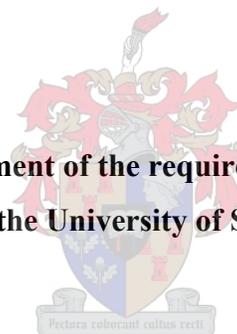


**MOLECULAR GENETIC ANALYSIS OF HUMAN IMMUNODEFICIENCY
VIRUS ANTIRETROVIRAL THERAPY RESPONSE IN
SOUTH AFRICA – A PHARMACOGENETICS STUDY**

BY

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**Thesis presented in partial fulfilment of the requirements for the degree of Master of
Science at the University of Stellenbosch**



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Co-Supervisor: Dr R Hillermann-Rebello

March 2007

DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature:

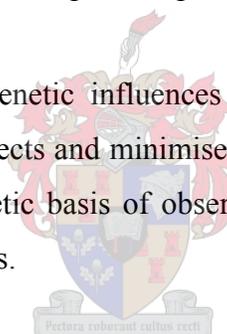
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ABSTRACT

The results of pharmacotherapy can vary both within and between different populations and ethnic groups. Although numerous factors are believed responsible for observed discrepancies in drug response, genetic differences, most often in the form of single nucleotide polymorphisms (SNPs), between individuals and ethnic groups are an important and at times predominant factor. The response to antiretroviral (ARV) drugs for the treatment of human immunodeficiency virus (HIV)-infection is not dissimilar. Marked variations in both ARV efficacy and occurrence of adverse drug reactions (ADRs) have been observed on both an individual and ethnic group level, which are largely attributed to polymorphisms within genes involved in the metabolism and transport of these compounds – such genes include the *CYP2B6* and *CYP3A4* genes, both members of the cytochrome P450 (CYP) gene superfamily, and the multidrug-resistance 1 (*MDR1*) gene encoding an efflux transporter protein, phosphoglycoprotein (PGP).

An improved understanding of the genetic influences on ARV drug response could lead to improved therapies with fewer side-effects and minimised drug resistance. The main aim of this study was thus to investigate the genetic basis of observed differences in ARV therapy (ART) response in South African ethnic groups.

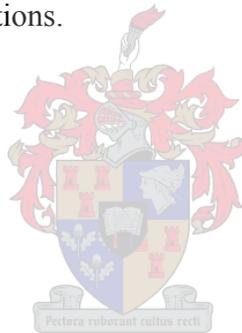


Deoxyribonucleic acid (DNA) samples were collected from 206 HIV-positive individuals of Mixed-Ancestry and Xhosa ethnicity that were currently or prospectively receiving ART. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis was employed to screen the A-392G SNP in *CYP3A4*, the G516T and A785G SNPs in *CYP2B6*, and the T-129C, C1236T, G2677T/A and C3435T SNPs in *MDR1*. Hardy-Weinberg equilibrium (HWE) and haplotype analyses were subsequently performed on the resultant SNP genotype and allele frequencies. The possible effects of ethnicity on ART response were examined by means of univariate two-way analysis of variables (ANOVA) testing. Univariate one-way ANOVA testing of the change in cluster of differentiation 4 (CD4)-cell count after six months of ART was used to analyse the possible effects of the seven polymorphisms on immune recovery.

All seven SNPs were found to be in HWE in both the Mixed-Ancestry and Xhosa ethnic groups. An extremely high level of linkage disequilibrium (LD) between the 516 and 785 loci in *CYP2B6*

was detected in both the Mixed-Ancestry ($r^2 = 0.97$; logarithm of the odds (LOD) score = 24.84) and Xhosa ($r^2 = 0.98$; LOD score = 41.31) ethnic groups. LD was also detected between the 1236, 2677 and 3435 loci in *MDR1* in the Mixed-Ancestry population, although none was found between any of the examined *MDR1* SNPs in the Xhosa population. Univariate two-way ANOVA testing found no apparent effect of ethnicity on immune recovery in response to ART. Univariate one-way ANOVA testing detected a discernible effect of genotype on immune recovery in the cases of the T-129C ($p = 0.03$) and G2677A ($p < 0.01$) polymorphisms in the *MDR1* gene.

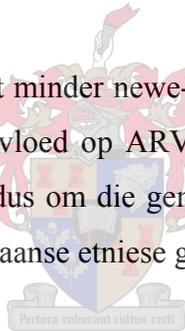
Therefore, this study identified no effect of ethnicity on ART response but did detect an association between the T-129C and G2677A SNPs in *MDR1* and the level of immune response to ART. These findings suggest that *MDR1* polymorphisms can be predictive of immune recovery after initiation of ART. This study thus represents an important step towards improved ART drug regimens in South African populations.



OPSOMMING

Farmakoterapie resultate verskil binne sowel as tussen verskillende populasies en etniese groepe. Hoewel talle faktore kan lei tot hierdie onvoorspelbare variasie in reaksies op medikasie is dit veral verskille op genetiese vlak, gewoonlik in die vorm van enkel basispaar polimorfismes (SNPs), wat 'n baie belangrike en soms oorheersende faktor is in die waargenome farmakologiese verskille tussen individue en etniese groepe. Pasiënte se reaksie op antiretrovirale (ARV) behandeling vir menslike immuuniteitsgebrek virus (MIV) is geen uitsondering nie. Merkbare verskille in beide die effektiwiteit en waargenome nuwe-effekte tussen individue, asook tussen verskillende etniese groepe, kan grootliks toegeskryf word aan genetiese polimorfismes in gene verantwoordelik vir die metabolisme en vervoer van hierdie produkte. Laasgenoemde gene sluit in die *CYP2B6* en *CYP3A4* gene, beide lede van die sitochroom P450 (CYP) geen-superfamilie, en die *MDR1* geen wat kodeer vir 'n uitskeidings-vervoerproteïen, fosfolipoproteïen (PGP).

Verbeterde terapeutiese behandeling, met minder nuwe-effekte en laer weerstand teen medikasie, kan bereik word indien die genetiese invloed op ARV behandeling beter verstaan word. Die hoofdoelstelling van hierdie studie was dus om die genetiese basis van waargenome verskille in ARV terapie (ART) reaksie in Suid Afrikaanse etniese groepe te bepaal.



Deoksiribonukleïensuur (DNS) monsters is van 206 HIV-positiewe individue van Gemengde-Etniese agtergrond asook Xhosa individue wat reeds of in die nabye toekoms ART sou ontvang. Polimerase ketting reaksie-restriksie ensiem vertering (PKR-RFLP) analise is gebruik om die individue vir die A-392G SNP in die *CYP3A4* geen, die G516T en A785G SNPs in die *CYP2B6* geen, en die T-129C, C1236T, G2677T/A en C3435T SNPs in die *MDR1* geen te ondersoek. Hardy-Weinberg ewewig (HWE) en haplotipe analise is uitgevoer op die SNP genotipe en alleelfrekwensie wat deur hierdie PKR-RFLP analise gevind is binne elke populasie groep. Die moontlike effek van etnisiteit op ARV behandeling is deur middel van eenveranderlike tweerigtingvariensieanalise toetsing bepaal. Eenveranderlike eenrigtingvariensieanalise toetsing van die veranderinge in die CD4-seltelling is gebruik om die effek wat die sewe polimorfismes op ARV terapie na ses maande gehad het vas te stel.

Al sewe SNPs was in HWE vir beide die Gemengde-Etniese en Xhosa populasies. 'n Baie hoë vlak van koppeling tussen die 516 en 785 loci in die *CYP2B6* geen is opgemerk in beide die Gemengde-Etniese ($r^2 = 0.97$; LOD score = 24.84) en Xhosa ($r^2 = 0.98$; LOD score = 41.31) groepe. Koppeling is ook tussen die 1236, 2677 en 3435 loci in die *MDR1* geen binne die Gemengde-Etniese pasiënte waargeneem, alhoewel geen koppeling tussen enige van die *MDR1* SNPs in die Xhosa groep gevind is nie. Eenveranderlike tweerigtingvariëansiële toetsing het geen merkbare effek van etnisiteit op immuun-herstelbaarheid in reaksie op ART aangedui nie. Eenveranderlike eenrigtingvariëansiële toetsing het wel getoon dat genotype die herstel van immuniteit affekteer in die geval van die T-129C ($p = 0.03$) en G2677A ($p < 0.01$) polimorfismes in die *MDR1* geen.

Hierdie studie het dus geen verskille tussen etniese groepe ten opsigte van reaksie op ART gevind nie. Daar is wel 'n assosiasie tussen die T-129C en G2677A SNPs en immuun-herstel in reaksie op ARV terapie gevind. Hierdie bevindinge suggereer dus dat *MDR1* polimorfismes 'n aanduiding kan gee van immuun-herstel in reaksie op ART. Die studie maak 'n belangrike bydrae tot verbeterde ART dosering in die Suid Afrikaanse populasie.



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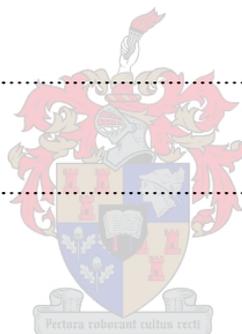
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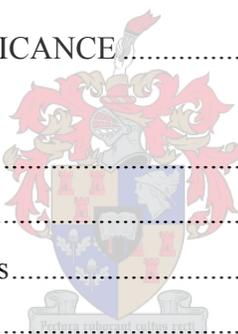
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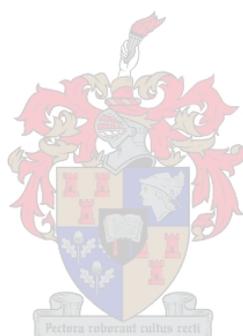
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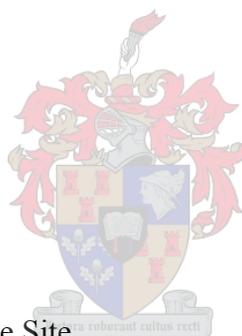
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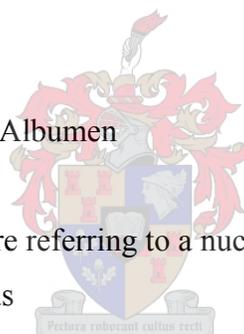
LIST OF ABBREVIATIONS, ACRONYMS, FORMULAE AND SYMBOLS

\approx	Almost Equal To
β	Beta
χ	Chi
\$	Dollar
γ	Gamma
>	Greater Than (except where referring to a nucleotide change)
\geq	Greater Than or Equal To
<	Less Than
\leq	Less Than or Equal To
μ	Mu (Micro)
μg	Microgram
μl	Microlitre
μM	Micromolar
%	Percent
\pm	Plus-Minus
\wedge	REase Cleavage Site



A	Adenine (where referring to a nucleotide)
AA	Acrylamide
ABC	Abacavir
ABCB1	ATP-Binding Cassette, Subfamily B, Member 1
ADC	AIDS Dementia Complex
ADR	Adverse Drug Reaction
AIDS	Acquired Immunodeficiency Syndrome
Ala	Alanine
AML	Acute Myeloid Leukaemia
ANOVA	Analysis of Variables
APS	Ammonium Persulphate ($\text{H}_8\text{N}_2\text{O}_8\text{S}_2$)
APV	Amprenavir

Arg	Arginine
ART	Antiretroviral Therapy
ARV	Antiretroviral
Asn	Asparagine
Asp	Aspartic Acid
ATP	Adenosine Triphosphate
ATZ	Atazanavir
AUC	Area Under the Curve
AZT	Zidovudine
BAA	Bisacrylamide
BCE	Before Common Era (dates before year 1)
BLAST	Basic Local Alignment Search Tool
BM	Body Mass
bp	Base Pair
BSA	Bovine Serum Albumen
C	Cytosine (where referring to a nucleotide)
°C	Degrees Celsius
CA	California
CD4	Cluster of Differentiation 4
CDC	Centres for Disease Control
cDNA	Complimentary DNA
CH ₃ CH ₂ OH	Ethanol
CH ₃ COONa	Sodium Acetate
C ₆ H ₁₆ N ₂	N, N, N', N'-Tetramethylethylenediamine
C ₄ H ₁₁ NO ₃	Tris(hydroxymethyl)aminomethane
C ₁₀ H ₁₆ N ₂ O ₈	Ethylenediaminetetraacetic Acid
C ₁₂ H ₂₅ OSO ₃ Na	Sodium Dodecyl Sulphate
C _{max}	Maximum Serum Concentration
CMBI	Centre for Molecular and Biomolecular Informatics
CMV	Cytomegalovirus



CNS	Central Nervous System
cSNP	Coding Single Nucleotide Polymorphism
CYP	Cytochrome P450
Cys	Cysteine
dbSNP	Single Nucleotide Polymorphism Database
ddH ₂ O	Double Distilled Water
dH ₂ O	Distilled Water
ddC	Zalcitabine
ddI	Didanosine/Dideoxyinosine
DE	Delaware
DLV	Delavirdine
DME	Drug-Metabolising Enzyme
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
DOH	Department of Health
d4T	Stavudine
EDTA	Ethylenediaminetetraacetic Acid (C ₁₀ H ₁₆ N ₂ O ₈)
EFV	Efavirenz
EM	Extensive Metaboliser
ETB	Extrapulmonary Tuberculosis
EtBr	Ethidium Bromide
EtOh	Ethanol (CH ₃ CH ₂ OH)
F	Forward Primer
fAPV	Fosamprenavir
FTC	Emtricitabine
G	Guanine (where referring to a nucleotide)
G6PD	Glucose-6-Phosphate Dehydrogenase
Gln	Glutamine

gp41	Glycoprotein 41
HAART	Highly Active Antiretroviral Therapy
H ₃ BO ₃	Boric Acid
HGP	Human Genome Project
His	Histidine
HIV	Human Immunodeficiency Virus
H ₈ N ₂ O ₈ S ₂	Ammonium Persulphate
HSV	Herpes Simplex Virus
http	Hypertext Transfer Protocol
HWE	Hardy-Weinberg Equilibrium
IDC	Infectious Diseases Clinic
IDV	Indinavir
Ile	Isoleucine
IM	Intermediate Metaboliser
Inc	Incorporated
indel	Insertion/Deletion
ins	Insertion
iSNP	Intergenic Single Nucleotide Polymorphism
kb	Kilobase
KCl	Potassium Chloride
kDa	Kilodalton
kg	Kilogram
KHCO ₃	Potassium Hydrogen Carbonate
KH ₂ PO ₄	Potassium Dihydrogen Orthophosphate
KS	Kaposi's Sarcoma
L	Litre
LD	Linkage Disequilibrium
LD	Lipodystrophy (where referring to a syndrome)



Leu	Leucine
LOD	Logarithm of the Odds
log	Logarithm
LPV/r	Lopinavir/Ritonavir
Lys	Lysine
M	Molar (moles per litre)
MA	Massachusetts
MD	Maryland
MDMA	Methylenedioxymethamphetamine
<i>MDR1</i>	Multidrug Resistance 1
mg	Milligram
MgCl ₂	Magnesium Chloride
MIV	Menslike Immunitetsgebrevirus
ml	Millilitre
mM	Millimolar
mm ³	Cubic Millimetre
MOTT	Mycobacteriosis Other Than Tuberculosis
Mr	Relative Molecular Mass
mRNA	Messenger Ribonucleic Acid
mtDNA	Mitochondrial DNA
n	Sample Size
N	Any Nucleotide (where referring to a nucleotide)
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
<i>NAT2</i>	<i>N</i> -acetyltransferase 2
NCBI	National Centre for Biotechnological Information
nd	No Data
NEB	New England Biolabs
NFSE	Nifedipine-Specific Element
NFV	Nelfinavir

NG	National Geographic
ng	Nanogram
NH ₄ Cl	Ammonium Chloride
NNRTI	Non-Nucleoside Reverse Transcriptase Inhibitor
NRTI	Nucleoside Reverse Transcriptase Inhibitor
NtRTI	Nucleotide Reverse Transcriptase Inhibitor
NVP	Nevirapine
<i>p</i>	Probability
PAA	Polyacrylamide
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCP	<i>Pneumocystis carinii</i> pneumonia
PCR	Polymerase Chain Reaction
PD	Pharmacodynamics
PGL	Persistent Generalized Lymphadenopathy
PGP	Phosphoglycoprotein
pH	Potential Hydrogen
Phe	Phenylalanine
PI	Protease Inhibitor
PK	Pharmacokinetics
PM	Poor Metaboliser
PML	Progressive Multifocal Leucoencephalopathy
PMPA	Tenofovir
PMTCT	Prevention of Mother to Child Transmission
pre-mRNA	Preliminary Messenger Ribonucleic Acid
Pro	Proline
pSNP	Perigenic Single Nucleotide Polymorphism
PTB	Pulmonary Tuberculosis
PTC	Phenylthiocarbamide
R	Reverse Primer

®	Registered Trademark
r^2	Correlation Coefficient
REase	Restriction Enzyme/Restriction Endonuclease
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
rpm	Revolutions per Minute
rSNP	Random Single Nucleotide Polymorphism
RT	Reverse Transcriptase
RTV	Ritonavir
SA	South Africa
SDS	Sodium Dodecyl Sulphate ($C_{12}H_{25}OSO_3Na$)
Ser	Serine
SIV	Simian Immunodeficiency Virus
SNP	Single Nucleotide Polymorphism
SQVh	Saquinavir (hard-gel capsule)
SQVs	Saquinavir (soft-gel capsule)
SSCP	Single Strand Conformation Polymorphism
SSRI	Selective Serotonin Re-uptake Inhibitor
T	Thymine (where referring to a nucleotide)
T20	Enfuvirtide
T_A	Annealing Temperature
<i>Taq</i>	<i>Thermus aquaticus</i>
TB	Tuberculosis
TBE	Tris Borate EDTA
3TC	Lamivudine
TCA	Tricyclic Antidepressant
TEMED	N, N, N', N'-tetramethylethylenediamine ($C_6H_{16}N_2$)
TFPGA	Tools for Population Genetic Analyses
Thr	Threonine
™	Trademark

TPV	Tipranavir
Tris	Tris(hydroxymethyl)aminomethane (C ₄ H ₁₁ NO ₃)
U	Unit (enzyme quantity)
UK	United Kingdom
UM	Ultra-rapid Metaboliser
UN	United Nations
URTI	Upper Respiratory Tract Infection
USA	United States of America
UTR	Untranslated Region
UV	Ultraviolet
V	Volts
VNTR	Variable Number of Tandem Repeats
vs	Versus
v/v	Volume per Volume
WHO	World Health Organisation
WI	Wisconsin
www	World Wide Web
w/v	Weight per Volume



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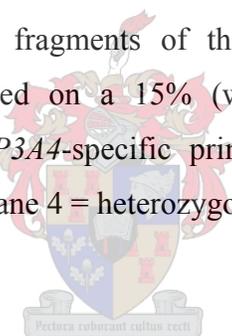
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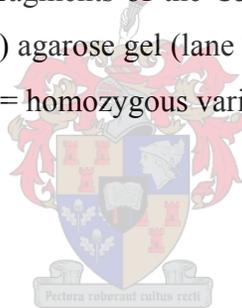
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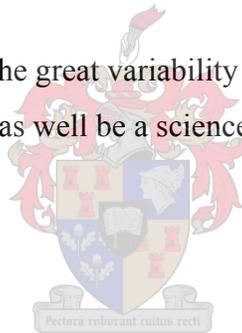
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“If it were not for the great variability among individuals,
medicine might as well be a science and not an art.”

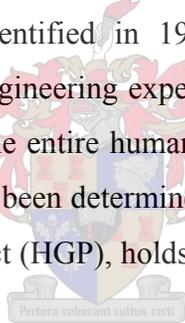


Sir William Osler, 1892
(quoted in Roses 2000)

CHAPTER ONE

INTRODUCTION

Modern genetics is a relatively young science. While the first instances of domestication and selective breeding of agricultural crops in the Middle East can be traced all the way back to 9 000 BCE, modern genetics traces its lineage to only the middle of the 19th century. During this period, Charles Darwin published his theory of natural selection in *The Origin of Species* and Gregor Mendel published the results of his investigations of the inheritance of “factors” in pea plants. It was in 1900, however, when Carl Correns, Hugo de Vries and Erich von Tschermak-Seysenegg independently discovered and verified Mendel’s principles, that modern genetics was truly born. Since then, massive breakthroughs have been made at an exponential rate within the field. Watson and Crick elucidated the structure of DNA in 1953, the first human chromosome abnormality, Down Syndrome, was identified in 1959, Stanley Cohen and Herbert Boyer performed the first successful genetic engineering experiment in 1973, and within less than fifty years of the discovery of its structure, the entire human genomic DNA sequence consisting of a staggering 3.3 billion base pairs (bp) had been determined by 2000. This latest breakthrough, the completion of the Human Genome Project (HGP), holds the greatest potential for human health of any scientific achievement.



The determination of the human genome sequence is considered one of the greatest accomplishments in the history of science, promising to revolutionise many seemingly disparate aspects of healthcare, ranging from diagnostics, disease management, epidemiology and treatment options. Despite the enormous significance and potential of the HGP, however, its completion was in effect a ‘race to the start line’. Although the complete sequence of the genome is now available, little is yet understood about the exact number of genes it contains, the number of proteins they encode, what the functions of all these proteins are and how they interact with each other, and a multitude of other relevant aspects of human molecular biology. Despite these and other shortcomings in our current understanding, the elucidation of the entire human genetic code will surely prove to have been the first step towards solving these mysteries and into an era of genomic medicine and healthcare. Of the many mysteries that the human genome sequence has

the potential to unravel, one of the most promising and widely touted is that of differential drug response and tailor-made drugs and drug regimens.

The consequences of differential drug response, both in terms of variations in efficacy and risk and severity of ADRs, include dire economic costs and mortality rates. In 1994, over one and a half million hospital admissions and one hundred thousand deaths in the United States of America (USA) alone were due to ADRs, making it one of the leading causes of hospitalisation and death (Lazarou *et al.* 1998). The economic costs are equally staggering: ADRs in the USA cost society approximately US\$10 billion annually while the total annual health expenditures related to lack of drug efficacy have been estimated to be a massive US\$170 billion (more than the total amount spent on prescription drugs annually within the entire country) (Gurwitz *et al.* 2006). It is abundantly clear that patients do respond differently to identical drug regimens (despite significant similarities in disease state) and that such differential response has serious healthcare and economic consequences. Less clear, unfortunately, are the causes of ADRs and variations in drug efficacy.

There is most certainly an extremely intricate interplay between genetic, nutritional, environmental and lifestyle factors that affect the risk and severity of undesirable side-effects and variations in efficacy of pharmaceutical drugs. Despite these numerous factors, however, an individual's response to pharmacotherapy is largely determined by pharmacokinetic (the process of absorption, distribution, metabolism and excretion of a drug) (PK) and pharmacodynamic (mechanisms by which drugs affect their target sites in the body to produce their desired therapeutic effects and adverse side effects) (PD) factors that often depend on the individual's genotype. This realisation of the seemingly large role that host genetics plays on drug response has increasingly gained momentum since the early 1950s, when researchers realised that some ADRs were caused by genetically determined variations in enzyme activity. This growing awareness, coupled with the rapid advances made in molecular biology within the past few decades and the recent completion of the HGP, has given rise to the field of pharmacogenetics. Pharmacogenetics blends important aspects of pharmacology and genetics with the aim of better understanding the genetic basis of differential drug response on both an interindividual and interpopulation level. The long-term goal of pharmacogenetics is thus a more customised approach to the administration of drug therapies with a subsequent decrease in ADRs and increase

in levels of efficacy. It is likely, however, that the eventual practical application of successfully investigating and applying pharmacogenetics in limiting ADRs and variations in efficacy will be restricted to diseases with dire prognosis. In such disease states, there exists a high medical need for drug therapies which offer unique potential advantages and for which the tolerance, even for relatively severe side-effects, is therefore much greater than for other drugs. This concept applies particularly well to HIV infection and any consequent treatment with ARVs.

The world in the 21st century is distorted by Acquired Immune Deficiency Syndrome (AIDS). The number of people worldwide infected with HIV, the causative agent of AIDS, is estimated at 38.6 million (as of the end of 2005). Of this 38.6 million, however, approximately 25 million (65%) live in sub-Saharan Africa. Sub-Saharan Africa is home to just over 10% of the world’s population – and almost two-thirds of all people living with HIV (UNAIDS 2006; UNAIDS 2004). The cartogram in Figure 1.01, in which each country’s size reflects the number of people infected with HIV/AIDS, clearly illustrates this global disproportionate distribution of the disease.

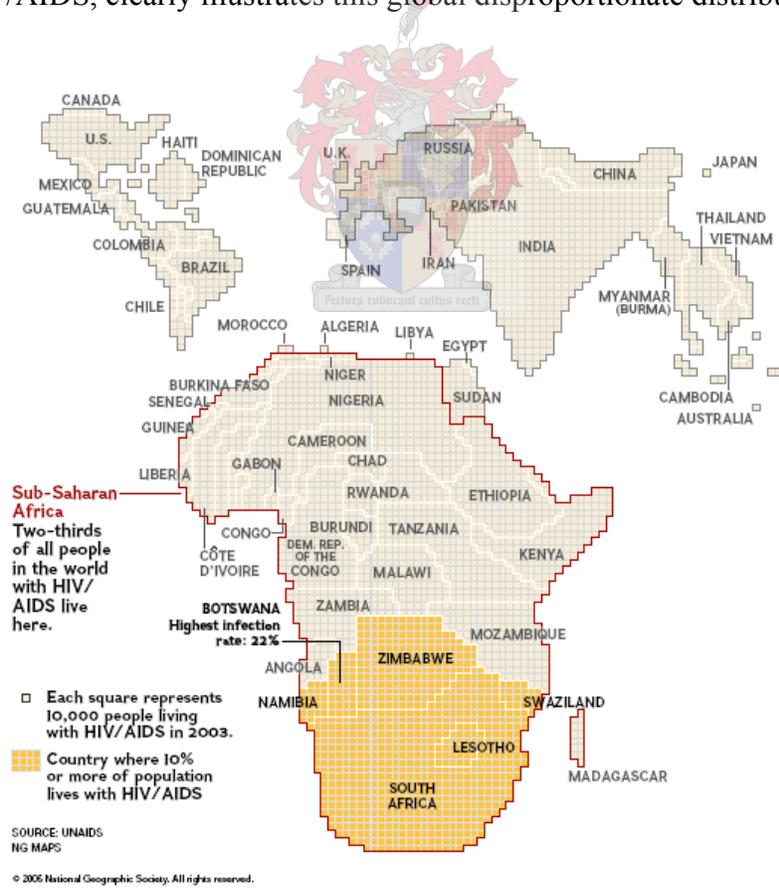


Figure 1.01: Cartogram representation of global HIV/AIDS prevalence and distribution (Source: UNAIDS, NG Maps in Mendel 2005)

Approximately 8 500 people in sub-Saharan Africa contract HIV daily, while another 6 300 die of the disease. In South Africa (SA), 5.5 million people are infected with the disease, more than any other country in the world, while a staggering 27.9% of pregnant women in SA tested positive for HIV in 2004. Beyond this incredibly large number of HIV-infected individuals within SA, is the enormous number who requires ARVs in order to prevent progression of the illness and certain death and/or mother-to-child transfer of the virus – 983 000 South Africans need ARVs, while only 117 000 (< 12%) currently receive them.

ART was first made available with the introduction of zidovudine in 1987, since when a range of drugs in numerous classes have been developed. The use of combinations of these drugs has provided potent ART which has had a dramatic impact on the prognosis of HIV-infection – morbidity and mortality rates have been significantly reduced in HIV-positive patients undergoing combination ART. Unfortunately, however, not all of the effects of these drugs have proved favourable. A significant proportion of patients experience a variety of toxic side-effects, some of which can be life-threatening, and/or little or no response to the treatment. Indeed, none of the many clinical studies so far performed on ART have demonstrated 100% response rates in terms of control of viral replication or CD4 cell recovery. Furthermore, there appears to be a 1-log variance in C_{max} values (maximum serum concentration that a drug achieves after administration) across a cohort of patients taking ARVs and large discrepancies in ARV drug metabolism and response between different ethnic groups.

The basis for the varied responses to ARVs is, similar to responses to numerous other drug classes, significantly influenced by host genetic factors. Various polymorphisms in genes involved in the metabolism and transport of ARVs have previously been associated with varied clinical responses. These genes include the CYP superfamily of genes, responsible for the metabolism of the vast majority of clinically administered drugs, and the Multidrug Resistance 1 (*MDR1*) gene, responsible for the energy-dependent efflux of substances from within cells and membranes. Although research examining the influences of allelic variants of these genes on ARV drug response has been performed globally in many ethnic groups, particularly Caucasians, Asians and African-Americans, little has been conducted in groups indigenous to SA's ethnically diverse population. Moreover, the fact that these ethnic groups constitute a massive segment of the population and have been documented to respond differently to ARVs, makes it imperative for

improved treatment that research of this nature is conducted. The increasing need for and usage of ARV drugs in SA further underscores the importance of such research. The aim of this study was therefore to examine the potential effects of several SNPs within genes involved in ARV drug transport and metabolism on ART response in South African ethnic groups.



CHAPTER TWO

LITERATURE REVIEW

2.1 PHARMACOGENETICS

The results of drug therapy can vary both within a population (Johnson 2003; Ingelman-Sundberg 2001) and between different populations or ethnic groups (Westlind-Johnsson *et al.* 2006; Meyer 2004; Xie *et al.* 2001; Meyer and Zanger 1997). Plasma drug concentrations within two individuals of equal weight on identical drug dosage can vary by more than 600-fold (Eichelbaum *et al.* 2006). Therefore, the same drug administered in the same dosage to patients with identical disease state may lead to the desired effect in the majority of treated patients, but can prove ineffective in a significant proportion of others and may even produce ADRs (any noxious, unintended or undesirable effects) in some (Meyer 2004; Meisel *et al.* 2000; Evans and Relling 1999; Nebert 1999). Apart from the detrimental effects of therapeutic failure or inefficacy, the unfavourable effects of ADRs can vary in intensity and severity, ranging from merely bothersome to potentially life-threatening (Lindpaintner 2003).

Various factors have in the past been held accountable for the observed differences in drug response and include age, concomitant diseases, gender, interactions with other drugs, misdosing, renal and hepatic function, as well as lifestyle variables such as smoking and alcohol consumption (Oscarson 2003; Schwartz 2003; Bachmann 2002; Meisel *et al.* 2000; Evans and Relling 1999). There is, however, an ever-increasing body of evidence that suggests that genetic differences between individuals and even entire populations can be an important and at times predominant factor influencing drug response variability (Evans and Relling 2004; Lesko and Woodcock 2004; Meyer 2000; Evans and Relling 1999). In fact, it is estimated that genetics is responsible for 15% to 95% (depending on the drug or class of drug) of the observed interindividual variability in drug disposition and effects (Eichelbaum *et al.* 2006; McGee 2006a; Evans and McLeod 2003). This increasing awareness of the significant role that genetic polymorphisms play in drug response variability, together with rapid developments in genomic technologies and the completion of the HGP, has given rise to the field of pharmacogenetics.

Pharmacogenetics is the study of the role of inheritance in interindividual and interpopulation variations in drug response (Robert *et al.* 2005; Meyer 2004; Vogel 1959). The rationale and ultimate aim of pharmacogenetics is the possibility that knowledge of an individual's genetic make-up could be used to enhance drug therapy by maximising drug efficacy while minimising drug toxicity (Lesko and Woodcock *et al.* 2004; Weinshilboun and Wang 2004; Meyer 2000; Linder *et al.* 1997). The ultimate goal of pharmacogenetics, therefore, is targeted pharmacological treatment of patients based on their genetic determinants of drug efficacy and toxicity, so that they are more likely to respond favourably with fewer or no unfavourable side-effects (Evans and Johnson 2001; Evans and Relling 1999).

As the influence of pharmacogenetics on drug discovery and development and drug treatment regimens increases, there will undoubtedly be a move away from the current approach of standardised treatments towards more individualised, 'tailor-made' therapies (Liggett 2004; Roses 2000). Despite the fact that this concept of individualised drug treatment seems futuristic and is largely the product of recent advances in human molecular biology, the scientific foundation on which it is based has a relatively long history.

2.1.1 A Brief History of Pharmacogenetics

Although often viewed as a new discipline, the scientific premise of pharmacogenetics has been recognised since the end of the 19th century. In the late 1800s, Sir Archibald Garrod noted that a subset of psychiatric patients developed porphyria in response to treatment with the hypnotic drug sulphonal (Lindpainter 2003; Garrod 1909), and in 1914 proposed that enzymes were somehow implicated in the detoxification of exogenous substances (Garrod 1914). These observations, along with those by physiological chemists regarding the excretion of drugs in different forms from those in which they were administered, led Garrod to conclude that the ability to transform drugs into non-toxic conjugates served as a protective mechanism against any poisonous effects and was in fact mediated by enzymes. Furthermore, due to the rediscovery of Mendel's Laws around 1900 and the subsequent flurry of research, Garrod, along with other researchers, anticipated the connection of enzymes with the genetic material. Garrod was thus ahead of his contemporaries in recognising that unexpected drug responses could be attributed to the failure of enzymes to detoxify these substances, and that these enzymatic inefficiencies could be genetic in origin (Weber and Cronin 2000).

Despite the insightful observations of Garrod, the first experimental identification and study of a pharmacogenetic trait was made during the 1930s and involved not individual response to a drug, but variation in the ability to taste a foreign chemical (Meyer 2004). It was noted that some individuals expressed an inability to taste ('taste-blindness') phenylthiocarbamide (PTC) (Fox 1932), which was subsequently found to be inherited in an autosomal-recessive manner and to vary in frequency in populations of different ethnicities (Snyder 1932). This study of 'taste blindness' was the forerunner of pharmacogenetic studies and, as such, was the first study to document an association between ethnicity and the response to chemical compounds. Further progress within the field slowed, however, until the 1950s when several breakthroughs and the development of new technologies led to further confluence of pharmacology, genetics and biochemistry (Weber and Cronin 2000).

During the 1950s, researchers realised that certain ADRs were in fact caused by genetically determined variations in enzyme activity (Johnson 2003; Meyer 2000) – it was discovered that prolonged muscle relaxation and apnea after suxamethonium treatment (an adjunct to anaesthesia) was due to altered enzyme kinetics of a pseudocholinesterase and is inherited as an autosomal-recessive trait (Kalow and Staron 1957), and that haemolytic anaemia from the antimalarial drug primaquine was resultant of a variant form of the glucose-6-phosphate dehydrogenase (G6PD) enzyme (Carson *et al.* 1956). It was in 1957, however, when Arno Motulsky's seminal paper, concerned with interindividual differences in drug response due to the unique genetic constitution of individuals, was published (Motulsky 1957), that pharmacogenetics as an experimental science was finally established (Meyer 2004). The term 'pharmacogenetics' was subsequently coined by Vogel in 1959 (Vogel 1959).

Further progress within pharmacogenetics was made during subsequent decades, characterised by the development of a community of researchers interested in pharmacogenetics and an increasing awareness of gene-drug interactions (Meyer 2004; Weber and Cronin 2000). However, with the advent of polymerase chain reaction (PCR) technology in the mid-1980s (Saiki *et al.* 1985), progress within the field accelerated markedly. In the late 1980s, the first CYP gene, *CYP2D6*, was cloned and characterised (Gonzalez *et al.* 1988), followed by the cloning and characterisation of several other drug metabolism genes, as well as some receptor and transporter genes (Nebert and Vessel 2004).

Developments within the field experienced further advancement with the inception and completion of the HGP, in 1990 and 2003 respectively, and consequent increased availability of gene sequences (Robert *et al.* 2005; Lerer 2004; Weber and Cronin 2000), the concurrent increase of data on genomic variation (Hoehe *et al.* 2003), numerous technological advances (Meyer 2004; Johnson and Evans 2002; Weber and Cronin 2000) and the elucidation of entire pathways that may be relevant to drug response (Goldstein *et al.* 2003). Also assisting progress within pharmacogenetics in more recent years is the increasing interest in pharmacogenetic research by physicians, geneticists, regulatory agencies and, to varying degrees, the pharmaceutical industry – this industry has shown relatively limited interest in pharmacogenetics due to the inherent nature of pharmacogenetics to segment potential drug markets (Breckenridge *et al.* 2004; Hosford *et al.* 2004; Meyer 2004; Weinshilboum and Wang 2004). Furthermore, there has been considerable investment from, and collaborations and alliances between, numerous biotechnology, genomics and pharmaceutical companies (Webster *et al.* 2004). This modern climate of substantial investment, financial and otherwise, and interest in pharmacogenetics is helping improve our understanding of the role that genetic polymorphisms play in drug response.

2.1.2 Genetic Polymorphisms

A genetic polymorphism is defined as a deoxyribonucleic acid (DNA) sequence variant which is stable within a population and occurs with a frequency equal to or greater than 1% (Lash *et al.* 2003; Bachmann 2002; Nebert 1999). There is a considerable level of variability between individuals at the genetic level, as manifested by the polymorphisms present within their genome (Oscarson 2003; Sachidanandam *et al.* 2001). Over 90% of these polymorphisms are believed to be accounted for by changes in a single nucleotide, namely SNPs, with the remainder of the variation caused by insertions and deletions (indels), variable number tandem repeats (VNTRs) and microsatellites (Marsh and McLeod 2006; Quirk *et al.* 2004). However, unlike many other previously characterised polymorphisms, such as VNTRs and microsatellites, SNPs are often found within the coding and regulatory regions of genes and thus can have functional consequences for gene expression and gene product functionality (Campbell *et al.* 2000; Gray *et al.* 2000).

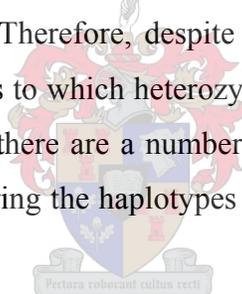
Initial estimates of 1 420 000 (Sachidanandam *et al.* 2001) to 3 000 000 (Roses 2002) SNPs within the human genome have since been significantly exceeded. The largest public SNP

database, the Single Nucleotide Polymorphism database (dbSNP), currently has > 27 000 000 submissions with more than 12 000 000 validated polymorphisms (Build 126, May 2006) (www.ncbi.nlm.nih.gov/projects/SNP/index.html). All of these SNPs can be characterised in terms of their minor allele frequency (rare SNPs < 0.01; polymorphic SNPs > 0.01; common SNPs > 0.05) (Nebert and Vessel 2004), but are also classifiable into three groups according to their position within the genome (Nebert 1999).

The vast majority of SNPs, so-called intergenic or random SNPs (iSNPs/rSNPs), are situated in the non-coding areas between genes (so-called 'junk DNA') and thus have no known function or effect on gene expression and gene product functionality. Perigenic SNPs (pSNPs) occur within or in the immediate vicinity of genes and include SNPs located within introns, non-coding regions of messenger ribonucleic acid (mRNA), upstream regulatory regions from the furthest upstream functional enhancer to the transcription initiation site, as well as silent codon polymorphisms (i.e. synonymous changes) and SNPs within 100 bp downstream of the last exon of a gene. pSNPs are thus similar to iSNPs in that they are non-coding, but differ in that they can still affect gene expression levels or incur functional changes to the gene product (Nebert 1999). Recent studies have indeed suggested that the presence of sequence variants, such as pSNPs, within intronic regions could affect basic preliminary-mRNA (pre-mRNA) splicing mechanisms and thereby cause altered levels of normal transcripts (Pagani *et al.* 2003). Should a pSNP occur within the regulatory region of a gene, it could affect the binding of transcription factors, altering the level of transcription and hence the amount of gene product within an individual (Ball *et al.* 1999). Furthermore, a pSNP within the 3'-untranslated region (UTR) following the coding sequence may affect the intracellular stability of the mRNA gene transcript (Quirk *et al.* 2004).

SNPs within the coding regions of a gene which do cause changes in the amino acid sequence of the encoded protein are known as coding SNPs (cSNPs) which, due to greater selective pressures against changes at positions dictating amino acid sequence, are generally less common than iSNPs or synonymous changes in coding sequence (Gray *et al.* 2000). A change in the amino acid sequence of a protein can have significant structural consequences, depending on the nature and location of the alteration, which in turn can exert considerable influence on the functionality of the protein, as well as its affinity for its intended substrates.

Despite these myriad effects that single SNPs can have, it should however be noted that single SNP approaches to genotype-phenotype correlations have severe limitations, and that it is in fact patterns of sequence variations that significantly influence the risk for disease and differential drug response (Clark *et al.* 1998; Nickerson *et al.* 1998). It has been demonstrated that gene-based haplotypes (i.e. specific combinations of SNPs throughout the genome) are superior to the use of individual SNPs for predicting association between genomic variation and phenotype (Johnne *et al.* 2002; Drysdale *et al.* 2000; Judson *et al.* 2000). Therefore, when trying to ascertain the genotypic cause of a particular phenotypic trait, it is important to consider not only individual SNPs that may be of interest, but rather combinations of SNPs as well as the different haplotypes – for SNPs that are in LD – that they exhibit. The determination of these different haplotypes that underlie a specific genotype is vital in the elucidation of the functionality of different forms of a gene (i.e. the form of a gene on each chromosome) (Hoehe *et al.* 2003). Such determination is, however, complicated by the uncertainty of the phase of heterozygous SNPs – in other words, whether two particular variants reside on the same chromosome (*'cis'*) or on separate chromosomes (*'trans'*). Therefore, despite having determined the genotype of an individual, there remains uncertainty as to which heterozygous SNP variants came from the same chromosome. Fortunately, however, there are a number of computational techniques that have been developed that can assist in inferring the haplotypes from the genotype data (Halldórsson *et al.* 2004).



The different types of SNPs are thus multiple, as are their effects. Depending on their location within the genome and their patterns of co-occurrence (i.e. haplotypes), SNPs can alter expression levels of a gene as well as the functionality of the encoded protein product or its affinity for its intended substrates. These effects of SNPs can, as is the case with many other phenotypic characteristics, greatly affect the manner in which a patient responds to drug therapy.

2.1.3 The Effects of Genetic Polymorphisms on Drug Response

Genetic polymorphisms within genes encoding drug targets, drug transporters and drug-metabolising enzymes (DMEs) can affect the PK and PD characteristics of drug compounds (Johnson 2003; Steimer and Potter 2002). The therapeutic index of a drug (the difference between the minimum effective dose and maximum tolerated dose) and the quantitative role of a drug transporter or DME in the drug's kinetics determine the clinical relevance of such genetic

polymorphisms (Meyer 2000) – e.g. the narrower a drug’s therapeutic index, the greater the clinical effects of changes in its PK and PD characteristics. As outlined in Figure 2.01, the clinical effects of these genetic polymorphisms on the PK and PD of pharmaceutical drugs can lead to variable drug efficacy or risk of toxicity and ADRs.

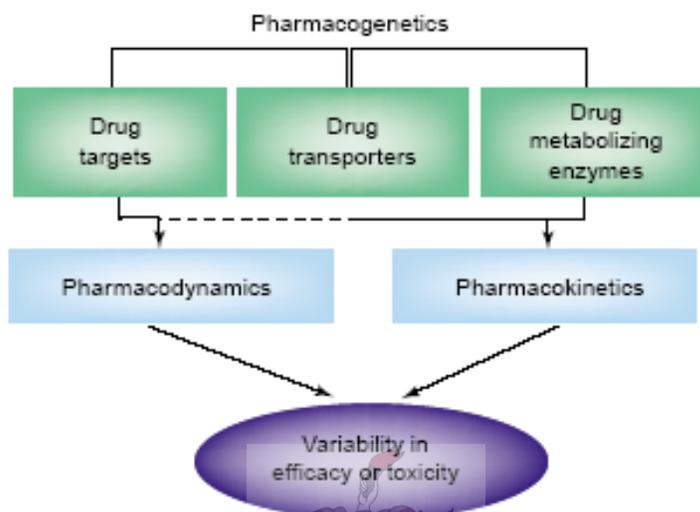


Figure 2.01: Key components in pharmacogenetics (the broken line illustrates that drug transporters are occasionally also the drug target, in addition to affecting drug PK characteristics)
(Source: Johnson 2003)

The effects on the PK nature of a drug include changes in drug disposition (absorption, distribution, metabolism and excretion) (Wilkinson 2005; Goldstein *et al.* 2003), with a consequent undesirable concentration of the drug and/or drug metabolites at the intended site of action. This non-optimal concentration of the drug and/or drug metabolites at the intended site of action can, in turn, result in either a lack of efficacy or ADRs (Lindpainter 2003; Meyer and Zanger 1997). The causes of variations in drug PK include polymorphisms in genes involved in the metabolism and transport of drug compounds, such as the CYP gene superfamily (Ingelman-Sundberg 2005; Wilkinson 2005) and *MDR1* gene (Ambudkar *et al.* 1999), respectively. The factors affecting drug PD characteristics include sequence variations in genes affecting how the drug target molecule, or another downstream member of the drug target molecule’s mechanistic pathway, respond to the drug compound (Johnson 2003). This can result in interindividual differences in drug response, despite the presence of appropriate concentrations of the intended drug compound and/or drug metabolites at the intended site of action (Lindpaintner 2003).

The effects of genetic polymorphisms on drug response are thus multifaceted, as are the nature of the polymorphisms. As previously mentioned, genetic polymorphisms include sequence variations within the intronic, regulatory and coding regions of genes that influence the PK and PD characteristics of drug compounds. Also of importance, however, are gene duplications that can result in increased enzyme quantity and thus enzyme activity (Ingelman-Sundberg 2004; Evans and Johnson 2001; Johansson *et al.* 1993) with a subsequent lack of therapeutic effect – as can occur with the *CYP2D6* gene and treatment with the antidepressant drug nortriptyline (Dalen *et al.* 1998) (see Section 2.1.6). The effects of these polymorphisms include alterations in the level of transcription of a gene or functionality and activity of the protein product, thereby altering the PK and/or PD characteristics of a drug and hence the clinical response to it.

The variations in clinical response to drug therapy ascribable to genetically-determined changes in drug PK (due to altered levels of drug metabolism) allow the classification of patients into four clinical groups (Ingelman-Sundberg 2004; Meyer 2000; Ingelman-Sundberg *et al.* 1999; Ingelman-Sundberg 1998). These four groups include:

- extensive metabolisers (EMs), who are either homo- or heterozygous for the wild-type or normal-activity enzymes and display a level of drug metabolism observed in the majority of patients;
- poor metabolisers (PMs), who carry two loss-of-function alleles and therefore have a severely impaired level of drug metabolism;
- intermediate metabolisers (ImS), who carry two decreased-activity alleles, resulting in decreased enzyme activity and subsequent level of drug metabolism (relative to EMs);
- ultra-rapid metabolisers (UMs), who have duplicated or multi-duplicated active copies of a gene and thus exhibit a considerably higher level of drug metabolism relative to EMs.

The two extremes of these four groups, namely PMs and UMs, clearly illustrate the clinical importance and effects of genotype on phenotype in terms of drug metabolism and response, as evident in Table 2.01.

Table 2.01: The clinical effects of genotypic influences on phenotype in terms of drug metabolism
(Sources: Ingelman-Sundberg 2004; Bean 2000; Ingelman-Sundberg 1998)

Poor Metabolisers	Ultra-rapid Metabolisers
Decreased rate of metabolism; increased drug bioavailability	Increased rate of metabolism; decreased drug bioavailability
Exaggerated response at standard dosage; side-effects, toxic effects (ADRs)	Lack of therapeutic effect at standard dosage; explanation for suspected poor adherence
Active metabolite not formed (in the case of a prodrug); loss of therapeutic efficacy	Excess of active metabolite formed (in the case of a prodrug); side-effects, toxic effects (ADRs)

The consequences of either a markedly decreased or increased level of drug metabolism (or drug transport) can thus ultimately manifest in unintended and undesirable side-effects, or ADRs, and variations in levels of therapeutic efficacy.

2.1.4 The Costs of ADRs and Variable Therapeutic Efficacy

Unanticipated and undesirable responses to pharmaceutical drugs, both in the forms of ADRs and lack of adequate response, constitute considerable economic, social and healthcare burdens (Eichelbaum *et al.* 2006; Haramburu *et al.* 2000; Schenkel 2000). Most major drugs are effective in only 25 to 60% of patients (Spear *et al.* 2001) while ADRs are estimated to be the fifth leading cause of death in the USA, responsible for over one and half million hospital admissions and one hundred thousand deaths per year (Lazarou *et al.* 1998). The monetary costs of ADRs to the USA economy are equally dramatic, with an approximate US\$10 billion spent annually on ADR related events, while the costs related to lack of therapeutic efficacy are estimated at a massive US\$170 billion (Gurwitz *et al.* 2006). Although similar data concerning mortality rates and economic costs of ADRs in SA are currently not available, there is no evidence to suggest that the number of deaths and level of costs, relative to its population size and healthcare system, are any lower.

2.1.5 Clinical Applications and Benefits of Pharmacogenetics

There are currently two main approaches to establishing the correct treatment and dosage regimen for the pharmacological management of a condition or disease (Johnson 2003). The first approach relies on trial-and-error and is used in the treatment of diseases such as depression,

diabetes, hypertension and schizophrenia. There are usually numerous first-line therapy drugs that can be used to treat these diseases and the trial-and-error method of establishing which drug, or combination of drugs, and at what dosage to use in each patient is time-consuming and can take months to accomplish. The second approach is a more 'one-size-fits-all' method, wherein the treatment employed is essentially the same for all patients. Examples of diseases and conditions treated in this manner include most cancers, heart failure and post-transplantation patients. In both approaches, however, a certain proportion of patients will undoubtedly experience a lack of efficacy or ADRs from a given drug.

There are therefore currently two main goals for the clinical application of pharmacogenetics (Johnson 2003; Johnson and Evans 2002), namely:

- the ability to predict which patients are at a higher risk of ADRs and which should, therefore, receive a lower dose of a drug or a different drug altogether;
- the ability to predict which patients are most likely to obtain the desired therapeutic effect(s) from a drug.

The subsequent stratification of patient eligibility for a drug or drug dosage level, based on genotypic markers, stemming from these two goals is clearly illustrated in Figure 2.02. For patients treated with either of the two methods of pharmacological treatment (i.e. trial-and-error and 'one-size-fits-all'), there are numerous benefits of the full, or even partial, realisation of these two goals and consequent stratification of patient populations. These benefits include a shorter time period in which the disease is not properly controlled (e.g. ART for HIV-infection), a decreased risk for negative consequences of the disease not being properly controlled (e.g. suicide in patients suffering from depression), fewer follow-up visits to the physician due to ineffective treatment, the avoidance of the use of ineffective therapies and drug toxicities, and an overall reduction in healthcare costs resulting from all of the above factors (Johnson 2003; Lindpaintner 2003).

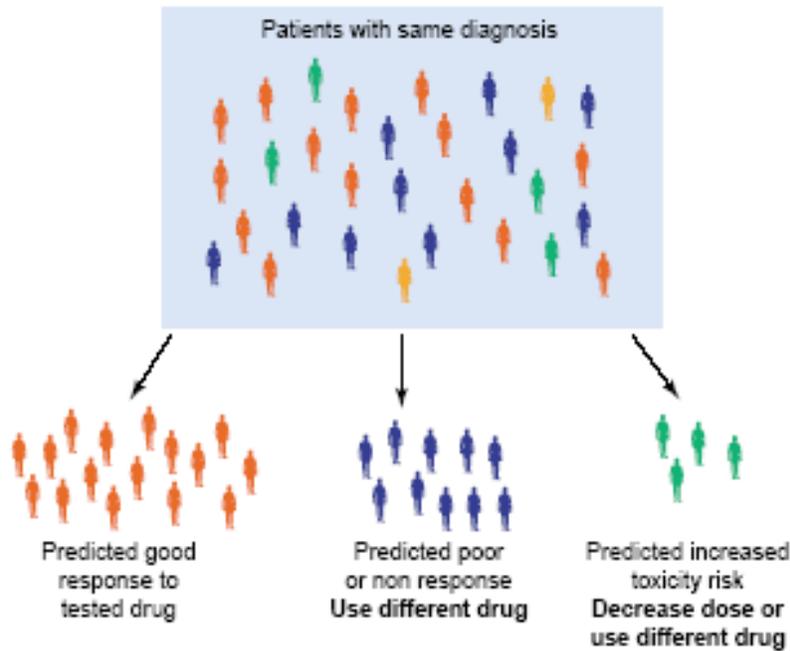


Figure 2.02: Customisation of pharmacological treatments through pharmacogenetic testing
(Source: Johnson 2003)

2.1.6 Population Differences in Pharmacogenetic Traits

The globalisation of drug development and clinical use has led to an increased awareness that different ethnic groups may not respond in the same manner to therapy (Westlind-Johnson *et al.* 2006). Furthermore, the extent of functional nucleotide diversity within any given gene may vary markedly between ethnic groups (Hoehe *et al.* 2003). Accordingly, all pharmacogenetic variations so far characterised have been found to occur at markedly dissimilar frequencies within different ethnic groups (Ingelman-Sundberg *et al.* 2001; Meyer 2000; Evans and Relling 1999; Ingelman-Sundberg *et al.* 1999).

Perhaps one of the most well-known and best characterised examples of inter-ethnic differences in a pharmacogenetic trait is the variable biotransformation efficiency of the antituberculosis drug isoniazid (Meyer 2004; Oscarson 2003), due to deleterious mutations within the *N*-acetyltransferase 2 (*NAT2*) gene. Decreased ability to convert isoniazid to acetylisoniazid (slow-acetylator status – analogous to the previously mentioned poor metaboliser phenotype) is associated with isoniazid toxicity, characterised by peripheral neuropathy (Lash *et al.* 2003; Hughes *et al.* 1954) and hepatotoxicity (Goldstein *et al.* 2003). Slow acetylator status has relevance beyond that of isoniazid metabolism, however, as such individuals metabolise a number

of other drugs (e.g. sulphonamides, phenelzine, dapsone, procainamide and hydralazine) at a slower rate, resulting in higher plasma concentrations and increased risk of ADRs. The frequency of *NAT2* slow acetylators varies markedly between different population groups – the majority of populations in North America and Europe contain 40 to 70% slow acetylators, whereas Asian populations have far fewer with only 10 to 30% exhibiting the slow acetylator phenotype (Meyer and Zanger 1997).

A similarly well-characterised pharmacogenetic trait which also exhibits considerable variation between different ethnic groups is the activity of the important DME, debrisoquine hydroxylase, encoded by the *CYP2D6* gene (Ingelman-Sundberg *et al.* 1999). The plasma concentration of the tricyclic antidepressant (TCA) nortriptyline can display 30- to 40-fold variability because of interindividual differences in the ability to metabolise the drug (Hammer and Sjöqvist 1967). The level of *CYP2D6* activity, and hence nortriptyline metabolism, is influenced by the presence of inactivating mutations (giving rise to the PM phenotype – see Section 2.1.3) (Skoda *et al.* 1988) or number of functional gene duplications (giving rise to the UM phenotype – see Section 2.1.3) (Ingelman-Sundberg 2001) of *CYP2D6*.

The gene dosage effect of multiple *CYP2D6* gene copies is salient, with the rate of clearance of nortriptyline (or other *CYP2D6* substrates) directly proportional to the number of *CYP2D6* gene copies present within an individual (Dalen *et al.* 1998). The frequency of individuals carrying alleles with inactive or multiple copies of the *CYP2D6* gene varies considerably in different parts of the world. Approximately 7% of Caucasians carry two non-functional variants of *CYP2D6*, while only 1-3% of Asians and people of African descent do so (Johnson 2003). Furthermore, 29 and 20% of Ethiopians and Saudi Arabians, respectively, carry alleles with multiple *CYP2D6* gene copies (presumably due to dietary selective pressures), while multi-duplicated copies are present in only 2 to 8% of the populations of Ghana, Zimbabwe and Tanzania, and in less than 2% of Asians and Scandinavians (Ingelman-Sundberg 1999; Ingelman-Sundberg *et al.* 1999; Droll *et al.* 1998; Ingelman-Sundberg 1998; McLellan *et al.* 1997). Such variations in the frequency of alleles containing inactive and multi-duplicated copies of the *CYP2D6* gene obviously portends to serious implications for treatments with *CYP2D6* substrates.

The considerable ethnic diversity ('gene geography') of pharmacogenetic traits, as illustrated in the above examples, clearly emphasises that ethnic origin is an important consideration and constraint in pharmacogenetic studies and pharmacotherapy. Furthermore, such genetic variation stresses the unreliability of extrapolating data concerning pharmacogenetic characteristics and drug responses from one ethnic group to another. Therefore, there exists a definite need to conduct pharmacogenetic studies in multiple cohorts that have specific drug responses and ADRs but differ on the basis of ethnicity (Hosford *et al.* 2004). The importance of this study, in which the clinical response to ARV drugs within South African ethnic groups is examined, is thus readily apparent, particularly in light of the scale of the Human Immunodeficiency Virus (HIV) /Acquired Immune Deficiency Syndrome (AIDS) epidemic within the country.

2.2 HIV/AIDS

2.2.1 A Brief History of HIV/AIDS

Despite the existence of numerous theories, the exact time, place and nature of origin of HIV is unknown and likely to remain a mystery. It is, however, generally accepted that the virus originated in West-Central Africa around the 1940s or early 1950s (Zhu *et al.* 1998), and is descended from a similar virus, Simian Immunodeficiency Virus (SIV), which presumably crossed the species barrier from chimpanzees to humans (Gao *et al.* 1999). Throughout the subsequent 30 to 40 years after this event, however, the virus went largely undetected in human populations. It was not until the mid- to late 1970s, when travel within the USA and internationally increased greatly, that the current epidemic was unleashed. By 1980, HIV is estimated to have spread to five continents (Africa, Australia, North America, South America and Europe) and, due to a complete lack of awareness and preventative action, infected an estimated 100 000 – 300 000 people.

In July 1981, a sudden outbreak of a particularly aggressive form of a rare cancer, Kaposi's Sarcoma (KS), was detected amongst homosexual men in New York, USA (Hymes *et al.* 1981) – the so-called 'gay cancer', as it was originally labelled. At approximately the same time, New York and Los Angeles experienced a dramatic influx of seemingly healthy young men into emergency rooms, presenting with fever, flu-like symptoms and *Pneumocystis carinii* pneumonia (PCP). Both outbreaks were seemingly the consequence of men who were severely immunocompromised. It was a year later, however, that the Centres for Disease Control (CDC)

linked the causative illness to blood and coined the term: Acquired Immune Deficiency Syndrome (AIDS) (Marx 1982). The causative agent of AIDS, HIV, was subsequently identified in 1983 by researchers at the Institute Pasteur in France (Barre-Sinoussi *et al.* 1983), and a year later a USA scientist, Robert Gallo, confirmed that HIV was indeed the causative agent (Popovic *et al.* 1984).

Since the 1980s, the HIV epidemic has spread at an alarming rate. Within less than 25 years since its discovery, the virus has spread to every part of the globe and infected an estimated 65 million people and killed more than 25 million (UNAIDS 2006). The remaining 40 million are those individuals currently living with HIV. However, as is strikingly evident in Figure 1.01, there exists a massively disproportionate global distribution of these HIV-infected individuals. Of these approximately 40 million individuals infected with HIV worldwide, as of the end of 2005, an estimated 25 million (65%) live in sub-Saharan Africa. In 2003 alone, between 2.6 and 3.7 million people within the region were infected with HIV and 2.0 to 2.5 million died of AIDS. Within sub-Saharan Africa, however, Southern Africa is the hardest hit by the epidemic.

Despite signs of the epidemic levelling off or even decreasing – in terms of adult (15-49 years of age) prevalence rate – within other regions of Africa, such as Kenya and Zimbabwe, Southern Africa shows few signs of improvement and continues to experience a growing epidemic. All seven countries within Southern Africa have HIV prevalence rates above 16%, with some countries such as Swaziland and Botswana as staggeringly high as 33.4 and 24.1%, respectively (UNAIDS 2006). The scale of the epidemic in SA is not dissimilar, and remains one of the worst in the world with no immediate indications of improvement. In 2005, approximately 5.5 million South Africans, including 18.8% of adults, were HIV-positive. In 2004, almost one in three women (27.9%) attending public antenatal clinics in SA was HIV-positive. Although already at such an overwhelming level, the scale of the epidemic in SA is expected to worsen still, as the number of reported AIDS-related deaths have shown an unabated rise (Groenewald *et al.* 2005; Bradshaw *et al.* 2004) and trends over time indicate a gradual increase in HIV prevalence (UNAIDS 2006; UNAIDS 2004).

2.2.2 ART for HIV/AIDS

Since the discovery of HIV/AIDS in the early 1980s, a number of ARV drugs with numerous methods of action, although all with the ultimate aim of suppression of viral replication, have

become available – the first of these being zidovudine in 1987 (Quirk *et al.* 2004; Gulick 1997). Zidovudine, along with other drugs within its class (i.e. with the same drug target and/or method of action), was initially used in patients as monotherapy against HIV-infection. Unfortunately, however, the use of a single drug to treat HIV-infection was later found to cause suboptimal suppression of viral replication (Gallant 2002; Barry *et al.* 1997). This lack of inhibition of HIV replication, coupled with the low fidelity of the reverse transcriptase catalysed DNA polymerisation reaction due to the virus's lack of proofreading polymerases (an average of one mutation occurs per replication cycle) and massive viral turnover (HIV replicates approximately 10 billion times per day), quickly led to the emergence of drug-resistant HIV variants (Gallant 2002; Balzarini 1999; Strachan and Read 1999; Ho *et al.* 1995; Wei *et al.* 1995). With the development of additional drugs that exerted their effects at a different stage of the HIV life cycle than zidovudine and other drugs within its class, combination therapy (the use of two or more drugs concurrently) subsequently proved to be a more beneficial and effective approach than monotherapy (Gallant 2002; Beach 1998; Gulick 1997).

The currently employed method of ART, available since 1996, involves a combination ('cocktail') of at least three ARV drugs from two or more classes, a practice referred to as highly active antiretroviral therapy (HAART) (Wynn *et al.* 2004; Brinkman *et al.* 1999). The objective of ART is to suppress the replication of HIV to such low levels that the CD4-cell count increases, viral load (i.e. HIV-RNA copy number in the plasma) decreases and remains at an undetectable level using the most sensitive routinely available assays (< 50 copies/ml), and the emergence of HIV variants that are drug-resistant is minimised (Yeni 2006; Beach 1998). Although ART does not eradicate the virus (Finzi *et al.* 1999), it enables HIV-infection to be treated and conceptualised more as a chronic, manageable disease than an inevitable and unstoppable progression towards illness and death (i.e. ARV drugs are virastatic and not viracidal) (Wynn *et al.* 2004; Gallant 2002; Li and Chan 1999). The use of ART, although not without negative aspects such as complicated regimens, ADRs, drug interactions and high costs, has thereby markedly decreased morbidity and mortality in patients with HIV-infection (Romanelli and Hoven 2006; Carr and Cooper 2000; Barry *et al.* 1998; Palella *et al.* 1998).

2.2.3 The Status of ART in Developing Countries

The use of ARV drugs over the past three years (end 2002 to end 2005) in low- and middle-income countries (North Africa and the Middle East; Eastern Europe and Central Asia; East, South and South-East Asia; Latin America and the Caribbean; and sub-Saharan Africa) has dramatically increased (UNAIDS 2006; UNAIDS 2004). Between 2001 and 2005, the number of HIV-infected individuals receiving ART within these resource-limited regions increased from 240 000 to roughly 1.3 million. This five-fold increase in ART usage in these low- and middle-income regions, as well as the level of usage within each specific region, is illustrated in Figure 2.03. Undoubtedly contributing to this hugely increased level of ARV usage is the substantial increase in their availability during the past three years – the number of sites providing ARVs increased from approximately 500 in 2004 to more than 5 000 by the end of 2005.

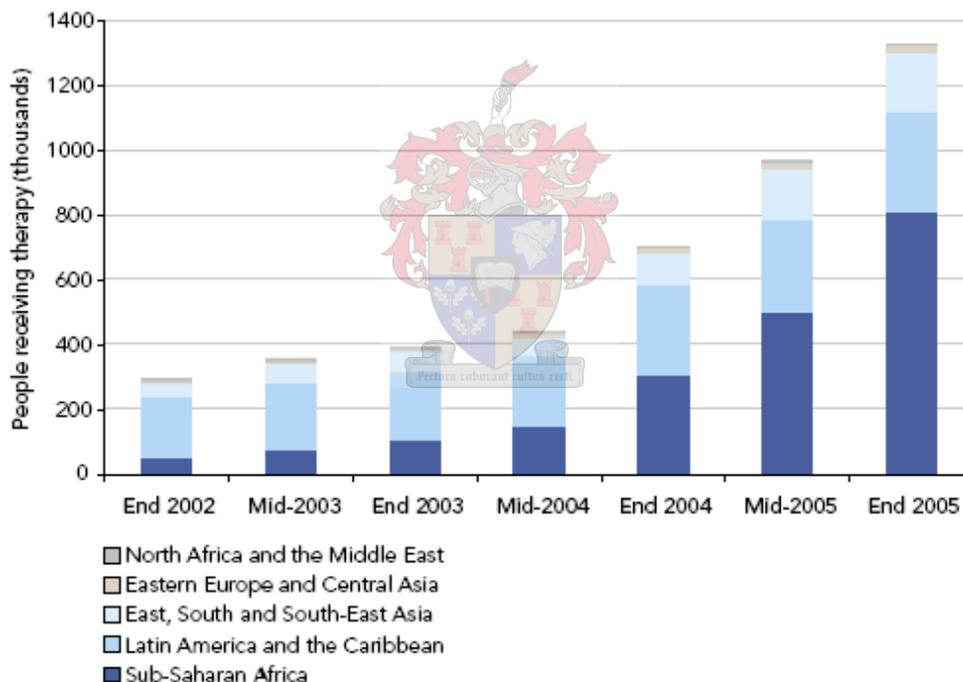


Figure 2.03: The number of people receiving ART in low- and middle-income regions from end 2002 to end 2005
(Source: UNAIDS 2006)

As is clearly apparent in Figure 2.03, the majority of the increase in ARV usage has occurred in sub-Saharan Africa, both in terms of absolute numbers as well as relative to any increases within other regions – no doubt a necessary consequence of the enormous scale of the epidemic within this region. The number of people within sub-Saharan Africa receiving ART increased more

than eight-fold (from 100 000 to 810 000) between end 2003 and end 2005, and more than doubled in 2005 alone. The majority of this trend is attributable to markedly improved treatment access in a few countries, notably Botswana, Kenya, Uganda, Zambia, and SA. However, despite this significant improvement in the number of HIV-infected persons within sub-Saharan Africa receiving ART, the level of ART coverage expressed as a percentage of those who require it remains exceedingly low. For example, by 2005 the level of ART coverage in SA had increased from a persistent figure of < 10% to 10-24.9%. Although this constitutes a significant improvement in terms of absolute numbers of people receiving treatment, it remains a low level of coverage of the total number of people who are in need of ARVs – an estimated 983 000 South Africans currently need ARV drugs, while only 117 000 receive them. This insufficient treatment coverage in SA is reflected by a worldwide inadequate level of coverage: globally, ARV drugs still reach only one in five who require them (UNAIDS 2006).

Despite the current shortcomings in treatment coverage, there is nonetheless a rapidly increasing level of ARV usage in SA and within other countries of sub-Saharan Africa. Furthermore, the persistent inadequate level of coverage and the persistently high rates of prevalence and infection within SA and many other countries within sub-Saharan Africa portends an ever greater need for ARV drugs. The increasing usage and need for ARVs, combined with the realisation that different ethnic groups can respond differently to pharmaceutical drugs (see Section 2.1.6), emphasises the necessity of research into any possible genetic basis for observed variations in efficacy and risk for ADRs within individuals of sub-Saharan Africa.

2.2.4 Classes of ARV Drugs and Methods of Action

There are currently, as of mid-2006, 21 ARV drugs within four drug classes (Table 2.02) available for the treatment of HIV-infection (although the production of zalcitabine was discontinued in February 2006) (Hanson and Hicks 2006; Wynn *et al.* 2004; Flepp *et al.* 2001; <http://hivinsite.ucsf.edu/InSite>). These four drug classes include nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs/NtRIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs) and entry inhibitors.

Table 2.02: Currently available ARV drugs for the treatment of HIV infection (as of mid-2006)

(yellow highlight = drugs administered in this study)

ARV Drug Class	ARV Drug	
Nucleoside/Nucleotide Reverse Transcriptase Inhibitors (NRTIs/NtRTIs)	Abacavir (ABC; Ziagen [®])	
	Didanosine (ddI; Videx [®])	
	Emtricitabine (FTC; Emtriva [®])	
	Lamivudine (3TC; Epivir [®])	
	Stavudine (d4T; Zerit [®])	
	Tenofovir (PMPA; Viread [®])	
	Zalcitabine (ddC; Hivid [®]) (production discontinued)	
	Zidovudine (AZT; Retrovir [®])	
	Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)	Delavirdine (DLV; Rescriptor [®])
		Efavirenz (EFV; Stocrin [®] , Sustiva [®])
Nevirapine (NVP; Viramune [®])		
Protease Inhibitors (PIs)	Amprenavir (APV; Agenerase [®])	
	Atazanavir (ATZ; Reyataz [®])	
	Fosamprenavir (fAPV; Lexiva [®] , Telzir [®])	
	Indinavir (IDV; Crixivan [®])	
	Lopinavir/Ritonavir (LPV/r; Kaletra [®])	
	Nelfinavir (NFV; Viracept [®])	
	Ritonavir (RTV; Norvir [®])	
	Saquinavir (soft-gel capsule: SQVs; Fortovase [®] /hard-gel capsule: SQVh; Invirase [®])	
	Tipranavir (TPV; Aptivus [®])	
	Entry Inhibitors	Enfuvirtide (T20; Fuzeon [®])

Each of the classes of ARV drug has a specific drug target and/or method of action. Before these drug targets and methods of action can be understood, however, basic knowledge of the life cycle of HIV is necessary.

HIV is a retrovirus whose genome consists of two identical strands of ribonucleic acid (RNA). The virus fuses with the CD4 receptor molecules present in the plasma membrane of T-lymphocyte cells (Wyatt and Sodroski 1998) and subsequently enters the cell by means of endocytosis (Li and Chan 1999). The viral coat proteins are discarded and proviral complementary DNA (cDNA) synthesised from the viral RNA by viral reverse transcriptase (RT). Viral integrase subsequently facilitates the integration of the proviral cDNA into the host cell genome inside the nucleus, after which the viral cDNA is transcribed into viral mRNA and genomic RNA. These RNA molecules exit the nucleus and enter the cytoplasm where the mRNA is subsequently translated into long precursor viral proteins (Wynn *et al.* 2004; Gallant 2002), which must subsequently be cleaved by viral protease before mature viral particles can be assembled (Flexner 1998). The resultant viral proteins accumulate, along with the viral genomic RNA, near the inner surface of the host cell plasma membrane. The viral proteins and genomic RNA are assembled into mature viruses which bud from the host cell (which eventually lyses and dies) and are then free to infect other T-lymphocyte cells (Strachan and Read 1999).

This cycle of infectivity is repeated indefinitely and eventually results in devastation of the host's immune system, causing a dramatic increase in susceptibility to opportunistic infections that inevitably leads to illness and death. Four distinct classes of ARV drugs have, however, been developed which disrupt the HIV life cycle and inhibit its replication, thus halting this otherwise inevitable progression of the disease. Each of these drug classes exerts its effect by targeting various stages of the HIV life cycle.

2.2.4.1 NRTIs/NtRTIs

Nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs/NtRTIs) exert their effect by competitively inhibiting viral RT (Li and Chan 1999). These agents, with the exception of the only NtRTI, tenofovir, are dideoxynucleoside analogues of endogenous nucleosides that undergo *in vivo* phosphorylation before becoming active triphosphates (tenofovir only requires conversion to the diphosphate form (Pillero 2004)). These drugs are thus administered in an inactive form

(i.e. in the form of a prodrug) and thus require phosphorylation within target cells before acquiring their antiretroviral effects. Once phosphorylated and in the active form, these defective nucleoside triphosphates lacking a 3'-hydroxyl group (Brinkman *et al.* 1998) compete for incorporation into the viral cDNA and, having done so, subsequently inhibit cDNA synthesis by means of chain termination (Carr and Cooper 2000; Mitsuya and Broder 1986; <http://www.merck.com/mrkshared/mmanual/section13/chapter154/154b.jsp>) – this process is analogous to dideoxy sequencing of DNA (Sanger *et al.* 1977). The resultant viral particles are subsequently destroyed by the host's cellular enzymes (Gallant 2002).

2.2.4.2 NNRTIs

The non-nucleoside reverse transcriptase inhibitor (NNRTI) class of ARV drugs contains structurally dissimilar agents which are noncompetitive inhibitors of HIV RT – they are, however, only effective against HIV-1, as the protein structure of HIV-2 RT differs to a degree that affects the NNRTI inhibitory activity (Li and Chan 1999). These agents exert their effects by disrupting the RT enzyme's catalytic site, thereby blocking polymerase activity and preventing cDNA synthesis from the viral RNA genome (Wynn *et al.* 2004; <http://www.merck.com/mrkshared/mmanual/section13/chapter154/154b.jsp>).

2.2.4.3 PIs

Protease inhibitors (PIs) act at a very late stage of the HIV life cycle, directly preceding viral assembly and budding from the host cell (Gallant 2002). These drugs exert their effect by competitively inhibiting the HIV protease enzyme needed to cleave the lengthy precursor viral proteins into nascent active viral particles, thereby preventing the completion of the virus life cycle (Wynn *et al.* 2004; Flexner 1998). PIs, therefore, cause an infected lymphocyte cell to produce non-infectious viral particles instead of new viruses.

2.2.4.4 Entry Inhibitors

Entry inhibitors make up the latest class of currently available ARV drug. Drugs within this class impede any of the different processes associated with virus entry into target cells (McGee 2006b). The virastatic effect of the only current member of the entry inhibitor drug class, enfuvirtide, is exerted by binding of the drug compound to an HIV envelope protein, glycoprotein 41 (gp41), which is responsible for the direct fusion of viral and cellular membranes and the subsequent

introduction of the viral core into the target cell (Yeni 2006; Wyatt and Sodroski 1998). Enfuvirtide thereby interferes with the fusion of the viral envelope with the target cell membrane, thus inhibiting entry of the virus into the target cell (Leonard and Roy 2006), and is hence more specifically categorised as a fusion inhibitor (McGee 2006b). By preventing the entry of the viral core into the lymphocyte cell, the HIV life cycle is interrupted at an extremely early stage and the progression of the disease halted.

2.2.5 ART Toxicity and Variable Therapeutic Efficacy

Despite the benefits offered by combination ART, it is widely recognised that all of the ARV drug classes have multiple effects other than just the intended suppression of HIV replication (Carr and Cooper 2000). A significant proportion of patients experience a loss of immunological, virological or clinical benefit from current regimens – none of the many clinical studies of ART has demonstrated 100% response rates in terms of either control of viral replication or CD4-cell recovery (Quirk *et al.* 2004) – while others can experience adverse effects and toxicities (Haas *et al.* 2005; Struble *et al.* 2005). These ADRs can range in severity from mild discomfort to life-threatening (Shibuyama *et al.* 2006; Carr and Cooper 2000; Brinkman *et al.* 1998) and can decrease adherence to a selected regimen (Nischal 2005; Wynn *et al.* 2004; Chesney 2000) – adherence being a crucial factor in ART necessary for limitation of the development of drug-resistant HIV strains, which may drastically limit future treatment options and negatively impact patient quality of life and chances of survival (Cressey and Lallemand 2006; Dybul *et al.* 2002; Gallant 2002).

Several factors, some unique to the nature of ART, have coalesced to intensify the occurrence and severity of ART-related ADRs. Firstly, since current ART does not eradicate HIV (Finzi *et al.* 1999), treatment is for life (Cressey and Lallemand 2006). Such long-term drug usage can lead to adverse side-effects (Yeni 2004; Schambelan *et al.* 2002). Secondly, many ARV drugs, due to the severity and urgency of the HIV epidemic, experienced accelerated licensing often with little known about their long-term safety (all the more pertinent considering the first above-mentioned factor) (Carr and Cooper 2000). Thirdly, the incidence of drug-related toxicities is increasingly being documented as a consequence of the declining incidence of HIV-associated opportunistic diseases. And lastly, there is currently a relatively large selection of ARV drugs available (Table 2.02) with an enormous number of resultant possible combinations. These factors, along with the

individual PK and PD characteristics of each drug or drug class, have led to the emergence of adverse events, some of which are drug-class specific (Schambelan *et al.* 2002).

2.2.5.1 Mitochondrial Toxicity

A number of ADRs has been associated with long-term NRTI usage (Shibuyama *et al.* 2006; Yeni 2006; Gallant 2002; Carr and Cooper 2000; Carr *et al.* 2000; Brinkman *et al.* 1999; Brinkman *et al.* 1998). These ADRs and specific NRTI drug(s) associated with each of them include:

- myopathy (progressive atrophy and weakening of muscles) – zidovudine
- neuropathy (motor or sensory deficit due to nerve damage) – stavudine, didanosine and zalcitabine
- hepatic steatosis (abnormal lipid increase within the liver, indicative of sublethal cellular damage) and lactic acidemia/acidosis (accumulation of lactic acid within the body, possibly occurring in conjunction with severe liver enlargement) – didanosine, stavudine and zidovudine
- peripheral subcutaneous lipoatrophy (decrease in adipose tissue particularly in the limbs, buttocks and face) – possibly all NRTIs, although predominantly stavudine
- pancreatitis (inflammation of the pancreas) – didanosine

The severity of these ADRs range from relatively mild, such as myopathy, to potentially life-threatening, such as pancreatitis and lactic acidosis (Yeni 2006; ter Hofstede *et al.* 2000; Brinkman *et al.* 1998) – mortality rates of over 80% have been observed in patients with plasma lactate concentrations > 10 mM (normal plasma lactate concentration is approximately 1 mM) (Carr and Cooper 2000). Although lactic acidosis is relatively rare ($\pm 1\%$) (Boubaker *et al.* 2002; John 2001), lactic acidemia is more common ($\pm 15\%$) (Carr and Cooper 2000) and is often associated with mild increases in concentrations of liver enzymes, peripheral lipoatrophy, and mild constitutional symptoms such as nausea, lack of appetite, malaise, fatigue and difficulty in breathing (Carr *et al.* 2000). Despite this range in type and severity of ADRs, they are, however, all believed to be the result of a single molecular mechanism: a decreased mitochondrial energy-generating capacity due to impaired synthesis of mitochondrial enzymes, responsible for the generation of energy in the form of adenosine triphosphate (ATP) via oxidative phosphorylation, as a result of the capacity of NRTI drugs to inhibit host mitochondrial DNA polymerases (Yeni 2006; Brinkman *et al.* 1998).

DNA polymerase gamma (γ) is the only enzyme responsible for the replication of mitochondrial DNA (mtDNA). Unfortunately, however, NRTI drugs can inhibit DNA polymerase γ and thereby induce depletion of mtDNA (Brinkman *et al.* 1999). This subsequently results in depletion of mtDNA-encoded mitochondrial enzymes which ultimately lead to mitochondrial dysfunction (Dalakas *et al.* 1994) and the above-mentioned ADRs. The prevalence and severity of such mitochondrial toxicities increase with more prolonged therapy and their onset and offset are generally gradual, although they can manifest within only days of starting ART (Carr and Cooper 2000; Brinkman *et al.* 1998). Furthermore, NRTIs differ in their degree of mitochondrial toxicity: stavudine and zalcitabine exert the greatest level of toxicity, and abacavir and lamivudine the least, while zidovudine and didanosine exert an intermediate effect (Gallant 2002). Tenofovir, the only available NtRTI, avoids incorporation by DNA polymerase γ and hence causes little or no mitochondrial toxicity (Johnson *et al.* 2001).

Although diagnosis of mitochondrial toxicity is straightforward unless the patient is on concomitant therapy with overlapping toxicities, there is currently no diagnostic assay available to predict susceptibility to toxicity (Carr and Cooper 2000; Brinkman 1998). Treatment of mitochondrial toxicities includes cessation of the causative drug and any other drugs that may aggravate the condition, as well as the use of agents traditionally used in the treatment of congenital mitochondrial diseases. The efficacy of such agents in the treatment or prevention of NRTI-induced mitochondrial toxicity is, however, unknown.

2.2.5.2 Drug Hypersensitivity

Drug hypersensitivity reactions in response to ART typically manifest as a reddened, spotted skin rash, similar to that produced in measles, which can occur with or without fever (when fever does occur, it often precedes the onset of the rash) (Roujeau and Stern 1994). Such rash usually starts to appear one to three weeks after commencement of therapy and is most noticeable on the arms and torso. Hypersensitivity reactions can be caused by the NRTI abacavir (which although uncommon, can be severe and thus always necessitates immediate and permanent discontinuation of abacavir usage), all NNRTIs – most common and severe with nevirapine (Fagot *et al.* 2001) – and the PI amprenavir. Hypersensitivity reactions caused by NNRTIs and amprenavir are considerably more common than with abacavir yet are usually milder, normally presenting with rash without the accompanying systemic symptoms (Gallant 2002). Drug hypersensitivity among

patients treated with the other NRTI or PI drugs is otherwise rare (Yeni 2006; Gallant 2002; Carr and Cooper 2000). The incidence of hypersensitivity reactions can be fairly high and occurs in approximately 3-20% of patients (Coopman *et al.* 1993).

The exact cause of and mechanism involved in ARV drug hypersensitivity is unknown and there is currently no demographic, metabolic or immunological factor, including previous hypersensitivity reactions to other drugs, which can predict the level of risk for its development (Carr and Cooper 2000). Fortunately, patients experiencing hypersensitivity reactions can be treated symptomatically and need not discontinue therapy (excluding abacavir-induced hypersensitivity) (Gallant 2002). Furthermore, approximately 50% of such hypersensitivity reactions resolve spontaneously (Carr and Cooper 2000).

2.2.5.3 LD Syndrome

Lipodystrophy (LD) Syndrome is characterised by peripheral subcutaneous lipoatrophy in the limbs, buttocks and face, along with central fat accumulation in the abdomen (so-called ‘protease paunch’), breasts, dorsocervical area (so-called ‘buffalo hump’), around the neck or under the chin, or in the form of scattered subcutaneous lipomass (Wynn *et al.* 2004; Gervasoni *et al.* 1999; Safrin and Grunfeld 1999; Lo *et al.* 1998; Miller *et al.* 1998; Roth *et al.* 1998). Additional metabolic characteristics of the syndrome include hyperlipidaemia (excessively high blood cholesterol and/or triglyceride levels) and insulin resistance, which rarely (< 10%) leads to overt diabetes (Yeni 2006; Heath *et al.* 2001; Carr *et al.* 1999; Carr *et al.* 1998a). LD Syndrome occurs at a relatively high incidence among patients, increasing in frequency with the duration of therapy – approximately 50% after 10 months of therapy (Heath *et al.* 2001; Carr *et al.* 1998a) and 83% after 21 months (Carr *et al.* 1999). However, despite the broad spectrum of symptoms and relatively high frequency of the syndrome, it has been noted that not all afflicted patients develop all of the clinical features generally associated with it (Carr *et al.* 1998b).

As mentioned in Section 2.2.5.1, the use of NRTI ARVs can lead to mitochondrial toxicity which can in turn cause peripheral subcutaneous lipoatrophy. The concomitant use of PI drugs with NRTIs, as is often the case in currently employed ART regimens, can however lead to the development of LD Syndrome (Lesho and Gey 2003; Gallant 2002; Carr and Cooper 2000; Carr *et al.* 2000; Brinkman *et al.* 1999). Therefore, despite the fact that lipoatrophy is a characteristic

trait of LD Syndrome and is a resultant adverse effect of NRTI-induced mitochondrial toxicity, PIs potentially exacerbate the metabolic process responsible for lipoatrophy through additional mechanisms (Carr *et al.* 1998b), thus causing fat accumulation and the development of LD Syndrome. NRTIs are accordingly considered to be an essential initiating factor in the development of ART-related LD Syndrome (Brinkman *et al.* 1999), while PIs are a necessary factor for its subsequent development (Brinkman *et al.* 2000; Carr *et al.* 2000).

It should be noted, however, that occurrences of LD Syndrome or clinical symptoms associated with it, such as ‘buffalo hump’ in men, have been reported in patients not receiving PI drugs (Carr *et al.* 1999; Gervasoni *et al.* 1999, Madge *et al.* 1999; Lo *et al.* 1998). However, the vast majority of studies indicate the necessity of co-administration of PIs with NRTIs for the development of the syndrome (Carr *et al.* 2000; Brinkman *et al.* 1999), as well as a marked discrepancy in the frequency of LD Syndrome between patients receiving a PI-containing regimen and those that are PI-naïve (64% vs 3%, respectively) (Carr *et al.* 1998a).

The pathogenesis of LD Syndrome is unknown (Carr and Cooper 2000). The most comprehensive hypothetical explanation thus far offered for the syndrome involves the inhibition of regulatory proteins responsible for lipid and carbohydrate metabolism and adipocyte regulation (Carr *et al.* 1998b). These proteins have partial amino acid sequence homology (\pm 60%) to the catalytic site of HIV-1 protease, to which all PIs bind. Therefore, currently prescribed PIs may bind to and inhibit these proteins, thus causing disturbances in lipid metabolism, with the primary event being apoptosis and reduced peripheral adipocyte differentiation, which in turn leads to the clinical and metabolic side-effects characteristic of LD Syndrome.

Possible risk factors for LD Syndrome have as yet not been elucidated (Carr and Cooper 2000), although studies to date have suggested a number of factors which include low bodyweight before commencement of therapy, the total duration of ART and the use of stavudine (Carr *et al.* 2000; Carr *et al.* 1999; Saint-Marc *et al.* 1999; Carr *et al.* 1998a). There are currently no criteria with which to diagnose or rate the severity of LD Syndrome (complicated by the above-mentioned fact that not all afflicted patients present with all the major features) or any form of proven treatment for any component of the syndrome (Wynn *et al.* 2004; Carr and Cooper 2000; Carr *et al.* 1999).

2.2.5.4 ARV Drug and Drug Class Specific ADRs

Apart from the major, well-characterised ARV-induced ADRs detailed in the above sections, a number of other adverse effects has been detected. The majority of these ADRs are associated with specific ARV drugs or classes of ARV drug. Due to the considerable number of adverse effects and the relatively large number of currently available ARVs, the following synopsis will place emphasis on the ARV drugs used within this study, namely: the NRTIs didanosine, lamivudine, stavudine and zidovudine; the NNRTIs efavirenz and nevirapine; and the PI combination drug lopinavir/ritonavir.

Adverse effects of NRTIs include mild to moderate short-term toxicities such as diarrhoea, nausea and gastrointestinal distress (Gallant 2002). This is particularly evident in treatment with zidovudine and non-enteric-coated formulations of didanosine. Treatment with zidovudine has also been associated with macrocytic anaemia, anorexia, fatigue, headache, malaise and vomiting (Carr and Cooper 2000). The adverse effects of lamivudine can also include headache and a dry mouth (<http://hivinsite.ucsf.edu/InSite>). Treatment with tenofovir has demonstrated little or no mitochondrial toxicity and no highly prevalent and significant adverse side-effects, even after two years of treatment with the drug (Squires *et al.* 2003).

The most frequent toxicity associated with NNRTIs is adverse effects on the liver such as elevated liver enzymes, hepatotoxicity, hepatic necrosis and liver failure (Gallant 2002). Hepatotoxicity and liver failure, which can prove life-threatening, have been attributed to nevirapine usage, particularly in patients with higher CD4-cell counts (nevirapine should not be initiated in women with CD4 > 250 cells/mm³ or in men with CD4 > 400 cells/mm³) (<http://hivinsite.ucsf.edu/InSite>).

A number of unique adverse side-effects have been attributed to efavirenz and comprise effects within the central nervous system (CNS), such as dizziness, difficulty concentrating, fatigue, impaired concentration, insomnia, mania, somnolence, and vivid dreams or nightmares (Shibuyama *et al.* 2006; Lochet *et al.* 2003; Fumaz *et al.* 2002; Marzolini *et al.* 2001; Carr and Cooper 2000; Adkins and Noble 1998; <http://hivinsite.ucsf.edu/InSite>). These ADRs can occur in up to 40% of patients in the initial few days to weeks after starting efavirenz therapy, but are severe enough to necessitate cessation of drug usage in only 3% (Lochet *et al.* 2003; Fumaz *et al.* 2002; Carr and Cooper 2000) – most of these symptoms diminish with continued therapy and

resolve spontaneously within two to four weeks (Gallant 2002). Efavirenz has also demonstrated teratogenicity in animal studies and is hence contraindicated in pregnancy or when pregnancy is possible (Saitoh *et al.* 2005a; Carr and Cooper 2000).

Class-wide adverse side-effects of PIs include diarrhoea (most commonly with nelfinavir, full-dose ritonavir, and soft-gel saquinavir), fatigue, headaches, hepatotoxicity, malaise, nausea, sexual dysfunction and vomiting (Wynn *et al.* 2004; Lesho and Gey 2003; Gallant 2002; Beach 1998; Markowitz *et al.* 1998; Picard *et al.* 1998; <http://hivinsite.ucsf.edu/InSite>). Adverse effects attributable to specific drugs within this class include the development of rash with amprenavir and dose-dependent perioral paresthesias (numbness around the mouth) with ritonavir (Gallant 2002; Danner *et al.* 1995).

2.3 PHARMACOGENETICS AND ART

As discussed in Section 2.2.5, there exists significant variation among HIV-positive patients in their response to ART. This lack of ART-response uniformity manifests as variations in the levels of drug efficacy, as evaluated by CD4-cell count and viral load measurements, and occurrence and severity of ADRs (Haas *et al.* 2005; Struble *et al.* 2005; Carr and Cooper 2000; Brinkman *et al.* 1998). Numerous factors are considered responsible for these observed variations and include adherence to therapy, age, co-morbidities, concurrent medications, nutritional status, disease state and stage of the disease (Cressey and Lallemand 2006; Wyles and Gerber 2005; Quirk *et al.* 2004; Tang and Kaslow 2003).

However, even if these factors are controlled for within a patient cohort, complete and uniform response to ART may still not be observed. The underlying cause for these differences in ART response is thus similar to responses to numerous other clinical drugs (such as those mentioned in Section 2.1.6) in that it is increasingly being attributed to a genetic basis (Rodríguez-Nóvoa *et al.* 2006; Cressey and Lallemand 2006; Haas *et al.* 2005; Evans and McLeod 2003; Bean 2000). This hypothesis is supported by reports that patients from distinct ethnic groups have significantly different clinical response and ARV PK (Kappelhoff *et al.* 2005; Pfister *et al.* 2003; Wegner *et al.* 2002)

The majority of studies to date concerning genetic data and ART response have relied upon a pharmacogenetic approach: *in vitro* and animal data are used to make educated suppositions regarding the selection of target genes in which to identify common polymorphisms (Quirk *et al.* 2004). Any potential association between these polymorphisms and clinical pharmacological responses are then subsequently investigated. In other, less frequent instances, clinically relevant polymorphisms in target genes have been discovered through DNA re-sequencing and genome-wide screening methods. These studies have thus identified a number of genes which potentially affect ARV drug response.

As discussed in Section 2.1.3, genetic polymorphisms within genes encoding DMEs and drug transporters can affect the PK and PD properties of drug compounds and hence the subsequent clinical response to them (Haas 2005; Johnson 2003; Steimer and Potter 2002). The clinical response to ART is no exception and numerous genes involved in ARV drug metabolism and transport have been implicated in variable ART efficacy and ARV-induced ADRs (Haas 2005; Haas *et al.* 2005; Quirk *et al.* 2004) – these genes include members of the CYP gene family and the *MDR1* gene, respectively.

2.3.1 The CYP Gene Superfamily

Prior to reaching systemic circulation, orally administered drugs, such as ARVs, must first pass through the small intestine where they are absorbed and transported, via the portal vein system, to the liver (Wilkinson 2005; Williams and Sinko 1999). These drugs are then metabolised by microsomal DMEs located within the liver and, to a much lesser extent, the small intestine (Ingelman-Sundberg *et al.* 1999) – this enzymatic metabolism of drug compounds within these organs includes a variety of sequential or competitive chemical processes involving oxidation, reduction and hydrolysis (phase I reactions) or glucuronidation, sulfation, acetylation and methylation (phase II reactions) (Wilkinson 2005).

The general aim and resultant effect of these enzymatic processes is to increase the polarity of resulting metabolites and hence their level of water-solubility, thereby enhancing the body's ability to expel them (Chang *et al.* 1993). The small intestine and liver thus both contribute to pre-systemic extraction (i.e. first-pass metabolism) of orally administered drugs, which can consequently reduce the portion of the dose that reaches the systemic circulation and in so doing

influence drug effects (Wilkinson 2005). Drugs that undergo high levels of such first-pass metabolism often have systemic drug levels with high interindividual variability resulting from variations in the activity levels of these DMEs (Thummel *et al.* 1997; Hellriegel *et al.* 1996). The most common such DMEs are the CYP enzymes that are the product of the CYP gene superfamily.

The CYP gene superfamily encodes a large group of haem thiolate monooxygenase proteins that catalyse the oxidative biotransformation (phase I reaction) of a diverse range of both endogenous compounds such as fatty acids, prostaglandins, retinoids, steroids and vitamins, and exogenous compounds such as carcinogens, toxins and pharmaceutical drugs (Wilkinson 2005; Nebert and Russell 2002; Sata *et al.* 2000; Nelson *et al.* 1996; Gonzalez and Gelboin 1994). Although the CYP enzymes are distributed throughout the human body, they are most abundant and active in tissues that often come into contact with exogenous compounds (as could be expected, considering their function). Such tissues include the kidneys, lungs and skin; they are, however, found predominantly within organs involved in drug and toxin metabolism and detoxification, namely the liver and to a lesser extent the enterocytes in the epithelium of the small intestine (Lamba *et al.* 2002a; Ingelman-Sundberg *et al.* 1999).

The evolutionary development of the CYP genes has been attributed to dietary selective pressure exerted by toxic metabolites formed by plants as a means of protection against predation (Nebert 2000; Schuler 1996; Gonzalez and Nebert 1990). There are over a 6 000 different members of the CYP superfamily with at least one or more P450 enzymes so far detected in every species of organism on earth, including humans, all other animals, plants, fungi and even archaeobacteria (Anzenbacher and Adithan 2001; <http://drnelson.utmem.edu/cytochromeP450.html>). These P450 enzymes are categorised into families and subfamilies based upon their amino acid sequence similarities, with enzymes with > 40% sequence homology belonging to the same family and those with > 55% homology belonging to the same subfamily.

There are currently 18 families and 43 subfamilies within the CYP superfamily, with a total of 57 putatively functional genes and 58 pseudogenes (highly homologous but non-functional copies of active genes) identified in humans (Nelson *et al.* 2004; <http://drnelson.utmem.edu/cytochromeP450.html>). The nomenclature system for the CYP

superfamily is as follows: the Arabic numeral directly following the italicised '*CYP*' denotes the specific family to which the gene belongs, the subsequent letter indicates the subfamily, and the final Arabic numeral represents the specific gene within the subfamily (Wilkinson 2005; <http://www.cypalleles.ki.se/>). An allelic variant is indicated by an asterisk followed by an Arabic numeral and a letter. A CYP name followed by a 'P' indicates that it is a pseudogene (<http://drnelson.utmem.edu/cytochromeP450.html>).

The CYP enzymes are of particular interest in pharmacogenetic studies as they are believed to be responsible for the metabolism of the vast majority of all clinically administered drugs (Wilkinson 2005; Lamba *et al.* 2002a), as well as due to the fact that there exist extremely variable hepatic CYP expression and enzyme activity levels. Furthermore, approximately 40% of human CYP-dependent drug metabolism is carried out by polymorphic enzymes (Ingelman-Sundberg *et al.* 1999) – an important realisation, considering that differences in drug metabolism and efficacy between individuals and different ethnic groups have been attributed to polymorphisms in the CYP genes (Ozawa *et al.* 2004; Steimer and Potter 2002; Ingelman-Sundberg *et al.* 1999).

Each individual CYP enzyme has a distinctive and unique substrate specificity, often to a certain region of a drug molecule, to a particular enantiomer, or to both (Wilkinson 2005). Despite this specificity, a considerable amount of overlap can occur, such that a single CYP enzyme may be predominantly responsible for all the oxidative metabolism of a particular drug or a variety of CYP enzymes may contribute in equal or varying degrees.

As discussed in Section 2.1.3, polymorphisms within the regulatory, intronic and coding regions of CYP genes can affect the PK characteristics of a drug by increasing, decreasing or eliminating the presence and activity of the enzyme responsible for its metabolism (Ingelman-Sundberg 2005; Wilkinson 2005; Johnson 2003). This change in drug PK characteristics can result in an undesirable concentration of the drug and/or drug metabolites at the intended site of action which can, in turn, result in either a lack of therapeutic response or the build-up of the drug to toxic levels resulting in ADRs (Lindpainter 2003; Meyer and Zanger 1997). Such influences on the metabolism of ARVs is a critically important consideration in that apart from the occurrence of ADRs resulting from decreased enzyme activity and drug metabolism, increased enzyme activity and drug metabolism could potentially lead to sub-therapeutic drug systemic levels (Cressey and

Lallemant 2006). Sub-therapeutic systemic levels could, in turn, lead to a lack of therapeutic effect at standard dosages and a consequent development of drug-resistance. The development of resistance to ARVs, as mentioned in Section 2.2.5, may drastically limit future treatment options and negatively impact patient quality of life and chances of survival.

Of the four ARV drug classes currently available for the treatment of HIV-infection, two of these, namely the NNRTIs and PIs, are substrates of CYP enzymes. Metabolism of NRTIs/NtRTIs is not carried out by CYP enzymes but is dependent on intracellular enzymes such as nucleoside kinases, 5'-nucleotidases, purine and pyrimidine nucleoside monophosphate kinases, and other similar enzymes (Zapor *et al.* 2004; Li and Chan 1999). The remaining class of ARV drug not metabolised by CYP enzymes is the entry inhibitors – enfuvirtide, currently the only member within this drug class, is a synthetic peptide and, as such, is believed to be metabolised in the liver and kidneys by peptidases with recycling of the resultant amino acids (Zapor *et al.* 2004). The major CYP enzymes responsible for the metabolism of each of the individual drugs within the NNRTI and PI drug classes are listed in Table 2.03 (Smith *et al.* 2005; DiCenzo *et al.* 2004; Hirani *et al.* 2004; Tsuchiya *et al.* 2004; Wynn *et al.* 2004; Zapor *et al.* 2004; Ward *et al.* 2003; Flexner 2000; Erickson *et al.* 1999; Li and Chan 1999; Riska *et al.* 1999; Adkins and Noble 1998; Beach 1998; Barry *et al.* 1997; <http://hivinsite.ucsf.edu/InSite>).



Table 2.03: The major CYP enzymes responsible for NNRTI and PI ARV drug metabolism

(yellow highlight = drugs administered in this study)

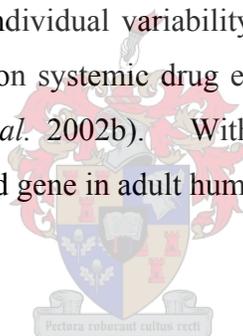
ARV Drug Class	ARV Drug	CYP Enzyme(s)
Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)	Delavirdine	CYP3A4 (possibly also CYP2D6 and CYP2C9)
	Efavirenz	CYP2B6 (primarily), CYP3A4
	Nevirapine	CYP3A4 (primarily), CYP2B6
Protease Inhibitors (PIs)	Amprenavir	CYP3A4 (75%), CYP2B6, CYP2C9
	Atazanavir	CYP3A4
	Fosamprenavir	CYP3A4
	Indinavir	CYP3A4 (88-90%)
	Lopinavir/Ritonavir	CYP3A4
	Nelfinavir	CYP2C19 (> 78%), CYP3A4
	Ritonavir	CYP3A4 (> 95%), CYP2D6
	Saquinavir	CYP3A4 (> 97%)
	Tipranavir	CYP3A4

As clearly evident in Table 2.03, CYP3A4 plays a dominant role in the metabolism of the majority of NNRTI and PI ARVs (exceptions being efavirenz and nelfinavir which are primarily metabolised by CYP2C19 and CYP2B6, respectively). The examination of the *CYP3A4* gene is thus crucial in any pharmacogenetic study aimed at elucidating the genetic basis for variable ART response and was thus included in this study. Furthermore, as evident in Table 2.03, CYP2B6 plays a dominant role in the metabolism of efavirenz and, to a lesser extent, nevirapine and amprenavir. As such, *CYP2B6* is of significant interest when examining the genetic basis for varied responses to these drugs. Therefore, because both efavirenz and nevirapine were administered to patients in this study (as indicated in Table 2.03), the *CYP2B6* gene was also examined.

2.3.1.1 The *CYP3A* Subfamily

The *CYP3A* subfamily, consisting of *CYP3A4*, *CYP3A43*, *CYP3A5* and *CYP3A7*, is the most abundant of the CYPs and is responsible for 30-50% of total hepatic CYP enzyme content (Wilkinson 2005; Shimada *et al.* 1994; Cholerton *et al.* 1992) as well as an even larger percentage of total small intestinal CYP content (de Waziers *et al.* 1990). The *CYP3A* subfamily thus plays a dominant role in the metabolic elimination of more drugs than any other biotransformation enzyme (Lamba *et al.* 2002b) and is estimated to be involved in the metabolism of 45-60% of all CYP-metabolised drugs (Lamba *et al.* 2002a; Murayama *et al.* 2002).

The apparent metabolic versatility of CYP3A enzymes may be due both to a characteristically large active site, which thereby facilitates the binding of structurally diverse molecules (Guengerich 1999), as well as the ability of these enzymes to accommodate multiple ligands (different molecules or two or more of the same molecule) within the active site (Lamba *et al.* 2002b). There is considerable interindividual variability in CYP3A activity (Wilkinson 2005) which can have a considerable effect on systemic drug exposure and, potentially, drug efficacy and occurrence of ADRs (Lamba *et al.* 2002b). Within the *CYP3A* subfamily, *CYP3A4* is considered the predominantly expressed gene in adult human liver and small intestine (Paine *et al.* 2006; Dai *et al.* 2001).



2.3.1.2 The *CYP3A4* Gene

The *CYP3A4* gene is located on chromosome 7, between bands q21.3 and q22.1, spans 27.2 kb and consists of thirteen exons. The mRNA transcript is 2 768 nucleotides in length and is subsequently translated into a protein product of 502 amino acids with a size of 57.3 kDa (Hashimoto *et al.* 1993; Inoue *et al.* 1992; <http://www.genatlas.org/>).

The *CYP3A4* gene is the predominant and most abundantly expressed CYP gene in adult human liver (Paine *et al.* 2006; Dai *et al.* 2001) and encodes the single most important enzyme involved in the oxidative metabolism of pharmaceutical drugs (Evans and Relling 1999; Barry *et al.* 1997). *CYP3A4* is so abundantly expressed, in fact, that the liver owes its characteristic colour to the CYP3A4 enzyme, nifedipine oxidase (Imaoka *et al.* 1996; Maurel 1996). The CYP3A4 enzyme is estimated to be responsible for the metabolism of approximately 50% of currently prescribed drugs that are eliminated by enzyme-catalysed processes (Guengerich 1999; Rebeck *et al.* 1998),

some of which include benzodiazepines, calcium channel blockers, cisapride, cyclosporine, erythromycin, haloperidol, local anaesthetics, opioids and pimoziide (Yamazaki *et al.* 1996; Vickers *et al.* 1995). CYP3A4 is also, as indicated in Section 2.3.1 and Table 2.03, predominantly responsible for the metabolism of the large majority of NNRTI and PI ARVs. Due to this long list of drugs metabolised by the *CYP3A4* gene product, the gene is of major interest in clinical medicine (Murray 1999).

The level of expression of *CYP3A4* is highly variable among individuals and has been observed to vary by as much as 40-fold in liver and small intestine tissues (Westlind *et al.* 1999; Paine *et al.* 1997; de Waziers *et al.* 1994; Shimada *et al.* 1994; Shimada *et al.* 1989). Significant interindividual differences in intrinsic CYP3A4-dependent clearance of probe substrates (such as cyclosporine, midazolam and triazolam) have also been noted (Lamba *et al.* 2002b; Lown *et al.* 1995). Moreover, differences in CYP3A4-mediated drug metabolism between different ethnic groups have also been established (Xie *et al.* 2001). These apparent discrepancies in *CYP3A4* expression and enzyme catalytic activity may be attributable to environmental, pathological, hormonal and dietary factors, and gene induction (Lamba *et al.* 2002b). However, much of this variability in *CYP3A4* expression and enzyme catalytic activity is attributed to a genetic basis – it has in fact been suggested that as much as 90% of the observed interindividual variability is due to genetic polymorphisms (Lamba *et al.* 2002a; Murayama *et al.* 2002; Paine *et al.* 1997).

To date, 38 allelic variants of *CYP3A4* have been identified. Seventeen of these variants are located in the promoter region, with the remaining 21 located within the exonic and intronic regions of the gene (Westlind-Johnsson *et al.* 2006; <http://www.cypalleles.ki.se/>). Until recently, there had been no reports of a *CYP3A4* null allele – Westlind-Johnsson *et al.* (2006) identified a novel allele (*CYP3A4*20*) containing a premature stop codon in exon 13 which consequently yields a truncated protein with no functional activity. The *CYP3A4*20* allele was identified by Westlind-Johnsson *et al.* (2006) in a heterozygous state within individuals of a single family of Brazilian descent; the allele has subsequently not been detected in any other individual and is thus believed to be extremely rare.

Despite the significant number of polymorphisms so far described within the *CYP3A4* gene, few functional polymorphisms have been identified. Indeed, of the known structural variants, none

involve changes of amino acids encoding the putative substrate-binding domain and very little evidence suggests that coding sequence variation forms the basis of observed discrepancies in CYP3A4 activity (Wilkinson 2005; Lamba *et al.* 2002a; Lamba *et al.* 2002b). The few coding sequence variants that have been associated with any measurable changes in CYP3A4 activity, such as *CYP3A4*17* and *CYP3A4*18*, have resulted in only minuscule differences in catalytic activity (Dai *et al.* 2001). Furthermore, no variant allele has yet been shown to alter CYP3A4 function *in vivo* (Westlind-Johnsson *et al.* 2006). These findings, along with the observed low allelic frequencies of known functional *CYP3A4* variants, suggest that coding sequence variants may contribute to, but are not likely to be the major cause of, interindividual differences in hepatic CYP3A-dependant drug clearance (Schuetz 2004; Lamba *et al.* 2002a; Lamba *et al.* 2002b).

A number of intronic SNPs have been identified in the *CYP3A4* gene, most of which are rare (< 1% allelic frequency) (Lamba *et al.* 2002b). Although certain intronic polymorphisms do occur with a high enough frequency to warrant interest, analysis of hepatic CYP3A4 content and catalytic activity in Caucasian American and African-American liver banks revealed no association between intronic polymorphisms and expression phenotypes (Lamba *et al.* 2002a). It remains to be determined, however, whether the physiological changes that occur in an organ donor mask any true association. Furthermore, no intronic SNPs have been linked to altered *CYP3A4* expression or CYP3A4 activity *in vivo* (Lamba *et al.* 2002b). The lack of evidence that coding or intronic sequence variants within *CYP3A4* contribute significantly to variable CYP3A4 content and activity has led to a growing consensus that such variability is caused by regulatory polymorphisms within the gene (Schuetz 2004).

A number of SNPs have been identified in the 5'-UTR of *CYP3A4*. By far the most common and best characterised of these is the A-392G transition in the putative nifedipine-specific element (NFSE), designated the *CYP3A4*1B* allele (occasionally referred to as *CYP3A4-V*) (Rebbeck *et al.* 1998). A number of studies have examined the possible effects of this polymorphism on transcriptional activity and *in vivo* catalytic activity. Amirimani *et al.* (1999) examined the effect of the *CYP3A4*1B* allele on luciferase reporter transcription in hepatoma and breast cancer cell lines. The study found that luciferase expression from the mutant -392G promoter construct occurred at a rate that was 1.4- to 1.9-fold higher than that observed for the wild-type -392A

construct. This finding was corroborated by clinical observations of 1.6- and 2.1-fold higher levels of nifedipine oxidation and hepatic CYP3A4 content in individuals carrying at least one variant -392G allele (Ando *et al.* 1999) – it should be noted, however, that these observed differences were deemed not to be statistically significant. Subsequent studies with larger samples sizes have failed to detect any clear association of the *CYP3A4*1B* allele with CYP3A4-specific content or catalytic activity within tissue banks from primarily Caucasian donors (Lamba *et al.* 2002a; Westlind *et al.* 1999).

Numerous studies have examined the functional significance of the *CYP3A4*1B* allele *in vivo*, albeit with populations of relatively limited size. Ball *et al.* (1999) examined the metabolism of the CYP3A probe substrates erythromycin and nifedipine in healthy African-American volunteers and found no difference in the mean erythromycin demethylation rate or mean oral nifedipine clearance in individuals homozygous for the wild-type or variant *CYP3A4*1B* alleles. These findings are perhaps confounded by the fact that erythromycin is also a substrate for hepatic PGP and hence its metabolism reflects factors other than simply CYP3A4-mediated metabolism (Kinirons *et al.* 1999). However, Wandel *et al.* (2000) found that the *CYP3A4*1B* allele did indeed have functional consequences on the CYP3A4 enzyme – a 30% lower systemic midazolam clearance for individuals homozygous for the variant -392G allele compared to those homozygous for the wild-type A-392 allele was detected. The study failed, however, to detect an association of this variant with oral clearance of midazolam – i.e. the genotype-phenotype relationship is only present with respect to hepatic CYP3A4, whose catalytic activity determines the systemic clearance of midazolam. Wandel *et al.* (2000) subsequently concluded that the functional effects of the *CYP3A4*1B* allele, although not potentially large, are possibly overridden in the enterocytes within the small intestine. Hesselink *et al.* (2004) have since detected a modest decrease in systemic cyclosporine clearance associated with this polymorphism.

This apparent uncertainty of any major PK consequence for the *CYP3A4*1B* allele is seemingly paradoxical to findings linking the polymorphism to altered clinical outcomes. Felix *et al.* (1998) and Rebbeck *et al.* (1998) noted that the *CYP3A4*1B* allele was associated with prostate tumours of higher clinical stage and grade in men with prostate cancer – a higher frequency of this allele being found in patients with prostate cancer of the more invasive clinical stage compared to patients with a lower level clinical stage. Rebbeck *et al.* (1998) went on to postulate that the

variant -392G polymorphism may lower *CYP3A4* expression, thereby decreasing testosterone 6 β -hydroxylase activity and increasing testosterone levels which, in turn, could affect androgen-mediated prostate carcinogenesis. These findings were subsequently confirmed by Paris *et al.* (1999) in African-American men with prostate cancer, and by Tayeb *et al.* (2002) in Caucasian men with benign prostatic hyperplasia who went on to develop prostate cancer.

Felix *et al.* (1998) reported that individuals homozygous for the *CYP3A4* wild-type allele were at an increased risk of developing secondary leukaemia following treatment of the primary cancer with epipodophyllotoxin therapy, than were individuals with a *CYP3A4*1B* allele. Felix *et al.* (1998) went on to hypothesise that the variant *CYP3A4*1B* allele confers reduced *CYP3A4* activity and a consequent decrease in the production of toxic epipodophyllotoxin metabolites. This hypothesis is, however, at odds with *in vitro* data indicating increased *CYP3A4* expression associated with the *CYP3A4*1B* allele (Amirimani *et al.* 1999) and other studies which have failed to detect any association of the allele with changes in hepatic *CYP3A4* content or catalytic activity (Lamba *et al.* 2002a; Westlind *et al.* 1999).

The *CYP3A4*1B* allele has also been associated with early onset puberty in girls – girls homozygous for the allele were significantly more likely to enter puberty prematurely than those homozygous for the wild-type allele – which is a recognised risk factor for the development of breast cancer (Kadlubar *et al.* 2003). Kadlubar *et al.* (2003) ascribed this association to increased gene transcription for the variant -392G allele, which would increase levels of the *CYP3A4* enzyme and thereby enhance steroidogenesis and breast maturation. Once again, this hypothesis is at odds with the findings of numerous other studies.

The *CYP3A4*1B* allele varies in frequency between different ethnic groups: 0% in Taiwanese (Walker *et al.* 1998), Chinese (Hsieh *et al.* 2001), Chinese Americans and Japanese Americans (Ball *et al.* 1999); 2-9.6% in Caucasians (Kuehl *et al.* 2001); 9.3-11% in Hispanic Americans; and 35-67% in African-Americans (Lamba *et al.* 2002a; Zuehl *et al.* 2001). Interestingly, this allele is absent in Africans from North Sahara (Lamba *et al.* 2002b). The increased risk of prostate cancer in carriers of the *CYP3A4*1B* allele is perhaps reflected by the higher risk-profile for the disease and higher *CYP3A4*1B* frequency in African-American men – African-American men have both a higher frequency of the *CYP3A4*1B* allele and frequency of prostate cancer than any

other group of men in the United States (Moinpour *et al.* 2000). Accordingly, Rebbeck *et al.* (1998) noted that African-American prostate cancer patients were more likely to be homozygous for the *CYP3A4*1B* allele than matched healthy controls. Despite the matching of backgrounds of cases and controls in some studies, the increased frequency of the *CYP3A4*1B* allele within prostate cancer patients may yet prove to be a case of association and not causation. Accordingly, population stratification as an explanation for these findings has been offered (Kittles *et al.* 2002).

The clinical outcomes thus far associated with the *CYP3A4*1B* allele do indicate a convincing association of the allele with altered enzyme kinetics and consequent disease risk. However, the lack of compelling *in vivo* evidence for altered CYP3A probe substrate disposition for this polymorphism suggests the possibility of population stratification or LD between *CYP3A4*1B* and another genetic polymorphism that is the true cause of the clinical phenotype. *CYP3A5* is currently considered as a possible candidate gene in this hypothesis (Lamba *et al.* 2002b; Kuehl *et al.* 2001) – Kuehl *et al.* (2001) demonstrated that the *CYP3A4*1B* and *CYP3A5*1* allele can be present in the same individual and hypothesised that *CYP3A5*1* might influence local or systemic functional CYP3A activity. Indeed, African-Americans frequently carry both the *CYP3A5*1* and *CYP3A4*1B* alleles and LD has been established between the two alleles (Kuehl *et al.* 2001).

Perhaps due to the current level of uncertainty regarding the PK and clinical effects of the *CYP3A4*1B* allele, few studies have examined its effects, if any, on NNRTI- and PI-containing ART response. Furthermore, the studies that have examined the effects of the *CYP3A4*1B* allele have yielded contradictory findings. Fellay *et al.* (2002) found no association between the *CYP3A4*1B* allele and plasma drug concentrations of efavirenz or nelfinavir. However, Haas *et al.* (2004) detected a weak association of *CYP3A4*1B* with a lower rate of efavirenz clearance and increased AUC, while Saitoh *et al.* (2005b) found that -392AA homozygous HIV-infected children had a higher efavirenz AUC than children with the -392GG or -392GA genotypes.

In anticipation of imminent large-scale implementation of ART in the SA, Chelule *et al.* (2003) examined the frequency of the *CYP3A4*1B* allele within African (83.6%), Indian (11.0%) and Caucasian (57.1%) ethnic groups. Chelule *et al.* (2003) concluded that the genotypic distribution of *CYP3A4*1B* is highly variable and that phenotypic studies are needed in order to assess the

clinical relevance of the allele in South African populations, especially in African patients homozygous for the variant allele. The authors also stated that studies assessing the relationships between host genetic factors and therapeutic efficacy may be required in order to ensure the optimisation of NNRTI- and PI-containing ART regimens for South African ethnic groups.

2.3.1.3 The *CYP2B* Subfamily

The human *CYP2* family is large and consists of numerous subfamilies that are physically clustered together in the genome. *CYP2B* is one such subfamily and is found within a cluster of six subfamilies (*CYP2A*, *-2B*, *-2F*, *-2G*, *-2S*, and *-2T*) on chromosome 19 (Hoffman *et al.* 2001). The *CYP2B* subfamily consists of only two known loci, namely the *CYP2B6* gene and *CYP2B7P* pseudogene (Jacob *et al.* 2004) – *CYP2B6* is thus the sole member of this subfamily expressed in humans.

2.3.1.4 The *CYP2B6* Gene

The *CYP2B6* gene is located on chromosome 19, between bands q12 and q13.2, (Hoffman *et al.* 2001) spans 27.1 kilobases (kb) and consists of nine exons. The mRNA transcript is 3 052 nucleotides in length and is subsequently translated into a protein product of 491 amino acids with a size of 563 kiloDaltons (kDa) (<http://www.genatlas.org/>).

Despite the fact that the *CYP2B6* gene only constitutes 3-6% of total human hepatic P450 content (Stresser and Kupfer 1999; Ekins *et al.* 1997), it nevertheless catalyses the oxidation of a relatively large number of structurally diverse compounds. In fact, an estimated 70 substrates are either partially or wholly metabolised by the *CYP2B6* gene product (Ekins *et al.* 1999; Ekins *et al.* 1998). These compounds include therapeutic agents such as amitriptyline, the antiestrogenic breast cancer drug tamoxifen, the antineoplastic drug cyclophosphamide, diazepam (Valium), ifosfamide, lidocaine, *S*-mephenytoin, verapamil, and, as indicated in Section 2.3.1 and Table 2.03, the ARV drugs efavirenz and nevirapine (Ward *et al.* 2003; Lang *et al.* 2001; Ekins *et al.* 1999; Heyn *et al.* 1996; Imaoka *et al.* 1996; Ono *et al.* 1996; White *et al.* 1995; Chang *et al.* 1993).

Numerous studies have indicated that hepatic *CYP2B6* expression and enzyme catalytic activity are highly variable both within individual ethnic groups and between different groups (Stresser

and Kupfer 1999; Ekins *et al.* 1998). For example, *in vitro* studies have reported a large degree of interindividual variability in hepatic *CYP2B6* expression at the mRNA, protein, and catalytic activity level (Lang *et al.* 2001; Code *et al.* 1997; Chang *et al.* 1993) – as much as a 20- to 288-fold interindividual and interpopulation difference in *CYP2B6* expression and enzyme activity levels in liver microsomes has been detected (Shimada *et al.* 1994). Such findings suggest considerable interindividual and interpopulation differences in the systemic exposure to a range of drugs metabolised by *CYP2B6* (Lamba *et al.* 2003), which can thereby have profound effects on the metabolism and consequent efficacy and risk for ADRs of these drugs (Guan *et al.* 2006; Zukunft *et al.* 2005). Although the underlying cause for this wide variation in *CYP2B6* expression and enzyme activity can partially be attributed to environmental and nutritional factors (e.g. gene induction due to dietary intake), it is mainly ascribed to genetic polymorphisms (predominantly in the form of SNPs) within the *CYP2B6* gene (Guan *et al.* 2006; Lang *et al.* 2001).

The *CYP2B6* gene has been found to be highly polymorphic within its exons and introns, as well as in its promoter region (Zukunft *et al.* 2005). Numerous SNPs within the gene have been described, many of which alter the amino acid sequence and thus possibly the function and activity of the encoded *CYP2B6* enzyme, although no *CYP2B6* null allele has thus far been identified (Lang *et al.* 2001; <http://www.cypalleles.ki.se/>). The first systematic search for genetic polymorphisms in *CYP2B6* identified nine SNPs in 35 German Caucasians, five of which cause amino acid substitutions (Lang *et al.* 2001). Lang *et al.* (2001) documented six *CYP2B6* alleles, namely: *CYP2B6**2 (C64T), *CYP2B6**3 (C777A), *CYP2B6**4 (A785G), *CYP2B6**5 (C1459T), *CYP2B6**6 (G516T, A785G) and *CYP2B6**7 (G516T, A785G, C1459T). Lamba *et al.* (2003) subsequently identified another two *CYP2B6* alleles, namely *CYP2B6**8 (A415G) and *CYP2B6**9 (G516T).

More recently, numerous SNPs in the *CYP2B6* promoter region, such as C-1848A, G-801T, T-750C, T-82C, have been reported (Zukunft *et al.* 2005; Lamba *et al.* 2003), as well as missense SNPs (A76T, A83G, C85A, G86C, C15618T, G18038A, C21498A), and silent and intronic SNPs defining six novel alleles (*CYP2B6**17A, *17B, *18, *19, *20 and *21) (Klein *et al.* 2005). Furthermore, several rare nonsynonymous SNPs have recently been found which result in absent or non-functional *CYP2B6* protein (Lang *et al.* 2004). Despite this significant number of

polymorphisms within *CYP2B6*, a great deal is yet unknown of their specific influence on the expression of the gene or functionality of the CYP2B6 enzyme, nor is much known regarding their association with altered metabolism of and clinical response to CYP2B6 substrates (Bumpus *et al.* 2005; Jinno *et al.* 2003).

Although there is a high level of polymorphism within *CYP2B6*, only a small number of the multitude of SNPs so far characterised within the gene has demonstrated an association with altered *CYP2B6* expression and/or CYP2B6 enzyme catalytic activity. A few of the common nonsynonymous polymorphisms, such as those of alleles *CYP2B6**5 (C1459T; Arg487Cys) and *CYP2B6**6 (G516T, A785G; Gln172His, Lys262Arg), are associated with decreased hepatic *CYP2B6* expression (Hesse *et al.* 2004; Lang *et al.* 2001) or altered function (Ariyoshi *et al.* 2001). The G516T SNP has been linked with an increased 7-ethoxycoumarin *O*-deethylase activity *in vitro* (Ariyoshi *et al.* 2001), while the *CYP2B6**6 allele has been associated with a significantly higher K_m value than that of the wild-type protein (Jinno *et al.* 2003). Jinno *et al.* (2003) also found that variants other than *CYP2B6**6 also containing the A785G SNP (*CYP2B6**4 and *CYP2B6**7) had increased V_{max} and V_{max}/K_m with 7-ethoxy-4-trifluoromethylcoumarin as substrate, while the kinetic parameters of the protein product of *CYP2B6**2 and *CYP2B6**3 remained unchanged by their corresponding amino acid substitutions. These effects of the A785G SNP were recently corroborated by Bumpus *et al.* (2005) who found that the polymorphism results in a dramatically altered ability to metabolise a number of CYP2B6 drug substrates compared with the wild-type enzyme. Therefore, of the multitude of SNPs thus far identified within *CYP2B6* and of those in any way associated with altered gene expression and/or protein catalytic activity, the nonsynonymous SNPs G516T and A785G appear to have the most significant impact on the PK characteristics of CYP2B6 substrates.

An *in vitro* study has suggested that CYP2B6 is the principal catalyst of efavirenz metabolism, while CYP3A4 plays a lesser role (Ward *et al.* 2003). Studies have also demonstrated that the plasma concentration of efavirenz can predict its efficacy and likelihood of causing CNS side-effects (such as those discussed in Section 2.2.5.4) – efavirenz possesses a narrow therapeutic range as patients with plasma levels below 1 $\mu\text{g/ml}$ exhibit greater risk of drug resistance development and treatment failure (Langmann *et al.* 2002; Marzolini *et al.* 2001) while those with levels above 4 $\mu\text{g/ml}$ are at increased risk of CNS side-effects (Gallego *et al.* 2004; Marzolini *et*

al. 2001). The cause of such interindividual variations in efavirenz plasma concentration is attributed to variations in the hepatic metabolism of the drug (Rodríguez-Nóvoa *et al.* 2006). Therefore, interindividual variations in efavirenz efficacy and risk of CNS toxicity is related to varied plasma levels of the drug, which is attributable to varied hepatic metabolism and which, in turn, is attributable to variable CYP2B6 activity. Variable CYP2B6 activity, as mentioned above, is principally ascribed to polymorphisms within the *CYP2B6* gene.

Haas *et al.* (2004) noted a significant association between the *CYP2B6* G516T SNP and the rate of plasma clearance of efavirenz and CNS toxicity. Median area under the curve (AUC) (a measure of the level of exposure and absorption of a drug within the body) of efavirenz was approximately three times higher in 516TT homozygotes than in 516GG homozygotes (i.e. the TT genotype is associated with a lower rate of efavirenz plasma clearance) within African-American and Caucasian American ethnic groups. The GT and TT genotypes were also significantly associated with a greater number of CNS-related ADRs at the initiation of therapy, which gradually abated by 24 weeks after commencement of treatment, despite continuously elevated efavirenz levels. Haas *et al.* (2004) suggested that due to the fact that no differences in PK profiles between Caucasian Americans and African-Americans for efavirenz were found after adjusting for *CYP2B6* genotype, the *CYP2B6* G516T polymorphism may thus explain previously noted racial disparities in efavirenz PK and efficacy – the rate of plasma clearance of efavirenz appears to be slower in African-Americans than in Caucasian Americans (Pfister *et al.* 2003), with the rate of clearance 32% higher in Caucasian Americans compared to African-Americans or Hispanics (Haas *et al.* 2004). This hypothesis is further substantiated by corresponding differences in the observed frequency of the 516TT genotype in African-Americans (20%) and Caucasian Americans (3%) and a follow-up study which demonstrated similar results (Haas *et al.* 2005). Although CYP2B6 plays a lesser role in its metabolism than that of efavirenz, the *CYP2B6* 516TT genotype has also been associated with increased plasma levels of nevirapine (Rodríguez-Nóvoa *et al.* 2006).

Numerous other studies have found similar results regarding the *CYP2B6* G516T SNP and plasma efavirenz levels and related CNS toxicity. Tsuchiya *et al.* (2004) demonstrated that homozygous *CYP2B6**6 correlates with considerably higher plasma efavirenz concentrations, while Lang *et al.* (2001) found that *CYP2B6**6 and *CYP2B6**7 are strongly associated with elevated efavirenz

plasma concentrations and a consequently higher rate of related ADRs. Rodríguez-Nóvoa *et al.* (2005) have since confirmed these findings but also found that the G516T SNP is the main determinant of functional effects of these alleles, as evident in Figure 2.04.

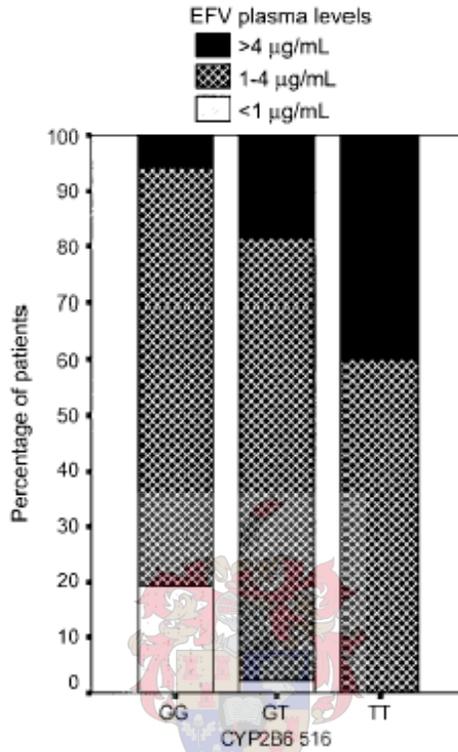


Figure 2.04: Distribution of efavirenz plasma concentrations among individuals with different *CYP2B6* 516 genotypes

(Source: Rodríguez-Nóvoa *et al.* 2005)

Rotger *et al.* (2005) also confirmed the clinical significance of the G516T SNP by detecting an association of the 516TT genotype with greater plasma and intracellular exposure to efavirenz, as did Ribaudó *et al.* (2006). Rodríguez-Nóvoa *et al.* (2005) also noted that efavirenz plasma levels are significantly affected by *CYP2B6* genotype, with the rate of CNS toxicities more frequent in patients carrying 516GT or 516TT genotypes as opposed to those homozygous for the wild-type. Moreover, Wang *et al.* (2006) found higher plasma efavirenz concentrations in carriers of the *CYP2B6* G516T polymorphism as well as in those carrying the A785G polymorphism.

Due to the considerable number of congruent studies, there is currently little doubt that the *CYP2B6* G516 SNP, and to a perhaps lesser extent the A785G SNP, are indeed associated with

differences in efavirenz plasma levels and CNS toxicities in most populations (Rodríguez-Nóvoa *et al.* 2006). The lowering of efavirenz doses in patients homozygous for the G516T variant in order to minimise the occurrence of ADRs, without compromising the efficacy of the drug, may thus be warranted.

2.3.2 The *MDR1* Gene

The human *MDR1* gene, also known as the ATP-binding cassette, subfamily B, member 1 (*ABCB1*) gene, is located on chromosome 7, band q21.1, spans 209.4 kb and consists of 29 exons (Gottesman *et al.* 1995; Callen *et al.* 1987). The mRNA transcript is 4 643 nucleotides in length and is subsequently translated into an integral transmembrane protein, known as PGP, of 1 280 amino acids with a size of 141.5 kDa (Alexandrova 1998; <http://www.genatlas.org/>).

PGP, a member of the large ATP-binding cassette superfamily of transport proteins also known as traffic ATPases (Song *et al.* 2002), is a phosphorylated and glycosylated protein consisting of two halves with a high degree of similarity, twelve membrane-spanning domains and two nucleotide binding sites (Xie *et al.* 2001; Ambudkar *et al.* 1999; Alexandrova 1998). PGP functions as an energy-dependent transmembrane efflux pump and is thus responsible for the export of xenobiotics from the inside of cells and membranes to the outside (Xie *et al.* 2001; Bradley *et al.* 1988). The physiological role of PGP is thus the protection of cells from toxic compounds and metabolites by the excretion of these substances into bile, urine and the intestinal lumen (Marzolini *et al.* 2004; Hoffmeyer *et al.* 2000).

PGP is found in a number of locations within the body, some of which include areas that are vitally important in drug absorption and disposition. Such areas include the small intestine, where it is thought to participate in drug absorption after drug ingestion, as well as the liver, kidneys and at the level of the blood-brain barrier where it acts to limit CNS penetration of many drug compounds and toxins (Kim 2003; Schuetz *et al.* 1996). PGP exhibits broad substrate specificity and there appears to be few distinct structural features of PGP substrates, although most tend to be large, lipophilic, amphipathic and contain one or more aromatic rings (Woodahl and Ho 2004; Schwab *et al.* 2003; Benet *et al.* 1999). Substrates of PGP include hormones and plant-derived chemicals, as well as many clinically used drugs (Kim 2003). Drug classes that are PGP substrates include antiarrhythmics, antifungals, β -adrenoceptor antagonists, calcium-channel

blockers, cardiac drugs, chemotherapeutic agents, hormones, immunosuppressants, as well as NNRTIs and PIs used in ART (Cressey and Lallemand 2006; Marzolini *et al.* 2004; Woodahl and Ho 2004; Wynn *et al.* 2004; Schwab *et al.* 2003; Stormer *et al.* 2002; Fischer *et al.* 1998; Kim *et al.* 1998).

PGP activity varies markedly among individuals (Hinoshita *et al.* 2000), which can consequently affect the efficacy and risk of toxicity of drug treatments on an interindividual basis through dissimilar drug dispositions (Anglicheau *et al.* 2004; Marzolini *et al.* 2004; Taniguchi *et al.* 2003). The overall activity level of PGP is dependant on two factors: the level of expression of the *MDR1* gene, which controls the quantity of PGP synthesised within cells, and the functionality of the resultant PGP, which subsequently determines which substrates are recognised and transported with what effectiveness (Hoffmeyer *et al.* 2000).

The first factor affecting PGP activity, the level of expression of *MDR1*, has been intensively analysed and can be attributed in part to gene amplifications (Schoenlein *et al.* 1992; Chen *et al.* 1986). However, it is accepted that other factors affecting expression levels must also exist (Chin *et al.* 1990). Such additional factors include polymorphisms within the *MDR1* gene that may be associated with or even causative of the observed variance in expression levels (Hoffmeyer *et al.* 2000). Furthermore, polymorphisms in promoter and/or enhancer regions of the gene could also influence expression levels, as could polymorphisms in sequences that influence mRNA stability (Taniguchi *et al.* 2003; Chen *et al.* 1990; Chin *et al.* 1990). The second factor, the functionality of PGP, is defined by the amino acid sequence of the protein that is encoded by the *MDR1* gene (Hoffmeyer *et al.* 2000). Polymorphisms that change amino acids can alter the substrate spectrum of PGP as well as the effectiveness of PGP-mediated transport (Ambudkar *et al.* 1999).

Hoffmeyer *et al.* (2000), the first to conduct a major screening of the *MDR1* gene, examined 188 healthy Caucasian individuals and identified fifteen SNPs. These fifteen SNPs included: seven intronic polymorphisms within introns 4, 6, 12, 16 and 17, close to exon-intron boundaries; three SNPs at wobble positions causing no amino acid changes, one in exon 12 (C1236T) and two in exon 26 (C3435T, C3396T); three resulting in amino acid changes, one in exon 2 (A61G; Asn21Asp), in exon 5 (T307C; Phe103Leu), and in exon 11 (G1199A; Ser400Asn); as well as one directly preceding the ATG translation start codon and a non-coding SNP in exon 1 (T-129C). A

subsequent analysis of *MDR1* in German Caucasians revealed two further rare polymorphisms, namely G2677A (Ala893Thr) and A3320C (Gln1107Pro) (Cascorbi *et al.* 2001). A number of subsequent pharmacogenetic studies determined the frequencies of several of these SNPs and identified additional SNPs within *MDR1* (Cascorbi *et al.* 2001; Kim *et al.* 2001). To date, at least 29 SNPs within the *MDR1* gene have been described in detail (Marzolini *et al.* 2004; Woodahl and Ho 2004). Of these 29 SNPs, nineteen are located in the exonic regions, nine within the introns, and one within the promoter region. Despite this relatively large number of SNPs, a significant proportion of which result in amino acid changes, few polymorphisms have thus far been definitively associated with changes in levels of *MDR1* expression or PGP activity (Marzolini *et al.* 2004; Woodahl and Ho 2004; Schwab *et al.* 2003).

In vitro studies examining the influence of *MDR1* polymorphisms on gene expression and PGP efflux activity levels have yielded inconsistent results. Factors that have been proposed to account for these discrepancies in *in vitro* findings include the selection of probe substrates (changes in *MDR1* sequence can alter substrate efflux levels some drugs but not of others) (Loo and Clarke 1993) and that cells used to express variant *MDR1* genes can also have variable intrinsic transporters which contribute to the efflux of PGP substrates (Woodahl and Ho 2004).

Following the study by Hoffmeyer *et al.* (2000), a multitude of *in vivo* studies examined possible association of *MDR1* polymorphisms with changes in gene expression and PGP function/activity, and consequent changes in drug disposition profiles. However, similar to findings of *in vitro* studies, much of the resultant data from *in vivo* studies have proved contradictory and inconclusive. A proposed cause of these inconsistent findings is the fact that direct comparisons of *in vivo* disposition data are difficult, since many of the drugs selected as probe substrates are often also substrates of DMEs or other transporter proteins (Woodahl and Ho 2004). However, despite such contradictory and inconclusive findings, *in vivo* drug disposition studies have fared better than *in vitro* studies in elucidating the effects, if any, of *MDR1* sequence variants on levels of gene expression and PGP activity. *In vivo* studies have detected association between a number of *MDR1* SNPs and altered gene expression and PGP activity.

Only one of the fifteen SNPs identified by Hoffmeyer *et al.* (2000) demonstrated any correlation with altered *MDR1* expression: the synonymous (wobble) C3435T transition in exon 26. The

C3435T SNP is by far the most extensively studied and best characterised polymorphism within the *MDR1* gene. Hoffmeyer *et al.* (2000) found that 3435TT homozygous individuals had a more than two-fold lower intestinal *MDR1* expression level than 3435CC homozygous individuals, while heterozygous individuals displayed an intermediate phenotype. The 3435TT homozygous individuals were subsequently shown also to have increased plasma concentration of digoxin – a PGP substrate (Greiner *et al.* 1999) – after oral administration, which suggests greater drug absorption and bioavailability within such individuals due to lower intestinal PGP levels and consequent intestinal drug efflux ability. The effect of the *MDR1* 3435 genotype on gene expression and subsequent PGP activity is clearly illustrated in Figure 2.05.

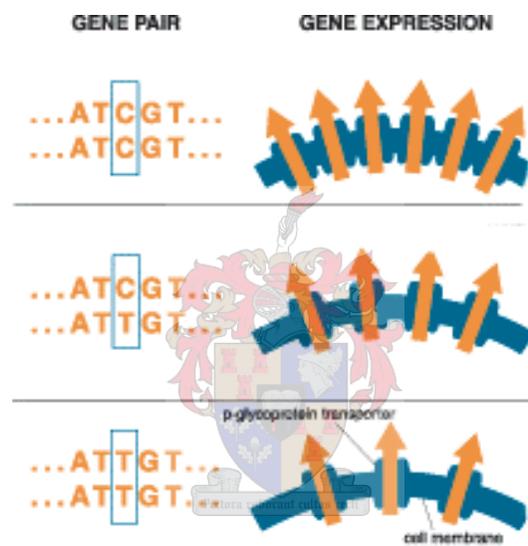


Figure 2.05: The purported effect of the C3435T SNP on *MDR1* expression and subsequent PGP activity
(Source: Schmelz 2002)

A subsequent study by Fellay *et al.* (2002) confirmed the association between *MDR1* 3435TT genotype and a lower level of *MDR1* expression, while subsequent studies by Verstuyft *et al.* (2003), Johne *et al.* (2002) and Kurata *et al.* (2002) confirmed the association of the 3435T allele with higher plasma digoxin levels. However, findings by Gerloff *et al.* (2002) have contradicted these observations. Despite any such contradictory findings, the C3435T SNP has consistently and repeatedly been associated with altered PK characteristics of PGP-substrates and changes in *MDR1* expression levels in a multitude of studies (Balram *et al.* 2003; Verstuyft *et al.* 2003; Fellay *et al.* 2002; Horinouchi *et al.* 2002; Illmer *et al.* 2002; Johne *et al.* 2002; Kurata *et al.* 2002; Moriya *et al.* 2002; Kim *et al.* 2001; Sakaeda *et al.* 2001; Hoffmeyer *et al.* 2000).

Considering that the C3435T SNP is synonymous and thus does not result in a change in amino acid sequence of the encoded PGP product, it seems improbable that it should demonstrate such significant association with altered *MDR1* expression and PGP activity. Although the molecular basis of this seeming paradox is not yet completely understood, a number of hypotheses have been suggested: LD between the C3435T SNP and other, potentially unidentified, causative mutations elsewhere within the *MDR1* gene or in another nearby gene (Schwab *et al.* 2003; Kim *et al.* 2001; Tanabe *et al.* 2001; Hoffmeyer *et al.* 2000); the Ile encoded by the synonymous SNP may have reduced translation efficiency (Sparreboom *et al.* 2003); silent codon changes may yet prove to alter downstream mRNA splicing by allele-specific differences in RNA folding (Shen *et al.* 1999). Perhaps the most probable and substantiated of these suppositions is LD between the C3435T SNP and other polymorphisms within the *MDR1* gene.

As discussed in Section 2.1.2, single SNP approaches to genotype-phenotype correlations have significant limitations and it is instead gene-based haplotypes that both greatly affect drug response and better predict association between genomic variation and phenotype (Drysdale *et al.* 2000; Judson *et al.* 2000; Clark *et al.* 1998; Nickerson *et al.* 1998). Such is the case with the *MDR1* gene in that previously observed phenotypic associations with the C3435T SNP are believed to be attributable to LD between this polymorphism and other SNPs within the gene (Marzolini *et al.* 2004; Kim *et al.* 2001). Kim *et al.* (2001) demonstrated that LD exists between the C3435T SNP and the G2677T/A SNP in exon 21 of *MDR1*. The existence of distinct LD between these polymorphisms has subsequently been confirmed in several other studies (Anglicheau *et al.* 2003; Horinouchi *et al.* 2002; Siegmund *et al.* 2002).

The *MDR1* G2677T/A polymorphism is unusual in that three allelic variants are found at the same locus, resulting in three different possible amino acids at position 893. The G2677T transition leads to an amino acid change of Ala to Ser, while the G2677A transition causes an Ala to Thr alteration in the intracellular domain between the tenth and eleventh transmembrane spanning domains of PGP (Yi *et al.* 2004; Kim *et al.* 2001). Tanabe *et al.* (2001) found that *MDR1* expression levels within placental tissue were lowest for 2677 homozygous variant (T or A) individuals, intermediate in heterozygous individuals, and highest in homozygous wild-type individuals. Yi *et al.* (2004) found that the variant 2677A allele significantly affects

fexofenadine disposition, with a markedly lower AUC for 2677AA homozygous individuals as compared to individuals with any other genotype for this SNP. Furthermore, Illmer *et al.* (2002) and Moriya *et al.* (2002) both found that intestinal mRNA expression levels were lower in 2677GG homozygous individuals than in heterozygous or homozygous variant (T or A) individuals. Although occasionally contradictory, a number of other studies have also detected a discernible effect of the G2677T/A polymorphism on PGP activity (Kurata *et al.* 2002; Siegmund *et al.* 2002; Kim *et al.* 2001).

It has been established that a synonymous SNP in exon 12 (C1236T) is linked to both the G2677T/A and C3435T polymorphisms (Anglicheau *et al.* 2003; Johne *et al.* 2002; Kim *et al.* 2001). More than 50% of individuals thus far studied have demonstrated strong LD at these three loci (Marzolini *et al.* 2004). The C1236T polymorphism, a synonymous (wobble) SNP in exon 12 of *MDR1*, has been examined in a number of studies (Illmer *et al.* 2002; Cascorbi *et al.* 2001; Kim *et al.* 2001; Hoffmeyer *et al.* 2000). Illmer *et al.* (2002) found that the overall survival rate in acute myeloid leukaemia (AML) patients was higher when the C1236T genotype was considered along with those of the G2677T/A and C3435T genotypes. However, no other significant associations of the C1236T SNP with any other phenotypic traits have since been demonstrated.

It has been suggested that apart from the functional consequences of the G2677T/A SNP, the C1236T, G2677T/A and C3435T SNPs may be in LD with polymorphisms within regulatory regions of the *MDR1* gene (Illmer *et al.* 2002). One potential candidate for such LD is the non-coding T-129C SNP in the untranslated exon 1b. The expression of the *MDR1* gene in placental tissue of Japanese women was significantly correlated to the T-129C SNP (Tanabe *et al.* 2001). Tanabe *et al.* (2001) noted a two-fold lower expression level of *MDR1* in heterozygous individuals compared to -129TT homozygous individuals. Furthermore, Taniguchi *et al.* (2003) not only found that the T-129C SNP was associated with altered *MDR1* expression, but also identified a DNA binding protein that binds to the nucleotide sequence carrying the -129T allele but not to that carrying the -129C allele. However, Horinouchi *et al.* (2002) found no evidence of LD between the -129 locus and either the 2677 or 3435 loci in Japanese subjects. Recently, Koyama *et al.* (2006) found that the T-129C polymorphism, but not G2677T/A or C3435T, was

associated with lower expression of *MDR1* mRNA in both colorectal adenocarcinomas and adjacent non-cancerous colorectal tissues.

A number of studies have examined the possible effects of *MDR1* sequence variants on ART response. In a landmark study, Fellay *et al.* (2002) detected an association of the C3435T SNP with differences in plasma drug concentrations of not only nelfinavir, a well-defined substrate of PGP, but also of efavirenz, which is not generally considered a PGP substrate. Fellay *et al.* (2002) found that individuals with the 3435TT genotype had decreased drug plasma concentrations compared to 3435CC homozygous individuals, while heterozygous individuals had intermediate drug plasma levels. To investigate whether the *MDR1* C3435T SNP was not only predictive of plasma drug concentration but also of treatment response, Fellay *et al.* (2002) also assessed CD4-cell count and viral load within patients after initiation of ART. Patients with the various 3435 genotypes had comparable CD4-cell counts prior to the initiation of therapy. After initiation of ART, all 3435 genotype groups of patients had similar rates of decrease in viral load. However, 3435TT homozygous patients had a higher mean CD4-cell count at 6 months than patients with either the 3435CC or 3435CT genotypes, as illustrated in Figure 2.06. Interestingly, patients with the 3435CC genotype had the lowest level of CD4-cell recovery, while 3435CT heterozygous patients had an intermediate level of CD4-cell recovery.

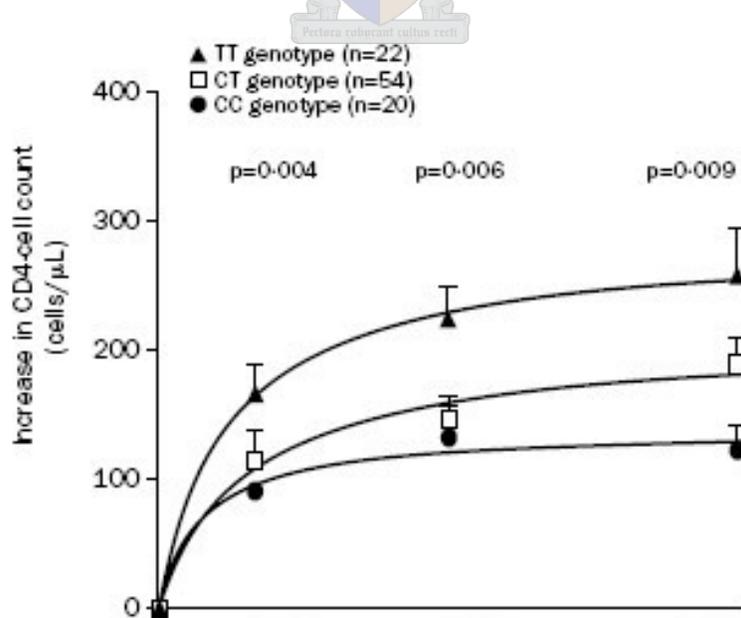


Figure 2.06: CD4-cell recovery according to *MDR1* 3435 genotype

(Source: Fellay *et al.* 2002)

Fellay *et al.* (2002) hypothesised that the low expression of *MDR1* and subsequently decreased PGP activity associated with the 3435TT genotype could allow for greater penetration of ARV drugs in cells susceptible to HIV infection, such as CD4 lymphocytes. Such increased penetration of ARV drugs within infected cells could thus lead to greater inhibition of HIV replication and a consequent improvement in the rate and extent of immune recovery.

A subsequent study by Nasi *et al.* (2003), failed to confirm the findings of Fellay *et al.* (2002) – the *MDR1* C3435T polymorphism was found not to influence the immunological or virological responses to ART in drug naïve HIV-positive adults. Furthermore, Winzer *et al.* (2005) also found no significant differences in the virological and immunological response with respect to the 2677 and 3435 genotypes and the 2677/3435 haplotype during the first 48 weeks of ART. Saitoh *et al.* (2005b), however, found that HIV-infected children with the *MDR1* 3435CT genotype had a more rapid virological response to ART at week eight than those with the 3435CC genotype. These virological findings were consistent with observed higher plasma concentrations and lower clearance rates of nelfinavir within 3435CT individuals than in 3435CC individuals. Explanations for the seemingly contradictory effects of the *MDR1* C3435T SNP on nelfinavir plasma concentration within the Fellay *et al.* (2002) and Saitoh *et al.* (2005b) studies include differences between the two studies, such as age of the study population and concomitant medications which could influence PGP activity.

Perhaps clarifying these contrasting results and confirming the effects of the C3435T SNP on nelfinavir plasma levels is a study by Zhu *et al.* (2004) which measured the intracellular concentrations of nelfinavir in lymphoblastoid cells and found significantly higher drug concentrations in cells with the 3435TT genotype than in those with the 3435CC or 3435CT genotypes. These findings support the supposed effect of the C3435T SNP on *MDR1* expression and subsequent PGP activity (illustrated in Figure 2.05) and the observation by Fellay *et al.* (2002) of an improved immune recovery in 3435TT homozygous individuals under nelfinavir treatment.

There exists considerable variability in the allele and genotype frequencies of *MDR1* polymorphisms between different ethnic groups. For example, the frequency of the variant 2677A allele ranges from 3.3% to 36% in Asians, to 1.9% to 10% in Caucasians, compared with

as low as 0.5% within Africans (Yi *et al.* 2004; Gerloff *et al.* 2002; Johnne *et al.* 2002; Cascorbi *et al.* 2001). The frequency of the C3435T SNP also varies markedly between different ethnic groups. Within European and American Caucasians, the frequency of 3435CC and 3435TT homozygous individuals is approximately 25% for each (3435C and 3435T frequency each ≈ 0.5). In Africans, however, the 3435C allele is found at a considerably higher frequency with 67-83% of individuals being 3435CC homozygotes and less than 6% being 3435TT homozygotes (Marzolini *et al.* 2004; Schwab *et al.* 2003; Fellay *et al.* 2002; Ameyaw *et al.* 2001; Kim *et al.* 2001; Schaeffeler *et al.* 2001). It has thus been hypothesised that due to the apparent functional consequences of the C3435T SNP (reduced *MDRI* expression) and the role of PGP in defence against numerous toxins, including bacterial and viral particles, that the considerably higher frequency of the 3435C allele and 3435CC genotype in Africans may be attributable to a selective advantage (Schaeffeler *et al.* 2001). The significantly higher frequency of the *MDRI* high expression 3435C allele and 3435CC genotype in Africans compared to Caucasians or Japanese (Ameyaw *et al.* 2001) may protect against development of gastrointestinal tract infections, which are endemic in tropical countries. This variable C3435T frequency between ethnic groups, particularly Africans and Caucasians, has been highlighted as a major consideration in treatment with drugs that are substrates of PGP (Schwab *et al.* 2004). Therefore, inter-ethnic variability in *MDRI* C3435T and other SNP frequencies could well translate into an important determinant factor for the pharmacokinetics and consequent use of ART (Chelule *et al.* 2003; Fellay *et al.* 2002).

2.4 PREVIOUSLY PUBLISHED SNP FREQUENCIES

The frequencies of the polymorphisms examined in this study, namely the A-392G (*CYP3A4*1B*) SNP in *CYP3A4*, the G516T and A785G SNPs in *CYP2B6*, and the T-129C, C1236T, G2677T/A and C3435T SNPs in *MDRI*, have previously been examined in numerous ethnic groups within a number of other studies. The frequencies of these SNPs within the Caucasian, African-American and African (Gabon, Ghana, Guinea-Bissau, Kenya, Nigeria, Senegal, SA, Sudan) ethnic groups as elucidated in previous such studies are presented in Table 2.04.

Table 2.04: Previously published frequencies of the SNPs examined in this study (nd = no data available)

(Sources: Haas *et al.* 2005; Haas *et al.* 2004; Marzolini *et al.* 2004; Yi *et al.* 2004; Cavaco *et al.* 2003; Chelule *et al.* 2003; Schwab *et al.* 2003; Fellay *et al.* 2002; Gerloff *et al.* 2002; Johnne *et al.* 2002; Kittles *et al.* 2002; Lamba *et al.* 2002a; Ameyaw *et al.* 2001; Cascorbi *et al.* 2001; Kim *et al.* 2001; Kuehl *et al.* 2001; Schaefer *et al.* 2001; Zuehl *et al.* 2001; Hoffmeyer *et al.* 2000; Moinpour *et al.* 2000; Tayeb *et al.* 2000; Ball *et al.* 1999; Walker *et al.* 1998)

	<i>CYP3A4</i>	<i>CYP2B6</i>		<i>MDR1</i>			
	A-392G	G516T	A785G	T-129C	C1236T	G2677T/A	C3435T
Caucasian	A: 90-98% G: 2-9.6%	G: 71-78% T: 22-29% (3% TT)	A: 70-80% G: 20-30%	T: 95% C: 5%	C: 58-66% T: 34-42%	G: 52-56% T: 38-46% A: 3-10%	C: 50% T: 50% (25% CC, 50% CT, 25% TT)
African-American	A: 33-65% G: 35-67%	G: 62% T: 38% (20% TT)	nd	nd	C: 85% T: 15%	G: 85% T: 15% A: 0.5%	C: 74-84% T: 16-26%
African	A: 20-30% G: 70-80%	nd	nd	nd	nd	nd	C: 74-90% T: 10-26% (67-84% CC, 3-4% TT)

2.5 RELEVANCE OF THE STUDY

The importance and relevance of this study is apparent when considering the following important factors (discussed in detail in the preceding sections):

- the scale of the HIV/AIDS epidemic in SA is enormous and shows few signs of abating (Section 2.2.1; Figure 1.01);
- the massive HIV prevalence and infection rates in SA are responsible for rapidly increasing levels of need and consequent usage of ARV drugs (Section 2.2.3; Figure 2.03);
- there exist clear variations in clinical responses to ARV drugs, both in terms of drug efficacy and the occurrence and severity of a multitude of ARV-related ADRs (Section 2.2.5);
- variations in ARV drug efficacy and the occurrence of related ADRs are influenced by numerous factors, but are believed to be largely attributable to DNA sequence variants (most

often in the form of SNPs) within genes responsible for drug metabolism and transport (Sections 2.1, 2.1.3 and 2.3; Figure 2.01);

- there exist significant differences in the presence and frequency of DNA sequence variants between ethnic groups, which can thus lead to marked differences in drug response between such groups (Section 2.1.6);
- an improved understanding of the presence, frequency and possible effects of DNA sequence variants – and their resultant haplotypes (Section 2.1.2) – within genes relevant to ARV metabolism (*CYP3A4* and *CYP2B6*) (Sections 2.3.1.2 and 2.3.1.4, respectively) and transport (*MDR1*) (Section 2.3.2) within South African ethnic groups could potentially aid in the customisation of ART on both an individual and population/ethnic group level (Section 2.1.5);
- subsequent customisation of ART has the potential to improve the efficacy of ARV drugs and to minimise the occurrence and/or severity of related ADRs (Section 2.1.5; Figure 2.02);
- improvement in therapy efficacy and minimisation of ADRs will improve patient adherence to therapy, thereby limiting the development of drug-resistant HIV strains and improving patient quality of life and chances of survival (Section 2.2.5).

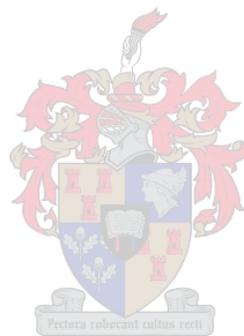
2.6 AIMS OF THE STUDY

The **general aim** of the study was to investigate the genetic basis for any observed differences in ART response, in terms of both drug efficacy and ADR occurrence, in South African ethnic groups.

The **specific aims** of the study were:

- the determination of the frequencies of the A-392G (*CYP3A4*1B*) SNP in *CYP3A4*, the G516T and A785G SNPs in *CYP2B6*, and the T-129C, C1236T, G2677T/A and C3435T SNPs in *MDR1* within the Mixed-Ancestry and Xhosa subpopulations of the patient cohort;
- the determination of the extent of LD between the G516T and A785G SNPs in *CYP2B6* and the T-129C, C1236T, G2677T/A and C3435T SNPs in *MDR1* within the Mixed-Ancestry and Xhosa subpopulations of the patient cohort;
- haplotype analysis of the genotypic data to determine the prevalent haplotypes (frequency > 0.01) within the *CYP2B6* and *MDR1* genes in the Mixed-Ancestry and Xhosa subpopulations of the patient cohort;

- ANOVA testing of the genotypic and clinical data to determine if any association exists between the examined polymorphisms and altered ARV drug response in terms of drug efficacy (CD4-cell count) and occurrence of ADRs.



CHAPTER THREE

MATERIALS AND METHODS

3.1 PATIENT COHORT AND ART

3.1.1 Study Population

The study population consisted of HIV-positive patients already enrolled on ARV treatment, new patients referred for evaluation of treatment, and prospective patients for ARV treatment, according to the National Department of Health (DOH) of SA (2004) National ARV Treatment Guidelines (<http://www.doh.gov.za/docs/factsheets/guidelines/artguidelines04/>), at the Infectious Diseases Clinic (IDC) at TC Newman Community Centre and antenatal clinic of Paarl Hospital in the Western Cape. Approximately 70% of the patients at these hospitals are from the Xhosa population while the remainder are primarily from the Mixed-Ancestry (Coloured) population. The Xhosa are representative of the Nguni-speaking tribes (du Toit *et al.* 1988) and are originally from the eastern coast of SA, but have subsequently migrated to other areas within the country. The Mixed-Ancestry (Coloured) population is of Asian, Western European and Southern and Eastern African ancestry (Nurse *et al.* 1995).

Exclusion criteria for the study included concurrent medication that could either potentially lead to drug-drug interactions with any of the administered ARVs or which were known inhibitors or inducers of the CYP enzymes responsible for ARV metabolism. Patients were also excluded from the study or ANOVA testing within the study if they were suffering from any conditions (e.g. kidney failure or liver cirrhosis) or exhibited any behaviour (e.g. poor treatment compliance or excessive alcohol consumption) that could in any way have affected the therapeutic efficacy of the prescribed ARV drugs.

An application form for the registration of a research project, along with the necessary documentation (checklist; information and informed consent document for DNA analysis and storage in Afrikaans, English and Xhosa; literature review; research project budget) for ethical approval of the project, were written and submitted to the Committee for Human Research of the Faculty of Health Sciences at Stellenbosch University. The recruitment and participation of patients in the study, as well as all work that was to be carried out, was subsequently approved by

the committee (project number: N04/10/181). All patients who participated in the study thus completed an information and informed consent document for DNA analysis and storage that was available in Afrikaans, English (Appendix A) and Xhosa.

3.1.2 ART Regimens

HIV-positive patients qualified for treatment if they had a CD4-cell count < 200 cells/mm³ and/or were at Clinical Stage IV of the disease according to the World Health Organisation (WHO) Clinical Staging System for HIV and AIDS for Adults and Adolescents (Appendix B), and expressed willingness and readiness to begin ART. After extensive counselling by trained HIV counsellors, patients were started on first-line therapy consisting of a combination of three simultaneously administered drugs, namely:

- lamivudine – 150 mg every twelve hours
- stavudine – 40 mg every twelve hours (or 30 mg every twelve hours if < 60 kg)
- efavirenz – 600 mg at night (or 400 mg if < 40 kg)

Patients who were pregnant received only two drugs – nevirapine and zidovudine – for prevention of mother-to-child transmission (PMTCT), but also qualified for triple therapy if their CD4 count was < 200 cells/mm³ and/or were at Clinical Stage IV of the disease. This triple therapy regimen was identical to that above, except that efavirenz was replaced with nevirapine due to the teratogenic effects of efavirenz (Saitoh *et al.* 2005a; Carr and Cooper 2000). Nevirapine was administered at 200 mg daily for two weeks, followed by 200 mg every twelve hours. If these patients did indeed qualify and were started on triple therapy, they were also invited to participate in the study.

If patients experienced an increase in viral load after six months, a repeat viral load measurement was performed after another three months. If there was still no response to the ART, the treatment regimen was changed to second-line therapy. This also consisted of a combination of three simultaneously administered drugs, namely:

- didanosine – 400 mg once a day (250 mg daily if < 60 kg), taken alone, dissolved in water, on an empty stomach
- zidovudine – 300 mg every twelve hours
- lopinavir/ritonavir – 400 mg/100 mg every twelve hours

3.1.3 Assessment of ART Efficacy and Toxicity

Drug response in all patients was measured after six months from initiation of treatment with a CD4-cell count and viral load measurement, with subsequent measurements taken every six months (except in cases where patients experienced an increase in viral load after six months despite ART, in which case a repeat viral load measurement was usually performed after another three months). Side-effects were assessed at two, four, eight and twelve weeks, and thereafter every three months for 36 months. All clinical information was collected at the clinics as part of patients' routine care and was kept blind until the completion of genotyping of all samples.

3.2 COLLECTION OF GENOMIC DNA SAMPLES

After explanation of and counselling about the study, patients were invited to participate by donating venous blood sampled with the routine bloods collected at the clinic for the management of their disease. A total of 225 whole blood samples were collected from patients. All samples that were collected were assigned an identification number that served as the sole means of identification for the laboratory personnel performing the genotyping.

Approximately 10 ml of whole blood was collected in a glass vacutainer tube (Becton Dickinson Vacutainer Systems, Preanalytical Solutions, Plymouth, UK), containing ethylenediaminetetraacetic acid (EDTA, $C_{10}H_{16}N_2O_8$) as anticoagulant and preservative. The freezing of blood samples was preferably avoided due to the decreased yield of DNA associated with the freezing process. Blood samples were, therefore, stored at 4°C until the DNA isolation process could be performed within three to five days of collection. However, if the DNA isolation process was unable to proceed within this time period, then samples were frozen at -20°C until the process could be carried out.

3.2.1 Genomic DNA Isolation from Whole Blood Samples

Total genomic DNA was isolated from leukocytes of whole blood samples by means of a modified salting-out method previously described by Miller *et al.* (1988).

The vacutainer tube containing the blood was shaken and the contents poured into a 50 ml centrifugation tube (Greiner Labortechnik, Kremsmuenster, Austria). Approximately 30 ml cold lysis buffer (8.3 mg/ml ammonium chloride (NH_4Cl), 1.1 mg/ml potassium hydrogen carbonate

(KHCO_3), 30 $\mu\text{g/ml}$ EDTA, pH 7.4) was added to the blood sample – if the blood volume was less than 10 ml, then the lysis buffer was added in a 3:1 (lysis buffer: blood) ratio. This mixture was kept on ice for 15 to 20 minutes and gently inverted every 5 minutes. This blood and lysis buffer mixture was then centrifuged at 1 500 rpm for 10 minutes in a Unicen 20 centrifuge with swing-out rotor (Orto Alresa, Madrid, Spain). The supernatant was discarded and the pellet resuspended in 10 ml phosphate buffered saline (PBS) solution (2 mg/ml potassium chloride (KCl), 8 mg/ml sodium chloride (NaCl), 1.14 mg/ml disodium hydrogen orthophosphate anhydrous (Na_2HPO_4), 0.2 mg/ml potassium dihydrogen orthophosphate (KH_2PO_4)). This mixture was then centrifuged at 1 500 rpm for 10 minutes and the supernatant again discarded. The pellet was resuspended in 3 ml nuclear lysis buffer (1.211 mg/ml tris(hydroxymethyl)aminomethane (tris, $\text{C}_4\text{H}_{11}\text{NO}_3$), 23.4 mg/ml NaCl, 0.6 mg/ml EDTA, pH 8.2), 15 μl proteinase K (20 mg/ml) (New England Biolabs Inc, Beverly, MA, USA), and 300 μl 10% (w/v) sodium dodecyl sulphate (SDS). This mixture was shaken well and incubated overnight in a water bath at 55°C.

After the overnight incubation, 2 ml of saturated sodium chloride (NaCl) solution (6 M) was added and the mixture shaken for 1 minute, after which it was centrifuged at 3 500 rpm for 30 minutes. The resulting supernatant was transferred to a new 50 ml centrifugation tube, taking care not to carry over any of the foam or pellet with it, and then shaken for 15 seconds. This was followed by centrifugation at 2 500 rpm for 15 minutes. The supernatant was again transferred to a new 50 ml centrifugation tube with care being exercised not to transfer any of the pellet or foam along with it. Twice the volume ice-cold ethanol (EtOH) (99%) ($\text{C}_2\text{H}_5\text{OH}$) was added and the solution left to stand until a white precipitant had formed. The precipitant was transferred to a 1.5 ml Eppendorf® microcentrifuge tube (Eppendorf, Hamburg, Germany) containing 500 μl EtOH (70%). This was subsequently centrifuged at 14 000 rpm for 10 minutes at 4°C (Beckman GS-15R, Beckman Coulter, Fullerton, CA, USA), after which the EtOH was poured off and the pellet allowed to air dry. Depending on the size of the pellet, 200-1 000 μl double-distilled water (ddH_2O) (SABAX) (Adcock Ingram, Johannesburg, SA) was added and the pellet dissolved by very gentle shaking on a vortex shaker for approximately 30 minutes prior to overnight storage at 4°C. Isolated genomic DNA samples were subsequently stored at -80°C.

See Appendix C for more information regarding the preparation of the reagents and solutions used for genomic DNA isolation from whole blood samples.

3.2.2 Quantification and Purity Analysis of Isolated Genomic DNA

The quantity and purity of DNA isolated from blood samples were assessed with a NanoDrop[®] ND-1000 spectrophotometer (Rockland, DE, USA) for small volume UV analysis. In order to ensure complete sample homogeneity, which is critical when measuring genomic DNA concentration and purity with this instrument, samples were very gently shaken on a vortex shaker for approximately 30 minutes before measurements taken. A sample volume of 1.5 to 2 μ l was pipetted onto the fibre optic measurement surface.

The quantification of the isolated genomic DNA solutions allowed for the making up of working solutions. Working solutions of genomic DNA were made up to a standard concentration of 50 ng/ μ l with ddH₂O (SABAX), except in cases where the sample had an initial concentration of less than 50 ng/ μ l, in which case an undiluted aliquot was taken as a working solution. All working solutions of genomic DNA were stored at -20°C until genotype analysis.

The purity and integrity of isolated genomic DNA were also assessed by means of agarose gel electrophoresis. A sample volume of 5 μ l (20-50 ng/ μ l) was resolved on a 1% (w/v) agarose gel, as was performed for the visualisation of polymerase chain reaction (PCR) amplification products detailed in section 3.6.1.

3.3 GENOTYPING OF SNPs

In order to facilitate the accurate genotyping of the patient DNA samples for the selected SNPs, PCR-RFLP was employed due to its affordability, ease of use and reliability. This method of genotyping entails the restriction enzyme (REase) digestion of polymerase chain reaction (PCR) amplification product. The subsequent digestion, or lack of digestion, of PCR amplification product due to the presence or absence of an SNP within the REase recognition site allows for accurate and reliable genotyping and the consequent determination of SNP frequencies within a sample cohort. The classification of an SNP genotype as ‘wild-type’ or ‘variant’ was done according to accepted nomenclature and the relevant reference sequences available from the

National Centre for Biotechnological Information (NCBI) Entrez Nucleotides Database (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide>).

3.3.1 DNA Amplification Using PCR

The relevant genomic target regions, containing the SNPs of interest, were amplified by means of primer-directed PCR using thermostable DNA polymerase, as originally described by Saiki *et al.* (1988; 1985). This primer-directed PCR method facilitates the *in vitro* amplification of single-copy genomic DNA sequences by a factor of more than ten million with extremely high sequence specificity.

3.3.1.1 Design of PCR Primers

Previously published primers were used for the amplification of *CYP2B6* exon 4 (G516T; Lamba *et al.* 2003), *CYP2B6* exon 5 (A785G; Jacob *et al.* 2004), *MDR1* promoter region (T-129C; Cavaco *et al.* 2003), *MDR1* exon 12 (C1236T; Hoffmeyer *et al.* 2000), *MDR1* exon 21 (G2677T/A; Cavaco *et al.* 2003) and *MDR1* exon 26 (C3435T; Hoffmeyer *et al.* 2000).

Primers for the amplification of the *CYP3A4* promoter region containing the A-392G polymorphism were designed using Primer Designer Version 1.01 and the PRIMER3 programme for primer design (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The relevant reference sequence (accession number: AF280107) was obtained from the NCBI Entrez Nucleotides Database. Primer sequence homology and specificity were checked by using the NCBI Basic Local Alignment Search Tool (BLAST) (<http://www.ncbi.nlm.nih.gov/BLAST/>).

The sequences of the primers used and their product sizes are presented in Table 3.01 – lowercase letters are indicative of mismatched nucleotides incorporated into the primer sequence for the generation of REase recognition sites.

Table 3.01: Primers used for PCR of relevant amplicons (F = forward primer; R = reverse primer; lower case = mismatched nucleotide)

Gene	SNP	Wild-type	Variant	Primer Sequence	Product Size
<i>CYP3A4</i>	A-392G	ggcaAgaga	ggcaGgaga	F: 5'-GGACAGCCATAGAGACAAGGGgA-3' R: 5'-TACTGGGGAGTCCAAGGGTTCTG-3'	107 bp
	A-392G	ggcaAgaga	ggcaGgaga	F: 5'-GGAGCTCACCTCTGTTTCAGG-3' R: 5'-GGCTATGTGCATGGAGCTTT-3' (nested-PCR – see Section 3.3.1.2 and Figure 3.01)	630 bp
<i>CYP2B6</i>	G516T	tccaGtcca	tccaTtcca	F: 5'-GGTCTGCCCATCTATAAAC-3' R: 5'-CTGATTCTTCACATGTCTGCG-3' (Lamba <i>et al.</i> 2003)	526 bp
	A785G	cccaAggac	cccaGggac	F: 5'-GTAGTCCTAACATGTCAG-3' R: 5'-AGAGCCTACA GTGCTCCCA-3' (Jacob <i>et al.</i> 2004)	1 474 bp
<i>MDR1</i>	T-129C	cgagTagcg	cgagCagcg	F: 5'-TCTCGAGGAATCAGCATTCAGTCAATCC-3' R: 5'-CTAAAGGAAACGAAGAGCGGCCTCTG-3' (Cavaco <i>et al.</i> 2003)	197 bp
	C1236T	agggCctga	agggTctga	F: 5'-TTTAAACCTAGTGAACAGTCAGTTCC-3' R: 5'-ATATCCTGTCCATCAACACTGACC-3' (Hoffmeyer <i>et al.</i> 2000)	345 bp
	G2677T/A	aggtGctgg	aggt(T/A)ctgg	F: 5'-GTACCCATCATTGCAATAGCA-3' R: 5'-TTTAGTTTGACTCACCTTCCgAG-3' (Cavaco <i>et al.</i> 2003)	108 bp
	C3435T	agatCgtga	agatTgtga	F: 5'-CTACCACATGCATACATCAGAAAC-3' R: 5'-CAGATGCTTGATACAGGTAAGGG-3' (Hoffmeyer <i>et al.</i> 2000)	581 bp

3.3.1.2 PCR Parameters and Programmes

GoTaq[®] Flexi DNA Polymerase, reaction buffer and MgCl₂ (Promega Corporation, Madison, WI, USA) were used for the PCR amplification of *CYP3A4* promoter region (A-392G) (both primer pairs), *CYP2B6* exon 4 (G516T), *MDR1* promoter region (T-129C), *MDR1* exon 12 (C1236T), *MDR1* exon 21 (G2677T/A) and *MDR1* exon 26 (C3435T). A 25 µl reaction volume was used containing 2 µl of genomic DNA (20-50 ng/µl), 5 µl of 5X Green *GoTaq*[®] reaction buffer, 4.0 µl MgCl₂ (25 mM), 2.0 µl dNTPs (2.5 mM), 1 µl of each primer (10 µM), 0.1 µl of *GoTaq*[®] DNA polymerase (5 U/µl), and 9.9 µl of ddH₂O (SABAX).

Due to repeated difficulties with amplification of the *CYP2B6* exon 5 (A785G) template using *GoTaq*[®] Flexi DNA Polymerase and an apparent superior robustness of the *BIOTAQ*[™] DNA Polymerase with difficult templates and/or contaminated template solutions, *BIOTAQ*[™] DNA

Polymerase, reaction buffer and MgCl₂ (Bioline, Randolph, MA, USA) were used for the amplification of *CYP2B6* exon 5 (A785G). A 25 µl reaction volume was used containing 2 µl of genomic DNA (20-50 ng/µl), 2.5 µl of 10X NH₄ reaction buffer, 2.0 µl MgCl₂ (50 mM), 2.5 µl dNTPs (2.5 mM), 1 µl of each primer (10 µM), 0.15 µl of *BIOTAQ*TM DNA polymerase (5 U/µl), and 13.85 µl of ddH₂O (SABAX).

PCR programme parameters for amplification of the *CYP3A4* promoter region (A-392G) (both primer pairs), *CYP2B6* exon 4 (G516T), *MDR1* promoter region (T-129C), *MDR1* exon 12 (C1236T), and *MDR1* exon 26 (C3435T) consisted of a two-step programme of initial denaturation at 94°C for 4 minutes, followed by 15 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 45 seconds, 20 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds and extension at 72°C for 45 seconds, and a final extension at 72°C for 5 minutes.

PCR programme parameters for the amplification of *MDR1* exon 21 (G2677T/A) were identical to those above, except that the annealing temperatures (T_A) of 60°C and 54°C for the successive cycling steps were lowered to 58°C and 53°C, respectively. PCR programme parameters for amplification of *CYP2B6* exon 5 (A785G) consisted of a two-step programme of initial denaturation at 94°C for 4 minutes, followed by 15 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 90 seconds, 20 cycles of denaturation at 94°C for 30 seconds, annealing at 52°C for 30 seconds and extension at 72°C for 90 seconds, and a final extension at 72°C for 5 minutes.

Due to sequence homology of the primers designed to amplify the *CYP3A4* promoter region, while incorporating an REase recognition site containing the A-392G SNP, with other members of the CYP3A subfamily (*CYP3A43*, *CYP3A5* and *CYP3A7*), nested-PCR was performed to confirm that this genotyping method was indeed accurate and reliable. Primers that were previously confirmed to be entirely *CYP3A4*-specific by means of DNA sequencing of PCR product (highlighted pink in Figure 3.01), were used to synthesise a 630 bp PCR product containing the amplicon and SNP (highlighted red in Figure 3.01) of interest. This PCR product then served as template for PCR amplification with the less *CYP3A4*-specific primers (highlighted

green in Figure 3.01) which generate an REase recognition site containing the A-392G SNP within the 107 bp amplification product.

```
ATAAGACTAGACTATGCCCTTGAGGAGCTCACCTCTGTTTCAGGGAACAGGCGTGAAACACAAT
GGTGGTAAAGAGGAAAGAGGACAATAGGATTGCATGAAGGGGATGGAAAGTGCCAGGGGAGGAA
ATGGTTACATCTGTGTGAGGAGTTTGGTGAGGAAAGACTCTAAGAGAAGGCTCTGTCTGTCTGGG
TTTGGAAGGATGTGTAGGAGTCTTCTAGGGGGCACAGGCACACTCCAGGCATAGGTAAAGATCTG
TAGGTGTGGCTTGTGGGATGAATTTCAAGTATTTTGAATGAGGACAGCCATAGAGACAAGGGC
AAGAGAGAGGCGATTTAATAGATTTTATGCCAATGGCTCCACTTGAGTTTCTGATAAGAACCAG
AACCCTTGGACTCCCCAGTAAACATTGATTGAGTTGTTTATGATACCTCATAGAATATGAACTCAA
AGGAGGTCAGTGAGTGGTGTGTGTGATTCTTTGCCAACTTCCAAGGTGGAGAAGCCTCTTCCA
ACTGCAGGCAGAGCACAGGTGGCCCTGCTACTGGCTGCAGCTCCAGCCCTGCCTCCTTCTCTAGC
ATATAACAATCCAACAGCCTCACTGAATCACTGCTGTGCAGGGCAGGAAAGCTCCATGCACATA
GCCCAGCAAAGAGCAACACAGAGCTGAAAGGA
```

Figure 3.01: Nested-PCR amplification of the *CYP3A4* A-392G SNP (pink highlight = nested-PCR primers; green highlight = mismatch nucleotide primers; red highlight = A-392G SNP; underlined = mismatched nucleotide)

As mentioned above, the PCR parameters and programme employed for both of these primer pairs were identical. Nested-PCR amplification was performed with 2 µl of a 1 in 500 dilution of PCR amplification product as template. Nested-PCR and subsequent RFLP analysis was not performed on all of the patient samples. It was, however, used only as a means of verifying that the non-specific amplification experienced with the *CYP3A4* primers in no way affected the reliability or accuracy of this genotyping method for the *CYP3A4* A-392G SNP. Nested-PCR was, therefore, only performed in a small subset of patients samples (\pm 10% of the patient cohort) that had already been genotyped, in order to confirm that the correct genotypes had indeed been established.

dNTPs used in PCR amplifications were manufactured by Bioline (Randolph, MA, USA) and PCR primers synthesised by Inqaba Biotec® (Inqaba Biotechnical Industries, Pretoria, SA). All PCR amplifications were carried out in a Perkin Elmer GeneAmp PCR Thermal Cycler 2700 (Applied Biosystems, Warrington, WA, USA).

3.3.2 RFLP Analysis

REases used previously in other studies for the genotyping of *MDR1* T-129C (*MspAII*; Cavaco *et al.* 2003), *MDR1* G2677T/A (*Alw2II*; Cavaco *et al.* 2003) and *MDR1* C3435T (*MboI*; Balram *et al.* 2003) were used. Appropriate REases for the genotyping of the *CYP3A4* A-392G, *CYP2B6*

G516T, *CYP2B6* A785G, *MDR1* C1236T and *MDR1* G2677A SNPs were selected with the aid of New England Biolabs (NEB) cutter V2.0 (<http://tools.neb.com/NEBcutter2/index.php>) and Webcutter 2.0 (<http://www.carolina.com/cgi-bin/carolinaup.pl>).

The REases employed in RFLP analysis in this study, along with their incubation temperatures and recognition sequences, are listed in Table 3.02.

Table 3.02: REases used for PCR-RFLP analysis (^ = REase cleavage site; N = any nucleotide)

Gene	SNP	REase	Incubation Temperature	Recognition Sequence
<i>CYP3A4</i>	A-392G	<i>MboII</i>	37°C	GAAGA(NNNNNNNN)^
<i>CYP2B6</i>	G516T	<i>BseNI</i>	65°C	ACTGG(1/-1)^
	A785G	<i>StyI</i>	37°C	C^C(A/T)(A/T)GG
<i>MDR1</i>	T-129C	<i>MspAII</i> (Cavaco <i>et al.</i> 2003)	37°C	C(C/A)G^C(T/G)G
	C1236T	<i>Eco0109I</i>	37°C	(A/G)G^G(N)CC(C/T)
	G2677T/A	<i>Alw2II</i> (Cavaco <i>et al.</i> 2003)	37°C	G(T/A)GC(T/A)^C
	G2677A	<i>RsaI</i>	37°C	GT^AC
	C3435T	<i>MboI</i> (Balram <i>et al.</i> 2003)	37°C	^GATC

MboII, *BseNI*, *StyI*, *Alw2II* and *MboI* were supplied by Fermentas Life Sciences (Burlington, Ontario, Canada), while *MspAII*, *Eco0109I* and *RsaI* by New England Biolabs (Beverly, MA, USA).

The *MDR1* G2677T/A polymorphism in exon 21 presented a unique challenge in terms of the method needed to genotype all six possible genotypes correctly and to thus accurately determine the presence and frequency of all three nucleotide variants. Initially, *Alw2II* was used to detect which samples were not homozygous for the wild-type allele – i.e. those that carried either a thymine (G/T) or adenine variant (G/A), or two copies of each (TT and AA) or a single copy of each (TA), at this position. These samples were subsequently digested with *RsaI*, which only digests the PCR fragment in the presence of the adenine variant, in order to distinguish between

the thymine and adenine nucleotides. This subsequently allowed for the correct determination of the presence and frequencies of the three possible nucleotides at this position.

A total reaction volume of 10 to 15 μ l, depending on the concentration of PCR product, was used in REase digestion reactions. In all cases, the enzyme reaction buffer and bovine serum albumen (BSA), when required, were used at final concentrations of 1X, along with approximately 5 U of REase, with the volume made up with ddH₂O (SABAX). Digestion reactions were incubated at their required incubation temperatures overnight (\pm 16 hours) to ensure complete digestion of PCR product.

The resultant fragment sizes from the REase digestion of PCR products are listed in Table 3.03.

Table 3.03: PCR-RFLP fragment sizes

Gene	SNP	Fragment Sizes
<i>CYP3A4</i>	A-392G	wild-type: 2 fragments (73 bp and 34 bp) variant: 1 fragment (107 bp)
<i>CYP2B6</i>	G516T	wild-type: 3 fragments (268 bp, 236 bp and 22 bp) variant: 2 fragments (504 bp and 22 bp)
	A785G	wild-type: 4 fragments (907 bp, 340 bp, 171 bp and 56 bp) variant: 3 fragments (1 078 bp, 340 bp and 56 bp)
<i>MDR1</i>	T-129C	wild-type: 2 fragments (180 bp and 17 bp) variant: 3 fragments (148 bp, 32 bp and 17 bp)
	C1236T	wild-type: two fragments (256 bp and 89 bp) variant: 1 fragment (345 bp)
	G2677T/A	wild-type: 2 fragments (87 bp and 21 bp) variant (T or A): 1 fragment (108 bp)
	G2677A	G or T: 2 fragments (106 bp and 2 bp) A: 3 fragments (82 bp, 24 bp and 2 bp)
	C3435T	wild-type: 3 fragments (211 bp, 198 bp and 172 bp) variant: 2 fragments (370 bp and 211 bp)

3.3.3 Visualisation of PCR Product and REase Digestion Fragments

PCR amplification products were visualised by means of agarose gel electrophoresis in order to allow for size estimation and thus confirmation of amplification of the desired genomic target region. REase digestion fragments that were of sufficient size (> 100 bp) and size differential between fragments (> 30 bp) were also visualised on agarose gel. These included those of *CYP2B6* G516T and *CYP2B6* A785G, and *MDR1* T-129C, *MDR1* C1236T and *MDR1* C3435T,

which were visualised on varying percentages (w/v) of agarose gel depending on the total size of and size differential between fragments. The REase digestion fragments of *CYP3A4* A-392G, *MDR1* G2677T/A and *MDR1* G2677A were of insufficient size and size differential to allow for visualisation on agarose gel. These fragments were consequently visualised by means of polyacrylamide (PAA) gel electrophoresis (PAGE), which displays a considerably higher level of resolution than agarose gel electrophoresis.

O'GeneRuler™ 100 bp DNA ladder (Fermentas Life Sciences, Burlington, Ontario, Canada) was used for size estimation of PCR amplification products, which served as confirmation that amplification of the desired genomic target region had occurred, as well as for quantification of PCR product prior to REase digestion reactions. O'GeneRuler™ 100 bp DNA ladder was also used for size estimation of all REase digestion fragments, allowing for accurate and reliable genotyping of samples. O'GeneRuler™ 100 bp DNA ladder is thus evident in lane 1 of all agarose and PAA gel photos. All agarose and PAA gels were visualised under ultraviolet (UV) light and photographed with a MultiGenius BioImaging Gel Documentation and Analysis System (Syngene, Frederick, MD, USA).

3.3.3.1 Agarose Gel Electrophoresis

All PCR products were resolved by electrophoresis in 1% (w/v) agarose gel at 120 volts (V). The REase digestion fragments of *CYP2B6* G516T and *CYP2B6* A785G were resolved on 1.8% (w/v) and 1.4% (w/v) agarose gel, respectively, while those of *MDR1* C1236T in 1.6% (w/v) and *MDR1* T-129C and *MDR1* C3435T in 2.0% (w/v) agarose gel. All REase digestion fragments were resolved at 80 V, so as to ensure sufficient resolution to allow for accurate genotyping.

All agarose gels were made with and resolved in 1X tris borate ethylenediaminetetraacetic acid (TBE) buffer, which was made and stored as a 5X stock solution and diluted to the required working concentration as was needed. In order to facilitate the visualisation of DNA within the agarose gel under UV light, 1 µg of ethidium bromide (EtBr) per ml agarose solution was added – i.e. 0.01% (v/v) EtBr stock solution (10 mg/ml). See Appendix C for more information regarding the preparation of EtBr and TBE buffer.

3.3.3.2 PAGE

The REase digestion fragments of *CYP3A4* A-392G as well as *MDR1* G2677T/A and *MDR1* G2677A were resolved on vertical, non-denaturing 15% (w/v) PAA gel at 200 V in a Hoefer Mighty Small SE 250 mini-vertical unit (Hoefer Scientific Instruments, San Francisco, CA, USA).

15% (w/v) PAA gels were made from a mixture containing 37.5% (v/v) PAA (5% cross-linkage – acrylamide (AA): bisacrylamide (BAA) ratio of 19:1; 40% (w/v)), 20% (v/v) 5X TBE buffer, and 42.5% (v/v) dH₂O. 200 µl of 10% (w/v) ammonium persulphate (APS, H₈N₂O₈S₂) and 25 µl of N, N, N', N'-tetramethylethylenediamine (TEMED) was subsequently added to approximately 10 ml of this mixture directly preceding the pouring of gels. Electrophoresis was performed in 1X TBE buffer for approximately 90 minutes. In order to facilitate the visualisation of DNA within the PAA gel under UV light, gels were soaked for approximately 10 minutes in 1X TBE buffer containing 1 µg of EtBr per ml – i.e. 0.01% (v/v) EtBr stock solution (10 mg/ml). Gels were subsequently destained by soaking in dH₂O for approximately 3 minutes. See Appendix C for more information regarding the preparation of acrylamide, EtBr and TBE buffer.

3.4 STATISTICAL ANALYSES

3.4.1 HWE Analysis

SNP allele and genotype frequencies were calculated with the use of Microsoft® Office Excel 2003. SNP and allele genotype frequencies were calculated for the population cohort and also the Mixed-Ancestry and Xhosa subpopulations. Testing for HWE in the Mixed-Ancestry and Xhosa subpopulations was subsequently performed by means of Fisher's exact test (Haldane 1954), using the Tools for Population Genetic Analyses (TFPGA) software programme (<http://www.marksgeneticsoftware.net/tfpga.htm>) (Miller 1997). Testing for HWE of the triallelic G2677T/A polymorphism in *MDR1* was also performed by means of Fisher's exact test using TFPGA, employing the conventional Monte Carlo method of estimating the *p*-value. The results of testing for HWE using TFPGA were verified with the GENETPOP population genetics software package (<http://wbiomed.curtin.edu.au/genepop/>). In all instances of testing for HWE, a *p* value ≤ 0.05 was deemed statistically significant.

3.4.2 Haplotype Analysis

The level of LD and most prevalent haplotypes (frequency > 0.01) of the examined SNPs in the *CYP2B6* and *MDR1* genes within the Mixed-Ancestry and Xhosa subpopulations were determined with Haploview, version 3.31 (<http://www.broad.mit.edu/mpg/haploview/>) (Barrett *et al.* 2005). However, due to the inability of Haploview to analyse triallelic SNPs and the exceedingly low frequency of the G2677A variant in the Mixed-Ancestry and Xhosa subpopulations (± 0.01), analysis of the *MDR1* gene included only the G2677T variant. Due to the fact that the EM algorithm used within the Haploview software programme assumes HWE, only polymorphisms that were in HWE could be used in haplotype analysis.

Due to the relatively small sample sizes, the correlation coefficient (r^2) value was used as indication of the level of LD between SNPs, rather than the D' value which can falsely indicate strong apparent LD in small sample sizes. An r^2 -value ≥ 0.4 was considered a reliable indication of LD. Furthermore, a logarithm of the odds (LOD) score of ≥ 3 (i.e. the likelihood of observing the result if the two loci are not linked is less than 1 in 1 000) was also accepted as a significant indication of LD. The occurrence and frequency of the most prevalent haplotypes (frequency > 0.01) of the examined SNPs within *CYP2B6* and *MDR1* in the Mixed-Ancestry and Xhosa subpopulations, as determined with Haploview, were verified with SNPHAP, version 1.3.1 (<http://www-gene.cimr.cam.ac.uk/clayton/software/snphap.txt>).

3.4.3 ANOVA Testing for Significance

ANOVA testing was performed on patients within the patient cohort which had commenced ART, had been on such treatment for sufficient time (three to six months), which did not meet any of the exclusion criteria and for which sufficient clinical data were supplied. The follow-up CD4-cell count measurements allowed for determination of patient response to treatment. Univariate two-way ANOVA testing of the change in CD4-cell count after six months of ART was performed on the separate Mixed-Ancestry and Xhosa subpopulations of the patient cohort. Univariate one-way ANOVA testing of the change in CD4-cell count after six months of ART was subsequently performed on the entire patient cohort as a whole (i.e. the Mixed-Ancestry and Xhosa subpopulations pooled together), as no significant difference in the change in CD4-cell count according to ethnicity was observed. In all instances of ANOVA testing, a p value ≤ 0.05 was deemed statistically significant.

CHAPTER FOUR

RESULTS

4.1 COLLECTION OF PATIENT DNA SAMPLES

A total of 225 whole blood samples were collected from HIV-positive patients already enrolled on ARV treatment, new patients referred for evaluation of treatment, and prospective patients for ARV treatment at the IDC at TC Newman Community Centre and antenatal clinic of Paarl Hospital in the Western Cape. Genomic DNA isolation from these whole blood samples was performed which yielded 206 DNA samples of sufficient yield and purity ($A_{260}/A_{280} \approx 1.8$; $A_{260}/A_{230} \approx 2.0$) for subsequent PCR-RFLP analysis.

4.2 GENOTYPING OF SNPs

4.2.1 Non-specific Amplification of *CYP3A4*-homologous Genes

Non-specific amplification products were evident when the 107 bp PCR amplification product containing the *CYP3A4* A-392G polymorphism was digested with *Mbo*II and resolved on 15% (w/v) PAA gel (Figure 4.01).

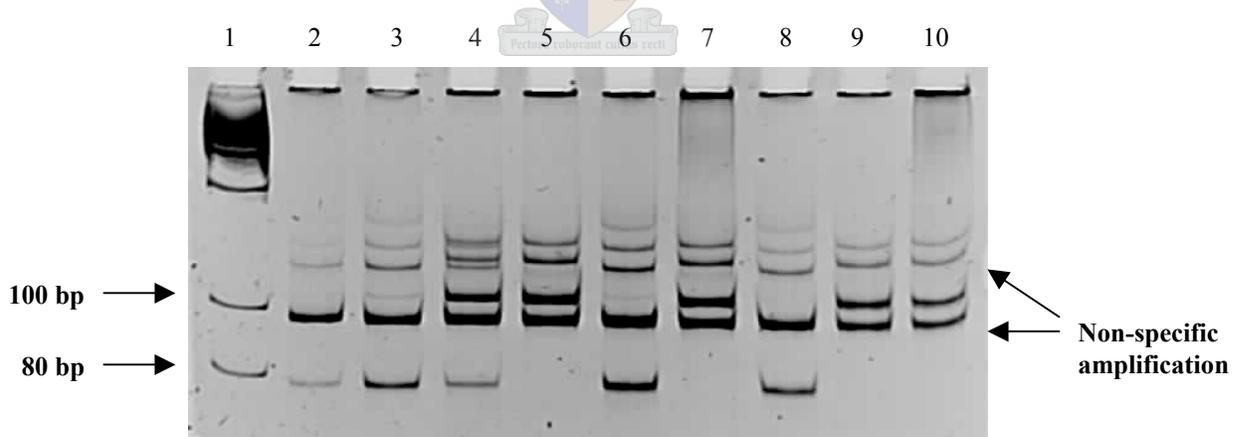


Figure 4.01: *Mbo*II REase digestion fragments of the A-392G SNP in the *CYP3A4* promoter region, resolved on a 15% (w/v) PAA gel (lane 2 = homozygous wild-type; lane 4 = heterozygous; lane 5 = homozygous variant)

This non-specific amplification is most likely due to sequence homology of the *CYP3A4* gene with other members of the *CYP3A* subfamily of genes (*CYP3A43*, *CYP3A5*, *CYP3A7*). In particular, a fragment of almost identical size is produced which is indistinguishable from the

CYP3A4 fragment when resolved on 1% (w/v) agarose gel, but clearly evident when resolved on 15% (w/v) PAA gel. As discussed in Section 3.3.1.2, nested-PCR amplification of this amplicon was thus performed with these mismatch primers, but with PCR amplification product as template. The resultant REase digestion fragments of the nested-PCR amplification product (Figure 4.02) exhibited a complete absence of the non-specific amplification products that were previously observed.

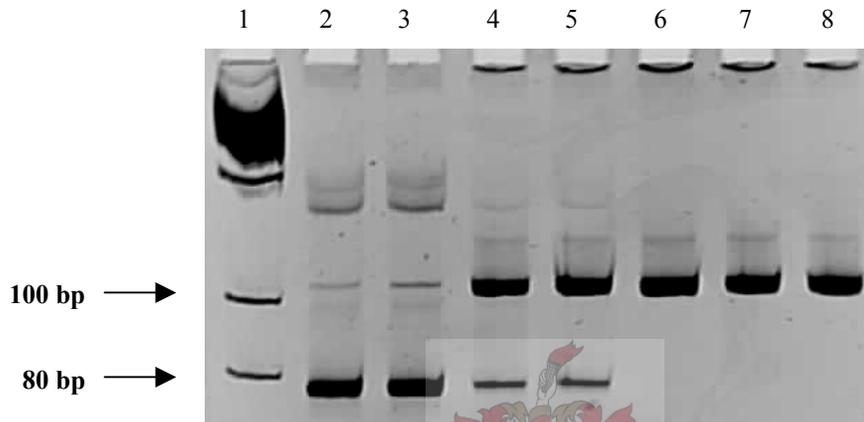


Figure 4.02: *Mbo*II REase digestion fragments of the A-392G SNP in the *CYP3A4* promoter region, resolved on a 15% (w/v) PAA gel, after nested-PCR amplification using *CYP3A4*-specific primers (Section 3.5.1.2) (lane 2 = homozygous wild-type; lane 4 = heterozygous; lane 6 = homozygous variant)

The small subset of patients ($\pm 10\%$ of the patient cohort) that was subsequently re-genotyped using this nested-PCR method exhibited no change in their formerly established genotype. It was thus evident that the non-specific amplification experienced with the mismatch *CYP3A4* primers for typing the A-392G SNP in no way affected the accuracy or reliability of this method of genotyping.

4.2.2 Visualisation of REase Digestion Fragments

The visualisation of REase digestion fragments on varying percentages of agarose (w/v) and 15% PAA (w/v) gel (depending on the total sizes and size differential of the resultant fragments) demonstrated sufficient resolution to allow for accurate genotyping of each of the seven SNPs. Examples of PCR-RFLP agarose and PAA gel photos of all the polymorphisms (except that of *CYP3A4* A-392G, already presented in Section 4.2.1) and their resultant genotypes and band patterns are presented in Appendix D. The subsequent genotyping of the patient samples allowed

for the accurate determination of the genotype and allele frequencies of the seven SNPs within the patient cohort.

4.2.3 SNP Genotype and Allele Frequencies in the Patient Cohort

The determination of the genotype for each of the seven SNPs in each of the 206 patient DNA samples allowed for subsequent elucidation of the genotype and allele frequencies in the patient cohort. The genotype and allele frequencies of the seven SNPs examined within this study in the patient cohort are presented in Table 4.01.

Table 4.01: Genotype and allele frequencies in the patient cohort

	<i>CYP3A4</i>	<i>CYP2B6</i>		<i>MDR1</i>				
	A-392G	G516T	A785G	T-129C	C1236T	G2677T/A		C3435T
Genotype								
Homozygous wild-type	18	86	86	130	141	GG	174	151
Heterozygous	85	107	107	67	61	GT	26	49
Homozygous variant	103	13	13	9	4	GA	4	6
						TT	2	
						TA	0	
						AA	0	
Total	206	206	206	206	206		206	206
Genotype Frequencies								
Homozygous wild-type	8.74%	41.75%	41.75%	63.11%	68.45%	GG	84.47%	73.30%
Heterozygous	41.26%	51.94%	51.94%	32.52%	29.61%	GT	12.62%	23.79%
Homozygous variant	50.00%	6.31%	6.31%	4.37%	1.94%	GA	1.94%	2.91%
						TT	0.97%	
						TA	0.00%	
						AA	0.00%	
Allele Frequencies								
Wild-Type	29.37%	67.72%	67.72%	79.37%	83.25%	G	91.75%	85.19%
Variant	70.63%	32.28%	32.28%	20.63%	16.75%	T	7.28%	14.81%
						A	0.97%	

4.3 CLINICAL DATA

After the completion of genotyping of all patient DNA samples, the clinical data were made available. 44 of the 206 patients did not qualify for ART. Two patients demonstrated poor treatment compliance and another stopped treatment for five months – these patients thus experienced marked deterioration in disease state and consequently were excluded from any subsequent ANOVA testing. Four patients died before treatment could commence, one patient died ten days after commencement of treatment, two patients did not return to the clinic for

treatment, and two patients were disqualified from treatment due to excessive alcohol consumption which could have affected kidney and/or liver function and subsequent ARV drug metabolism and response.

Although meticulous patient information files were kept by the clinical staff, certain details of numerous patients were not obtainable and/or not provided. Of the 206 patients within the patient cohort, clinical data were not supplied for seven patients while fourteen patients had only gender and/or ethnicity data and a further two patients had incomplete data. Furthermore, although 70 patients were found to be of Mixed-Ancestry and 112 of Xhosa ethnicity, the ethnicities of the remaining 24 patients were not obtainable or provided.

A total of 126 patients remained after exclusion of those not yet started on ART, those who demonstrated poor treatment compliance or met other exclusion criteria, those who died before the commencement of treatment or shortly thereafter (\pm two weeks), and those for whom sufficient clinical data regarding treatment response was lacking. These 126 patients were thus used in subsequent ANOVA testing of their *CYP3A4*, *CYP2B6* and *MDR1* genotypes and ART response. However, of these 126 patients, clinical information concerning ethnicity of sixteen patients was found to be missing. Therefore, univariate two-way ANOVA testing on the Mixed-Ancestry and Xhosa subpopulations could only be performed on 39 and 71 patients, respectively.

The responses of the 126 patients to ART, in terms of CD4-cell recovery, were overwhelmingly favourable. The average change in CD4-cell count from the commencement of therapy was an increase of approximately 341% after six months and an increase of approximately 613% after one year. The median values for the change in CD4-cell count after six months and one year of ART were increases of approximately 188% and 363%, respectively. The poorest responses after six months and one year of ART were decreases in CD4-cell count of approximately 22% and 17%, respectively. The most favourable responses after six months and one year of ART were increases in CD4-cell count of approximately 1 891% and 3 717%, respectively. According to the criteria detailed in Section 3.1.3, 80 patients were good responders to ART and 23 were found to be poor responders, while the responses of the remaining 23 patients (in terms of immune recovery) were not ascertainable due to their too recent commencement of therapy.

The most commonly observed ARV-induced ADRs within these 126 patients at two weeks after commencement of ART were peripheral neuropathy (n = 9), skin rash (n = 5), nausea (n = 2), fatigue (n = 2), back pain (n = 1), dizziness (n = 1), double vision (n = 1), pruritis (n = 1) and convulsions (n = 1). The most commonly observed ARV-induced ADRs within these 126 patients at three months after commencement of ART were peripheral neuropathy (n = 9), skin rash (n = 4), nausea (n = 1), fatigue (n = 1), double vision (n = 1), pruritis (n = 1), convulsions (n = 1), liver enlargement (n = 1) and lipodystrophy (n = 1). A total of 18 patients developed lactic acidosis as a result of mitochondrial toxicity from NRTI usage. Of the patients who experienced lactic acidosis, one consequently developed cortical blindness, which necessitated cessation of all ART, while one died from the disorder.

Apart from the single death of a patient as a result of lactic acidosis, a further three patients died during the course of the study. One patient died from severe tuberculosis (TB) while two died of other AIDS-related causes.

4.4 SNP GENOTYPE AND ALLELE FREQUENCIES IN THE MIXED-ANCESTRY AND XHOSA SUBPOPULATIONS

After completion of genotyping of the patient cohort and subsequent collection of the clinical data, the SNP genotype and allele frequencies within the Mixed-Ancestry and Xhosa subpopulations could be determined. These genotype and allele frequencies within the Mixed-Ancestry and Xhosa subpopulations are presented in Tables 4.02 and 4.03, respectively.

Table 4.02: Genotype and allele frequencies in the Mixed-Ancestry subpopulation of the patient cohort

	<i>CYP3A4</i>	<i>CYP2B6</i>		<i>MDR1</i>				
	A-392G	G516T	A785G	T-129C	C1236T	G2677T/A	C3435T	
Genotype								
Homozygous wild-type	12	32	31	41	39	GG	47	45
Heterozygous	36	34	35	24	28	GT	19	21
Homozygous variant	22	4	4	5	3	GA	2	4
						TT	2	
						TA	0	
						AA	0	
Total	70	70	70	70	70		70	70
Genotype Frequencies								
Homozygous wild-type	17.14%	45.71%	44.29%	58.57%	55.71%	GG	67.14%	64.29%
Heterozygous	51.43%	48.57%	50.00%	34.29%	40.00%	GT	27.14%	30.00%
Homozygous variant	31.43%	5.71%	5.71%	7.14%	4.29%	GA	2.86%	5.71%
						TT	2.86%	
						TA	0.00%	
						AA	0.00%	
Allele Frequencies								
Wild-Type	42.86%	70.00%	69.29%	75.71%	75.71%	G	82.14%	79.29%
Variant	57.14%	30.00%	30.71%	24.29%	24.29%	T	16.43%	20.71%
						A	1.43%	

Table 4.03: Genotype and allele frequencies in the Xhosa subpopulation of the patient cohort

	<i>CYP3A4</i>	<i>CYP2B6</i>		<i>MDR1</i>				
	A-392G	G516T	A785G	T-129C	C1236T	G2677T/A	C3435T	
Genotype								
Homozygous wild-type	5	47	48	74	84	GG	105	91
Heterozygous	40	58	57	34	27	GT	5	20
Homozygous variant	67	7	7	4	1	GA	2	1
						TT	0	
						TA	0	
						AA	0	
Total	112	112	112	112	112		112	112
Genotype Frequencies								
Homozygous wild-type	4.46%	41.96%	42.86%	66.07%	75.00%	GG	93.75%	81.25%
Heterozygous	35.71%	51.79%	50.89%	30.36%	24.11%	GT	4.46%	17.86%
Homozygous variant	59.82%	6.25%	6.25%	3.57%	0.89%	GA	1.79%	0.89%
						TT	0.00%	
						TA	0.00%	
						AA	0.00%	
Allele Frequencies								
Wild-Type	22.32%	67.86%	68.30%	81.25%	87.05%	G	96.88%	90.18%
Variant	77.68%	32.14%	31.70%	18.75%	12.95%	T	2.23%	9.82%
						A	0.89%	

As is evident in Tables 4.02 and 4.03, there exist marked differences in genotype and allele frequencies between the Mixed-Ancestry and Xhosa subpopulations. Although the G516T and A785G SNPs in *CYP2B6* have similar variant allele frequencies ($\pm 30\%$) and genotype frequency distributions within both subpopulations, there are considerable differences in the genotype and allele frequencies of the other SNPs.

The largest difference in SNP variant frequency is that of A-392G in *CYP3A4* – an approximate 20% disparity between the Mixed-Ancestry and Xhosa subpopulations (57.14% vs 77.68%, respectively). The two subpopulations demonstrated a consequent marked difference in their respective genotype frequencies for this polymorphism, with almost double the proportion of homozygous variant patients within the Xhosa subpopulation (59.82%) than in the Mixed-Ancestry subpopulation (31.43%). Furthermore, there were considerable differences in genotype and allele frequencies of the T-129C, C1236T, G2677T/A and C3435T polymorphisms in the *MDR1* gene.

4.5 HWE ANALYSIS

After determination of the SNP genotype and allele frequencies within the Mixed-Ancestry and Xhosa subpopulations, testing for HWE could be performed. The results of the subsequent Fisher's exact test for HWE using TFPGA in the Mixed-Ancestry and Xhosa patients of the patient cohort are presented in Tables 4.04 and 4.05, respectively.

Table 4.04: Fisher's exact test for HWE in the Mixed-Ancestry subpopulation of the patient cohort

	<i>CYP3A4</i>	<i>CYP2B6</i>		<i>MDR1</i>				
	A-392G	G516T	A785G	T-129C	C1236T	G2677T/A	C3435T	
Observed Genotype								
Homozygous wild-type	12	32	31	41	39	GG	47	45
Heterozygous	36	34	35	24	28	GT	19	21
Homozygous variant	22	4	4	5	3	GA	2	4
						TT	2	
						TA	0	
						AA	0	
Total	70	70	70	70	70		70	70
Expected Genotype								
Homozygous Wild-type	12.86	34.30	33.60	40.13	40.13	GG	47.23	44.00
Heterozygous	34.29	29.40	29.79	25.74	25.74	GT	18.89	22.99
Homozygous Variant	22.86	6.30	6.60	4.13	4.13	GA	1.89	3.00
						TT	1.64	
						TA	0.33	
						AA	0.01	
<i>p</i> -value	0.8085	0.2610	0.2580	0.5275	0.7441		1.0000	0.4695

Table 4.05: Fisher's exact test for HWE in the Xhosa subpopulation of the patient cohort

	<i>CYP3A4</i>	<i>CYP2B6</i>		<i>MDR1</i>				
	A-392G	G516T	A785G	T-129C	C1236T	G2677T/A	C3435T	
Observed Genotype								
Homozygous wild-type	5	47	48	74	84	GG	105	91
Heterozygous	40	58	57	34	27	GT	5	20
Homozygous variant	67	7	7	4	1	GA	2	1
						TT	0	
						TA	0	
						AA	0	
Total	112	112	112	112	112		112	112
Expected Genotype								
Homozygous Wild-type	5.58	51.57	52.25	73.94	84.88	GG	105.11	91.08
Heterozygous	38.84	48.86	48.50	34.13	25.25	GT	4.84	19.84
Homozygous Variant	67.58	11.57	11.25	3.94	1.88	GA	1.94	1.08
						TT	0.06	
						TA	0.65	
						AA	0.01	
<i>p</i> -value	1.0000	0.0809	0.0823	1.0000	0.6896		1.0000	1.0000

All seven SNPs within both the Mixed-Ancestry and Xhosa subpopulations of the patient cohort were found to be in HWE. Interestingly, however, when the Mixed-Ancestry and Xhosa patients were grouped together into a single cohort and subsequently tested for HWE by means of Fisher's exact test using TFPGA, the G516T and A785G polymorphisms within the *CYP2B6* gene were not in HWE ($p = 0.0245$ for each of the SNPs). The results of testing for HWE using TFPGA were confirmed to be accurate by subsequent verification of the results with the GENEPOP population genetics software package.

4.6 HAPLOTYPE ANALYSIS

4.6.1 The *CYP2B6* Gene

The results of haplotype analysis of the G516T and A785G SNPs within the *CYP2B6* gene indicate an exceedingly high level of LD between these two loci in both the Mixed-Ancestry and Xhosa subpopulations. The r^2 -values, LOD scores and most prevalent haplotypes (frequency > 0.01) of these two polymorphisms within the Mixed-Ancestry and Xhosa subpopulations are presented in Table 4.06.

Table 4.06: Haplotype analysis of G516T and A785G in *CYP2B6* in the Mixed-Ancestry and Xhosa subpopulations of the patient cohort

	Mixed-Ancestry	Xhosa
r^2 -value	0.97	0.98
LOD score	24.84	41.31
GA	69.30%	67.90%
TG	30.00%	31.70%

As evident in Table 4.06, the r^2 -value and LOD scores within both subpopulations are considerably higher than 0.4 and 3, respectively, thus indicating a significantly high level of LD between these two loci in both subpopulations of the patient cohort. In fact, only a single patient within the Mixed-Ancestry subpopulation was found with a copy of the A785G variant allele without a concurrent occurrence of the G516T variant allele (heterozygous for the A785G variant allele while homozygous for the G516T wild-type allele). Similarly, only a single patient within the Xhosa subpopulation was found with the G516T variant allele without a concurrent occurrence of the A785G variant allele (heterozygous for the G516T variant allele while homozygous for the A785G wild-type allele). In other words, all patients within both subpopulations (except for a single patient in each) exhibited an identical genotype for both

CYP2B6 polymorphisms (homozygous wild-type for both polymorphisms, heterozygous for both polymorphisms, or homozygous variant for both polymorphisms) – the phase of heterozygous individuals was thus almost certainly deducible – thus indicating an extremely high level of LD between these two loci.

The occurrence and frequency of the most prevalent haplotypes (frequency > 0.01) of the G516T and A785G SNPs within *CYP2B6* in the Mixed-Ancestry and Xhosa subpopulations, as determined with Haploview, version 3.31, were confirmed to be accurate by subsequent verification of the results with SNP-HAP, version 1.3.1.

4.6.2 The *MDR1* Gene

The results of haplotype analysis of the T-129C, C1236T, G2677T and C3435T SNPs within the *MDR1* gene varied markedly between the Mixed-Ancestry and Xhosa subpopulations. Haplotype analysis indicates the presence of LD between three of the four loci within the Mixed-Ancestry subpopulation and the absence of LD between any of the four loci within the Xhosa subpopulation.

Haplotype analysis of the *MDR1* gene in the Mixed-Ancestry subpopulation indicates an absence of LD between the -129 and 1236 (r^2 -value = 0.010; LOD score = 0.12), 2677 (r^2 -value = 0.063; LOD score = 0.57) and 3435 loci (r^2 -value = 0.001; LOD score = 0.01). There thus appears to be no LD between the -129 locus and any of the three other loci within *MDR1* in the Mixed-Ancestry subpopulation. However, there does appear to be strong LD between the 1236 and the 2677 loci (r^2 -value = 0.535; LOD score = 9.44), as well as a significant indication of LD between the 1236 and 3435 loci (r^2 -value = 0.395; LOD score = 6.52). Also, there appears to be a significant level of LD between the 2677 and 3435 loci (r^2 -value = 0.437; LOD score = 7.52) within the Mixed-Ancestry subpopulation. The most prevalent haplotypes (frequency > 0.01) of the SNPs within the *MDR1* gene that displayed LD (C1236T, G2677T, C3435T) within the Mixed-Ancestry subpopulation are presented in Table 4.07.

Table 4.07: The most prevalent haplotypes of C1236T, G2677T and C3435T in *MDR1* in the Mixed-Ancestry subpopulation of the patient cohort

CGC	69.20%
TTT	13.80%
TGC	6.40%
CGT	5.00%
TGT	2.50%
TTC	2.20%

The occurrence and frequency of the most prevalent haplotypes (frequency > 0.01) of the C1236T, G2677T and C3435T SNPs within *MDR1* in the Mixed-Ancestry subpopulation, as determined with Haploview, version 3.31, were confirmed to be accurate by subsequent verification of the results with SNP-HAP, version 1.3.1.

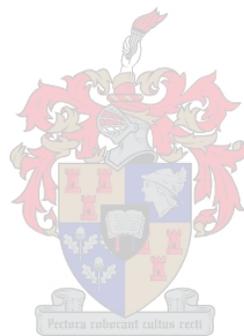
Haplotype analysis of the *MDR1* gene in the Xhosa subpopulation indicates an absence of LD between any of the four loci examined in this study. The -129 locus demonstrated no LD with 1236 (r^2 -value = 0.009; LOD score = 0.17), 2677 (r^2 -value = 0.005; LOD score = 0.45) or 3435 loci (r^2 -value = 0.025; LOD score = 0.58). The 1236 locus demonstrated no LD with the 2677 locus (r^2 -value = 0.037; LOD score = 0.83) and perhaps a weak level of LD with the 3435 locus (r^2 -value = 0.175; LOD score = 3.64). Furthermore, the 2677 locus demonstrated a lack of LD with the 3435 locus (r^2 -value = 0.058; LOD score = 1.16).

4.7 ANOVA TESTING FOR SIGNIFICANCE

Univariate two-way ANOVA testing for significance of the change in CD4-cell count after six months of ART detected no discernible effect of patient ethnicity on immune response to ART. Therefore, there were no statistically significant differences in the levels of immune recovery after the initiation of ART between the Mixed-Ancestry and Xhosa subpopulations of the patient cohort. Due to this lack of any effect of ethnicity on immune response to ART, one-way ANOVA testing of the change in CD4-cell count could subsequently be performed on the entire patient cohort as a whole (i.e. the Mixed-Ancestry and Xhosa subpopulations could be pooled together).

Univariate one-way ANOVA testing revealed no statistically significant association between the A-392G SNP in *CYP3A4* or the G516T and A785G SNPs in *CYP2B6* and changes in CD4-cell

count after six months of ART. Furthermore, no association was detected between the C1236T and C3435T SNPs in *MDR1* and the level of CD4-cell recovery after six months of ART. One-way ANOVA testing did, however, reveal a statistically significant association between the *MDR1* T-129C ($p = 0.03$) and G2677A ($p < 0.01$) polymorphisms and immune recovery after six months of ART. In the case of the T-129C SNP, there appeared to be a trend for a difference in the increase in CD4-cell count between homozygous wild-type and homozygous variant patients, with the latter experiencing a greater increase in CD4-cell count. In the case of the G2677A SNP, there appeared to be statistically significant differences in the increase in CD4-cell count between homozygous wild-type and 2677GA heterozygous patients ($p < 0.01$), and between 2677GT heterozygous patients and 2677GA heterozygous patients ($p < 0.04$) – in both cases, the 2677GA patients demonstrated greater increases in CD4-cell counts.



CHAPTER FIVE

DISCUSSION

5.1 CLINICAL DATA

Analogous to the need for unambiguous and reliable genotypic data, there is a vital requirement within any pharmacogenetic study for comprehensive data of the phenotypic traits that are to be assessed (Howard 2004; Weinshilboum and Wang 2004). This study, examining the genetic basis for any observed differences in ART response, was no exception and required accurate, adequate and comprehensive clinical data of the level of immune recovery and occurrence of ADRs. The reliability and relevance of any ANOVA testing within the study was certainly significantly influenced by the accuracy and comprehensiveness of the employed clinical data.

As mentioned in Section 4.3, although considerable data regarding the patients' response to ART was available in the form of CD4-cell count measurements and indications of any ARV-induced ADRs, many of the patients (38.84%) had to be excluded from ANOVA testing due to insufficient clinical data. Furthermore, the clinical data available for numerous patients that were included in ANOVA testing exhibited significant shortcomings in terms of comprehensiveness. The reasons for these instances of insufficient and inconclusive clinical data appeared to be the result of patient-specific and surveillance-specific factors. Patient-specific factors included poor adherence to treatment, unsanctioned cessation of treatment, death before the commencement of treatment, death shortly after (\pm two weeks) the commencement of treatment, lack of sufficient follow-up visits to the treatment facility, and possible non- and under-reporting of ADR occurrence. Surveillance-specific factors contributing to insufficient and inconclusive clinical data included incomplete patient records and difficulty in locating certain patient information folders.

The frequency of ADRs within the patient cohort was exceedingly low. For example, there were few or no reports of many of the characteristic CNS ADRs associated with efavirenz usage, as discussed in Section 2.2.5.4. In fact, only two patients (1.6%) reported any ADRs which could potentially be categorised as a CNS ADR resultant from efavirenz usage – a single patient experienced nausea while one other experience double vision. Such findings are incongruous

with other studies that have detected efavirenz-induced CNS ADRs in a far greater proportion of patients (9-40%) (Lochet *et al.* 2003; Fumaz *et al.* 2002; Carr and Cooper 2000). Lochet *et al.* (2003) assessed neuropsychiatric symptoms in patients receiving efavirenz and found that 19.3% of their 174 subjects complained of impaired concentration and memory, anxiety and sadness, while 9.2% experienced suicidal ideations. Furthermore, 23% of these 174 patients rated their global neuropsychiatric discomfort as moderate to severe after three months of treatment. Also, Fumaz *et al.* (2002) found that 13% of patients within their study reported mood swings, melancholy and irritability at week 48 of treatment with efavirenz. Therefore, despite these studies having detected efavirenz-induced CNS ADRs in a relatively large proportion of patients, this study failed to detect a similarly high incidence of such ADRs.

The incidence of drug hypersensitivity reactions caused by NNRTIs, normally presenting with a skin rash without the accompanying systemic symptoms (Gallant 2002), was also lower than could reasonably have been expected. Previous studies have found the incidence of such hypersensitivity reactions to be fairly high, occurring in approximately 3-20% of patients (Balzarini 2004; Zapor *et al.* 2004; Coopman *et al.* 1993). In this study, however, only four of 126 (3.2%) patients reported any form of skin rash.

The exceedingly low level of ADR occurrence within the patient cohort was unexpected. It can reasonably be assumed, however, that the individuals within the patient cohort (and the Mixed-Ancestry and Xhosa population groups as a whole) do not display any inherent resistance to ADR occurrence or an improved treatment response profile in terms of drug toxicity. The most likely explanation then for the lower-than-expected incidence of ADRs is under- and non-reporting of ADRs by patients, as discussed further in Section 5.6.2. Therefore, further studies are required in order to assess the occurrence of ARV-related ADRs within the Mixed-Ancestry and Xhosa populations. Such studies will lead to improved and more accurate estimations of ARV ADR incidence rates within these SA population groups.

5.2 SNP GENOTYPE AND ALLELE FREQUENCIES IN THE MIXED-ANCESTRY AND XHOSA SUBPOPULATIONS

The SNP genotype and allele frequencies differ markedly between the Mixed-Ancestry and Xhosa subpopulations of the patient cohort. A possible explanation for this marked difference in variant

allele frequency for this polymorphism between the two subpopulations of the patient cohort is the lineage of the Mixed-Ancestry population. As mentioned in Section 3.1.1, the Mixed-Ancestry population is of Asian, Western European and Southern and Eastern African ancestry (Nurse *et al.* 1995). Furthermore, as can be seen in Table 2.04, the Caucasian and African populations vary dramatically in the frequency of the *CYP3A4* -392G variant allele (2-9.6% and 70-80%, respectively) – perhaps prompting further consideration of the usage and definition of ‘wild-type’ and ‘variant’ allele nomenclature. Therefore, due to the admixture of Caucasian and African populations within the ancestry of the Mixed-Ancestry population, it could be surmised that the resultant frequency of this variant allele within this population would be lower than in African populations but higher than in Caucasian populations. As such, the frequency of the *CYP3A4* -392G variant allele is indeed lower in the Mixed-Ancestry subpopulation of the patient cohort than it is in the Xhosa subpopulation examined in this study, but is markedly higher than in the Caucasian populations examined in previous studies.

Interestingly, both the Mixed-Ancestry and Xhosa subpopulations of the patient cohort display a lower frequency of homozygous variant genotypes for the *CYP2B6* G516T and A785G SNPs than could be expected, considering the results of previous studies of African-Americans. As mentioned in Section 2.3.1.4 and evident in Table 2.04, there exists a significant difference in the frequency of the 516TT genotype in African-Americans (20%) and Caucasians (3%) (Haas *et al.* 2005; 2004). As such, the frequency of the 516TT genotype within a black African population, such as the Xhosa, could be expected to be more similar to that of African-Americans than Caucasians. However, the frequency of the 516TT genotype in the Xhosa subpopulation (5.71%) is considerably lower than the frequency previously detected in African-Americans (20%) and is in fact closer to the frequency within the Caucasian population (3%).

Also worth noting is the exceedingly low frequency of the *MDR1* C3435T variant allele in the Xhosa subpopulation of the patient cohort. Only a single Xhosa individual (0.89%) was found to be homozygous for the variant nucleotide at this position, significantly lower than the previously detected frequency of approximately 3-4% within other African populations (Ameyaw *et al.* 2001; Schaeffeler *et al.* 2001).

The lower than expected frequencies of the *CYP2B6* 516TT genotype and *MDR1* C3435T variant allele in the Xhosa subpopulation of the patient cohort thus demonstrates a vitally important consideration in all pharmacogenetic studies and pharmacological treatment regimens: the frequency of a genetic variant or genotype cannot be extrapolated from one population to another population of seemingly similar ethnicity and/or genetic make-up. This is all the more pertinent in the case of the *CYP2B6* G516T SNP, considering the functional and clinical effects of this polymorphism and 516TT genotype (Ribaudó *et al.* 2006; Rodríguez-Nóvoa *et al.* 2006, 2005; Rotger *et al.* 2005; Haas *et al.* 2004; Tsuchiya *et al.* 2004; Lang *et al.* 2001) and the documented effects of the C3435T polymorphism on PGP activity and immune recovery in response to ART (Verstuyft *et al.* 2003; Fellay *et al.* 2002; Johne *et al.* 2002; Kurata *et al.* 2002; Hoffmeyer *et al.* 2000).

5.3 HWE ANALYSIS

As mentioned in Section 4.5, the genotype frequencies of G516T and A785G in *CYP2B6* within the patient cohort as a whole did not conform to HWE. This may appear to be paradoxical as the genotype frequencies do obey the Hardy-Weinberg principle within each of the two subpopulations comprising the patient cohort. However, the reason for departure from HWE in the patient cohort as a whole is most likely due to the fact that, as evident in Tables 4.01 and 4.02, the Mixed-Ancestry and Xhosa subpopulations differ in respect of genotype and allele frequencies for these two polymorphisms, albeit to a relatively minor degree.

The admixture of these differing genotype and allele frequencies into a single patient cohort thus leads to statistically significant deviations of observed genotype frequencies from expected genotype frequencies (i.e. a lack of conformity to HWE). This effectively illustrates the importance of analysing a patient cohort or study population in terms of its subpopulations (based on ethnicity/genetic make-up), due to the possibility of varying genotype and allele frequencies within them which can thereby skew the results of subsequent statistical analyses and the resultant interpretations and conclusions.

5.4 HAPLOTYPE ANALYSIS

5.4.1 The *CYP2B6* Gene

The G516T and A785G SNPs in the *CYP2B6* gene exhibit an extremely high level of LD in both the Mixed-Ancestry and Xhosa subpopulations of the patient cohort. Indeed, the exceptionally high LOD score values of 24.84 and 41.31 and r^2 -values of 0.97 and 0.98 in the Mixed-Ancestry and Xhosa subpopulations, respectively, are clearly indicative of an exceedingly high level of LD between these two *CYP2B6* polymorphisms in both of these subpopulations. These findings are congruent with those of other studies, such as Ariyoshi *et al.* (2001) in Japanese individuals and Lang *et al.* (2001) in German Caucasian individuals, which have also documented a high level of LD between these two polymorphisms. This high level of LD between the 516 and 785 loci is not entirely unexpected, considering their relative close proximity to one another within the gene: the two loci are in consecutive exons (exons 4 and 5, respectively) and are separated by only 2 423 bp. This high level of LD between these two polymorphisms may simplify the method of genotyping these two loci, as it may be feasible to restrict genotyping to only one of the two SNPs, thereby inferring the genotype of the other polymorphism due to this extremely high level of LD.

5.4.2 The *MDR1* Gene

The results of haplotype analysis of the *MDR1* T-129C, C1236T, G2677T and C3435T SNPs in the Mixed-Ancestry subpopulation of the patient cohort are in many ways congruent with the findings of other studies in numerous other populations. In accordance with the findings of Anglicheau *et al.* (2003) (French Caucasian), Horinouchi *et al.* (2002) (Japanese), Siegmund *et al.* (2002) (German Caucasian) and Kim *et al.* (2001) (Caucasian American and African-American), significant LD was found to exist between the G2677T and C3435T polymorphisms. Furthermore, LD was found to exist between the C1236T, G2677T and C3435T polymorphisms, as was also detected by Anglicheau *et al.* (2003) (French Caucasian), Johne *et al.* (2002) (German Caucasian) and Kim *et al.* (2001) (Caucasian American and African-American).

As discussed in Section 2.3.2, the seemingly functional effects of the C1236T, G2677T/A and C3435T SNPs may be due to their being in LD with polymorphisms within regulatory regions of the *MDR1* gene: a potential candidate for such LD is thought to be the non-coding T-129C SNP in the untranslated exon 1b. However, congruent with the findings of Horinouchi *et al.* (2002) in

Japanese subjects, no LD was found to exist between the T-129C polymorphism and any of the other three *MDR1* polymorphisms examined in this study. Therefore, any association of the C1236T, G2677T/A and/or C3435T polymorphisms with altered *MDR1* expression and/or PGP function in the Mixed-Ancestry or Xhosa population groups is unlikely to be attributable to LD between any of these polymorphisms and the T-129C SNP.

The fact that LD within the *MDR1* gene is observed in the Mixed-Ancestry subpopulation of the patient but not within the Xhosa subpopulation is perhaps explainable in terms of the comparative evolutionary ages of the two populations. LD is considered an indication of the evolutionary age of a population in that if a population is relatively young, then insufficient generations may have passed to disrupt any LD within a gene. Therefore, the presence of LD within *MDR1* in the Mixed-Ancestry subpopulation of the patient cohort may perhaps be attributable to the younger evolutionary age of the population, compared to that of the Xhosa population, and a consequent shorter time for the disruption of any linkage. However, this is a fairly presumptuous explanation that requires further validation. Additional research into the level of LD within *MDR1* in the Mixed-Ancestry and Xhosa populations, as well as in other SA population groups, is therefore warranted. Such further research into gene-based haplotypes will potentially better predict association between genomic variation and ART response and, as is the case of the G516T and A785G SNPs in *CYP2B6* discussed above, simplify genotyping methods.

5.5 ANOVA TESTING FOR SIGNIFICANCE

Univariate two-way ANOVA testing for significance of the change in CD4-cell count after six months of ART detected no discernible effect of patient ethnicity on immune response to ART. This study is thus contradictory to those of Kappelhoff *et al.* (2005), Pfister *et al.* (2003) and Wegner *et al.* (2002), all of which found a statistically significant effect of patient ethnicity on ARV drug response. Possible explanations for these incongruous findings include the relative sample sizes within this study and these previous studies, as well as a greater degree of genetic similarity between the Mixed-Ancestry and Xhosa populations than between those examined in the above-mentioned studies. Therefore, further examination of the level of immune recovery in response to ART in SA population groups is warranted, as statistically significant differences in response between less genetically congruent population groups may be detected (e.g. between Caucasian and Xhosa populations).

Univariate one-way ANOVA testing for significance of the change in CD4-cell count after six months of ART detected a discernible effect of genotype only in the cases of the T-129C ($p = 0.03$) and G2677A ($p < 0.01$) polymorphisms in the *MDR1* gene. In the case of the T-129C SNP, homozygous variant patients experienced the greatest increase in CD4-cell count, while heterozygous patients displayed an intermediate level of immune recovery between that of the homozygous wild-type and variant patients. The T-129C polymorphism has previously been associated with decreased *MDR1* expression in numerous studies (Koyama *et al.* 2006; Tanabe *et al.* 2001). Therefore, it can be hypothesised that the decreased expression of *MDR1* and consequently decreased PGP activity leads to a lower level of intestinal drug efflux ability within individuals carrying the -129C allele. This lower level of intestinal drug efflux ability thus allows for greater drug absorption and bioavailability which, in the case of ART, causes a greater level of suppression of HIV replication and a consequently greater increase in CD-cell count.

This apparent association of the T-129C polymorphism with decreased *MDR1* expression and greater drug bioavailability and CD4-cell recovery is analogous to that previously associated with the C3435T polymorphism in studies by Verstuyft *et al.* (2003), Fellay *et al.* (2002), Johnes *et al.* (2002), Kurata *et al.* (2002), Hoffmeyer *et al.* (2000) and Greiner *et al.* (1999) (see Figures 2.05 and 2.06). The exact mechanism underlying the association between the T-129C SNP and transcriptional activity of *MDR1* is not known (Taniguchi *et al.* 2003). Taniguchi *et al.* (2003) did, however, identify a DNA binding protein which binds to the -129T wild-type allele but not to the -129C variant allele. The reduced *MDR1* transcriptional activity and consequent greater level of immune recovery in response to ART associated with this polymorphism is therefore most probably attributable to the inability of a transcriptional factor from binding to the variant allele. However, the possibility of LD between this polymorphism with other regulatory SNPs cannot be ruled out (Taniguchi *et al.* 2003), as neither can the possibility of association of the T-129C SNP (or other regulatory polymorphisms) with different epigenetic states that might modulate transcriptional activity (Tada *et al.* 2000).

In the case of the G2677A SNP in the *MDR1* gene, there appeared to be a statistically significant and marked increase in CD4-cell count within 2677GA heterozygous patients in comparison to patients with the homozygous wild-type or 2677GT heterozygous genotypes. This increased

level of immune response to ART may be attributable to decreased PGP activity resultant from the 2677A polymorphism and consequent substitution of the Ala at position 893 within PGP with Thr. This substitution of Ala (a hydrophobic amino acid) with Thr (a polar, hydrophilic amino acid) could thus have significant consequences on the functionality and/or activity of the encoded PGP gene product. It should be noted, however, that the G2677T polymorphism results in an Ala to Ser substitution, which is also a replacement of a hydrophobic amino acid with a polar, hydrophilic amino acid. Therefore, the exact cause of the functional differences between the two variant alleles on the encoded gene product remains unclear.

The altered PGP activity resultant from the 2677A polymorphism potentially lowers intestinal drug efflux ability, thereby increasing drug bioavailability and immune response to ART (i.e. similar to the effects of the T-129C polymorphism discussed above). Tanabe *et al.* (2001) found that *MDR1* expression levels within placental tissue were lower in 2677 homozygous variant (T or A) individuals than in heterozygous or homozygous wild-type individuals. Furthermore, a number of other studies have noted an effect of the 2677A variant on PGP activity and/or drug disposition (Yi *et al.* 2004; Illmer *et al.* 2002; Moriya *et al.* 2002), albeit with conflicting results. The reasons for the discrepancies concerning *MDR1* polymorphisms and PGP functionality and activity are unclear. Further research into the functional effects of *MDR1* sequence variants is thus warranted.

Despite the statistically significant increases in CD4-cell count after six months of ART associated with the T-129C and G2677A polymorphisms, the number of individuals within the patient cohort carrying either of the polymorphisms was relatively limited. For example, few patients were homozygous for the T-129C variant (n = 9) or heterozygous for the G2677A variant (n = 4). Therefore, although this is the first pharmacogenetic study to document an effect of the T-129C and G2677A SNPs on immune recovery in response to ART, any conclusions drawn from this study with regards to the possible effects of these two polymorphisms need to be viewed in light of the limited sample sizes. Further research with larger patient cohorts of Mixed-Ancestry and Xhosa individuals (along with other SA population groups) is thus warranted to elucidate the functional effects of these polymorphisms on *MDR1* expression and PGP activity, and consequent ART response.

5.6 LIMITATIONS OF THE STUDY

5.6.1 Sample Size

A distinct limiting factor in the ability of this study to detect possible association of any of the seven SNPs with altered ARV efficacy and/or related ADR occurrence is the small number of patients examined. Although blood samples were collected from a total of 225 patients, the genomic DNA isolation process provided only 206 DNA samples of sufficient yield and purity for subsequent PCR-RFLP analysis. However, of these 206 DNA samples, only 126 samples remained after patients were excluded due to poor treatment compliance or other exclusion criteria, death before or shortly after (\pm two weeks) the commencement of treatment, or insufficient clinical data regarding treatment response. Furthermore, the ethnicities of sixteen of these 126 patients were unknown, leaving only 39 Mixed-Ancestry and 71 Xhosa patients for subsequent ANOVA testing. Therefore, the already limited initial number of whole blood samples that was collected was reduced by exclusion criteria, incomplete clinical data and the division of samples into the separate population groups. This reduction in sample size is an important consideration and thus warrants collection of greater initial sample sizes within future ARV pharmacogenetic studies.

Due to the small number of patients within each of the population groups in the study and low variant allele frequencies for certain of the SNPs, there were extremely few individuals of particular genotypes. For example, only a single patient within the Xhosa subpopulation was found to be homozygous for the C1236T and C3435T SNPs within the *MDR1* gene, while none were found to be homozygous for the *MDR1* G2677T polymorphism. This limited number of individuals of particular genotypes can be considered a significant limiting factor in the detection of possible association of such genotypes with altered ARV drug response profiles.

5.6.2 ADR Reporting by Patients

A potentially drastic limiting factor of the study is the possibility of under- and non-reporting of ADRs. Patients who qualified for treatment were most often at a late clinical stage of the disease and were thus experiencing a host of conditions and illnesses resultant from their severely immunocompromised state of health (Appendix B). These conditions and illnesses may overshadow any ADRs that may otherwise (i.e. in a healthy, non-immunocompromised individual) be quite noticeable and apparent, thus masking the occurrence and prevalence of

ARV-related ADRs in HIV-positive patients who qualify for ART. However, this reason alone is unlikely to fully account for the seemingly low incidence levels of ART-induced ADRs within the study, as other studies with patients of comparable clinical stage of HIV-infection have noted significantly higher incidence rates, as discussed in Section 5.1.

Cultural differences between population groups could also be a likely explanation for the seemingly low level of ADR occurrence in the study, as could conditions and methods of patient management at the clinics from which the patients in this study were recruited. Individuals of Mixed-Ancestry and Xhosa ethnicity may be less likely to report any noticeable ADRs than individuals of Caucasian ethnicity (particularly those of developed nations in which many pharmacogenetic studies are performed). Furthermore, the high throughput and limited resources of the clinics from which the patients within the study were recruited may further limit the power of detection of any ART-induced ADRs that may be apparent within many of the patients.

5.6.3 HIV Genetic Factors

Although this study examined host genetic factors that may influence ART response, an important parameter which was not addressed that can significantly affect the clinical response to ART, particularly in terms of immune recovery, is the genetic make-up of HIV (Luber 2005). Patients who are receiving ART may experience a lack of immune recovery due not to altered levels of drug metabolism or transport, but as a result of drug resistance of their particular strain of HIV to the prescribed ARV drugs. Therefore, some patients within this study may experience a decreased level of immune response to ART not attributable to their *CYP3A4*, *CYP2B6*, or *MDR1* genotype, but to HIV genetic factors that cause drug resistance and a consequent lack of suppression of viral replication and subsequent immune recovery. This is a potentially significant confounding factor in this and similar studies and warrants further attention and consideration.

5.6.4 Inherent Complexities within the Study

There were a number of factors within this study that significantly contributed to the complexity of the study design as well as the interpretation of the results and the subsequent conclusions that could be drawn. First and foremost is the complexity of the ART regimens. The treatment regimens typically consisted of multiple drugs from three different ARV drug classes. As

discussed in Section 2.3.1, two of these drug classes (NNRTIs and PIs) are metabolised by CYP enzymes while the other (NRTIs) is metabolised by intracellular enzymes such as nucleoside kinases, 5'-nucleotidases, purine and pyrimidine nucleoside monophosphate kinases, and other similar enzymes (Zapor *et al.* 2004; Li and Chan 1999). Furthermore, as indicated in Table 2.03, although the NNRTIs and PIs used within this study (efavirenz, nevirapine and lopinavir/ritonavir, respectively) are metabolised by CYP enzymes, the metabolism of efavirenz and nevirapine is carried out to varying degrees by CYP3A4 and CYP2B6. In other words, although both drugs are metabolised by both enzymes, each enzyme contributes to each drug's metabolism to a different degree. Therefore, this multitude of drugs from multiple drug classes with multiple metabolic pathways makes any attempt at an elucidation of genetic causes of differential response extremely challenging.

Perhaps the most significantly complex and limiting factor of the study is the immense biochemical complexity of drug action and response. The absorption, distribution, metabolism and excretion of an exogenous compound such as a pharmaceutical drug are complex processes which undoubtedly involve a great multitude of genes (Nebert and Vesell 2004). Drug response is thus like many other phenotypic traits in that it is a complex, multi-factorial and epistatic trait and as such is seldom the product of, or affected by, a single gene or gene variant (Nebert 2000). Therefore, although this study examined numerous SNPs within several genes that play significant roles in ARV metabolism and transport, the ultimate effects of these polymorphisms may well be overshadowed by other genetic polymorphisms, both within the genes examined in this study as well as in other relevant genes.

CHAPTER SIX

CONCLUSIONS AND FUTURE RESEARCH

The general aim and all of the specific aims of the study, as listed in Section 2.6, were successfully achieved. A number of conclusions can be drawn from the accomplishment of these aims:

- there exists an essential requirement within any pharmacogenetic study (or any other association study) for accurate, complete and comprehensive data of the phenotypic traits that are to be investigated;
- ARV pharmacogenetic studies need to incorporate large patient cohorts, due to the possibility of a significant number of individuals having to be excluded due to numerous exclusion criteria and unavailable or incomplete clinical data;
- the frequencies of genetic sequence variants can vary markedly and unexpectedly between different population groups, thus making extrapolation of data from one population group to another unreliable and necessitating analysis of a patient cohort in terms of the separate population groups of which it is comprised;
- a high level of LD exists between the 516 and 785 loci within the *CYP2B6* gene in both the Mixed-Ancestry and Xhosa populations (perhaps allowing inference of the genotype of either polymorphism based on the genotype of the other, thereby necessitating the genotyping of only one of these two polymorphisms in future studies);
- significant LD exists between the 1236, 2677 and 3435 loci within the *MDR1* gene in the Mixed-Ancestry population but not in the Xhosa population, perhaps attributable to the comparative ages of the two populations;
- univariate two-way ANOVA testing for significance of the change in CD4-cell count after six months of ART detected no discernible effect of patient ethnicity on immune response to ART;
- univariate one-way ANOVA testing for significance of the change in CD4-cell count after six months of ART detected a discernible effect of genotype only in the cases of the T-129C ($p = 0.03$) and G2677A ($p < 0.01$) polymorphisms in the *MDR1* gene.

These conclusions constitute invaluable insights into the pharmacogenetics of ART in the Mixed-Ancestry and Xhosa populations of SA. Although the findings regarding the genetic basis of immune recovery in response to ART (i.e. T-129C and G2677A within *MDR1*) are not entirely conclusive and require further validation, they represent important initial steps towards a better understanding of the pharmacogenetics and customisation of ART in SA. Furthermore, these findings provide an excellent foundation for future studies. Areas of interest in such studies include:

- further examination of incidence levels of ARV-related ADRs within the Mixed-Ancestry and Xhosa populations;
- additional research into the level of LD within *MDR1* in the Mixed-Ancestry and Xhosa populations, as well as in other SA population groups;
- further examination of the level of immune recovery in response to ART in the Mixed-Ancestry and Xhosa populations, as well as in other SA population groups;
- further research to elucidate the functional effects of the T-129C and G2677T/A polymorphisms on *MDR1* expression and/or PGP activity;
- further research to confirm the effects of the T-129C and G2677A polymorphisms within *MDR1* on immune recovery in response to ART;
- further ARV pharmacogenetic studies with larger patient cohorts of Mixed-Ancestry and Xhosa individuals, as well as of other SA population groups.

Although the above-mentioned avenues of further research should help improve the understanding of the genetic basis for varied ARV drug response, currently employed technologies remain a limiting factor. Future technological advances, such as more affordable and faster whole-genome sequencing, SNP genotyping and genome-wide haplotype mapping, as well as advances in gene expression and proteomic analyses, are perhaps required before a complete understanding of the genetic basis of ARV drug response (and drug response in general) can be achieved. Despite such technological limitations, however, further work on the above-listed areas of interest should drastically aid in improving the understanding of pharmacogenetics of ART in SA. Such improvements in the understanding of ART pharmacogenetics will represent further steps towards improved ARV drug regimens with enhanced immune recovery rates, fewer ADRs and decreased levels of drug resistance.

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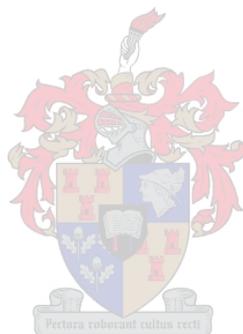
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APPENDICES

APPENDIX A

Information and Informed Consent Document for DNA Analysis and Storage (English)

FACULTY OF HEALTH SCIENCES
STELLENBOSCH UNIVERSITY

INFORMATION AND INFORMED CONSENT DOCUMENT FOR DNA ANALYSIS AND STORAGE

TITLE OF THE RESEARCH PROJECT: Molecular genetic analysis of varied clinical responses to antiretroviral drug compounds in South African populations – a pharmacogenetics study.....

REFERENCE NUMBER:

PRINCIPAL INVESTIGATOR:

Address:



Professor Louise Warnich.....
Department of Genetics
Stellenbosch University.....
Private Bag X1
Matieland.....
7602.....

DECLARATION BY OR ON BEHALF OF PARTICIPANT:

I, THE UNDERSIGNED, (name)

[ID No:] the participant/*in my capacity as of
the participant [ID No:] of.....

..... (address).

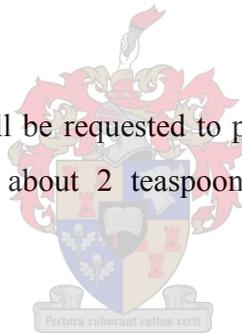
A. HEREBY CONFIRM AS FOLLOWS:

1. I/*The participant was invited to participate in the above-mentioned research project of the Department of Genetics, Stellenbosch University.
2. The following aspects have been explained to me/*the participant:

2.1 **Aim:** This project aims to better understand the genetic causes of observed differences in antiretroviral drug response and effectiveness in HIV-positive persons belonging to South African populations.

Additionally, this project aims to collect genetic material (blood and/or buccal swabs) to analyse for certain genetic variants – within the cytochrome P450 (CYP) gene family and MDR1 gene – and to store excess material for future research. When a large group of patients with similar diseases has been collected, meaningful research into the disease processes may become possible.

2.2 **Procedures:** I/*The participant will be requested to provide information about my/*his/*her medical history. Blood (10ml, about 2 teaspoonfuls) will be collected from me/*the participant.



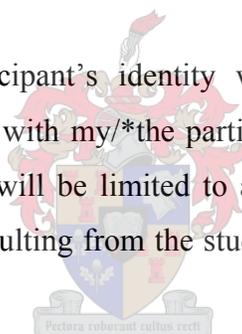
2.3 Genetic considerations:

- The blood or tissue may be used to create a cell line, which can grow indefinitely and can be used to synthesize more of my/*the participant's DNA at any time in the future;
- The DNA may be stored for several years until the technology for meaningful analysis becomes available;
- Your personal results will be made known to you only if they indicate that you may: 1) have a definite risk of developing a particular genetic disorder; 2) have a condition or predisposition to developing a condition that is treatable or avoidable (e.g. by lifestyle modification); 3) need genetic counselling. In addition, I/*the participant authorise(s) the investigator(s) to make the information available to ...Dr Stefan Gebhardt / Dr Nelis Grobbelaar * (*doctor's name*), the doctor involved in my/*the participant's care, as well as to the following family

members.....
..... (names)

- The DNA will be maintained indefinitely, unless I/*the participant request(s) to have it and/*or the stored clinical data destroyed by contacting the investigator conducting the present study,Professor Louise Warnich...(name)...(021).808.5888...(telephone number) or the Chairperson of the Committee for Human Research at(021) 938 9207.....(telephone number) if the former cannot be located;
- The analyses in the current study are specific to the condition mentioned above and cannot determine the entire genetic make-up of an individual;
- Genetic analyses may not be successful in revealing additional information regarding some families or some family members;
- Even under the best conditions, current technology of this type is not perfect and could lead to unreliable results.

2.4 Confidentiality: My/*The participant's identity will be kept confidential throughout. Information will not be associated with my/*the participant's name. The research staff will use only a coded number, access will be limited to authorized scientists and any scientific publications, lectures or reports resulting from the study will not identify me/*the participant by name.



2.5 Voluntary participation: Participation is voluntary and I/*the participant may decline participation, or withdraw from the study at any time without any loss of benefits to which I am/*the participant is otherwise entitled. Future management at this or any other institution will not be compromised by refusal or withdrawal.

2.6 Risks: There are no more than minimal medical or psychological risks associated with this study:

- I/*The participant may feel some pain associated with having blood withdrawn from a vein and may experience discomfort, bruising and/or slight bleeding at the site;
- As some insurance companies may mistakenly assume that my/*the participant's participation is an indication of a higher risk of a genetic disease which could hurt my/*the participant's access to health or other insurance, no information about me/*the participant

or my/*the participant's family will be shared with such companies as this investigation cannot be regarded as formal genetic testing for the presence or absence of certain genetic variants.

2.7 Benefits:

- Although there may not be any direct benefits to me/*the participant by participating at this stage, family members and future generations may benefit if the researchers succeed in scientifically delineating certain disease states and drug responses further. The identification of the genes, and their genetic variants, involved in such drug responses, could in the end lead to the development of methods for prevention and to forms of new treatment aimed at curing or alleviating these conditions;
- In the unlikely event that the research may lead to the development of commercial applications, I/*the participant or my/*the participant's heirs will not receive any compensation, but profits will be reinvested into supporting the cause of further research which may bring benefits to my/*the participant's family and to the community, such as health screening, medical treatment, educational promotions, etc;

2.8 Permission for further studies: Before my/*the participant's material is used in further projects in the future, the written approval of the Committee for Human Research, Faculty of Health Sciences, will be obtained.

3. The information conveyed above was explained to me/*the participant by (name) in Afrikaans/English/Xhosa/Other* and I am/*the participant is fluent in this language/* was translated and explained by (name).

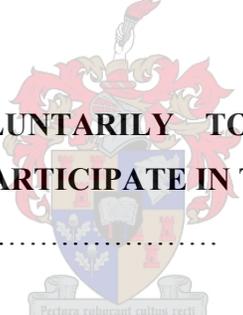
4. I/*The participant was afforded adequate time to pose any questions and all questions were answered to my/*the participant's full satisfaction.

5. I/*The participant was not pressurized to participate.

6. I/*The participant will not be paid for participation, but reimbursement of travel costs will be considered (if applicable).
7. I/*The participant will not incur any additional costs through participation.
8. I/*The participant have/*has received a copy of this document for my/*his/*her records.
9. The Committee for Human Research, Faculty of Health Sciences, Stellenbosch University, has approved recruitment and participation of individuals in this study on the basis of:
 - Guidelines on Ethics for Medical Research of the SA Medical Research Council;
 - Declaration of Helsinki;
 - International Guidelines : Council for International Organisations of Medical Sciences (CIOMS);
 - Applicable RSA legislation.

B. HEREBY CONSENT VOLUNTARILY TO PARTICIPATE/*ALLOW THE POTENTIAL PARTICIPANT TO PARTICIPATE IN THIS STUDY:

Signed/*Confirmed at on 20

(place)  *(date)*

.....

*Signature or right thumb print of participant/*representative of participant* *Signature of witness*

DECLARATION BY OR ON BEHALF OF INVESTIGATOR(S):

I, *(name)* declare that I explained the information in this document to *(name of the patient/*participant)* and/or his/her representative *(name of the representative)*;

she/*he was encouraged and afforded adequate time to ask me any questions; this conversation was conducted in Afrikaans/*English/*Xhosa/*Other and no translator was used/*was translated into *(language)* by *(name)*.

Signed at on 20

(place)

(date)

Signature of investigator/representative of investigator

Signature of witness

DECLARATION BY TRANSLATOR:

I, (name) confirm that I

- translated the contents of this document from English into (language);
explained the contents of this document to the participant/*the participant's representative;
also translated the questions posed by (name) as well as the answers given by (name), and
conveyed a factually correct version of what was related to me.

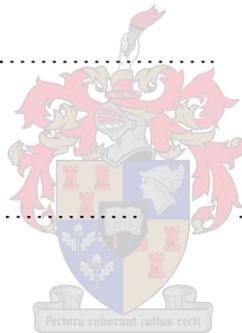
Signed at on 20

(place)

(date)

Signature of translator

Signature of witness



IMPORTANT MESSAGE TO PARTICIPANT/*REPRESENTATIVE OF PARTICIPANT:

Dear participant/*representative of participant,

Thank you very much for your/*the participant's participation in this study. Should, at any time during the study,

- an emergency arise as a result of the research, or
you require any further information with regard to the study, kindly contactDr Stefan Gebhardt.....(name) at ...(021) 872 1711 / 082 781 1516 Department of Obstetrics and Gynaecology, Paarl Hospital, Private Bag X 3012, Paarl, 7620..... (telephone number/address).

*Delete where not applicable

APPENDIX B

WHO Clinical Staging System of HIV and AIDS for Adults and Adolescents

Clinical Stage I

- 1 Asymptomatic
- 2 Persistent generalized lymphadenopathy (PGL)
- 3 Acute retroviral infection (sero-conversion illness)

And/or performance scale 1: asymptomatic, normal activity

Clinical Stage II

- 4 Unintentional weight loss (< 10% of body weight)
- 5 Minor mucocutaneous manifestations (e.g. seborrhoeic dermatitis, prurigo, fungal nail infections, recurrent oral ulcerations, angular cheilitis)
- 6 Herpes zoster within the last five years
- 7 Recurrent upper respiratory tract infection (URTI) (e.g. bacterial sinusitis)

And/or performance scale 2: symptomatic, normal activity

Clinical Stage III

- 8 Unintentional weight loss (> 10% of body weight)
- 9 Unexplained chronic diarrhoea > one month
- 10 Unexplained prolonged fever (intermittent or constant) > one month
- 11 Oral candidiasis (thrush)
- 12 Oral hairy leukoplakia
- 13 Pulmonary tuberculosis (PTB) within the last year
- 14 Severe bacterial infections (e.g. pneumonia, pyomyositis)
- 15 Vulvovaginal candidiasis > one month

And/or performance scale 3: bedridden < 50% of the day during the last month

Clinical Stage IV

- 16 HIV wasting syndrome †
- 17 *Pneumocystis carinii* pneumonia (PCP)

- 18 Central nervous system (CNS) toxoplasmosis
- 19 Cryptosporidiosis with diarrhoea > one month
- 20 Isosporiasis with diarrhoea
- 21 Cryptococcosis – non pulmonary
- 22 Cytomegalovirus (CMV) infection of an organ other than liver, spleen or lymph node
- 23 Herpes simplex virus (HSV) infection, visceral or > one month mucocutaneous
- 24 Progressive multifocal leucoencephalopathy (PML)
- 25 Any disseminated endemic mycosis (i.e. histoplasmosis, coccidiomycosis)
- 26 Candidiasis of oesophagus, trachea, bronchi
- 27 Atypical mycobacteriosis other than tuberculosis (MOTT), disseminated or lungs
- 28 Non-typhoidal Salmonella septicaemia
- 29 Extrapulmonary tuberculosis (ETB)
- 30 Lymphoma
- 31 Kaposi's sarcoma (KS)
- 32 HIV encephalopathy/AIDS dementia complex (ADC) ††
- 33 Invasive cervical carcinoma

And/or performance scale 4: bedridden > 50% of the day during the last month

† HIV wasting syndrome: weight loss of > 10% of body weight, plus either unexplained chronic diarrhoea (> one month) or chronic weakness and unexplained prolonged fever (> one month).

†† HIV encephalopathy: clinical findings of disabling cognitive and/or motor dysfunction interfering with activities of daily living, progressing over weeks to months, in the absence of a concurrent illness or condition other than HIV infection which could explain the findings.

APPENDIX C

Reagents and Solutions

Final volumes, where indicated, are made up with dH₂O.

3.2.1 Genomic DNA Isolation from Whole Blood Samples

Lysis Buffer (1 000 ml)

0.1552 M	NH ₄ Cl	8.3 g	(Mr = 53.49)
0.0110 M	KHCO ₃	1.1 g	(Mr = 100.12)
0.0001 M	EDTA	0.03 g	(Mr = 292.2)

Phosphate Buffered Saline (PBS) (1 000 ml)

0.0268 M	KCl	2 g	(Mr = 74.56)
0.1369 M	NaCl	8 g	(Mr = 58.44)
0.0080 M	Na ₂ HPO ₄	1.14 g	(Mr = 141.96)
0.0015 M	KH ₂ PO ₄	0.2 g	(Mr = 136.09)

Nuclear Lysis Buffer (1 000 ml)

0.0100 M	Tris	1.211 g	(Mr = 121.14)
0.4004 M	NaCl	23.4 g	(Mr = 58.44)
0.0021 M	EDTA	0.6 g	(Mr = 292.2)

10% Sodium Dodecyl Sulphate (SDS) (100 ml)

0.3468 M	SDS	10 g	(Mr = 288.38)
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Proteinase K

10 mg/ml dissolved in ddH₂O

Store at -20°C

Saturated Sodium Chloride (NaCl) Solution (6 M) (100 ml)

6 M	NaCl	35.1 g	(Mr = 58.44)
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3.3.3 Visualisation of PCR Product and REase Digestion Fragments

5X Tris Borate EDTA (TBE) Buffer (1 000 ml)

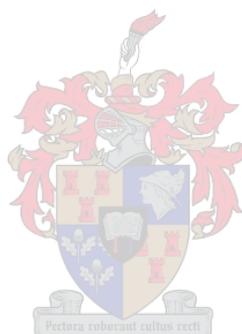
0.4458 M	Tris	54 g	(Mr = 121.14)
0.3639 M	Boric Acid	22.5 g	(Mr = 61.83)
0.0100 M	EDTA	3.7225 g	(Mr = 372.24)

Polyacrylamide (PAA) (5% cross-linkage – acrylamide:bisacrylamide = 19:1; 40% (w/v))

38% (w/v)	acrylamide
2% (w/v)	N, N'-methylenebisacrylamide

PAA Gel (15%)

37.5% (v/v)	PAA (5% cross-linkage – acrylamide:bisacrylamide ratio of 19:1; 40% (w/v))
20% (v/v)	5X TBE Buffer
42.5% (v/v)	dH ₂ O



APPENDIX D

PCR-RFLP Agarose and Polyacrylamide (PAA) Gel Photos

Molecular size marker visible in lane 1 of all gels is O'GeneRuler™ 100 bp DNA ladder
(Fermentas Life Sciences, Burlington, Ontario, Canada).

Annotation used: +/+ = homozygous wild-type; +/- = heterozygous; -/- = homozygous variant; A = adenine variant.

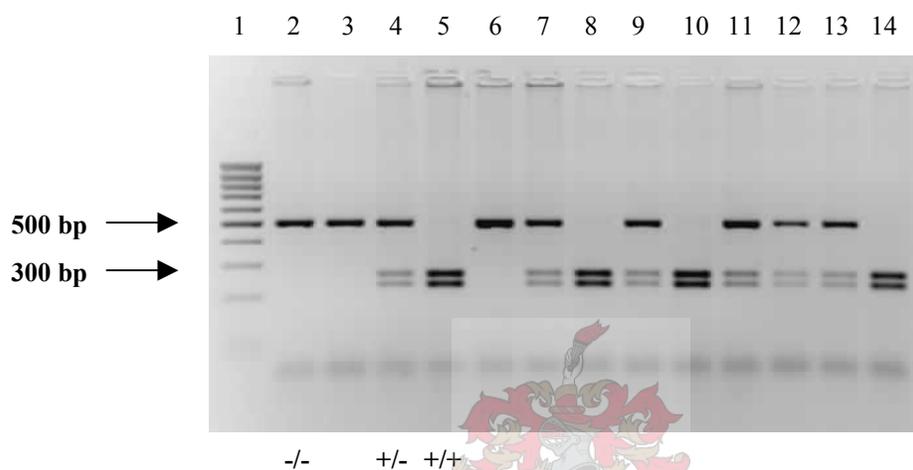


Figure D.01: *Bse*NI REase digestion fragments of the G516T SNP in exon 4 of *CYP2B6*, resolved on a 1.8% (w/v) agarose gel (lane 2 = homozygous variant; lane 4 = heterozygous; lane 5 = homozygous wild-type)

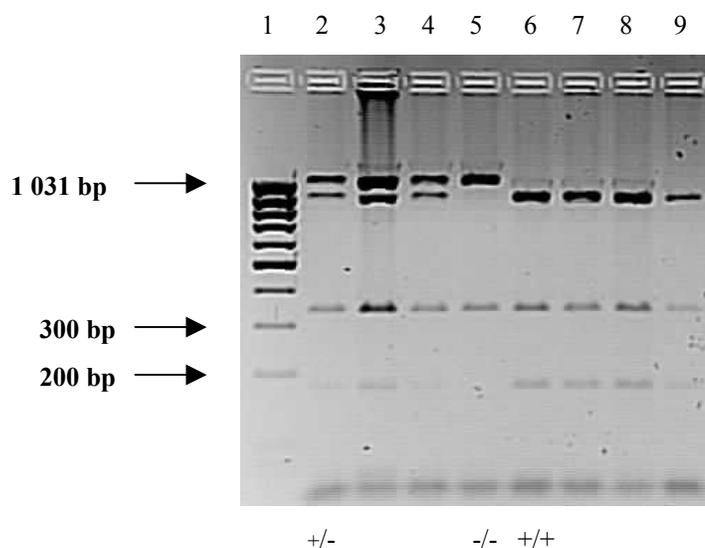


Figure D.02: *Sty*I REase digestion fragments of the A785G SNP in exon 5 of *CYP2B6*, resolved on a 1.6% (w/v) agarose gel (lane 2 = heterozygous; lane 5 = homozygous variant; lane 6 = homozygous wild-type)

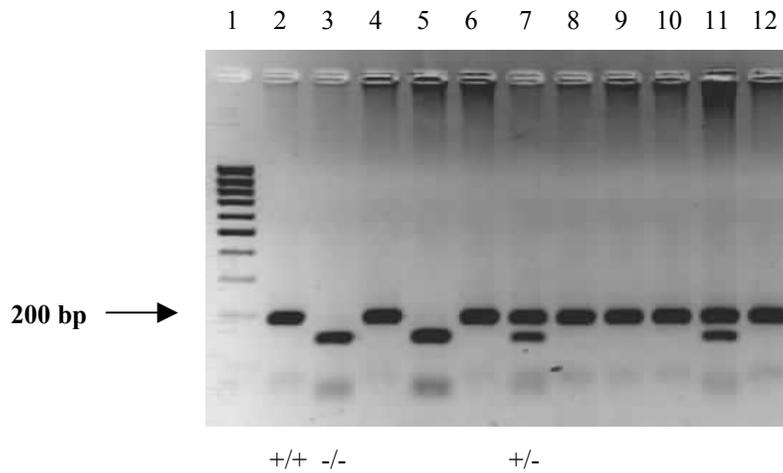


Figure D.03: *MspAII* REase digestion fragments of the T-129C SNP in the promoter region of *MDR1*, resolved on a 2.0% (w/v) agarose gel (lane 2 = homozygous wild-type; lane 3 = homozygous variant; lane 7 = heterozygous)

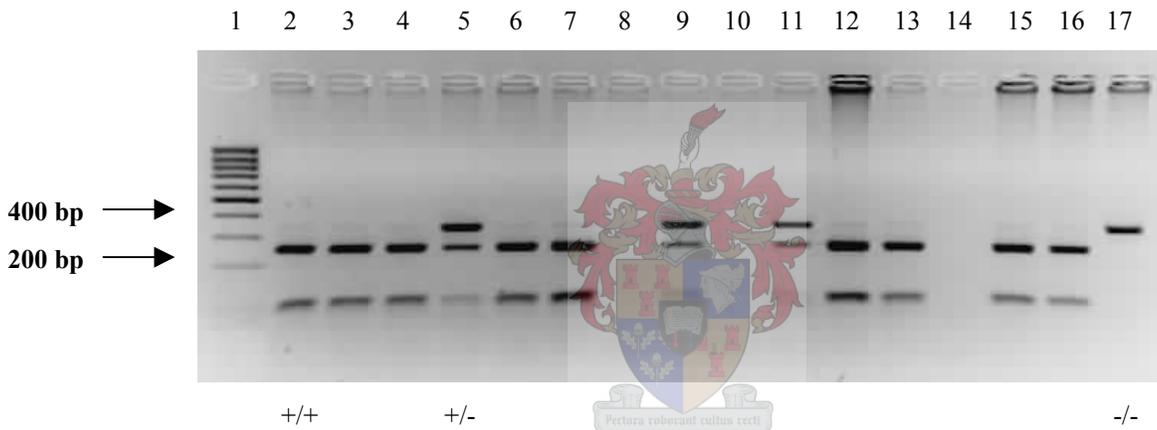


Figure D.04: *Eco0109I* REase digestion fragments of the C1236T SNP in exon 12 of *MDR1*, resolved on a 1.6% (w/v) agarose gel (lane 2 = homozygous wild-type; lane 5 = heterozygous; lane 17 = homozygous variant)

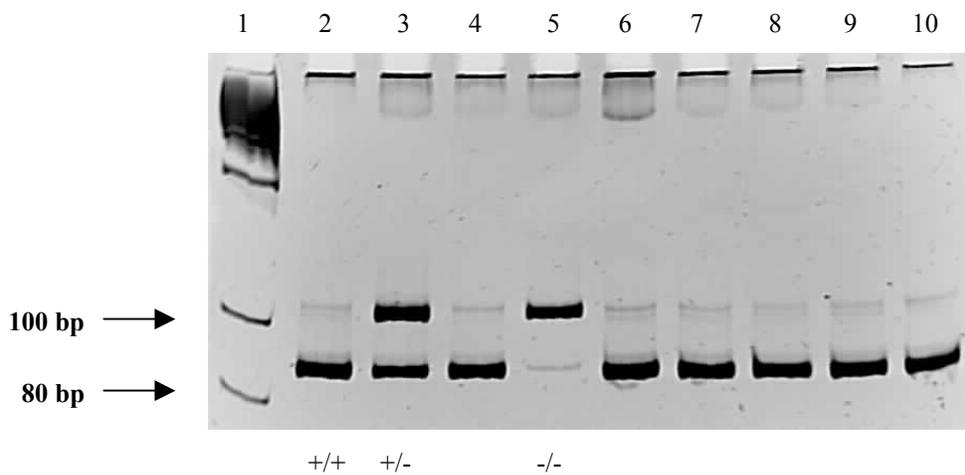


Figure D.05: *Alw2II* REase digestion fragments of the G2677T/A SNP in exon 21 of *MDR1*, resolved on a 15% (w/v) PAA gel (lane 2 = homozygous wild-type; lane 3 = heterozygous; lane 5 = homozygous variant)

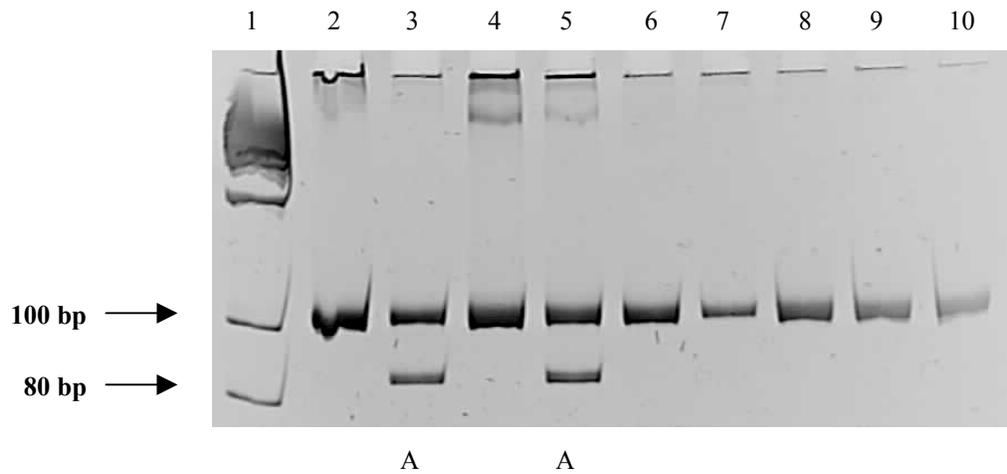


Figure D.06: *RsaI* REase digestion fragments of the G2677A SNP in exon 21 of *MDRI*, resolved on a 15% (w/v) PAA gel (lanes 3 and 5 = adenine variant (i.e. heterozygous for adenine nucleotide at this position))

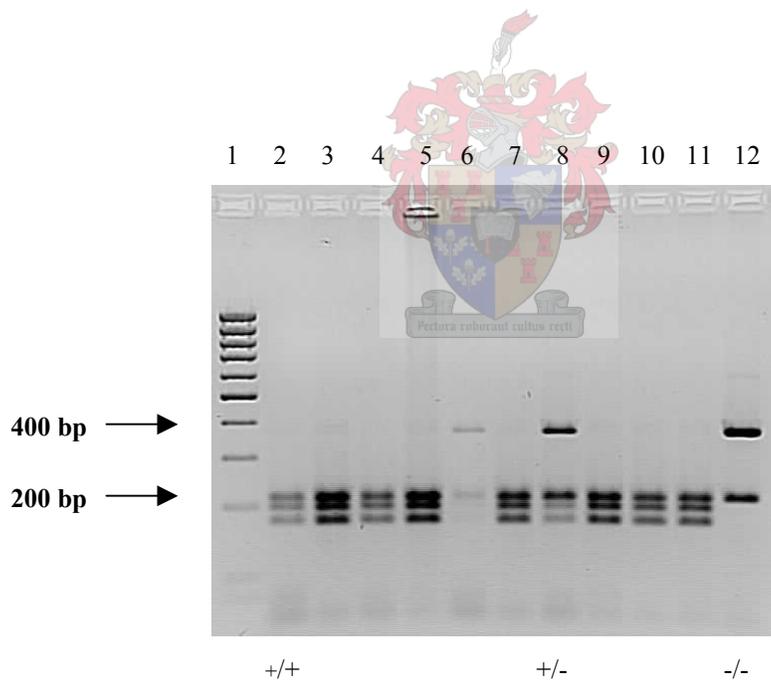


Figure D.07: *MboI* REase digestion fragments of the C3435T SNP in exon 26 of *MDRI*, resolved on a 2.0% (w/v) agarose gel (lane 2 = homozygous wild-type; lane 8 = heterozygous; lane 12 = homozygous variant)