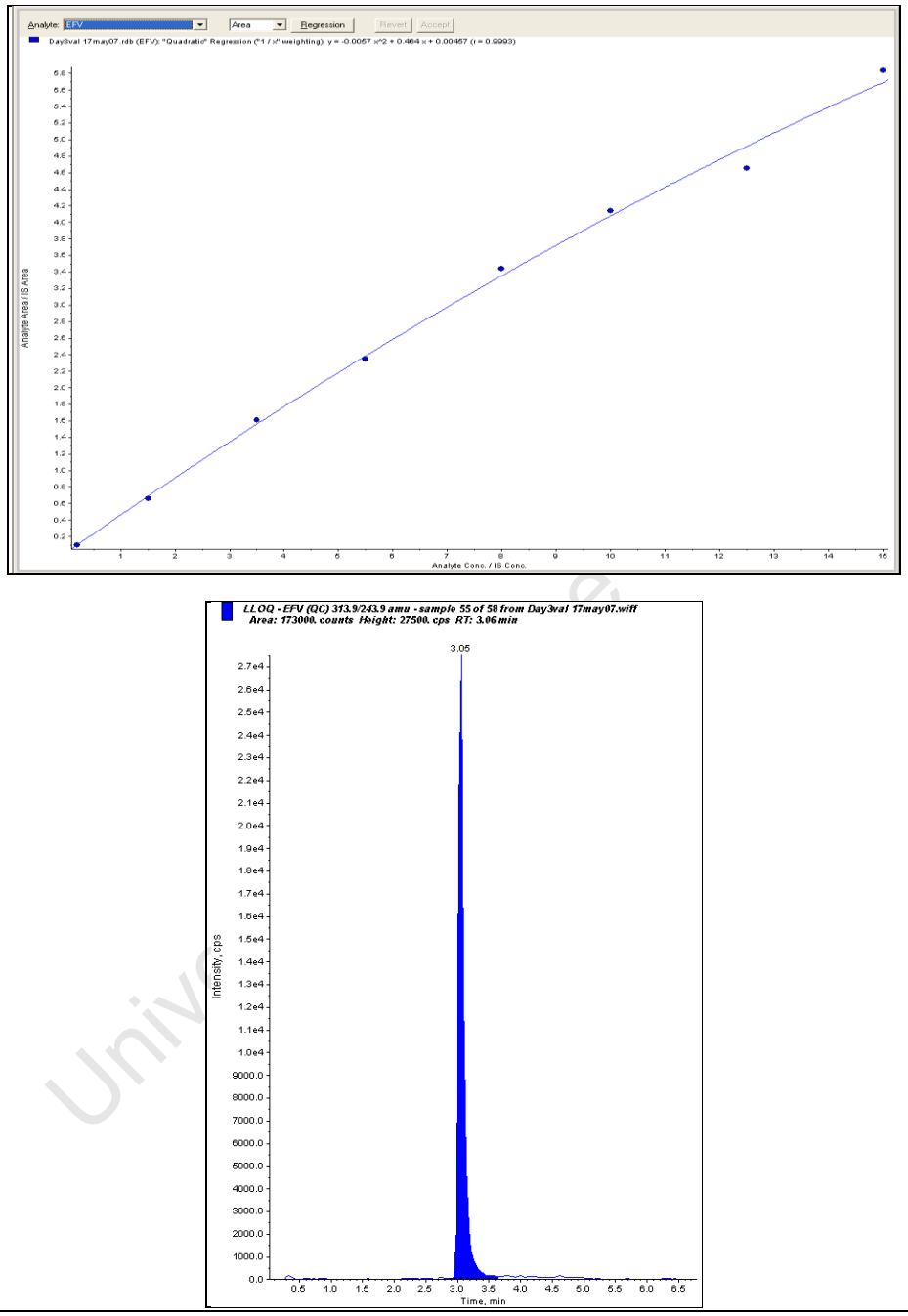


Figure A: EFV plasma calibration curve and chromatogram of plasma LLOQ (Adapted from Yuan Ren, PhD Thesis: Methods and Materials).

Appendix D: DNA integrity evaluation

The average (±SD) DNA concentration was 187.36 ng/µl (±394.60) with concentrations ranging from -1.3 ng/µl to 2438.0 ng/µl for the healthy research participants and HIV/AIDS individuals. Figure B shows the DNA integrity of DNA samples extracted from the plasma of 9 HIV/AIDS individuals using agarose gel electrophoresis.

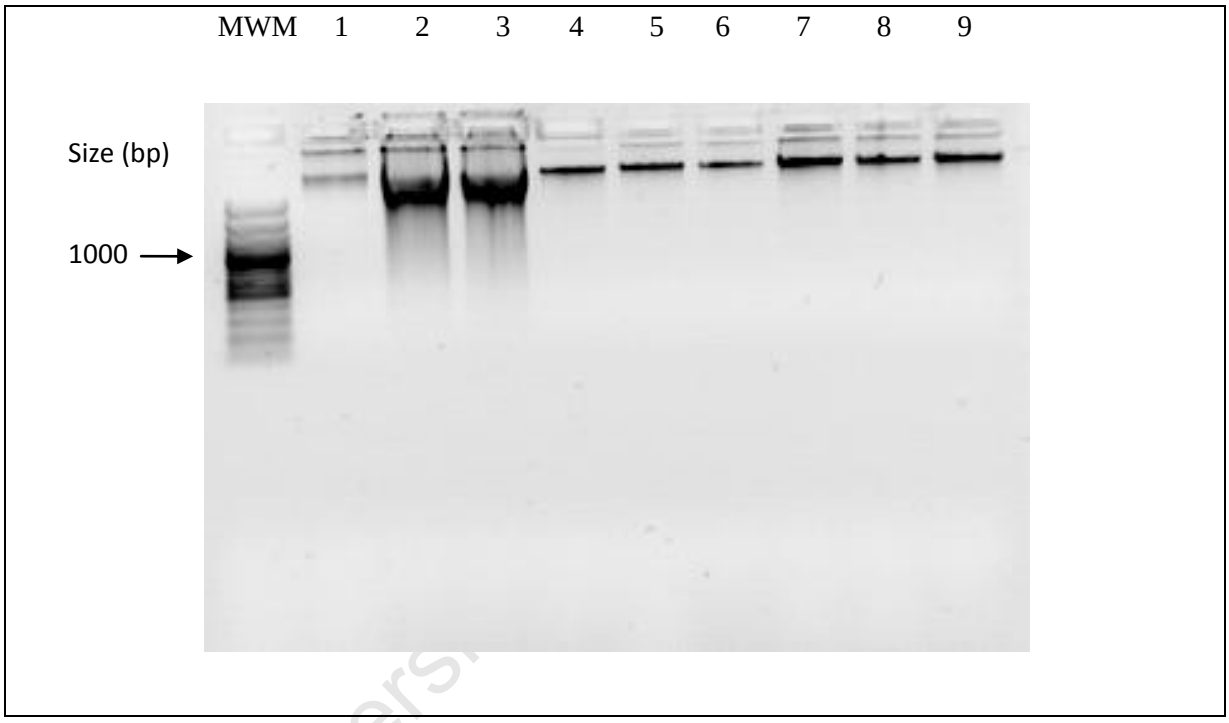
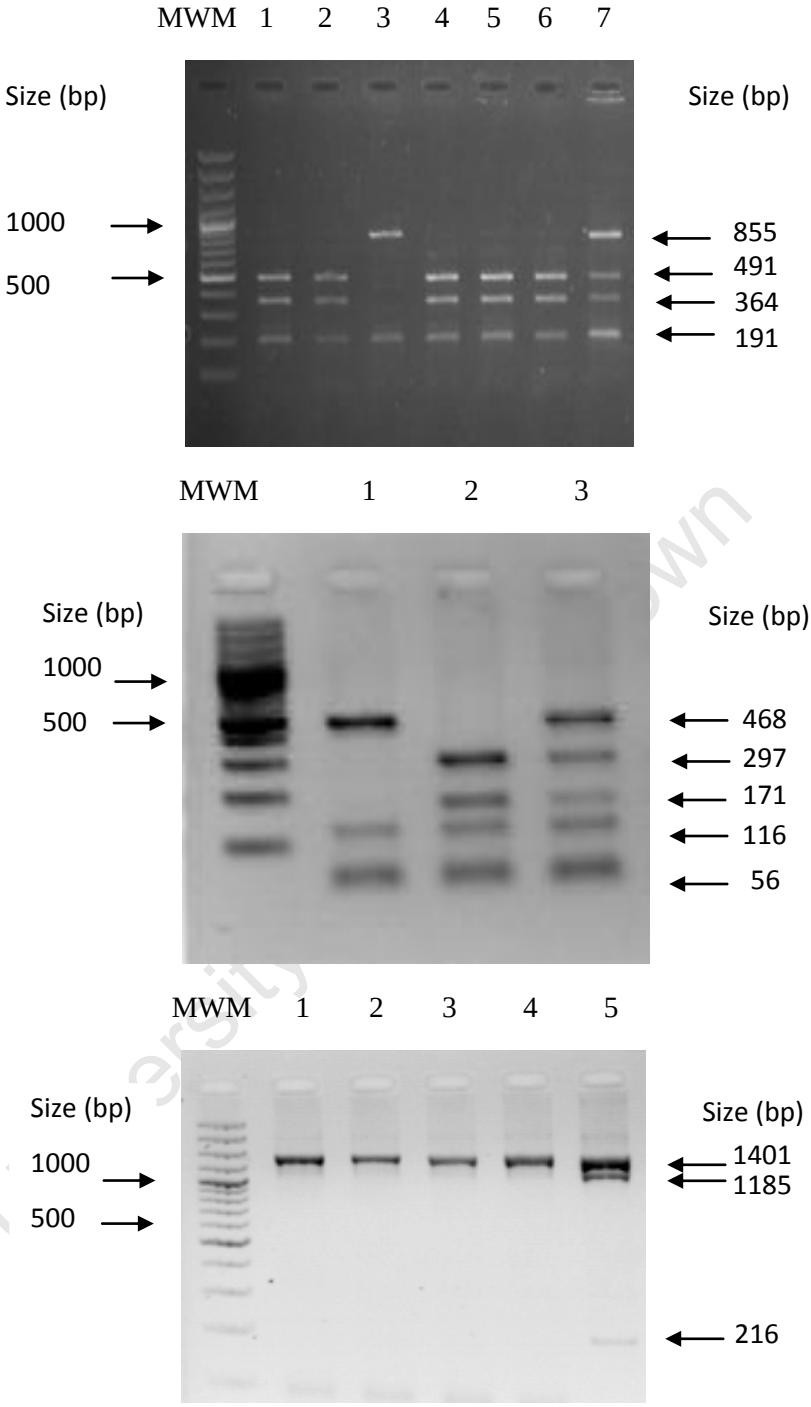


Figure B: Evaluation of DNA integrity using a 1% agarose gel. The 100bp plus molecular weight marker (MWM) was used and lanes 1 to 9 show variability in DNA concentration and quality of the DNA samples from 9 HIV/AIDS individuals.

Appendix E: PCR/RFLP genotyping results continued from section 3.2

Genotyping of the *CYP1A2 C-163A, CYP2A6 G1093A, CYP2B6 G516T, A785G* and *C1459T, CYP3A4 A-392G, CYP3A5 A6986G, G14690A* and *27131-2insT* genetic polymorphisms were performed using PCR-RFLP (Figure C). The *CYP2A6 G1093A* polymorphism created a *TaiI* restriction site and the *1093G* allele produced two bands (855 and 191 bp), while digestion of the *1093A* allele resulted in three bands (491, 364 and 191 bp). The *CYP2B6 A785G* polymorphism also abolished a restriction site (*StyI*) and *StyI* digestion of the PCR product from the *785A* allele produced four bands (297, 171, 116 and 56 bp) whereas the *785G* allele produced three bands (468, 116 and 56 bp). The *CYP2B6 C1459T* polymorphism created a *BglII* restriction site and the *1459C* allele generated a single band (1401 bp) whereas the *1459T* allele resulted in two bands of 1185 and 216 bp. The *CYP3A5 A6986G* polymorphism resulted in the abolishment of an *SspI* restriction site and digestion of the PCR product from the *6986A* allele produced three bands (148, 125 and 20 bp) whereas the *6986G* allele produced two bands (168 and 125 bp). The *CYP3A5 G14690A* polymorphism abolished a *DdeI* restriction site, while the *CYP3A5 27131-2insT* SNP created a *DdeI* restriction site. The *14690G* allele of the *G14690A* polymorphism produced four bands (230, 137, 103 and 25 bp), while the *14690A* allele produced three bands (230, 137 and 128 bp). *DdeI* digestion of the PCR product from the *27131-2insT* generated four bands of 41, 24, 22 and 20 bp. The allele without the *27131-2insT* produced three bands (61, 24 and 22 bp).


MWM 1 2 3 4 5 6 7
Size (bp)
1000
500
Size (bp)
855
491
364
191
MWM 1 2 3
Size (bp)
1000
500
Size (bp)
468
297
171
116
56
MWM 1 2 3 4 5
Size (bp)
1000
500
Size (bp)
1401
1185
216

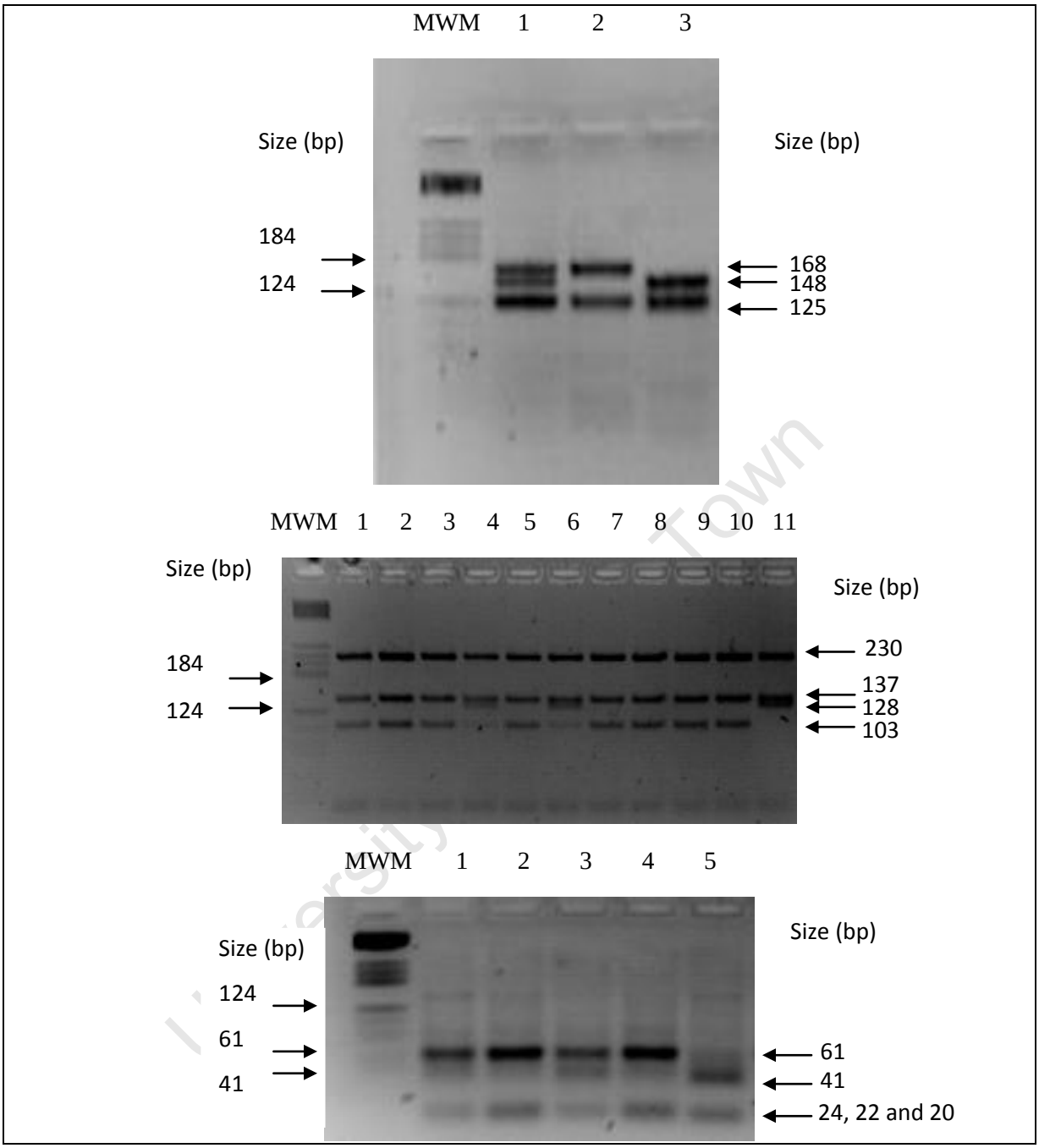


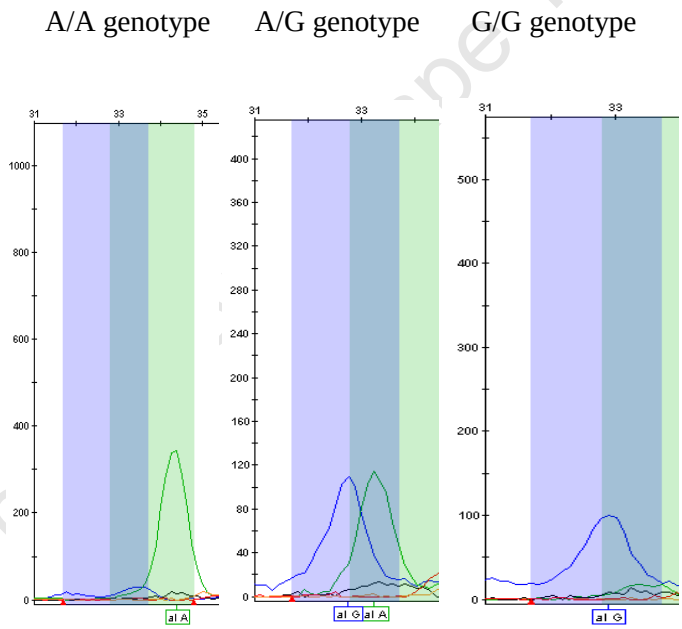
Figure C: PCR-RFLP genotyping of *CYP2A6 G1093A* shown on a 1.5% agarose gel. The 100bp plus molecular weight marker (MWM) was used and lanes 1 to 7 represent the different *G1093A* genotypes from 7 individuals; A/A = lanes 1, 2 4, 5 and 6, G/G = lane 3 and G/A = lane 7, respective. The 100bp plus molecular weight marker (MWM) was also used in PCR-RFLP genotyping of *CYP2B6 A785G* and lanes 1 to 3 represent the different *A785G* genotypes from 3 individuals; G/G = lane 1, A/A = lane 2 and G/A = lane 3. Genotyping of *CYP2B6 C1459T* is shown on a 1.5% agarose gel. The 100bp plus molecular weight marker (MWM) was used and the C/C genotype is shown in lanes 1 to 4 and the C/T genotype in lane 5. A 4.5% agarose gel was used to show the *CYP3A5 A6986G* genotypes from 3 individuals. The Roche DNA molecular weight marker V was used and the different genotypes are represented as A/G = lane 1, G/G = lane 2 and A/A =

lane 3. Genotyping of *CYP3A5 G14690A* is shown on a 4.5% agarose gel, with the Roche DNA molecular weight marker V and lanes 1 to 11 representing the different *G14690A* genotypes from 11 individuals. G/G = lanes 1, 2, 3, 5, 7, 8, 9 and 10, G/A = lanes 4 and 6 and A/A = lane 11. PCR-RFLP genotyping of *CYP3A5 27131-2insT* shown on a 5% agarose gel. The Roche DNA molecular weight marker V was used and lanes 1 to 5 represent the different genotypes from 5 individuals. The -/- genotype is represented in lanes 1 to 4 and the T/T genotype in lane 5.

Appendix F: SNaPshot genotyping results continued from section 3.3

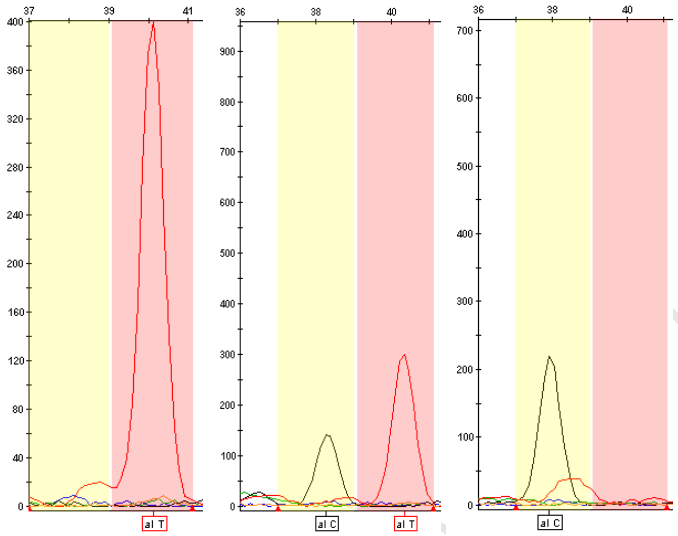
Genotyping of the *CYP2B6 A136G* and *T983C*, *CYP3A5 C3699T*, *G19386A* and *C31611T*, *ABCB1 A61G*, *C1236T*, *C3435T/A*, *T2677G/A* and *A4036G* genetic polymorphisms were performed using SNaPshot and is shown in Figure D below.

CYP2B6 A136G



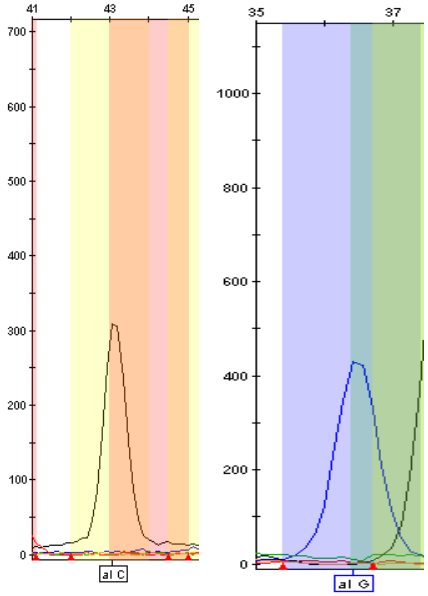
CYP2B6 T983C

T/T genotype T/C genotype C/C genotype



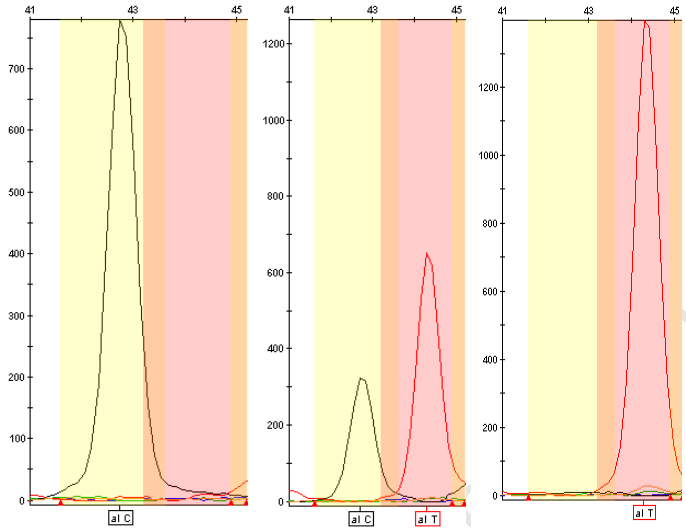
CYP3A5 C3699T and CYP3A5 G19386A

C/C genotype G/G genotype



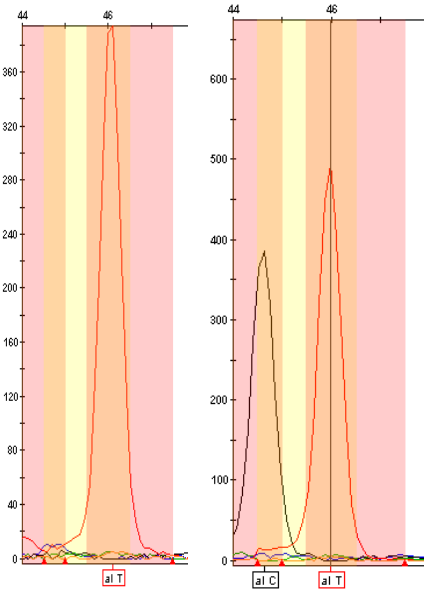
CYP3A5 C31611T

C/C genotype T/C genotype T/T genotype



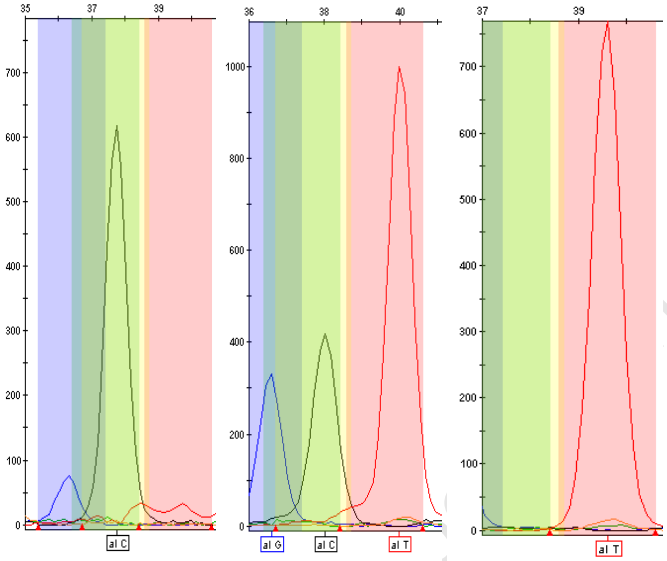
ABCB1 A61G

A/A genotype A/G genotype



ABCB1 C1236T

C/C genotype T/C genotype T/T genotype



ABCB1 C3435T/A

C/C genotype C/T genotype T/T genotype

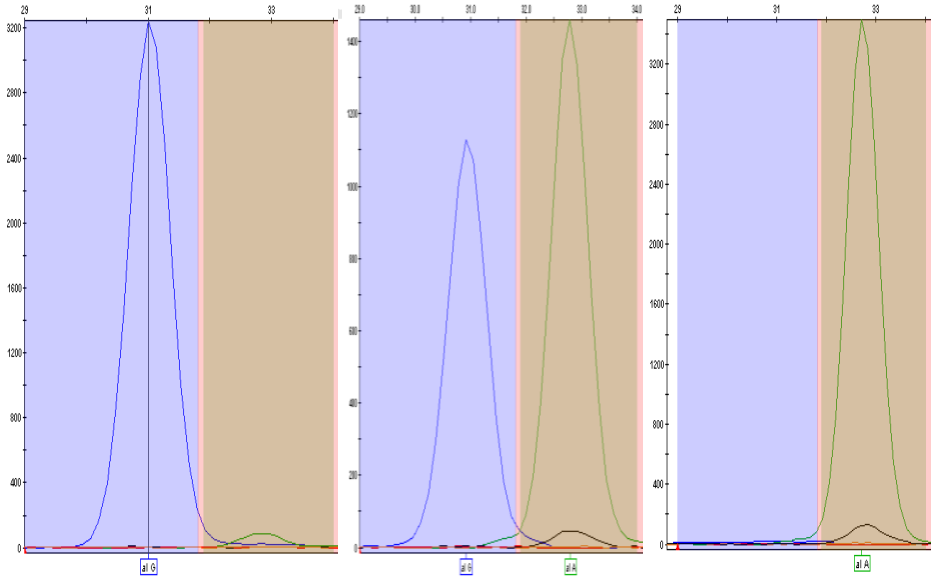


Figure D: SNaPshot genotyping of the *CYP2B6 A136G* and *T983C, CYP3A5 C3699T, G19386A* and *C31611T, ABCB1 A61G, C1236T* and *C3435T/A* genetic polymorphisms were performed.