The pharmacogenetics of Lopinavir in a cohort of black African HIV/AIDS patients

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Presented for M.Sc. (Med) in Human Genetics

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Table of Contents

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I thank the Lord for the journey and for carrying me through it. He has taught me more than I could have ever imagined.

ABBREVIATIONS

ABC	abacavir
ABCB1	ATP-binding cassette, sub-family B
ABCC2	ATP-binding cassette, sub-family C
ADME	absorption, distribution, metabolism, and excretion
AGP	alpha$_1$-acid glycoprotein
AIDS	acquired immune deficiency virus
Ala	alanine
ALT	alanine aminotransferase
ANOVA	Analysis of Variance
ART	antiretroviral therapy
ARV	antiretroviral
AST	aspartate aminotransferase
ATP	adenosine triphosphate
ATV	atazanavir
AUC	area under the concentration time curve
AZT	zidovudine
A_{260}	absorbance at 260 nm
A_{280}	absorbance at 280 nm
BLQ	below the lower limit of quantification
bp	base pairs
cART	combination antiretroviral therapy
CCD	charge-coupled device
CD4	cluster of differentiation 4
CEU	Utah residents with Northern and Western European ancestry from the CEPH collection
CHB	Han Chinese in Beijing, China
cm	centimetre
C_{max}	peak plasma concentration
C_{trough}	trough plasma concentration
CYP	cytochrome P450
ddI	didanosine
ddNTPs	dideoxynucleotides
dH_2O	distilled water

DLV	delavirdine
dNTPs	deoxynucleotides
DRV	darunavir
DTG	dolutegravir
d4T	stavudine
EDTA	ethylenediaminetetraacetic acid
EFV	efavirenz
et al.	et alia
EVG	elvitegravir
Exo	exonuclease
FPV	fosamprenavir
FTC	emtricitabine
g	gravitational force
HAART	highly active antiretroviral therapy
HCl	hydrochloride
Hi-Di	highly deionised
HIV	human immunodeficiency virus
hrs	hours
HWE	Hardy-Weinberg equilibrium
IDV	indinavir
kb	kilo base pairs
LC/MS/MS	liquid chromatography tandem mass spectrometry
LD	linkage disequilibrium
L/h	litres per hour
LPV	lopinavir
LPV/r	lopinavir/ritonavir
LWK	Luhya in Webuye, Kenya
M	molar
MDR1	multidrug resistance protein 1
Met	methionine
mg	milligrams
$MgCl_2$	magnesium chloride
min	minutes
mL	millilitres

mm^3	cubed millimetres
MRP1	multidrug resistance protein 1
MRP2	multidrug-associated resistance protein 2
mtDNA	mitochondrial DNA
MVC	maraviroc
NaCl	sodium chloride
NaOAc	sodium acetate
nDNA	nuclear DNA
NNRTI	non-nucleoside reverse transcriptase inhibitor
nm	nanometres
NRTI	nucleoside reverse transcriptase inhibitor
NR1I2	nuclear receptor subfamily 1, group I, member 2
NVP	nevirapine
OATP	organic anion transporter polypeptide
OATP1B1	organic anion-transporting polypeptide
ORM	orosomucoid
PCR	polymerase chain reaction
PEP	post-exposure prophylaxis
PrEP	pre-exposure prophylaxis
PI	protease inhibitor
PXR	pregnane X receptor
RAL	raltegravir
rcf	relative centrifugal force
RFLP	restriction fragment length polymorphism
RPM	revolutions per minute
RPV	rilpivirine
RTV	ritonavir
RXR	retinoid X receptor
s	seconds
SDS	sodium dodecyl sulphate
SLCO1B1	solute carrier organic anion transporter family, member 1B1
SNPs	single nucleotide polymorphisms
SQV	saquinavir
TB	tuberculosis

TBE	tris-borate EDTA
TDF	tenofovir disoproxil fumarate
TE	Tris EDTA
Thr	threonine
TPV	tipranavir
Tris	tris(hydroxymethyl)aminomethane
T-20	enfuvirtide
U	unit
UV	ultraviolet
V	volts
Val	valine
WHO	World Health Organisation
YRI	Yoruba in Ibadan, Nigeria
3TC	lamuvidine
µg/mL	micrograms per millilitre
µL	microliters
°C	degrees Celsius

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

The Sub-Saharan African region remains the most severely affected by the HIV/AIDS epidemic. At the end of 2011, The Joint United Nations Programme on HIV/AIDS (UNAIDS) estimated that about 5% of adults were living with the HIV in this region, accounting for 69% of the global HIV prevalence. Efforts to curb the epidemic are focused on managing HIV through prevention strategies, such as advocating the use of condoms or pre-exposure or post-exposure prophylactic treatment, and prolonging life through the use of antiretroviral (ARV) therapy. Drugs used in ARV therapy target different major steps of the HIV reproductive cycle. These are nucleoside and non-nucleoside reverse transcriptase inhibitors (NRTIs/NNRTIs); fusion/entry inhibitors; integrase inhibitors; and protease inhibitors (PIs). In South Africa PIs, specifically lopinavir (LPV) boosted with another PI, ritonavir (RTV) are used in second-line ARV regimens along with a backbone of 2 NRTIs. The use of ARVs is not without issues – patients often experience side-effects to the drugs such as nausea, diarrhoea, and lipodystrophy with LPV use, which may influence their adherence to treatment and eventually lead to treatment failure. Inter-individual variability exists in patients' response to treatment despite the standard dose of 400 mg/100 mg (LPV/RTV) that is given and this may be due to differences in transport or metabolism of the drug in the liver. High plasma drug levels (associated with side-effects or toxicity) may be a result of poor metabolism or conversely, low plasma drug levels (associated with failure to suppress the virus) may be a result of extensive metabolism of the drug. Proteins involved in the disposition of LPV include the drug metabolising enzymes, CYP3A4 and CYP3A5; the hepatic uptake transporter, OATP1B1; and the efflux transporter, MRP2. Variation in the genes encoding these proteins may influence their functioning and hence LPV disposition. The aim of the study was to identify significant single nucleotide polymorphisms (SNPs) in each gene; to genotype a cohort of HIV-infected patients from Malawi and South Africa to identify the frequency of those variants; and to correlate genotypes with LPV plasma levels and other clinical parameters. Blood was obtained from 86 HIV-infected participants on LPV treatment. Genotyping was performed using PCR-RFLP for *CYP3A4*1B; CYP3A5*3*; *CYP3A5*6* and *ABCC2* 1249G>A; TaqMan genotyping assay for *CYP3A4*22*; and sequencing to identify variants within *SLCO1B1* exon 5 and exon 6 to 7, including c.388A>G and c.521T>C. Thirty-two SNPs in four genes were identified in the study. LPV plasma levels of participants ranged from 0.0206 to 38.6 µg/mL. An association with LPV pharmacogenetics was observed for *CYP3A4*1B* and *SLCO1B1* rs4149049A>G. No other association was found between the variants determined through PCR-RFLP genotyping and LPV plasma levels. Notably, *CYP3A4*22* and *SLCO1B1* c.521T>C, which are suggested to influence the disposition of LPV in other populations, were not observed in our cohort. This highlights the need for further pharmacogenetic studies in sub-Saharan Africa in order to optimise HIV treatment available.

1. Introduction

1.1 Epidemiology of HIV/AIDS

Since the first HIV cases were reported in the early 1980s, the prevalence of Human Immunodeficiency Virus (HIV)-infected individuals increased to approximately 35 million cases worldwide by the end of 2013 [(1) Table 1.1]. Africa is the continent with the highest burden of the disease (Fig 1.1). HIV is classified into two types (HIV-1 and HIV-2) within which several subtypes have been described owing to the degree of genetic diversity present (2, 3). HIV-1 is the most prevalent type and is reported among infected patients in many regions of the world including Sub-Saharan Africa, the United States and Europe, whereas HIV-2 is prevalent in the West African region (2). For the remainder of this literature review, HIV-1 will be the focus and will be simply referred to as HIV.

Table 1.1 Summary of the global figures for the HIV/AIDS Epidemic in 2013

	Number of people living with HIV	People newly infected with HIV	AIDS deaths	Other Reference
Total (million)	35.0	2.1	1.5	
Adults (million)	31.8	1.9	1.3	
Women (million)	16.0	0.38	N/A	(1)
Children (<15 years) (million)	3.2	0.24	0.19	

Table adapted from the World Health Organisation (WHO) Data and Statistics. Global summary of the HIV/AIDS epidemic (2013)(4)
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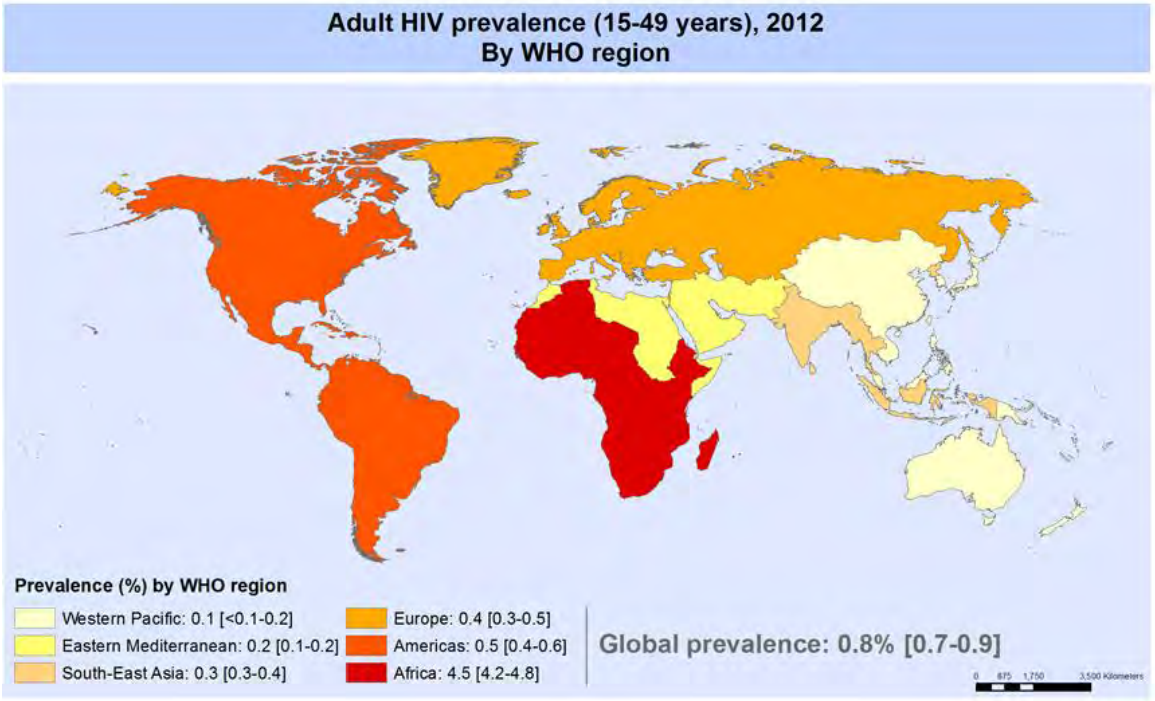

Figure 1.1 Prevalence of HIV in adults (15-49 years) in different regions of the world. Adapted with modification from WHO Global Health Observatory programme. Available at: http://www.who.int/gho/hiv/hiv_013.jpg?ua=1

The sub-Saharan African region, which includes countries such as South Africa, Malawi and Zimbabwe, remains the most affected by the HIV/AIDS epidemic. At the end of 2011, UNAIDS estimated that approximately 5% of adults were living with the HIV in this region, accounting for 69% of the global HIV prevalence (5). In support of this, the World Health Organisation (WHO) reported in 2012 that 71% of the worldwide HIV cases were found within the sub-saharan African region (http://www.who.int/gho/hiv/epidemic_status/cases_all_text/en/). The United Nations AIDS agency (5) reports that, although many challenges regarding the Acquired Immune Deficiency Syndrome (AIDS) epidemic still need to be addressed and overcome, there is an increase in encouraging news related to the global state of AIDS. For instance, at the end of 2012, a 34% decline in the number of newly infected people in the Sub-Saharan African region was reported (6), and the prevalence of HIV among young people (between 15 and 24) had decreased by 42% at the end of 2012 in Sub-Saharan Africa (7). Although these declines highlight the progress made in efforts aimed at HIV prevention (will be discussed below), the high prevalence of individuals living with HIV still warrants improving HIV treatment strategies.

This great burden of HIV warrants the continued promotion of research focusing on populations in this region, whereas currently much of the data reported is generated from studies carried out on either European/American or Asian populations.

1.2 Management of HIV/AIDS

The WHO estimates that currently half of the people living with HIV worldwide are unaware of their HIV status (8). This poses a large problem as the delay in HIV testing as well as poor counselling services available in many low to middle income countries lead to a delayed assessment of antiretroviral therapy (ART) eligibility and late access to treatment. Thus, when these infected individuals eventually begin treatment they would have been severely immunocompromised, resulting in a poorer prognosis. Furthermore, time that HIV-infected individuals take unaware of their status, increases their likelihood of spreading the virus (8). In order to improve HIV testing, the WHO has included in its consolidated guidelines, a discussion on self-testing and considerations for policy-makers (9). Although HIV self-testing is not a full-proof means of diagnosing one's HIV-status and does not rule out the need for testing by clinicians, researchers hope that more people will be encouraged to test as it allows for privacy; autonomy and greater convenience for patients. The need for HIV-testing goes hand-in-hand with the need for adequate counselling services. The WHO recommends that HIV testing and counselling not only be available in clinical settings, but also in community settings to allow for greater access to these services. As part of managing HIV and in order to improve the quality of life of individuals infected with the virus, services for emotional support; nutritional advice; advice on family planning for those with HIV are added recommendations (8).

1.2.1. Prevention

In many cases where an individual is at a higher risk for contracting HIV, it is important to consider means of prevention. HIV prophylaxis aims to aid in the management of HIV by reducing the number of people in high-risk situations who will contract the virus and, thereby, lowering the incidence of people who become infected with HIV. As a tool for prevention of HIV contraction, condoms have proved to be an effective barrier to HIV transmission. Johnson *et al*. (10) reported that in South Africa, condom use seemed to have accounted for a bigger proportion of the decrease in HIV incidence between 2000 and 2008. In South Africa, male circumcision has also been lauded as an effective means of reducing risk of acquiring HIV infections and is thought to be more cost-effective than ART in preventing HIV infections (11).

ART as a means of HIV prevention was initially used for the prevention of mother to child transmission (reviewed by Mutevedzi *et al*.(11) however, currently ART is also used to prevent HIV transmissions in other populations. Populations that are reported to benefit from pre-exposure prophylaxis (PrEP) as a form of prevention through the daily use of antiretroviral (ARV) drugs include serodiscordant couples

(one partner is HIV-infected while the other is HIV-negative); high risk heterosexual couples; men and transgender women who have sex with men and people who inject drugs (8, 12). Cohen *et al*. (12) reported that, for serodiscordant couples, early initiation of ART (when CD4 count is between 350 and 550 cells/mm^3) in HIV-infected patients was more effective in preventing the transmission of HIV to the uninfected partner than delayed initiation of ART (CD4 count 200 to 250 cells/mm^3). PrEP involves the use of tablets (Truvada, a combination of the ARV drugs emtricabine and tenofovir disoproxil fumarate) or a mucosal microbicide (in the form of a topical gel) (13, 14). However, mucosal microbicides have not shown to be as effective as condom use and ART in preventing HIV transmission (15). The challenges with prophylactic treatment include non-adherence to the prophylactic treatment (16); development of ARV resistance due to prolonged use; and mucosal safety (11, 17).

When an individual has already been exposed to HIV, post-exposure prophylaxis (PEP) is a means of preventing contraction of the virus. Within 72 hours of exposure to HIV, the individual is to be placed on a short-term ARV regimen for 28 days (8). PEP requires diligent use and as suggested by the WHO, ART is required for a certain period of time in order for it to be effective (18). Poor adherence to the various prevention strategies remains the greatest barrier to their efficacy (16, 18).

1.2.2. Treatment

None of the available ARV drugs are curative of HIV/AIDS, rather they merely act to prevent further infection of host cells by the virus. As there is no cure for HIV, the current goal of HIV treatment for those infected is maintaining viral suppression by achieving a viral load persistently below the level of detection (HIV RNA <20 to 75 copies/mL) and hence, improving immune function (19) . According to the updated AIDS Info guidelines (http://aidsinfo.nih.gov/guidelines) the CD4 count of HIV patients needs to be measured routinely and, for those on treatment, CD4 count is expected to increase by approximately 50 to 100 cells/mm^3 yearly until steady state is reached (>500 cells/mm^3; (8). In efforts to achieve the goals of treatment, combination ARV therapy (cART), has largely contributed towards decreased morbidity and mortality in HIV patients (5, 20).

Highly Active Antiretroviral Therapy (HAART) involves the use of 2 or more ARV drugs (cART) in order to suppress HIV viral replication in infected patients and its efficacy was first reported in 1997 (21). Gulick *et al*. (21) reported that the use of 3 ARV drugs: 2 nucleoside reverse transcriptase inhibitors (NRTIs), and 1 protease inhibitor (PI) in patients resulted in a greater decrease in HIV-RNA over 24 weeks compared to patients who were taking 2 NRTIs or on single NRTI-drug regimens. Since then,

cART has become the standard form of HIV ART with different classes of ARVs being combined in each regimen as is discussed further below.

The challenges with cART are often associated with high pill burden (which has recently been addressed by the new single pill containing combination of tenofovir (TDF), emtricitabine (FTC) and efavirenz (EFV) (22); drug-drug interactions (for example with drugs that are common substrates for major drug metabolising enzymes, such as cytochrome P450 which will be discussed later); adverse side effects; number of times per day that the drugs have to be taken and development of drug resistance. These factors reduce patient compliance in taking their medication, a form of poor adherence, which leads to reduced efficacy of treament and high likelihood of development of drug-resistant virions.

1.2.3. Co-morbid Diseases and Opportunistic Infections

The management of HIV not only involves medical intervention through ARV drugs, but also includes managing other illnesses that may arise due to the prolonged life span of patients such as diabetes and cardiovascular disease (23). The immune compromising effects of HIV makes patients susceptible to other opportunistic infections including tuberculosis (TB), cryptoccocal meningitis and *Pneumocystis carinii* pneumonia (24, 25). The introduction and availaibilty of ART has led to a decrease in the incidence of opportunistic infections (25).

1.3 Antiretroviral Therapy (ART)

The U.S. Food and Drug administration has approved more than 30 ARV drugs as of December 2014, for the treatment of HIV (26, 27). Each of these drugs target a specific step of the HIV life cycle (Fig. 1.2). The main steps in the HIV life cycle include: binding of the viral proteins to host cell co-receptors; fusion of the virus with the host cell; reverse transcription of viral RNA to DNA; integration of the viral DNA into the host genome; synthesis of viral proteins; and cleavage of precursor polypeptides by viral proteins to form mature, infectious virions [Fig. 1.2; (3, 26)]. Treatment of HIV involves the targeting of each of these steps in order to arrest the HIV replicative cycle and inhibit further infection of host cells. ARVs are divided into 5 classes (see Table 1.2), namely NRTIs; non-nucleoside reverse transcriptase inhibitors [NNRTIs; (28, 29)]; PIs (30); fusion inhibitors (31); entry inhibitors [e.g. CCR5 co-receptor antagonists (32, 33)]; and HIV integrase strand transfer inhibitors (34). Fusion Inhibitors, Entry Inhibitors and HIV integrase strand transfer inhibitors are the newest classes of inhibitors. Each drug acts by targeting and inhibiting a specific step in the replicative cycle of the virus [(29); Fig. 1.2].

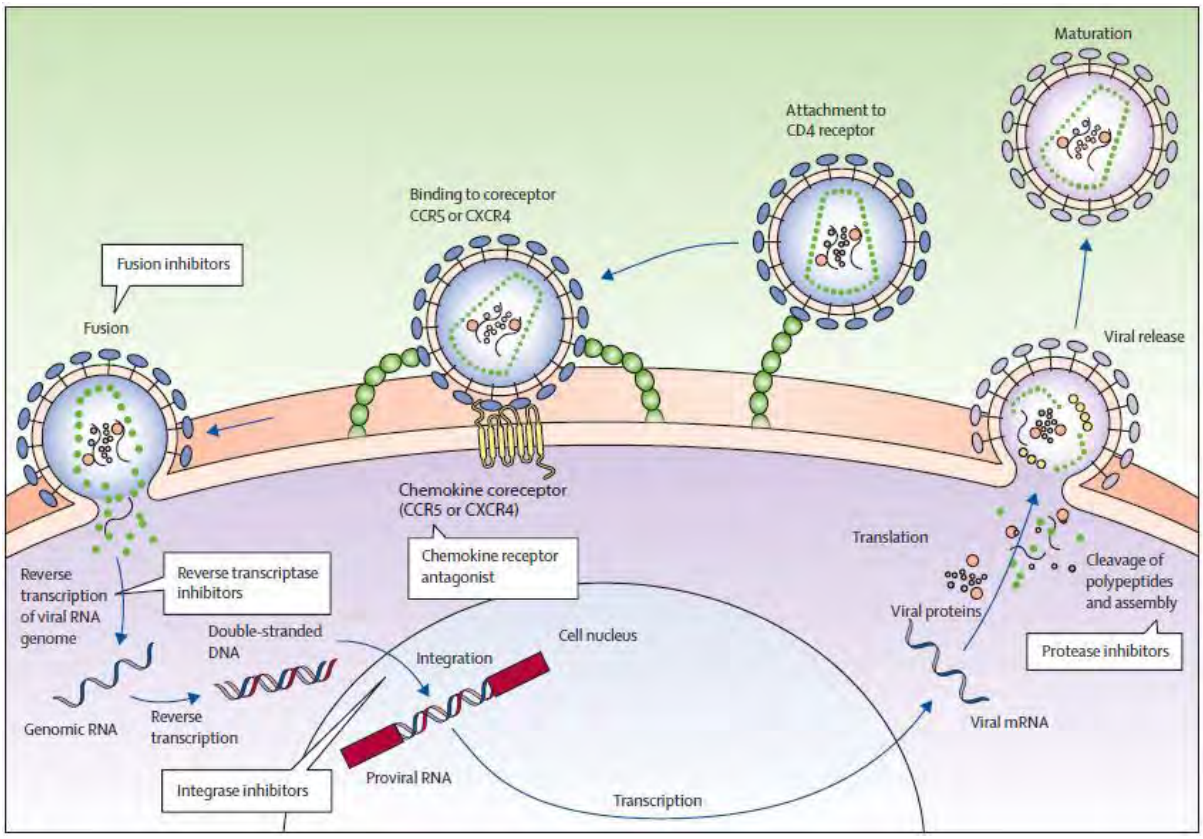

Figure 1.2 Replicative life cycle of HIV. Figure highlights targets for ARV action. Adapted without modification from Maartens *et al*. (26).

Table 1.2 Antiretroviral Drugs approved by the U.S. Food and Drug administration and recommended for use in the treatment of HIV in South Africa.

Class	Mode of action	Drug (Generic Name)	Reference
Nucleoside Reverse Transcriptase Inhibitors (NRTI)	Compete with analogous dNTPs as substrates for HIV reverse transcriptase enzyme. Incorporation of NRTI into the nascent viral DNA chain results in termination of synthesis	zidovudine (AZT) didanosine (ddI) stavudine (d4T) lamuvidine (3TC) abacavir (ABC) tenofovir disoproxil fumarate (TDF) emtricitabine (FTC)	(35, 36)
Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTI)	Target an allosteric site near the active site of the enzyme and, thereby, induce conformational changes in the enzyme affecting its activity	nevirapine (NVP) delavirdine (DLV) efavirenz (EFV) etravirine (ETV) rilpivirine (RPV)	(35, 36)
Protease Inhibitors (PIs)	Inhibit the HIV protease enzyme preventing it from cleaving the gag and gag-pol precursor proteins, inhibiting the production of mature, infectious virions	saquinavir (SQV) ritonavir (RTV) indinavir (IDV) nelfinavir (NFV) lopinavir (LPV) atazanavir (ATV) fosamprenavir (FPV) tipranavir (TPV) darunavir (DRV)	(36)
Fusion Inhibitors	Disrupt interactions of the HR1 and HR2 domains of the gp41 protein of the virus, preventing the fusion of the viral envelope to the host cell membrane	enfuvirtide (T-20)	(36)
Entry Inhibitors - CCR5 co-receptor antagonist	Block interactions of the viral proteins and the CCR5 co-receptor of the host cell to inhibit fusion of the virus to the host cell	maraviroc (MVC)	(36)
Integrase Strand Transfer Inhibitors	Act on the HIV integrase enzyme and the viral DNA ends to prevent the viral genome from being integrated into the host genome	raltegravir (RAL) dolutegravir (DTG) elvitegravir (EVG)	(36, 37)

1.3.1. ARV treatment regimens

According to the 2013 WHO guidelines (8), ART should be initiated in HIV-infected adults in WHO clinical stage 3 or 4 when an individual's CD4 count is below 350 cells/mm^3; or below 500 cells/mm^3 for HIV-infected individuals, regardless of WHO clinical stage. Exceptions are in the cases of patients

co-infected with HIV and TB; with HIV and hepatitis B virus or partners with HIV in serodiscordant couples – these patients are initiated on ARV regardless of their WHO clinical stage or CD4 count (38, 39). WHO clinical stage 3 or 4 is defined as severe or advanced HIV clinical disease characterised by symptoms such as weight loss of more than 10% of body weight; unexplained chronic diarrhoea, anaemia, persistent fever; pulmonary TB (WHO 2010 Interim WHO Clinical Staging of HIV/AIDS and HIV/AIDS case definitions for surveillance).

1.3.1.1 First-line ART

When patients begin ARV treatment (ART), they are first placed on a first-line regimen consisting of two NRTIs plus a NNRTI. The preferred fixed-dose combination recommended by the WHO includes TDF; lamivudine (3TC) or FTC; and EFV (i.e. TDF+3TC/FTC+EFV). The use of stavudine (d4T) in ART has been discontinued due to the toxicities associated with the drug including peripheral neuropathy, lactic acidosis and severe hepatomegaly (8, 40). d4T treatment in HIV/AIDS patients has been suggested to result in mitochondrial depletion, with patients showing a lower mitochondrial DNA to nuclear DNA ratio (mtDNA/nDNA) compared to healthy controls (41). The WHO suggests that patients still on d4T be switched to zidovudine (AZT). The preferred first-line regimen according the WHO as of 2013 is once daily TDF + 3TC (or FTC) + EFV and alternative regimens are (AZT + 3TC + EFV; AZT + 3TC + NVP; and TDF + 3TC (or FTC) + NVP) in ART-naïve patients (8). Pregnant women and women who are breastfeeding may experience adverse effects with NVP treatment, such as hepatotoxicity and severe skin rashes and regimens with EFV may be used rather than NVP.

In high income countries, viral load is routinely measured in order to monitor treatment efficacy, whereas in low to middle income countries CD4 count and clinical prognosis are often used to monitor treatment as these measures are more accessible than viral load testing (42). At treatment failure, genotypic resistance tests are useful in determining whether patients should continue on the current regimen (if showing no resistance) or whether they should switch to more expensive second-line treatment. Unfortunately, these tests are often not easily accessible in low to middle income countries, and thus some patients may be switched to second-line treatment unnecessarily (43). Therefore, as suggested by Levison *et al*. (43) genotypic resistance testing may be cost effective in South Africa through identifying patients with virologic failure who actually have the wild-type virus, and therefore can continue with first-line therapy.

1.3.1.2 Second-line ART

In order to assess how patients are faring on the treatment, viral load is measured or, if viral load is not available, CD4 count is measured (8). Measures of treatment failure are virologic, immunological and clinical. Virologic failure entails a viral load which exceeds 1000 copies/mL after 2 successive measurements (with a 3 month interval); immunological failure is noted when a patient presents with a low CD4+ cell count (<50 cells/mm^3); whilst clinical failure is noted when patients reach WHO clinical stage 4 (8). Patients presenting with first-line treatment failure are switched to a second-line ARV regimen which consists of a boosted PI; such as LPV/r (LPV boosted with RTV); plus a NRTI backbone as recommended by the WHO (8). The incidence of switching from first-line ART to second-line ART is predicted to be low in resource-limited countries (44) which may be partly due to the poor sensitivity of using immunological criteria to measure treatment failure rather than viral load. Delayed switching of treatment may influence patient response: morbidity and mortality.

In 23 resource-limited countries (Burkina Faso, Burundi, Cambodia, Cameroon, Côte d'Ivoire, Ethiopia, Guatemala, Haiti, India, Kenya, Lesotho, Malawi, Mozambique, Namibia, Nigeria, Russian Federation, Rwanda, Swaziland, Tanzania, Thailand, Uganda, Zambia, and Zimbabwe) 4% of HAART patients (which translates into 34 040 individuals) were predicted to be on second-line ART in 2007 (42, 45) however only 25% of those patients on second-line therapy were on regimens recommended by the WHO: including: ABC+ddI+LPV/r (24%) and TDF+ddI+LPV/r (1%). In 2012, the number of people in low-to-middle income countries on second-line therapy was estimated to be 500 000 (46). Renaud-Théry *et al*. (45) suggest that the use of second-line therapy may be more complicated than that of first-line therapy as the choice of second-line treatment is dependent on drugs used in first-line therapy; and the cost of second-line drugs may affect the ability of resource-limited countries to adhere to recommended drugs. For patients with no prior experience to PIs who are placed onto a second-line regimen with a boosted PI (such as LPV/r) the greatest predictor of viral suppression is adherence to treatment (47). In a study by Murphy *et al*. (47), adherence to treatment was the main predictor of viral suppression over a 12 month period while poor adherence could partially explain the reported treatment failure among 25% of the patients. This observation was also supported by a report on HIV infected adults and children who were followed up for 20 months while on second-line ART (48); and then later by Court *et al*. (46) who report an association between poor adherence and predicted virologic failure on second-line ART and, conversely, between adherence and virologic suppression. Court *et al*. (46) used the pharmacy refill tool which measures short-term adherence to treatment. Adherence may be the greatest predictor of viral suppression in patients on second-line ART with PIs (46, 49), however, adherence is not the only factor; there are other possible reasons for the failure of second-line treatment to improve patient treatment outcomes. As with genotypic resistance that is

known to lead to first-line treatment failure, resistance to PIs could possibly play a role in second-line treatment failure. This study evaluates the pharmacogenetic component that is associated with response to LPV.

1.3.1.3 Protease Inhibitors and their mechanism of action

Through binding to the viral protease, PIs prevent the cleavage of the gag and gag-pol precursors into smaller viral proteins, a process essential for the formation of mature virions (30, 50). As immature viral particles are released from the infected host cell, further infection of host cells by newly synthesised virions is prevented (51). There are several PIs (listed in Table 1.2 above) which include RTV and LPV. Figure 1.3 below is a schematic showing how PIs form a complex with the HIV viral protease. Specifically, LPV/r is recommended for use in second-line ARV regimens in Southern Africa (8, 52).

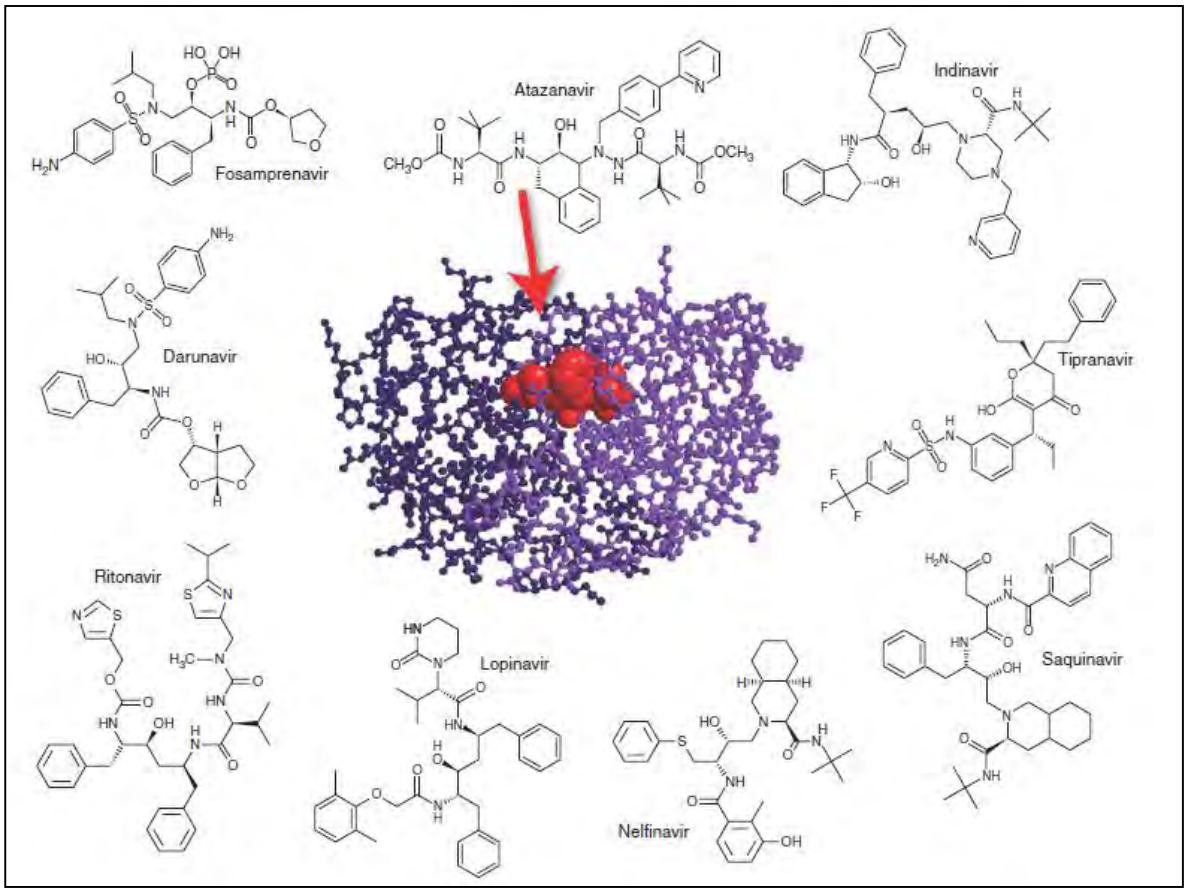

Figure 1.3 Various Protease Inhibitors and the crystal structure of HIV viral protease, complexed with the PI atazanivir. The arrow points to the site of the protease inhibitor within HIV protease. Figure adapted from Arts and Hazuda, 2012 (36), with no modifications.

The current regimens that include LPV are as follows: AZT+3TC + LPV/r for patients in whom TDF was used in first-line treatment or; TDF+3TC (or FTC) + LPV/r for patients in whom AZT was used in first-line treatment (8). The recommended alternative PI to LPV/r used in second-line therapy is atazanavir (ATV) which is also boosted with RTV. Other boosted PIs (darunavir, saquinavir (SQV/r); indinavir (IDV/r); fosamprenavir (FPV/r) are not available as fixed dose combinations and are associated with higher pill-burdens and with severe side effects. In resource-limited countries, LPV/r is currently the preferred option for second-line ART (47). However, due to the adverse side effects associated with LPV/r use, research aimed at making LPV/r more tolerable in patients or reducing the cost of newer PIs such as DRV is warranted, especially in developing nations (47, 53). Failure in second-line ART leads to recommending patients for third-line treatment, which is even more expensive than second-line ART and not as easily available in resource-limited settings.

Third-line treatment currently consists of raltegravir (an integrase inhibitor), DRV/r (PI) and; etravirine (an NNRTI) (8, 54). The WHO recommends that in countries where third-line ART is not readily available, patients who present with second-line treatment failure should continue on a tolerated regimen. This project focussed on LPV as the main component of second-line ART.

1.4 A focus on Lopinavir-Ritonavir use: benefits and associated toxicities

LPV/r is administered as a single drug (Kaletra) and is taken at a dose of 400mg/100mg twice daily (8). The use of LPV/r in ART may have other added benefits apart from the treatment of HIV. Ruel *et al*. (55) found that LPV/r used in ARV regimens in children also had anti-malarial effects. However, some adverse effects have been noted associated with LPV/r use and these include: hepatotoxicity, dyslipidaemia, diarrhoea and nausea [Table 1.3; (8, 56-60)]. Drug-induced hyperlipidaemia is of concern as it is associated with increased cardiovascular disease risk (58, 60, 61).

The effects of LPV on cardiac function as well as on metabolism and vascular function have been investigated in rat models (58, 62). Yeung *et al.* (62) suggested that LPV (and other PIs) may affect responses of vascular smooth muscle to relaxing agents and hence pose a risk for cardiovascular diseases, while Reyskens *et al.* (58) reported changes in the contractile force of the heart and myocardial oxidative stress induced by LPV/r.

Table 1.3 Some toxic events that are associated with LPV/r use.

Major Types of toxicity	Risk factors	References
Attenuated cardiac function reported in a rat model	PI-induced myocardial oxidative stress Decreased contractile force of the heart with PI treatment	(58)
Electrocardiographic abnormalities (PR and QT interval prolongation – indicate abnormal electrical activity of the heart)	People with pre-existing conduction system disease Concomitant use of other drugs that may prolong the PR and/or QT interval (e.g. ATV) Congenital long QT syndrome Hypokalaemia	(63, 64)
Hepatotoxicity	Underlying hepatic disease HBV and HCV co-infection Concomitant use of hepatotoxic drugs	(56)
Pancreatitis	Advanced HIV disease	
Lipoatrophy or metabolic syndrome, dyslipidaemia	Risk factors unknown	(58, 60)
Myocardial infarction	Partially explained by dyslipidaemia associated with PI use	(59, 60)
Hypertriglyceridaemia: increased non-HDL cholesterol levels	Higher baseline triglyceride levels Total duration of ARV treatment	(57)
Gastroenteric symptoms: nausea or diarrhoea		(57)

Adapted with modification from Table 7.15, WHO guidelines 2013(8). ATV: atazanavir; HDL: high-density lipoprotein; PI: protease inhibitor; PR and QT Interval indicate the rhythm of the heart.

1.4.1. Structure and functional activity of Lopinavir

LPV (also known as ABT-378) acts as a competitive inhibitor for HIV viral protease by forming a complex in the active site of the protease, thereby preventing the cleavage of the gag and gag-pol precursor proteins, by the affected protease, as illustrated in section 1.3.1 above (65, 66). LPV is the analogue of RTV (Fig. 1.4), and was developed to overcome the high resistance to treatment which was found to occur with RTV treatment. LPV shows greater efficacy against HIV than RTV and is associated with reduced risk of development of HIV resistance (67). Administered alone, LPV has a low bioavailability (68) due to high first-pass metabolism by cytochrome P450 (CYP) enzymes, CYP3A4 and CYP3A5 (69, 70) and efflux by MRP2 (multidrug resistance protein; (71). RTV is a potent inhibitor of CYP3A4 and, thus, the co-formulation of LPV with RTV (LPV/r, Kaletra) has allowed for improved bioavailability of LPV as RTV inhibits the metabolism of LPV by CYP3A4 (67) and has also been shown to inhibit the bio-activation of LPV by CYP3A4 (72). Figure 1.4 shows the comparative structures of RTV and LPV. The 2 molecules are similar in structure, except for an isopropyl group in RTV which is replaced by a cyclic urea moiety in LPV (Fig. 1.4). This cyclic group in LPV is shorter than the isopropyl group in RTV and, thus, interacts less with the valine at position 82 of HIV protease, a site prone to drug-resistant mutations. This contributes to the increased potency of LPV compared to RTV. Also, the cyclic urea in LPV allows for hydrogen bonding with aspartate 29 of HIV protease which results in

higher potency of LPV compared to RTV (66). Liu *et al*. (65) suggested that drug resistant mutations in HIV protease conferred less potency of LPV against the protease by weakening the interactions between LPV and the enzyme.

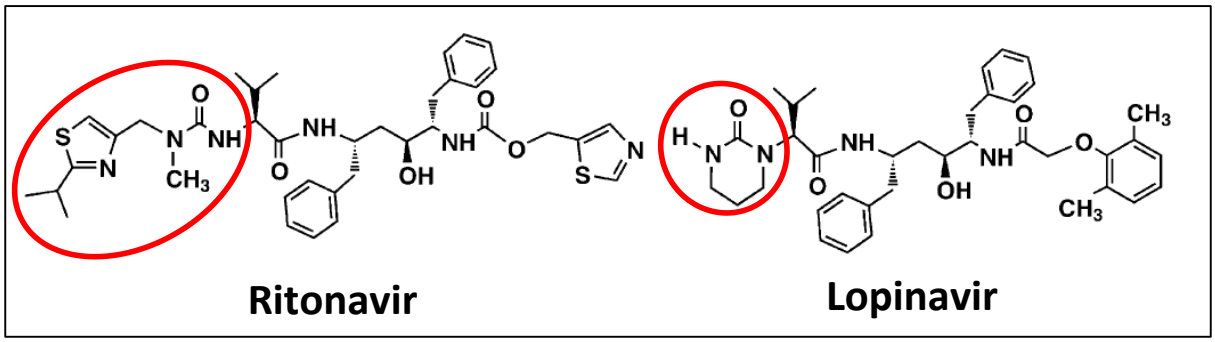

Figure 1.4 The structure of Ritonavir and Lopinavir. The isopropyl group and cyclic urea moiety are circled in red. Figure adapted from Stoll *et al*. (66).

1.5 Pharmacokinetics of Lopinavir

Hurst and Faulds (73) reviewed the steady state pharmacokinetics of LPV, which is administered orally and taken twice daily with food. The bioavailability of the drug has been shown to be increased when taken with food containing a moderate-to-high fat content (74). At a dose of 400/100 mg, the acquired peak plasma concentration (C_{max}) after administration of LPV is approximately 9.6 µg/mL and the C_{trough}=5.5 µg/mL (75). For twice daily administration, the elimination half-life is about 4-6 hrs, whilst the oral clearance of LPV is 6-7 L/h (73, 74). In this section, proteins which play a role in the pharmacokinetics of LPV, including its distribution, disposition, metabolism and transport will be discussed (Table 1.4).

Table 1.4 Genes and proteins associated with pharmacokinetics and disposition of Lopinavir.

Gene	Protein Encoded by Gene	Functional Significance	Reference
SLCO1B1	OATP1B1 (organic anion transporting polypeptide)	Solute carrier – localised to basolateral membrane of hepatocytes. Facilitates entry of substrate into cells.	(76)
CYP3A4	CYP3A4	Major phase I drug metabolising enzyme. Responsible for 30% drug metabolism in the body (along with CYP3A5). Found on the membranes of cells in the gut and the liver.	(77, 78)
CYP3A5	CYP3A5	Phase I drug metabolising enzyme. Main CYP3A isoform in the kidney, but also expressed in the liver. CYP3A5 is approximately 80% homologous to CYP3A4. Large inter-individual variability exists in the expression of CYP3A5.	(79, 80)
ABCC2	MRP2 (multidrug resistance-associate protein)	Membrane transporter – drives efflux of non-conjugated compounds across cell membranes using ATP-derived energy.	(81)
ABCB1	MDR1 (multidrug resistance protein; p-glycoprotein)	Efflux transporter, uses ATP-derived energy to drive the transport of substrates. Expressed in the apical membrane of hepatocytes.	(82, 83)
NR1I2	PXR (pregnane X receptor)	Ligand-activated nuclear receptor which induces the expression of certain drug metabolising enzymes, including CYP3A4, and transporters (e.g. MRP2 and MDR1).	(78, 84)
AGP (α_1- acid glycoprotein)	ORM (orosomucoid) – controlled by 2 loci: ORM1 and ORM2	Plasma carrier protein for basic and lipophilic endogenous compounds or xenobiotics. Inflammatory marker – levels are increase in states of immune activation. Plasma levels vary according to different disease states including cancer, depression, genetic factors, HIV.	(85-88)
	Human Serum Albumin	Binding of LPV	(87)

1.5.1. Lopinavir Distribution and Disposition

As with other PIs, LPV binds to the α_1-acid glycoprotein (AGP; also known as orosomucoid, ORM) within the plasma (89). ORM has a high affinity for lipophilic basic and neutral molecules, hence its binding of LPV which is a lipophilic, basic molecule (85, 90). Within the plasma, 99% of LPV is found bound to ORM; as reviewed by Ford *et al*. (89). However, the amount of drug bound to protein is influenced by protein concentrations (which may fluctuate), as well as on drug concentrations (85-87). ORM expression is controlled by 2 genes on chromosome 9q31–34.1, *AGP1* and *AGP2,* which encode ORM1 and ORM2, respectively. The binding of drugs to these 2 proteins is not well understood; some drugs bind preferentially to ORM1, whilst others to ORM2 (86). ORM1 is characterised by 3 codominant alleles: ORM1*F1; ORM1*F2; ORM1*S (91, 92) and ORM2 is monomorphic in most populations (92). The rs17650*S>*F polymorphism within *AGP* has been suggested to influence binding affinity of ORM to drug and was reported by Wang *et al*. (93) to

influence warfarin dosage requirements. The structure of the *AGP* gene has also been investigated African individuals (94-96).

The disposition of LPV may be influenced by its binding to the ORM proteins. Circulating levels of ORM vary with the inflammatory status within an individual and, therefore, can vary between HIV-infected individuals (77, 88). As PIs are highly bound to ORM, the metabolism or clearance of these drugs may be influenced by varying concentrations or different phenotypes of the binding protein as the amount of free drug will be affected. This has been reported by Colombo *et al.* (86) who found that ORM1 concentration had an influence on the disposition and clearance of LPV and IDV. However, the effect seen in the study was greater in IDV than in LPV. Contrary to the findings by Colombo *et al.* (86), it has later been reported that plasma drug binding protein concentration has little influence on antiviral activity of LPV/r (77). Although the total AUC_{12} (area under the concentration time curve) and LPV C_{max} were influenced by ORM concentration, the amount of free LPV in the plasma and hence the antiviral activity of LPV were unchanged. The authors suggested that other factors, such as drug clearance, may account for this poor association. In the instance of levels of free LPV increasing due to a decrease in ORM levels, the clearance of LPV may also increase so that the amount free LPV in the plasma remains unchanged.

In resource-limited settings, it has been proposed that ORM may be used as a marker for disease severity in HIV-1 infected mothers (88). It was reported that ORM concentration positively correlated with viral load and elevated ORM levels was associated with an increased risk in HIV-related deaths. However, it is not known whether the changing levels affect LPV levels or whether the allelic variation reported in Africans may influencing the binding of ORM substrates, including LPV.

1.5.2. Hepatic Uptake Transporters: The Organic Anion Transporter Polypeptide 1B1

Hepatic uptake transporters belong to the superfamily of solute carriers and are divided into the organic cation transporter and organic anion transporter families and the organic anion transporter polypeptide (OATP) family; reviewed by König *et al*. (97). Within the family, OATPs have ≥40% amino acid sequence similarity and in each subfamily, the proteins share more than 60% amino acid sequence identity (98). OATPs are a family of proteins which facilitate the uptake of drugs from portal blood into hepatocytes (99, 100). These proteins are involved in the active hepatic uptake of substrates through mechanisms independent of sodium, chloride and potassium gradients, membrane potential and ATP levels (82, 99, 101). These transporters are regarded as electroneutral anion exchangers, although the mechanism has not been elucidated (99, 102).

OATP1B1 has been implicated in the pharmacokinetics of several drugs including PIs and statins, which are used in the management of cardiovascular disease (103, 104). It is also important to mention that PIs are also substrates of OATP1A2, OATP1B3 (105), but OATP1B1 is most important for the hepatic uptake of LPV. OATP1B1 consists of 691 amino acids and putatively contains 12 transmembrane domains (99, 106). Figure 1.5 shows the predicted structure of OATP1B1 which is expressed mainly in the liver and located at the basolateral (or sinusoidal) membrane of hepatocytes (76, 107, 108).

Adequate functioning of the transporters and hence absorption of drugs is essential to ensure pharmacological action of orally administered drugs (82). The uptake of substrates into hepatocytes by OATP1B1 usually precedes the elimination of those substrates through metabolism or biliary excretion. Hence, impaired functioning of the transporter affects the pharmacokinetics of its drug substrates. The inhibition of OATP1B1 by certain drugs may play a role in drug-drug interactions which often lead to adverse drug reactions. For example, the anti-tuberculosis drug, rifampicin, has been shown to inhibit OATP1B1 and, hence, is not recommended for co-administration with PIs in patients with co-morbid TB and HIV infection (109). More specifically, LPV has been shown through *in silico* predictions and *in vitro* experiments, to be an inhibitor of OATP1B1 (110, 111).

1.5.3. Phase 1 Drug Metabolism: Cytochrome P450 Enzymes (CYP3A4 and CYP3A5)

Within the CYP3A subfamily of Cytochrome P450 (CYP) enzymes, CYP3A4 and CYP3A5 are the major isoforms expressed in the liver and small intestine, followed by CYP3A7 (expressed mainly in the foetal liver) and CYP3A43 (112). The genes encoding these enzymes are found on the same locus on chromosome 7 (80, 112). *CYP3A4* is suggested to have arisen from duplication of exons in *CYP3A5* (113) and are very similar in sequence and share common substrates. These enzymes are responsible for Phase I metabolism of xenobiotics (involving oxidation and hydrolysis of substrates), and CYP3A4 and CYP3A5 account for approximately 30% of the drug metabolism in the body and are thus the major drug metabolising enzymes in the body (114). Drug metabolising enzymes act by making xenobiotics more water-soluble.

CYPs are haemoproteins, and are specifically oxidases found on the membrane of the endoplasmic reticulum in cells of the gut and liver (115, 116). Upon binding of the ligand to the enzyme, CYPs undergo conformational changes (114). As mentioned earlier, CYP3A4 and CYP3A5 are involved in the metabolism of LPV (70, 108, 117) into three major metabolites: M1, an oxidised derivative of LPV, M3

and M4, which are both epimers (compounds with the same chemical formula, but which differ in spatial arrangement around a single carbon atom) of a hydroxylated product of LPV [Fig 1.5; (118)].

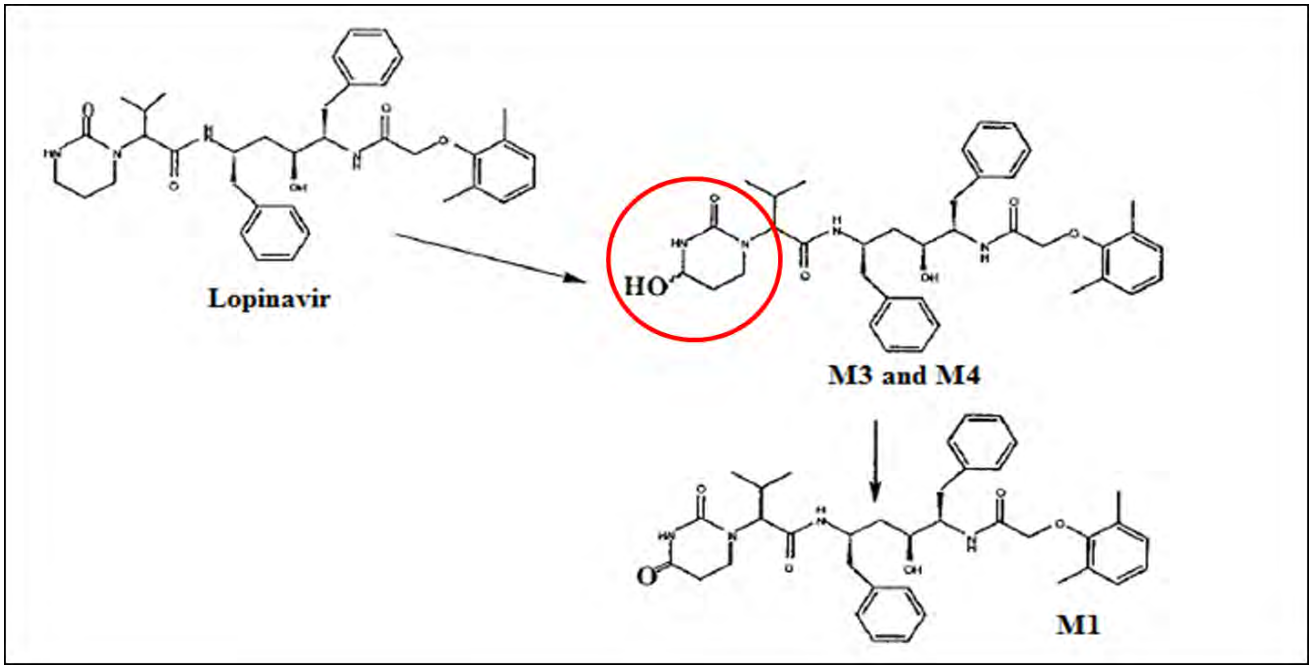

Figure 1.5 The major metabolites of Lopinavir metabolism (M1, M3 and M4). The cyclic urea unit around which M3 and M4 are epimers is circled in red. Figure adapted from Kumar *et al*. (118).

1.5.4. Efflux Transporters: Multidrug resistance protein (MRP2)

The multidrug resistance protein 2 (MRP2), encoded for by the *ABCC2* gene, is a member of the ATP-binding cassette superfamily (ABC) of transport proteins and is important in the biliary excretion of compounds from the liver; reviewed in Suzuki and Sugiyama (119). This 1541 amino acid protein is localised to the canalicular membrane of the liver (120, 121). It is also expressed on the apical membrane of enterocytes in the small intestine of rats (119, 122) and humans (123) and is also localised to the apical plasma membrane domain of the proximal tubule segments of the kidney in rats (124). MRP2 shows 49% sequence similarity to MRP1 (125) and as with other ABC transporter proteins, it contains the essential ATP-binding domain (121, 125). MRP2 has been shown to be involved in the ATP-dependent transport of glutathione, glucuronate and sulphate conjugates of lipophilic compounds (126). Products such as phase II metabolites of drugs are transported from within the liver across the hepatocyte canalicular membrane out of the liver (120). Loss of MRP2 function has been linked to Dubin Johnson Syndrome which is a hereditary disorder, characterised by conjugated hyperbilirubinemia and the accumulation of dark pigments in the liver (119, 127).

As with MDR1, MRP2 (but not MRP1 and other ATP-binding cassette transport proteins) has been demonstrated to be an efficient efflux transporter of PIs (71, 128). LPV has also been shown to be a substrate of P-glycoprotein (MDR1, coded for by the *ABCB1* gene (71). When investigating transport of LPV by MRP2 in this study, Agarwal *et al*. (71) reported that in the presence of an inhibitor of MRP2, the efflux ratio of LPV was reduced from 2.9 to 1 *in vitro*, thus demonstrating that LPV is a substrate of MRP2. Van Waterschoot *et al*. (129) however found that P-glycoprotein, rather than MRP2 was an efficient efflux transporter of LPV when investigating transport *in vitro* and suggested that, overall, CYP3A activity was a better indicator of LPV oral pharmacokinetics than that of the transporter proteins. A further understanding into the importance of MDR1 and MRP2 in the transport of PIs, including LPV, is required overall, and also specifically in the sub-Saharan African context. For this study, MRP2, for which less is known, was selected for investigation.

1.5.5. Pregnane X Receptor (PXR)

The pregnane X receptor (PXR) is a nuclear receptor involved in inducing the promoter activity of drug metabolising enzymes (78) as well as efflux transporter proteins (130). In the case of drugs such as LPV/r, nuclear receptors act as sensors of the drug which lead to increased expression of CYP3A4/5 and metabolism of the drug. After the drug binds to PXR, which is localised to the cytoplasm, PXR translocates to the nucleus where it forms a heterodimer with the Retinoid X Receptor (RXR). The heterodimer is then able to bind to nuclear response elements upstream of drug metabolising enzymes such as CYP3A4 and CYP3A5 (131). The efflux transporter proteins regulated by PXR include MRP2 and MDR1 (127, 130). The expression of *SLCO1B1*, which encodes the uptake transporter OATP1B1, is also regulated by PXR (106).

Figure 1.6 below shows the proteins mentioned above in section 1.5, involved in the ADME (Absorption, Distribution, Metabolism and Excretion) process, specifically for LPV and the stages at which these proteins are active.

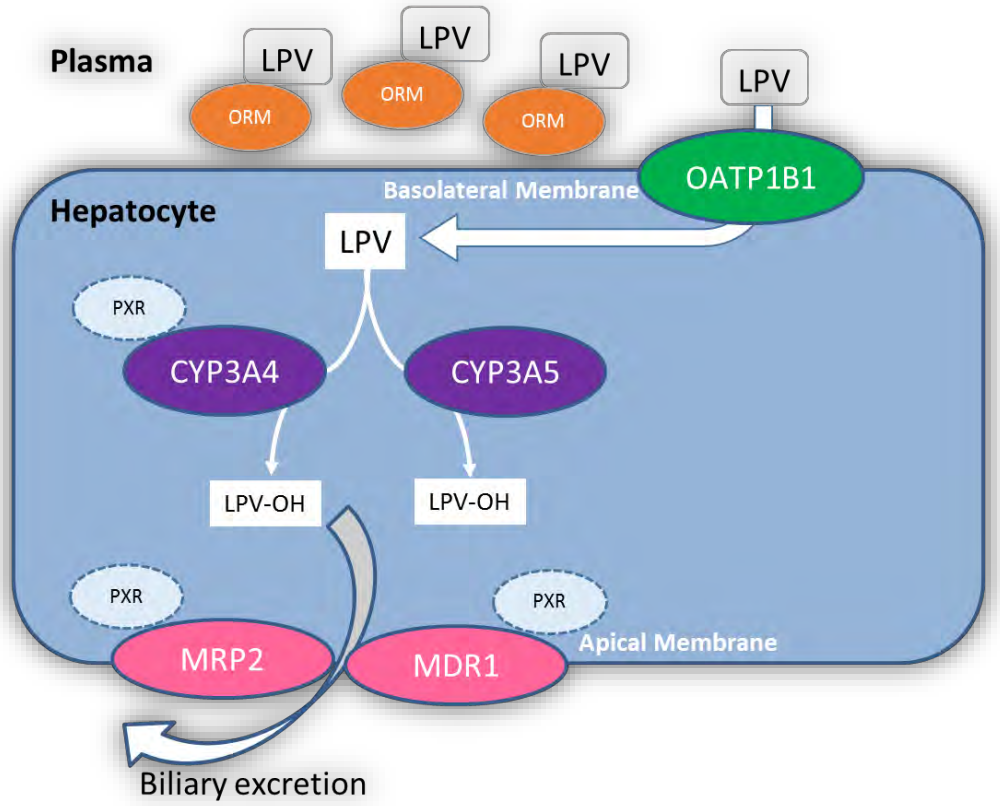

Figure 1.6 Schematic representation of some proteins involved in uptake, distribution metabolism and efflux of Lopinavir in the liver. LPV: Lopinavir; LPV-OH: hydroxylated products of Lopinavir; MDR1: multidrug transporter, P-glycoprotein; MRP2: multidrug resistance-associated protein 2; OATP1B1: organic anion transporter 1B1; ORM: orosomucoid; PXR: pregnane X receptor.

1.6 Lopinavir Pharmacogenetics

Variation in genes that encode proteins involved in the metabolism and disposition of drugs is often associated with differences in plasma drug levels and patient drug responses. Single nucleotide polymorphisms (SNPs) within genes may play a role in the inter-individual variability in drug disposition either through changes in enzyme activity or expression levels (78). For instance, Svärd *et al*. (78) reported that Val140Met (valine to methionine substitution at amino acid 140) and Ala370Thr (alanine to threonine substitution) SNPs in PXR decreased induction of *CYP3A4* promoter activity by LPV.

1.6.1. SLCO1B1

Genetic variation affecting the functioning of OATP1B1 may influence uptake of LPV into the liver, and hence its metabolism and clearance. The *SLCO1B1* gene encoding OATP1B1 is located on the short arm of chromosome 12 as with other genes of the *SLCO1* family (132). A highly reported SNP in literature is the rs4149056 (c.521T>C) SNP which leads to a Val to Ala change at amino acid 174 of

OATP1B1 (Fig 1.7). Although not much is known about OATP1B1 and the uptake of LPV, the rs4149056C allele has been associated with increased plasma concentrations of the drug in HIV-positive patients (79, 105, 133). Therefore, it could be suggested that when the functional activity of OATP1B1 is decreased, less drug is taken up into the liver and more free drug is available in the blood stream which may lead to toxic effects. However, in the study by Schipani *et al*. (79) only 2% of the patients were homozygous for the minor allele and the c.521T>C SNP is known to have a low frequency in African populations (1-2%); therefore its significance in these populations may be low.

Other SNPs in *SLCO1B1*, such as 388A>G (rs2306283), which results in an asparagine to aspartate substitution at amino acid 130 of OATP1B1 [Fig 1.7; (105)] may also be associated with the reduced activity of the protein contributing to joint effects. Four functionally distinct haplotypes are formed by combining the c.521T>C and c.388A>G SNP as these two SNPs are in linkage disequilibrium (LD), namely: c.521T/c.388A, *1A; c.521T; c.388G/c.521T, *1B; c.521C/c.388A, *5; c.521C/c.388G, *15. The *SLCO1B1*5* and *15 haplotypes have been associated with decreased activity of OATP1B1 *in vitro* (134). However, little or no information is available on the distribution and therefore possible effects of these haplotypes in African populations. Moreover, African populations are the most affected with HIV/AIDS, and therefore more likely to use drugs that are substrates of OATP1B1, which means that possible deleterious effects of variation will be more prominent in these populations.

Identifying the possible effect of SNPs in *SLCO1B1* also aids in understanding drug-drug interactions. When drugs which are common substrates of OATP1B1 are used together, drug-drug interactions have been reported to occur such as for rifampicin (used in TB treatment) and atorvastatin when each are co-administered with LPV (110). In South Africa, especially in the Western Cape, many individuals are co-infected with HIV and TB and are therefore at high risk for experiencing such drug-drug interactions (135).

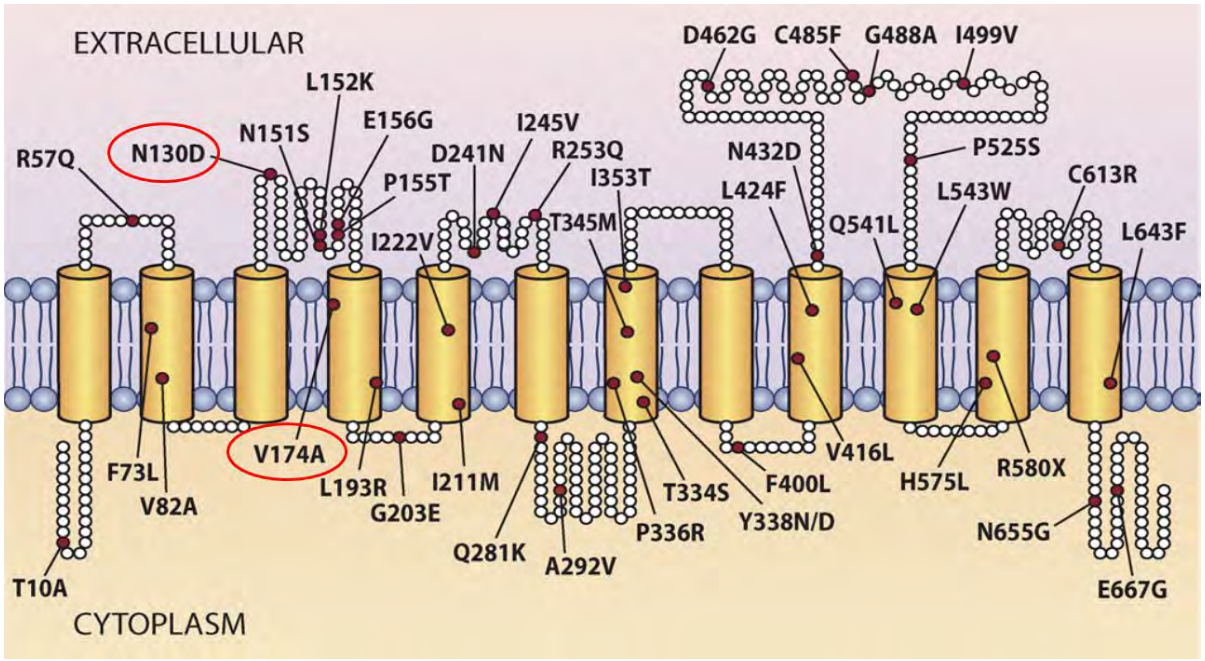

Figure 1.7 The predicted transmembrane structure of OATP1B1 encoded by *SLCO1B1*. The positions of non-synonymous SNPs of interest are circled in red. Figure adapted from Niemi *et al*. (106).

1.6.2. CYP3A4

The *CYP3A4* gene is located on chromosome 7q21-22 (136) and is 27 kb in length, comprised of 13 exons. Much inter-individual variability (up to 40 fold) has been characterised in the expression of *CYP3A4* both in the intestine (137) and in the liver (138). Few functional genetic variants have been found within *CYP3A4* (http://www.cypalleles.ki.se/cyp3a4.htm). It is also important to note that genetic variation is not homogenous across populations (139) and so a variant which may have a significant influence in protein function and hence drug disposition in one population, may have a weaker influence in another population.

The *CYP3A4*1B* allele is encoded for by the -392A>G SNP in the promoter region of *CYP3A4* (140, 141). As reviewed by Lamba *et al*. (138) and Werk *et al*. (112)(2014), there are conflicting reports on the functional significance of *CYP3A4*1B* allele. The SNP has been suggested to lie within a nifedipine (drug used to treat hypertension) specific binding element in *CYP3A4* (141, 142) and was previously hypothesised to affect expression and, possibly, activity of CYP3A4 (140). This hypothesis was due to an association that was found between carriers of the *CYP3A4*1B* allele and prostate cancer in Caucasian men. However, Rebbeck *et al*. (140) do state that the association may have been due to an over-representation of the SNP in their 230 cases compared to the 94 healthy controls. This may be likely, as subsequent research has failed to prove an effect of this variant of expression or activity of the enzyme. The effect of the SNP on transcriptional activity of *CYP3A4* was investigated through an

in vitro enzymatic activity assay where the hydroxylation of 6β-testosterone, a reaction catalysed by CYP3A4, was studied (141). Although much variability was shown in CYP3A4 activity, no differences were found between constructs harbouring the wild-type, *CYP3A4*1* allele and constructs harbouring the *CYP3A4*1B* allele. Further studies have failed to prove an association of this allele with activity of the enzyme. The allele frequency varies among populations, ranging from 0.03 to 0.05 in Caucasians and 0.46 to 0.82 in Africans and African Americans (139, 143).

A second SNP of possible functional significance is also found in a non-coding region of *CYP3A4*. After investigating various SNPs, Wang *et al*. (144) reported on an intron 6 SNP (*CYP3A4*22*, rs35599367C>T) to be functionally significant in a mixed population, representative of the population demographic in Columbia, Ohio. The study reported an association between *CYP3A4*22* and statin dosage in patients being treated for hypercholesterolemia. *In vitro* expression analysis showed lower levels of *CYP3A4*22* allele RNA compared to *CYP3A4*1* allele RNA. Subsequent research has found the *CYP3A4*22* allele to be associated with reduced protein levels and function in vitro (145). The intron 6 SNP is more prevalent in Caucasians (3 to 5%) than in Africans and African Americans (0 to 2%). Hence, this SNP seems to be less significant in African populations, it has not yet been characterised in a sub-Saharan African cohort. Characterisation of these variants in a sub-Saharan population and investigating possible associations with LPV plasma levels may aid in explaining inter-individual variability in treatment response.

1.6.3. CYP3A5

CYP3A5 is located on the same locus as *CYP3A4* on chromosome 7q21-22 and, as with *CYP3A4*, *CYP3A5* is also comprised of 13 exons, but is 32 kb long. The expression of CYP3A5 is polymorphic as individuals with the wild-type *CYP3A5*1* allele produces greater levels of full-length mRNA and express more CYP3A5 (146). The *CYP3A5*1* allele has been reported to be in LD with the *CYP3A4*1B* allele in Caucasians and African Americans (147, 148). Two SNPs that have been shown to influence this polymorphic expression are an intron 3 SNP (6986A>G) and a SNP in exon 7 (14690C>T or G>A) of *CYP3A5.*

The *CYP3A5*3* (6986G) allele results in a truncated protein through alternative splicing mechanisms which result in a premature stop codon after amino acid 102 (146). This leads to degradation of the mRNA and hence low levels of CYP3A5 expressed from the *CYP3A5*1* allele. The effect of this SNP on midazolam clearance and, hence, CYP3A5 activity has been investigated in a cohort of predominantly Caucasian cancer patients (149). The *CYP3A5*3* was more prevalent than the *CYP3A5*1* allele in the

study population. Although a significant association was found between carriers of the *CYP3A5*3* allele and reduced midazolam clearance, this result may be influenced by the low representation of *CYP3A5*1* allele carriers. Subsequent studies have not found a role for *CYP3A5**3 in midazolam clearance (148, 150, 151). The effect of the SNP has also been investigated on tacrolimus and cyclosporine clearance in Caucasian renal transplant patients (152) and on the metabolism of the PI, saquinavir, in HIV-seronegative black Tanzanian individuals (153). The *CYP3A5*3/*3* genotype was reported to be associated with taclorimus dose-requirements and plasma trough levels but not on cyclosporine dose-requirements (152).

Although LPV is also metabolised by CYP3A5, Rakhmanina *et al*. (108) found no association with the *CYP3A5*3* allele (allele frequency G=0.417) and LPV concentrations in a cohort of HIV-infected children (a majority of African-American population). Estrela *et al*. (154) suggest that the inhibitory effects of RTV on CYP3A5-mediated LPV metabolism may eliminate the effect of polymorphisms within *CYP3A5*. It will be interesting to determine the relevance of this SNP for LPV pharmacokinetics in a sub-Saharan African population where the G allele has been found to be even less prevalent at a frequency of 0.09 (155).

The *CYP3A5* 14690A allele (*CYP3A5*6*) results in the deletion of exon 7 from the protein (146). It is thought that the point mutation lies within an exon splicing enhancer region of exon 7, disrupting the splicing of the exon and leading to skipping of exon 7. The contribution of *CYP3A5*6* to low expression of CYP3A5 in African populations is not well understood.

The frequencies of *CYP3A5*3* and **6* vary across populations. The *CYP3A5*1* allele is more common in Africans than in Caucasians and Asians with the frequencies of *CYP3A5*3* being on average 23%, 85% and 29% respectively, and of *CYP3A5*6* being 17%, 0% and 1% respectively (139). These frequencies may suggest that the role of *CYP3A5*3* is more significant in Caucasians than in other populations.

Daly (156) reviewed other missense mutations within *CYP3A5* such as 27131-32insT (*7 allele); 12952T>C at the intron 5 junction (*5 allele); 14665A>G (*4 allele) detected in a Chinese population; and a few other rare SNPs, 3699C>T (*8 allele) and 19386G>A (*9 allele). Rare variants have been identified in African (157) and Japanese populations (158) but these were associated with the 6986A>G SNP.

1.6.4. ABCC2

The association of a few SNPs, including the *ABCC2* rs717620 SNP (-24C>T) with LPV/r induced viral suppression was investigated in 69 Caucasian patients, 38 in whom the virus was suppressed (159). The SNP was determined not to be a determinant of reduced viral suppression in these patients. A trend towards association was only present when the joint effects of the rs4149056 (in *SLCO1B1*), rs6945984 (in *CYP3A*) and rs717620 were determined in a univariate analysis, however the effect did not remain when a multivariate analysis was conducted. The SNP has been reported to result in decreased expression and activity of the protein (160). According to data from 1000 Genomes (http://spsmart.cesga.es/search.php?dataSet=engines), rs717620 SNP has been reported to have a low frequency in African populations (Yoruba population, Nigeria; and Luhya population, Kenya) of 0.033 compared to 0.211 in Caucasians. The study by Glass *et al*. (159), in which an association with rs717620C>T and LPV/r induced viral suppression was observed, was conducted in a Caucasian population and thus the effect reported may be more relevant in this population which has a higher frequency of the variant.

In a study on Chinese individuals with epilepsy investigating the effect of SNPs in *ABCC2* on epileptic drug resistance (161), it was reported that rs717620C>T was in LD with a SNP in exon 10 of the gene (c.1249G>A, rs2273697; D'=0.694). Although an association was identified with the -24T allele and drug resistance, no association was identified with the c.1249G>A SNP and drug resistance. The frequency of this variant is reported at 0.199 in African populations, and slightly lower at 0.134 in Chinese according to 1000 Genomes data (http://spsmart.cesga.es/search.php?dataSet=engines).

In an *in vitro* study investigating the effects of another SNP, rs8187710 (c.4544G>A), on MRP2 activity, this variant was suggested to decrease the efflux activity of the transporter (81). Polymorphisms that decrease the activity of this efflux transporter may result in drug failure associated with reduced excretion of the metabolised drug from the liver. The c.4544G>A SNP (which results in a cysteine to tyrosine transition at position 1515 of the protein) in *ABCC2* has been suggested to result in toxicity of certain drugs due to decreased cellular efflux, for example, of doxorubicin and flavopiridol (both used in cancer treatment) (162, 163). Interestingly, both these drugs are also substrates for the uptake transporter OATP1B1. Elens *et al*. (81) reported that this 4544G>A SNP resulted in increased cellular accumulation of LPV in transfected cells. In cells with the G variant, the intracellular accumulation of LPV was almost 3 times lower than in cells with the variant allele, suggesting that the SNP reduces the functional activity of the protein (81). It has been reported to be in a haplotype with rs17222723 (c.3563T>A) (164) and both are suggested to be associated with non-alcoholic fatty liver disease (165).

These are just a few of the SNPs which may possibly have an effect of the activity of ABCC2 and hence on its transport of LPV/r. It would be important to study the importance of this gene in LPV/r pharmacogenetics, identifying any important SNPs from the above mentioned or even SNPs not previously investigated. This would be of particular interest to us in a sub-Saharan African context.

1.7 Aims and Objectives

The aims of this study were, then, firstly, to identify variation in genes that may affect LPV disposition and compare the frequencies of the identified variants with those of other populations. The second aim was to identify the role of these variants with respect to plasma LPV levels to possibly be used as markers for LPV response within the specified populations.

In order to achieve these aims, the objectives were to:

- Recruit patients on LPV/r regimen
- Identify genes commonly implicated in LPV disposition from the literature
- Identify variation in the selected genes associated with variable LPV response
- Determine the genotypes of patients at loci of interest
- Determine the allele frequencies for the selected variants
- Correlate variation in the genes with the determined LPV concentrations from patient blood samples

2. Materials and methods

This was a cross-sectional study involving Bantu Africans recruited from South African and Malawian populations groups (N=86). The participants were adult HIV/AIDS patients on ART regiment with LPV/r.

2.1 Participants

Recruited participants were 86 unrelated, adult individuals with HIV/AIDS on ART regimen with LPV/r. The inclusion criteria included participants who identified themselves as Bantu African for at least three generations, were at least 18 years of age and had been on ARV treatment for at least six months. In addition, participants had to give informed written consent for participation and agree to the accessing of their medical records. The patients were placed on second-line ART after failing first-line ARV therapy, either due to adverse reactions experienced while on first-line treatment or failure of the treatment to suppress the virus (<20 to 75 copies/mL). First-line therapy was 3TC+AZT or 3TC+NVP+d4T or TDF. Fifty-two individuals from Malawi (Queen Elizabeth ART Clinic, Blantyre); and thirty-four individuals from South Africa (Helen Joseph Hospital, Thembalethu, Johannesburg) were recruited after giving written and verbal informed consent. Participants were on treatment for at least 6 months up to 9 years at the time of recruitment. The sample size was small due to there being few participants on second-line treatment. Blood samples were collected from these patients, in EDTA (ethylenediaminetetraacetic acid)-coated tubes and kept at -20°C until subsequent DNA extraction and genetic analysis, and for LPV plasma level detection. Patients' demographic information was obtained from their medical records and the CD4 count, viral load and liver enzyme levels were also available from clinical patient records. Ethical approval for the study was obtained both from the Malawi University College of Health Sciences and the University of Cape Town, Faculty of Health Sciences Human Research Ethics Committee (HREC REF: 439/2013).

2.2 DNA Isolation

2.2.1 DNA Isolation using GenElute™ Blood Genomic DNA Kit

For the blood samples collected from Malawi, the GenElute™ Blood Genomic DNA Kit (Sigma-Aldrich, St Louis, USA) was used for the extraction of DNA from the blood samples according to the manufacturer's instructions. For each sample, whole blood that had been collected in a tube coated with an anti-coagulant. 10 µL of proteinase K (20 mg/mL) was added to 250 µL of whole blood in a 1.5 mL eppendorf tube. 20 µL of RNase A solution was added to the mix (in order to obtain RNA-free genomic DNA); the contents were mixed by gently inverting the tube 6 to 8 times and the tubes were

then incubated for 2 min at room temperature. The cells were lysed by adding 1 volume (275 µL) of lysis solution C to the samples, followed by vortexing the tube for 15 s. The samples were then incubated at 55°C for 1 hour. 250 µL of the column preparation solution was added to the GenElute miniprep binding column and centrifuged at 12 000 x g (rcf) for 1 min; this solution allows DNA to bind efficiently to the column to obtain good yields after isolation. The eluted liquid was discarded and the column left to stand for approximately 10 min. In preparation for binding of the DNA, 275 µL of absolute ethanol (95% - 100%) was added to the lysate from the cell lysis step and mixed thoroughly by mixing for 5 to 10 s. 650 µL of the contents of the tube were transferred to the treated column; the column was centrifuged at ≥6500 x g for 1 min. The process was repeated twice, with the collection tube being discarded each time and replaced with a new one. 250 µL of prewash solution (diluted with 110 µL absolute ethanol prior to use; the solution helps to remove contaminants in blood samples that aren't fresh) was added to the column and centrifuged at ≥6500 g for 1 min. The collection tube containing the eluted liquid was discarded and replaced with a new 2 mL collection tube. 250 µL of wash solution (diluted with absolute ethanol prior to use) was added to the column and centrifuged for 4 min at maximum speed (12 000 g). It is important to ensure that there is no residual ethanol remaining in the column after this step which is just before the DNA elution step. Ethanol is thought to interfere with downstream processes such as polymerase chain reaction (PCR) and sequencing through inhibition of used enzymes. The collection tube containing the eluted liquid was discarded and replaced with a new 2 mL collection tube. After the ethanol wash of the column, the DNA is assumed to have bound to the column, thus, 200 µL of elution solution (10 mM Tris-HCl, 0.5 mM EDTA, pH 9.0; Tris-EDTA buffer prevents the degradation of the DNA) is added directly into the centre of the column. The solution was incubated overnight at room temperature to increase the efficiency; and the following day the column was centrifuged at ≥6500 g to elute the DNA. The collection tubes containing the eluted pure genomic DNA were stored at -20°C for long-term use. 50 µL aliquots of DNA were stored at -4°C as working solutions for downstream analyses.

2.2.2 DNA Isolation using a Salting out Extraction Method

For the blood samples collected from the South African participants, DNA was extracted using a salting out extraction method adapted from Gustafson *et al*.'s (166) protocol. According to this method, 4 mL of blood thawed at room temperature was diluted with phosphate buffered saline (PBS) after which samples were centrifuged at 2200 x g for 10 min. The supernatant was then poured off and the pellet re-suspended in 10 mL Sucrose Triton X-100 lysis buffer (see recipe in appendix). This buffer is made up 1 M Tris-HcL (pH 8; provides the necessary pH for the buffer), 1 M $MgCl_2$ (provides Mg^{2+} ions which act as co-factors for the enzymes in the solution), Triton X-100 (a detergent which solubilises proteins

by manipulating hydrophobic-hydrophilic interactions of membranes) and sucrose (increases the osmolarity of cells allowing water to flow in so that the cells burst). The tubes were placed on ice for 5 min and then centrifuged at 2200 g for 10 min. The supernatant was carefully poured off and the pellet resuspended in 3 mL of T20E5 (pH 8.0; see protocol for making the solution in appendix A) and the tubes inverted repeatedly to mix. 200 µl of 10% SDS (sodium dodecyl sulphate) was added and again the tubes were inverted, followed by the addition of 100 µL of proteinase K (final concentration of 250 µg/mL) and mixing of the solution. SDS breaks down cell membranes to release lipids and proteins, and proteinase K digests proteins, particularly DNase and RNase enzymes. The samples were incubated overnight in a water bath at 45°C. The following day, 1 mL of saturated NaCl was added to the samples to precipitate the proteins and each sample was mixed by vortexing for 15 s, and then centrifuged at 2400 g for 30 min. The DNA-containing supernatant was transferred to a new sterile polypropylene tube and 8 mL of 100% ethanol (kept at room temperature) was added and the tubes were gently agitated and the DNA was spooled using a glass Pasteur pipette. The DNA was transferred to an eppendorf tube and washed by the addition of 1 mL 70% ice-cold ethanol. The samples were left to air dry after which the DNA was dissolved in 150 µL of 1X TE buffer (pH 8.0).

2.2.3 DNA Integrity – NanoDrop™ Spectrophotometry

After DNA extraction, it is good laboratory practice to determine the quality of the isolated DNA by checking its integrity and purity. The integrity of extracted DNA was determined by analysis of 100-500 ng on a 1.5% agarose gel using electrophoresis, whilst the purity of the DNA was measured using a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). Through spectrophotometry using the NanoDrop, we were able to measure the absorbance of DNA at 260 nm in order to determine its concentration. The NanoDrop™ Spectrophotometer makes use of fibre optic technology and surface tension to measure the absorbance of nucleic acids, in this case DNA. 1uL of sample is loaded onto an optical surface and held in place between two optical surfaces that define path length in a vertical direction (167). The concentration of DNA isolated using the GenEulute Blood Genomic DNA kit ranged from 6.19 to 91.19 ng/µL, while that of the DNA isolated using the salting out extraction method ranged from 3.48 to 624.53 ng/µL. The A_{260}/A_{280} ratio indicates the purity of the DNA. For DNA that is pure from contaminants such as alcohol, protein and RNA, a ratio of 1.7-1.9 is expected. The 260/280 ratios obtained with spectrophotometry ranged from 1.5 to 2.1. The A_{260}/A_{230} ratio is a secondary measurement of DNA purity, a value below the expected 2.0-2.2 may indicate the presence of contaminants such as phenol, carbohydrates and EDTA which absorb light at a wavelength of 230 nm. The values obtained for the A_{260}/A_{280} ranged from 0.4 to 4. The values outside the

appropriate range may have been indication of alcohol, or protein impurity. However, this did not impact on downstream investigations.

2.2.4 DNA Integrity – Agarose Gel Electrophoresis

Agarose gel electrophoresis is a method used to analyse DNA by separating DNA molecules based on size through the pores in a gel. The percentage of the gel determines the size of the pores in the gel, and hence the size of DNA fragments that can be separated on the gel. Small fragments (50 to 300 bp) will require a high percentage gel (2 to 5%), whilst larger fragments (e.g. 500 to 1500 bp) require a lower percentage gel (0.8 to 1.5%). To make the gel, agarose powder was dissolved in 1X Tris-Borate EDTA (TBE) buffer and melted for 3 to 5 min depending on the concentration of the gel (high percentage gels required a longer heating time to dissolve the agarose). Once melted, the solution was allowed to cool to a temperature around 55°C after which the nucleic acid stain GR Green (5 µL/100 mL of solution) was added. Nucleic acid stain, GR Green, facilitates the visualisation of DNA under UV light. Once the GR Green had been homogenously mixed, the gel solution was poured into casting trays with combs inserted (to create wells in the gel), and the gel was left to set for approximately 30 min.

After the gel had set, the combs were removed creating wells where samples were loaded into the wells, for integrity evaluation. The gel was placed in a tank containing 1X TBE buffer, in which a current was run. We used the Enduro™ power pac (Labnet, Woodbridge, USA) with a maximum output of 300 V or 500 mA. The current allows the DNA to migrate from the cathode (negative end) of the tank towards the anode (positive end) of the tank, as DNA is negatively charged, thus the wells should be placed closest to the cathode. The voltage that is set determines the speed at which the DNA migrates; typically the standard is 5 V/cm (168). Small fragments migrate faster and further than large fragments through the gel. Before loading the samples into the wells, 3 µL of a 1X loading dye containing two dyes (Bromophenol Blue and xylene cyanol FF) and Glycerol (Thermo Scientific, Wilmington, USA) was mixed with 5 µL of the sample. The dyes allow the tracking of the DNA as it migrates on the gel; whereas, glycerol facilitates the sample to sink to the bottom of the wells. A molecular weight marker is often used in agarose gel electrophoresis to guide us on the size of the DNA molecules being separated. In this, case, the GeneRuler™ 100 bp plus ladder (Thermo Fisher Scientific, Wilmington, USA) was used as the molecular weight marker was loaded into one of the wells. After electrophoresis, agarose gels were placed in the FireReader V4 UV gel documentation machine (UVitec Limited, Cambridge, UK) so that the DNA fragments could be visualised under UV light, and an image was captured using the machine's CCD (charge-coupled device) camera.

2.3 Genetic Characterisation of Samples

The extracted DNA was used for genetic characterisation of genes that are known to play a role in the disposition of LPV and these include *CYP3A4, CYP3A5, SLCO1B1*, and *ABCC2* (Table 2.1). Each of these genes was annotated using the PerlV5 program and the SNPs to be genotyped were selected based on literature and with respect to their frequency in populations, as well as on their possible functional effects. The targeted SNPs include c.-392A>G (rs2740574) and a SNP in intron 6 (rs35599367C>T) in *CYP3A4*; c.6986A>G (rs776746) and 14690C>T (rs10264272) SNPs in *CYP3A5;* c.388A>G (rs2306283) and c.521T>C (rs4149056) in *SLCO1B1*; c.1249G>A (rs2273697), c.-1019A>G (rs2804402), and c.-1549G>A (rs1885301) in *ABCC2*.

Table 2.1 Variation in genes associated with Lopinavir disposition.

Gene	SNP	Allele	Possible Functional Effect of SNP	Variant Allele Frequency			
				Africans		Caucasian	Asian
				Sub-Saharan African	African American		
CYP3A4	rs2740574A>G	*1B	May influence drug plasma levels ↓ CYP3A4 protein levels	0.7-0.8	0.68	0.023	0.00
	rs35599367C>T	*22	May influence CYP3A4 expression and activity	0.00	N/D	0.025	0.00
CYP3A5	rs776746G>A	*3	Loss of CYP3A5 function	0.83-0.87	0.63-0.74	0.04-0.06	0.26-0.34
	rs10264272C>T	*6	Deletion of exon 7 of gene	0.14-0.25	0.115	0.00	0-0.01
SLCO1B1	rs4149056T>C	*5	Amino acid change in substrate recognition domain of transporter	0.009	0.022-0.083	0.08-0.194	0.10-0.16
	rs2306283A>G	*1B	Possibly associated with reduced function of SLCO1B1	0.81	0.73-0.81	0.30-0.44	0.65-0.8
ABCC2	rs717620C>T		Decreased promoter activity, mRNA expression and duodenal protein content	0.03-0.06	N/D	0.15-0.2	0.15-0.22
	rs2273697G>A		In LD with rs717620C>T	0.21-0.25	0.1-0.24	0.146-0.26	0.078-0.12
	rs8187710G>A		Decreased efflux activity of ABCC2	0.108-0.153	0.217	0.053-0.067	0.00
	rs17222723T>A		Haplotype formed with rs8187710G>A May be associated with non-alcoholic fatty liver disease	0.058	0.062-0.13	0.046-0.081	0.00

The online tools, Primer Blast (www.ncbi.nlm.nih.gov/tools/primer-blast/) and Oligo Analyzer® 3.1 (Integrated DNA Technologies Inc., USA; eu.idtdna.com/analyzer/Applications/OligoAnalyzer/) were used in the design and analysis of primers. Table 2.2 presents a summary of the methods and conditions used to genotype the selected genes.

Table 2.2 Primers and genotyping conditions for the targeted SNPs.

Gene	SNP/Region	Method	Primers	Size (bp)	T_a°
CYP3A4	c.-392A>G	PCR-RFLP	F: 5′-GGACAGCCATAGARACAAGGGCT-3′ R: 5′-AGGTTTCCATGGCCAAGTCT-3′	334	64°C
	c.522-191C>T	TaqMan	N/A		
		Sequencing	F: 5′-GCTCTGGGCATAGAGTCTGC-3′ R: 5′-ATG CAT GCA ACA GGA AAC CC-3′	364	59°C
CYP3A5	g.6896A>G	PCR-RFLP	F: 5′-CATCAGTTAGTAGACAGATGA-3′ R: 5′-GGTCCAAACAGGGAAGAAATA-3′	293	51°C
	g.14690G>A	PCR-RFLP	F: 5′-TGGAAGATGATTCAGCAGATA-3′ R: 5′-GTGGGGTGTTGACAGCTAAAG-3′	495	58°C
SLCO1B1	Exon 5	Sequencing	F: 5′-TATCTTTCTTGCTGGACACTTC-3′ R: 5′-CACACAGAAGATCACCTCAAG-3′ R2: 5′-CTAGTTCATATTGTTAAATGGT-3′†	1448	58°C
	Exon 6	Sequencing	F: 5′-ACAAGGAAACTGCAAAAGGA-3′ R: 5′-ATTATATTAAGCAAAGGACTATTGAAAGAG-3′ Internal R: 5′-AATCATCAATGTAAGAAAGCC-3′‡	1436	64°C
ABCC2	c.1249G>A	PCR-RFLP	F: 5′-GGGCAAAGAAGTGTGTGGAT-3′ R: 5′-ACATCAGGTTCACTGTTTCTCCCA-3′	303	61°
	-1632 to -1419	Sequencing	F: 5′-AAAAGTGTCTGTTCAAGTCC-3′ R: 5′-GTGAATTGTATGGACCTTGT-3′	214	51°C
	-1157 to -908	Sequencing	F: 5′-GGATACCGCATGGGTGGTTC-3′ R: 5′-ACTACAGGCACATGCCCACA-3′	249	60°C

N/A: Not applicable

†A second reverse primer was designed for sequencing of the SLCO1B1 exon 5 region (NCBI reference sequence: NC_000012.12), replacing the reverse primer used in the PCR amplification of that region.

‡ An internal reverse primer was designed for sequencing of the SLCO1B1 exon 6 and 7 region (NCBI reference sequence: NC_000012.12) to cover the gap not covered by sequencing with the forward and reverse primers.

2.3.1 Description of Genotyping Techniques Used in this project

2.3.1.1 Principle of PCR Restriction Fragment Length Polymorphism

PCR restriction fragment length polymorphism (PCR-RFLP) allows for the identification of specific variants through cleavage by DNA restriction enzymes which each recognise a specific restriction site (a sequence of nucleotides). RFLP is only used where the variant of interest creates or abolishes a restriction site making it possible to use patterns of restriction/digestion as markers for presence or absence of mutations. Fragments are separated by agarose electrophoresis to identify the fragment sizes achieved after enzymatic digestion, and the genotypes are thus determined.

NEBcutter (New England Biolabs® Inc., Beverly, USA) and RestrictionMapper (http://www.restrictionmapper.org/) were used to select suitable restriction enzymes for the characterisation of each of the SNPs. Briefly, an input sequence flanking the SNP of interest was pasted into the search box (typically the sequence of the amplified PCR fragment) and the program then generated an output of the possible restriction enzymes that would cleave that fragment, their restriction sites and the positions at which they would cleave (169). Each restriction enzyme used was selected based on the presence of the SNP of interest within the restriction site i.e. a restriction site was created or abolished in the presence of the SNP.

2.3.1.2 TaqMan® SNP Genotyping Assay

The TaqMan assay is a real-time PCR method that makes use of fluorescent probes to identify specific variants within genes (170). As with conventional PCR, primers are used to amplify the region of DNA containing the SNP of interest. The assay also contains 2 additional labelled minor groove binder probes which are short (20 nucleotides) sequence of oligonucleotides that are complementary to either the wild-type allele, or the variant allele. Each probe has a quencher dye at one end, and a reporter dye at the other end. The quencher dye absorbs baseline fluorescence emitted by the reporter dye when the two are in close proximity. When the probe binds to its complementary sequence, the probe is cleaved through the 5' nuclease activity of the *Taq* DNA polymerase as it extends the newly made strand. The reporter dye is able to release fluorescence. If the probe does not bind, it is not cleaved and the reporter and quencher dyes remain in close proximity and only low levels of fluorescence are emitted.

2.3.1.3 Sequencing to confirm genotypes or to identify novel variants

The Principle behind Cycle Sequencing

Cycle sequencing was performed to confirm the genotypes determined for *CYP3A4* and *CYP3A5* variants, and to identify novel variants in *SLCO1B1* exon 5, 6 and 7 and their exon-intron junctions. Cycle sequencing is based on the method published by Sanger *et al*. (171) involving dideoxy-nucleotides (ddNTPs) that terminate synthesis of newly formed chains at specific nucleotide residues. Unlike deoxynucleotides (dNTPs) used in the synthesis of DNA strands, ddNTPs lack a 3'-hydroxyl group essential in the formation of phosphodiester bonds during synthesis of the DNA strand. This prevents the chain from being further extended and results in the termination of synthesis of that strand. Cycle sequencing is a fluorescence-based method which uses fluorescent dyes to label the extension products (172, 173) and the products are analysed by capillary electrophoresis.

Sequencing Method

Following PCR amplification, products were purified using FastAp™ Thermosensitive alkaline phosphatase (Thermo Scientific, Wilmington, USA) and Exonuclease 1 (Exo 1; Thermo Scientific, Wilmington, USA) to remove unincorporated dNTPs and excess primers. FastAp™ is involved in the degradation of nucleotides through dephosphorylation, while Exo 1 is a hydrolytic enzyme which degrades single-stranded molecules in the reaction. 5 µL of PCR product was added to a mixture containing 1 µL (1 U) of FastAp™, 2 U of Exo 1 and 13.9 µL of distilled water (dH_2O; obtained from Sabax) to a total volume of 20 µL. The mixture was incubated at 37°C for an hour followed by 15 min at 75°C to inactivate the enzymes.

To sequence amplified fragments either to confirm the genotypes determined through RFLP and TaqMan or to identify novel variants, 4 µL of dH_2O, 2 µL of 5X Sequencing Buffer (Life Technologies, California, USA), 1 µL of BigDye® Terminator v3.1 Ready Reaction Mix (Life Technologies, California, USA), 1 µM primer (reverse or forward depending on the position of the SNP) and 2 µL of purified PCR product were added to each tube or well and mixed well by vortex pulsing. The Applied Biosystems®2720 Thermal Cycler (Life Technologies, California, USA) was used for sequencing with cycling conditions set at 98°C for 5 min followed by 35 cycles of 96°C for 30 s, 55°C for 15 s and 60°C for 4 min. Following the sequencing reaction, sequenced products were placed at 4°C or -20°C until purification of the products.

Ethanol Precipitation of Sequenced Products

Purification of the sequenced products is necessary to remove unincorporated dNTPs and labelled ddNTPs which may interfere with analysis of fluorescent signals during capillary electrophoresis. Sequenced products were purified by ethanol precipitation. For each 10 µL sequenced product, 22 µL cold absolute ethanol, and 1 µL of 3 M NaOAc (pH 5.21) was added to a 1.5 mL eppendorf tube. The sequenced product was then added to the tube, the tubes were vortexed and placed at -20°C overnight. The following day, the samples were centrifuged at 10 RPM for 10 min and the supernatant was then carefully removed by pipette. To each tube, 35 µL of 70% ethanol was added and again the tubes were vortexed and centrifuged at 10 RPM for 10 min. The supernatant was removed and the tubes were left open for at least 2 hrs (covered with paper towel) to allow the remaining ethanol to evaporate. Once dry, samples were resuspended in 10 µL dH_2O and placed in the fridge at 4°C until analysis by capillary electrophoresis.

Capillary Electrophoresis

The ABI Prism 3130*xl* Genetic Analyzer (Applied Biosystems®, Life Technologies, California, USA) was used for capillary electrophoresis. This is a 16-capillary electrophoresis instrument which allows for the sequencing of read lengths of more than 500 bases. A 36 cm capillary array was used to achieve up to 600 base length reads, with POP-7™ polymer. To prepare the plate for electrophoresis, 5 µL of sample was added to each well on a MicroAmp® Optical 96-well reaction plate (Applied Biosystems®, Life Technologies, California, USA) and 5 µL Hi-DI (highly deionised) Formamide (used as an injection solvent for capillary electrophoresis) was added to each sample. Samples are analysed in groups of 2 columns (16 wells) at a time, thus when analysis fewer than 16 samples, 10 µL of Hi-Di was added to the empty wells. The reaction plate was sealed with a septa mat to prevent evaporation of samples

while still allowing the capillaries access through the pierce-able septa. The sequenced products were denatured by placing the reaction plate in a thermal cycler at 94°C for 5 min.

2.3.2 Characterisation of variation in *SLCO1B1*

To identify novel SNPs within the region from exon 5 to 7 of *SLCO1B1* (see Fig. 2.1), the ABI Big Dye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies, California, USA) was used for sequencing and products were analysed on the ABI Prism 3130 Genetic Analyzer. For sequencing of the region containing exon 5, a 1448 bp fragment was amplified by PCR using the following primers, forward: 5'-TAT CTT TCT TGC TGG ACA CTT C-3'; reverse: 5'-CAC ACA GAA GAT CAC CTC AA- 3'. The PCR conditions used for amplification on the T100™ Thermal Cycler (Bio-Rad, Hercules, USA) were: 94°C for 3 min, 94°C for 30 s, followed by 35 cycles of 65°C for 30 s, 72°C for 1 min 30 s, and 72°C for 7 min.

For sequencing of the region containing exon 6 and exon 7, a 1436 bp fragment was amplified by PCR using the following primers, forward: 5'-ACA AGG AAA CTG CAA AAG GA-3'; reverse: 5'-ATT ATA TTA AGC AAA GGA CTA TTG AAA GAG-3'.The PCR conditions used for amplification were: 94°C for 3 min, 94°C for 30 s, followed by 35 cycles of 64°C for 30 s, 72°C for 1 min 30 s, and 72°C for 7 min.
Amplified fragments were purified for downstream sequencing, as described in section 2.3.1.3 above. To sequence the amplified fragments, 3.5 µL of dH_2O, 2 µL of 5X sequencing buffer (Life Technologies, California, USA), 2 µL of BigDye® Terminator v3.1 Ready Reaction Mix (Life Technologies, California, USA), 1 µM primer (refer to Table 2.2 for primers used for sequencing) and 1.5 µL of purified PCR product were added to each tube or well and mixed well by vortex pulsing. The Applied Biosystems®2720 Thermal Cycler (Life Technologies, California, USA) was used for sequencing with cycling conditions set at 98°C for 5 min followed by 35 cycles of 96°C for 30 s, and 60°C for 4 min and 15 s. Following the sequencing reaction, sequenced products were placed at 4°C or -20°C until purification of the products. The sequences were assembled and aligned using DNAstar Lasergene® SeqMan Pro™ software (DNASTAR Inc., Madison, USA); NCBI reference sequence: NC_000012.12.

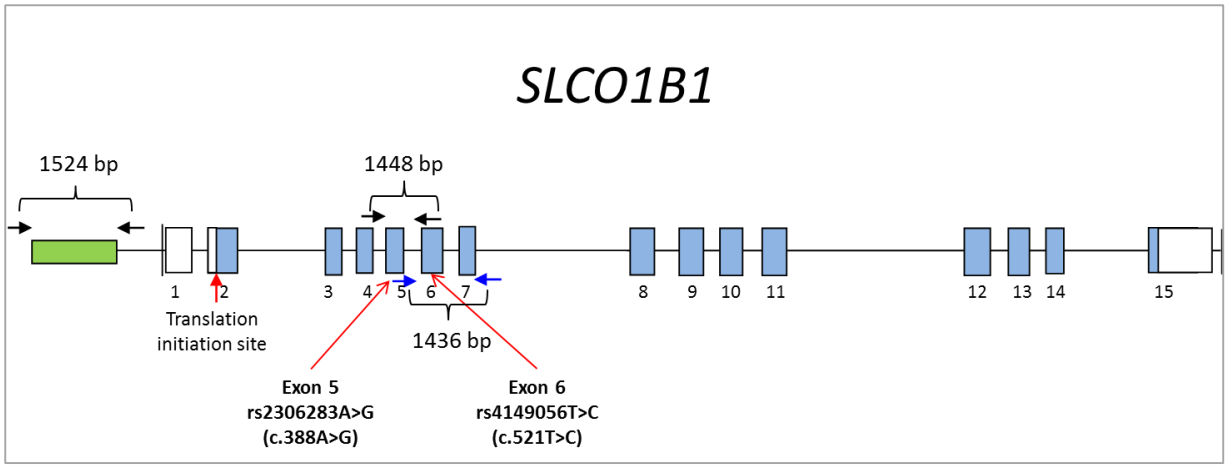

Figure 2.1 Schematic diagram of *SLCO1B1* gene showing the regions that were genotyped. The gene is 108.6 kb long ad has 14 coding exons (blue boxes). Brackets indicate the regions which were sequenced, with two known SNPs of interest in exon 5 and exon 6 shown with arrows.

2.3.3 Characterisation of variation in *CYP3A4* and *CYP3A5*

The CYP3A family genes (*CYP3A4, 3A7* and *3A5*) are located in a cluster on the same locus on chromosome 7 and a total of 4 SNPs (2 in *3A4* and 2 in *3A5*) were genotyped for in this project. *CYP3A4* is 27 kb in length, whilst *CYP3A5* is 32 kb in length (Figure 2.1).

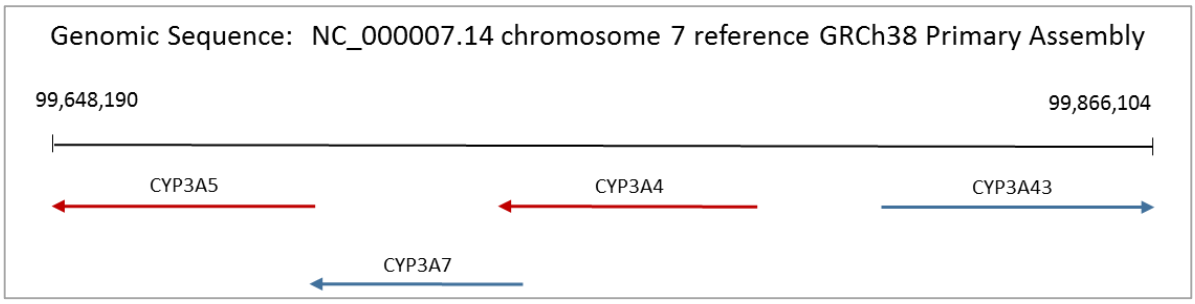

Figure 2.2 Schematic diagram of position and orientation of CYP3A family genes as on the NCBI gene database. Diagram not drawn to scale. Numbers above the black line indicate the position of the genes in the genome (Reference sequence: NC_000007.14).

PCR-RFLP was used to genotype the rs2740574A>G SNP in *CYP3A4*, and the rs10264272C>T and rs776746A>G SNPs in *CYP3A5*.

2.3.3.1. CYP3A4 rs2740574A>G SNP characterisation

The position of the genotyped SNPs within *CYP3A4* is indicated in Fig. 2.3. PCR-RFLP of a 448 bp fragment within the *CYP3A4* promoter containing the rs2740574A>G SNP was performed using primers and a restriction enzyme as reported by Tavira *et al*. (174) (Table 2.2). The Bio-rad T100™ Thermal Cycler (Bio-rad Laboratories Inc, Hercules, CA) was used for PCR amplification, set at the

following conditions: 94°C for 3 min, 35 cycles of 94°C for 30 s, 64°C for 60 s, 72°C for 1 min, 72°C for 10 min. The following primers were used for PCR, forward: 5'-GGA CAG CCA TAG ARA CAA GGG CT-3'; reverse: 5'-AGG TTT CCA TGG CCA AGT CT-3'. PCR conditions were carried out as follows: initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, annealing at 64°C for 60 s, and 72°C for 60 s; final extension at 72°C for 10 min. The T100™ Thermal Cycler (Bio-Rad, Hercules, USA) was used for the PCR reaction. The presence of the correct fragment was confirmed by agarose gel electrophoresis, on a 1.5% Agarose™ Gel using SeaKem® LE Agarose (Lonza, Rockland, USA). 3 µL of 1X loading dye was added to 5 µL of product and loaded into the well. Gels were run for 45 to 60 min at 100 V.

The PCR product was digested with *Fsp*BI (*Bfa*I) enzyme (Thermo Fisher Scientific, Wilmington, USA) by incubating overnight at 37°C. Digested products were then analysed on a 5% nusieve gel made up using Nusieve™ 3:1 agarose (Lonza, Rockland, USA) according to the suppliers instructions and electrophoresed for 120 min at 80 V, followed by 60 min at 120 V. In the presence of the A allele, the 448 bp fragment was cleaved twice to yield three fragments, 256, 170 and 22 bp. In the presence of the variant G allele, the 448 bp fragment was cleaved once to yield two fragments of 278 and 170 bp.

2.3.3.2. CYP3A4 Intron 6 rs35599367C>T SNP Characterisation

To genotype the intronic (rs35599367C>T; *CYP3A4*22*) SNP in *CYP3A4*, a TaqMan assay was performed using TaqMan® SNP Genotyping Assay (Assay ID: C__59013445_10) and TaqMan Universal Master Mix (Life Technologies, California, USA). See section 2.3.1.2 for a brief description of the technique.

To confirm the genotypes, sequencing was performed using the following primers, forward: 5'-GCT CTG GGC ATA GAG TCT GC- 3'; reverse: 5'-ATG CAT GCA ACA GGA AAC CC- 3'. PCR, to amplify a 364 bp fragment, was performed on the T100™ Thermal Cycler (Bio-Rad, Hercules, USA): initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 59°C for 30 s, and 72°C for 40 s; and final extension cycle of 72°C for 10 min. The amplified fragments were then separated on a 1.5% agarose gel to confirm amplification of the correct fragment. The post-PCR clean-up of the amplified fragments was performed using FastAp™ thermosensitive alkaline phosphatase and Exo 1 enzymes for the removal of unincorporated dNTPs and primers. Cycle sequencing was performed on the purified PCR product under these conditions: 98°C for 5 min, 96°C for 30 s, 58°C for 15 s and 60°C for 4 min. The sequenced products were purified by ethanol precipitation. For the sequencing analysis, 5 µL of each

product was added onto 96 well ABI plates, to which 5 µL Hi-Di formamide (Life Technologies, California, USA) was added to resuspend the DNA sample.

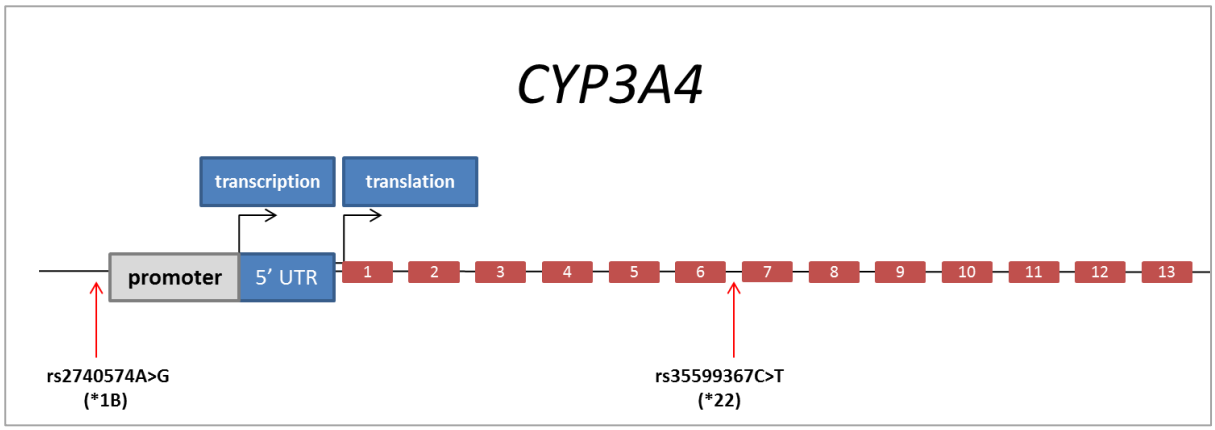

Figure 2.3 Schematic diagram of CYP3A4 gene. The position of SNPs that were genotyped are indicated with arrows. The gene is 27 kb in length, containing 13 exons.

2.3.3.3. CYP3A5 rs776746G>A SNP Characterisation

Figure 2.4 shows the position of SNPs genotyped for in *CYP3A5*. To genotype for *CYP3A5*3* (rs776746 A) allele, a 293 bp fragment was amplified under the following conditions (adapted from Van Schaik *et al*. (175) with modifications): initial denaturation at 94°C for 3 min, followed by 30 cycles at 94°C for 30 s, 51°C for 30 s, and 72°C for 30 s; and a final extension step at 72°C for 10 min. The Mycycler™ Thermal Cycler (Bio-Rad, Hercules, USA) was used for the PCR reaction. The amplified fragments were digested with the *Ssp*I restriction enzyme (Thermo Fisher Scientific, Wilmington, USA) incubated overnight at 37°C and then at 65°C for 5 min to inactivate the enzyme. The digested products were separated on a 4.5% nusieve gel for 120 min at 80 V, followed by 60 min at 120 V. In the presence of the G variant, the restriction site is abolished to yield 125 bp and 168 bp fragments; whilst for the A allele three fragments of 20, 125 and 148 bp were obtained.

2.3.3.4. CYP3A5 rs10264272C>T SNP Characterisation

In order to genotype for the *CYP3A5*6* (rs10264272T) allele, a 495 bp fragment was amplified using the following using forward primer: 5'-TGG AAG ATG ATT CAG ATA-3' and reverse 5'-GTG GGG TGT TGA CAG CTA AAG-3'. The conditions used for PCR were adapted from Van Schaik *et al*. (175) and modified as necessary: initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 40 s; and a final extension step of 72°C for 10 min; the reaction was performed on a MyCycler™ Thermal Cycler (Bio-Rad, Hercules, USA). The amplified fragment was digested with the *Hpy*F3I (*Dde*I) restriction enzyme (Thermo Fisher Scientific, Wilmington, USA) incubated overnight at 37°C and then at 65°C for 5 min to inactivate the enzyme. The digested

products were then separated on a 4.5% nusieve gel for 120 min at 80 V, followed by 60 min at 120 V. In the presence of the T variant, a restriction site was abolished to yield four fragments of 25, 103, 137 and 230 bp; whilst digestion of the C variant yielded three fragments of 128, 137 and 230 bp.

Sequencing was performed to confirm the genotypes identified, using the ABI Big Dye ® Terminator v3.1 Cycle Sequencing Kit (Life Technologies, California, USA). Sequenced products were analysed via capillary electrophoresis on the ABI Prism 3130 Genetic Analyzer (Life Technologies, California, USA). Genotypes for both the rs776746A>G and rs10264272C>T SNPs were confirmed by cycle sequencing and aligned to the *CYP3A5* reference sequence (NCBI Reference Sequence: NC_000007.14) using DNAstar Lasergene® SeqMan Pro™ software (DNASTAR Inc., Madison, USA).

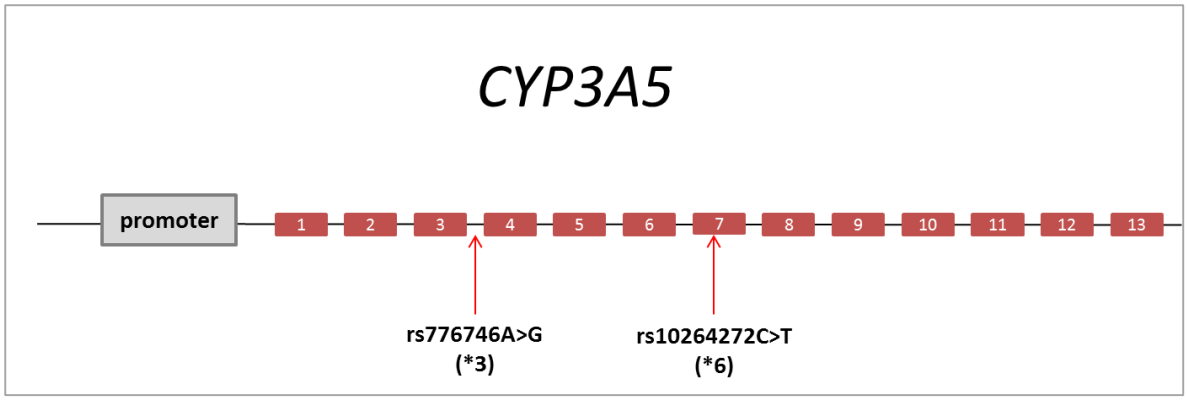

Figure 2.4 Schematic diagram of CYP3A5 gene. The position of SNPs that were genotyped are indicated with arrows. The gene is 32 kb in length, containing 13 exons.

2.3.4 Characterisation of variation in *ABCC2*

In order to genotype *ABCC2* c.1249G>A (rs2273697), PCR-RFLP was performed (Fig. 2.5). The genotyping conditions used were adapted from Qu *et al*. (161). PCR amplification was performed on a T100™ Thermal Cycler (Bio-Rad, Hercules, USA): initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 61°C for 30 min, and 72°C for 1 min; and a final extension step of 72°C for 5 min. The amplified fragments were separated on a 1.5% Agarose Gel at 80 V for 90 min to confirm amplification, a fragment of 303 bp was expected. The amplified fragment was digested using the *Nco*I restriction enzyme (Thermo Fisher Scientific, Wilmington, USA), incubated at 37°C overnight (± 16 hrs). Digested products were resolved on a 4% agarose gel at 80 V for 120 min. For the G allele, fragments of 95 and 208 bp were obtained after digestion. For the A allele, fragments of 26, 69 and 208 bp were obtained.

Two regions within the promoter of *ABCC2* were amplified by PCR and sequenced to identify known and novel SNPs. Primers used for PCR and sequencing were taken from a protocol by Fujita *et al.* (176). To amplify the 214 bp region from -1632 to -1419 upstream of *ABCC2* (including an known c.-1549G>A SNP), using the following primers, forward: 5'-AAA AGT GTC TGT TCA AGT CC-3' and reverse: 5'-GTG AAT TGT ATG GAC CTT GT-3' the PCR conditions were: initial denaturation at 94°C for 3 min, 94°C for 30 s, then 35 cycles of 51°C for 30 s, 72°C for 1 min and 72°C for 5 min. To amplify the 249 bp region from -1157 to -908 upstream of *ABCC2* (including the known c.-1019A>G SNP) the primers were as follows, forward: 5'-GGA TAC CGC ATG GGT TC-3' and reverse: 5'-ACT ACA GGC ACA TGC CCA CA-3'. PCR amplification conditions were an initial denaturation at 94°C for 3 min, 94°C for 30 s, then 35 cycles of 60°C for 30s, 72°C for 1 min and 72°C for 5 min. The T100™ Thermal Cycler (Bio-Rad, Hercules, USA) was used for PCR amplification of both fragments.

Sequencing was performed on the Applied Biosystems®2720 Thermal Cycler (Life Technologies, California, USA) as described in section 2.3.1.3 above. The sequences were assembled and aligned using DNAstar Lasergene® SeqMan Pro™ software (DNASTAR Inc., Madison, USA); NCBI Reference Sequence: NC_000010.11.

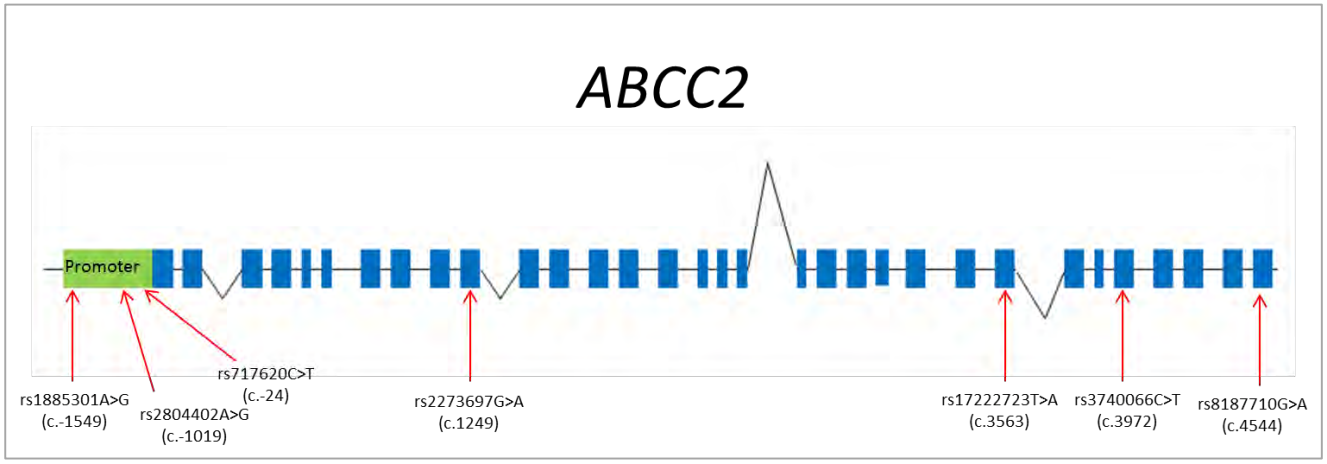

Figure 2.5 Schematic diagram of the ABCC2 gene. The gene is 69 kb long ad has 32 coding exons (blue boxes). Two known SNPs of interest are indicated with arrows.

2.4 Measurement of Lopinavir plasma levels

To measure the LPV plasma levels for the samples, the plasma samples were sent to the Division of Clinical Pharmacology, University of Cape Town for analysis. LPV concentrations were measured by liquid chromatography tandem mass spectrometry (LC-MS/MS). This method allows for the identification of proteins and peptides through providing information about the primary sequence and the post-translational modifications (177).

2.5 Statistical Analysis

A chi-squared analysis was used to determine whether differences in frequency distribution between populations were statistically significant; a χ^2 statistic and p-value were obtained. LD tests were performed using the SHEsis online software (178). GraphPad Prism 5 was used to obtain column statistics (mean, lower and upper confidence interval, and standard deviation), and used for genotype and LPV plasma level associations. The statistical analyses conducted using this program were t-test to compare two groups, and One-Way Analysis of Variance (ANOVA) to compare more than two groups. Fisher's Exact test was used when testing for an association between genotypes and LPV therapeutic range.

3. Results

This project sought to evaluate the pharmacogenetics of lopinavir (LPV) by correlating 32 genetic variants in four genes, *CYP3A4* (2 SNPs), *CYP3A5* (2 SNPs), *SLCO1B1* (22 SNPs) and *ABCC2* (6 SNPs) with plasma LPV concentration. The genetic analysis involved genotyping using PCR-RFLP for the following SNPs: *CYP3A4* rs2740574A>G; *CYP3A5* rs776746A>G and rs10264272C>T; and *ABCC2* rs2273697G>A. The rest of the SNPs were genotyped using targeted sequencing where sections of genes known to harbour mutations were selected for sequencing. The *CYP3A4*22* allele was however evaluated using SNP analysis by a TaqMan genotyping assay. The study was carried out in a total of 86 patients on second-line ARV. Samples were limited as, at the time of sampling, there were few people who were on second-line containing LPV. The patient cohort comprised of 57 females, and their age ranged from 19 to 60 years. LPV plasma concentrations were available for 64 of the 86 participant samples. Other clinical parameters such as CD4 count, viral load, liver enzyme levels [aspartate aminotransferase (AST) and alanine aminotransferase (ALT)] were collected and formed part of the analysis.

3.1. Frequency distribution of variant SNPs

A total of 32 SNPs in the four genes were genotyped for. Three were observed to be monomorphic, and these are *CYP3A4* rs35599367C>T; and *SLCO1B1* rs4149056T>C (see Table 3.1). The SNP at position c.-999 of *ABCC2* was not present in the annotated gene (using the PerlV5 program; NCBI Reference Sequence: NC_000010.11.) Most SNPs were in Hardy-Weinberg equilibrium (HWE), except for *CYP3A5* rs776746A>G; *SLCO1B1* rs4149049A>G, and rs4149053G>T. The SNPs in each gene were used to construct haplotypes as well as evaluate LD. Figures 3.1, 3.2 and 3.3 show the haplogroups for *ABCC2, SLCO1B1* exon 5, and exon 6 and 7.

For the four genes, CYP3A4, CYP3A5, SLCO1B1 and ABCC2, the most common alleles in each gene respectively were *CYP3A4*1B* (rs2740574G) (0.80); *CYP3A5*3* (rs776746G) (0.21); *SLCO1B1* rs2306283 (c.388A>G) (0.86); and *ABCC2* rs1885301 (c.-1549A>G) (0.64), while the least common were, *CYP3A4* rs35599367T (0.00), *CYP3A5*6* (rs10264272T) (0.20); *ABCC2* rs17222653A (0.014); and *SLCO1B1* rs4149056 (0.00) (Table 3.1). The frequencies for all the genotyped variants are presented in Table 3.1. Genotypes for all the samples (n=86) were only available for *CYP3A4*1B* which was genotyped first. For the remaining variants, certain samples could not be genotyped because either the DNA sample had been finished or could not amplify at that locus. Hence, the differences observed in the total number of samples genotype for each SNP.

When comparing the distribution of allelic variants in our cohort to that in other populations, Table 3.1 also shows that, for the SNPs investigated, our cohort differed significantly at 19/32 loci, when compared to Caucasian (CEU) populations. The Chinese (CHB) populations differed with this southern African cohort on 5 loci. The other African population group, Yoruba (YRI) differed with this cohort only on one loci, while there were no significant differences in the distribution of compared SNPS with the Luhya in Webuye, Kenya (LWK).

Table 3.1 The distribution of frequencies of genotyped SNPs in our study compared to other populations.

Allele	Our study	N[a]	HWE p-value	CEU freq. (n=87)	X^2	p-value	YRI freq. (n=88)	X^2	p-value	LWK freq. (n=97)	X^2	p-value	CHB freq. (n=97)	X^2	p-value
CYP3A4															
rs2740574G (*1B)	0.8	86	0.71	0.017	106.704	<0.0001*	0.767	0.118	0.7307	0.825	0.0591	0.8079	0.00	120.964	<0.0001*
rs35599367T (*22)	0.00	84	N/A	0.052	2.695	0.1007	0.00	N/A	N/A	0.00	N/A	N/A	0.00	N/A	N/A
CYP3A5															
rs776746G (*3)	0.21	83	**0.000031†**	0.954	94.2	<0.0001*	0.159	0.44	0.5073	0.124	1.829	0.1762	0.686	38.830	<0.0001*
rs10264272T (*6)	0.2	83	0.98	0.00	17.08	<0.0001*	0.165	0.156	0.6932	0.247	0.329	0.5664	0.00	19.049	<0.0001*
ABCC2															
rs2273697 c.1249A	0.12	84	0.2155	0.236	3.163	0.0753	0.250	3.969	**0.0464***	0.196	1.405	0.2358	0.134	0.0034	0.9536
rs17222653 c.-1536A	0.014	73	0.9055	0.00	0.0123	0.9116	0.00	0.0136	0.9070	0.005	0.428	0.8361	0.00	0.0278	0.8676
rs1885301 c.-1549G	0.64	74	0.5645	0.603	0.102	0.7496	0.597	0.159	0.6902	0.619	0.0148	0.9030	0.727	1.105	0.2931
rs7910642 c.-1023A	0.16	83	0.335	0.08	1.885	0.1697	0.114	0.427	0.5136	0.186	0.0684	0.7937	0.258	2.013	0.156
rs2804402 c.-1019G	0.31	83	0.264	0.397	1.05	0.3054	0.267	0.204	0.6515	0.304	0.0056	0.9401	0.273	0.145	0.7035
SLCO1B1															
rs182769903 c.360-66G	0.006	82	0.9557	N/D	N/A	N/A	N/D	N/A	N.A	N/D	N/A	N/A	N/D	N/A	N/A
rs2306283 c.388G	0.86	84	0.5958	0.41	35.263	<0.0001*	0.824	0.191	0.6618	0.825	0.192	0.6609	0.794	0.938	0.3328
rs11045818 c.411A	0.006	84	0.9562	0.213	16.478	<0.0001*	0.006	0.975	0.3233	0.00	0.423	0.5155	0.005	0.846	0.3576
rs11045819 c.463A	0.05	84	0.6468	0.213	8.488	**0.0036***	0.06	0.0015	0.9691	0.03	0.0734	0.7865	0.01	2.051	0.1521
rs77271279 c.481+1T	0.02	84	0.8677	0.00	0.303	0.5822	0.057	0.739	0.39	0.026	0.0518	0.82	0.00	0.387	0.5338
rs11045820 c.481+160T	0.006	84	0.9562	0.213	16.478	<0.0001*	0.006	0.975	0.3233	0.00	0.423	0.5155	0.005	0.846	0.3576
rs4149044 c.481+165T	0.66	84	0.6334	0.167	0.846	0.3576	0.614	0.219	0.6398	0.546	1.984	0.1590	0.51	3.564	0.059
rs4149045 c.481+189A	0.65	84	0.7593	0.167	39.417	<0.0001*	0.61	0.11	0.7405	0.546	1.611	0.2043	0.51	3.06	0.0802
rs4149046 c.481+191A	0.02	84	0.8231	0.494	47.337	<0.0001*	0.028	0.0241	0.8765	0.021	0.229	0.6325	0.21	13.069	**0.0003***
rs189925961 c.481+242T	0.006	84	0.9562	N/D	N/A	N/A	N/D	N/A	N/A	N/D	N/A	N/A	N/D	N/A	N/A

Table 3.1 continued

Allele	Our study	N[a]	HWE p-value	CEU freq. (n=87)	X^2	p-value	YRI freq. (n=88)	X^2	p-value	LWK freq. (n=97)	X^2	p-value	CHB freq. (n=97)	X^2	p-value
rs4149048 c.481+520G	0.63	82	0.4339	0.167	36.062	<0.0001*	0.614	0.0032	0.9548	0.55	0.968	0.3251	0.51	2.138	0.1437
rs4149049 c.482-823G	0.22	81	**0.0006†**	0.029	12.64	0.0004*	0.142	1.254	0.2629	0.18	0.228	0.6328	0.28	0.511	0.4749
rs4149050 c.482-522C	0.66	80	0.4433	0.19	36.006	<0.0001*	0.619	0.153	0.6593	0.557	1.537	0.2150	0.515	3.213	0.073
rs4149051 c.482-453G	0.66	80	0.4433	0.19	36.006	<0.0001*	0.619	0.153	0.6593	0.567	1.226	0.2682	0.515	3.213	0.073
rs4149052 c.482-451G	0.66	80	0.4433	0.19	36.006	<0.0001*	0.619	0.153	0.6593	0.567	1.226	0.2682	0.515	3.213	0.073
rs4149053 c.482-375T	0.56	79	0.0405	0.19	22.856	<0.0001*	0.557	0.0137	0.9068	0.5	0.411	0.5214	0.515	0.197	0.6575
rs4149054 c.482-331A	0.54	80	0.0501	0.19	20.725	<0.0001*	0.54	0.024	0.8768	0.49	0.261	0.6092	0.52	0.0325	0.8569
rs141555703 c.482-272A	0.08	79	0.4255	N/D	N/A	N/A	N/D	N/A	N/A	N/D	N/A	N/A	N/D	N/A	N/A
rs67496683 c.482-120_482-115del	0.62	81	0.0961	N/D	N/A	N/A	N/D	N/A	N/A	N/D	N/A	N/A	N/D	N/A	N/A
rs4149056 c.521C	0.00	81	N/A	0.14	10.604	**0.0011***	0.011	0.0054	0.9414	0.021	0.365	0.5457	0.149	11.214	**0.0008***
rs4149057 c.571C	0.09	81	0.5782	0.69	60.409	<0.0001*	0.153	1.024	0.3115	0.052	0.487	0.4853	0.206	3.728	0.0535
rs2291075 c.597T	0.59	81	0.2396	0.402	5.203	0.0225*	0.614	0.026	0.8720	0.521	0.594	0.4410	0.521	0.594	0.441

X^2: Chi-squared statistic for comparison of allele frequencies obtained in study compared to selected populations.

†: Frequencies not in HWE

*: p<0.05 (frequency of allele in population is significantly different to that obtained in the study)

N[a]: Not all samples were successfully genotyped for each of the SNPs due to difficulties or lack of adequate amounts

HWE: Hardy-Weinberg equilibrium

CEU: Utah residents with N & W European ancestry from the CEPH collection

YRI: Yoruba in Ibadan, Nigeria

LWK: Luhya in Webuye, Kenya

CHB: Han Chinese in Beijing, China

N/D: No data

N/A: Not applicable

3.2. Linkage Disequilibrium Analysis

Haplotypes were calculated for the SNPS that were genotyped in the same gene. Figures 3.1, 3.2 and 3.3 show the LD plots for the two genes, *ABCC2* and *SLCO1B1*. The SNPs in *ABCC2* had a very high D' and the low r^2 and this was mainly due to differences in allele frequencies (Fig. 3.1). Figure 3.2 presents the LD plots for *SLCO1B1* exon 5 to 7 and surrounding introns. It appears, the intronic region starting from position c.481 (i.e. rs77271279 c.481+1T), is an unstable region thus, so many SNPs. Most of these present with very high D' but also have low r2 as shown for *ABCC2* (see Fig. 3.2).

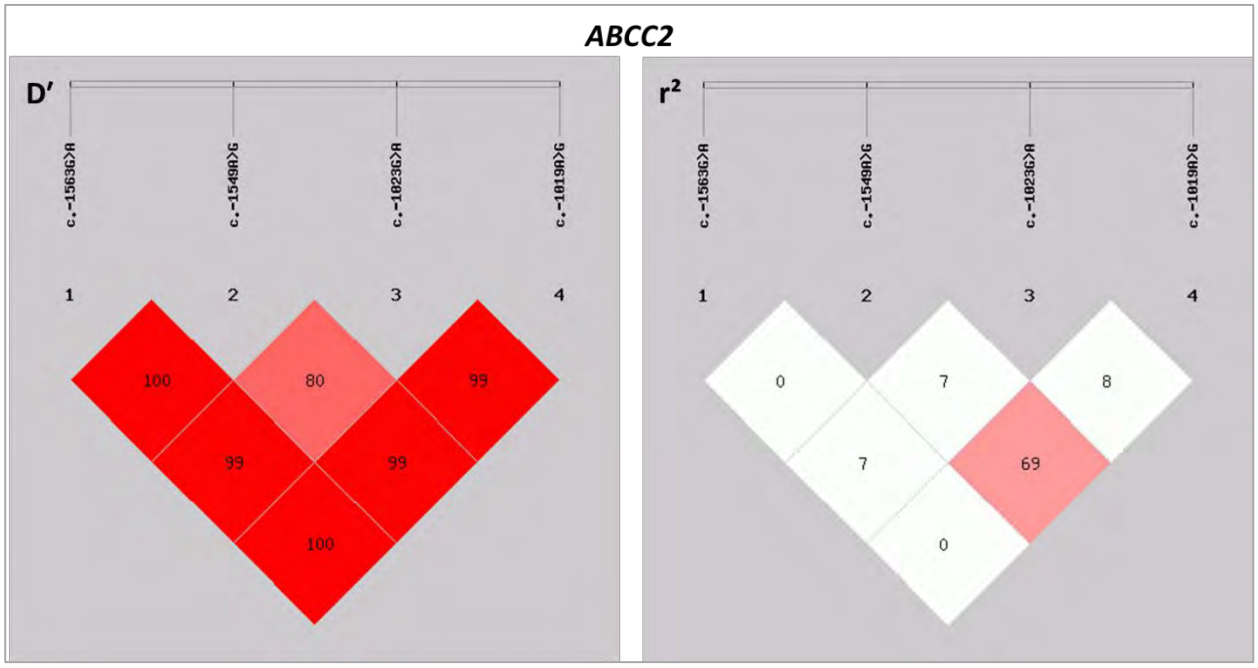

Figure 3.1 Linkage Disequilibrium plot for c.-1563G>A (rs17222653); c.-1549A>G (rs1885301); c.-1023G>A (rs7910642); and c.-1019A>G (rs2804402) SNPs in *ABCC2*.

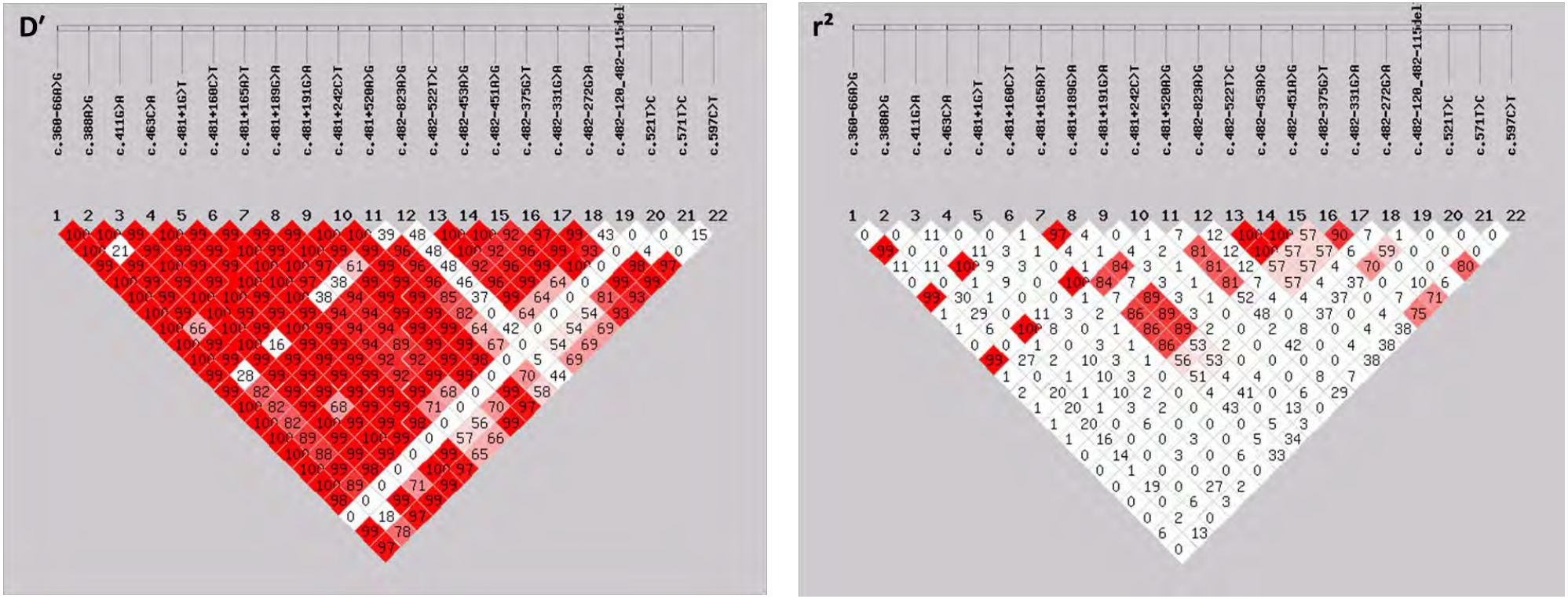

Figure 3.2 Linkage Disequilibrium plot for SNPs genotyped in *SLCO1B1* exon 5, and exon 6 to 7 and their intron-exon junctions.

3.3. Effects of genetic variants on Lopinavir therapeutic levels

From the 85 patient samples which were genotyped, plasma LPV levels were available for 74. Ten samples out the 74 had LPV levels below the lower limit of quantification (BLQ) of 0.0195 µg/mL. Samples with BLQ values were excluded from further genotype-plasma LPV level association analysis and were excluded from determination of the distribution of LPV concentrations (Fig. 3.3 A and B). The excluded patient samples were not limited to either one of the populations. Plasma LPV levels were thus available for 64 patient samples. The mean of LPV levels was 9.401 µg/mL with a standard deviation of 6.760 µg/mL. The C_{min} and C_{max} values of 5.5 and 9.6 µg/mL, which indicate the therapeutic range are shown in Fig. 3.3 A. Most of the LPV levels obtained in our study fell outside the therapeutic range – 28% (n=18/64) were below the therapeutic range, while 39% (n=25/64) were above the therapeutic range. Only 33% (n=21/64) were within the therapeutic range. When samples were divided according to the sex of the individuals (Fig. 3.3 B), males (n=21) had a mean LPV concentration of 10.78 µg/mL, with a standard deviation of 7.053 µg/mL, while females (n=43) had a mean LPV concentration of 8.727 µg/mL, with a standard deviation of 6.591 µg/mL. There was no significant difference between the mean LPV concentration in males and in females (p=0.4029; Fig. 3.3 B).

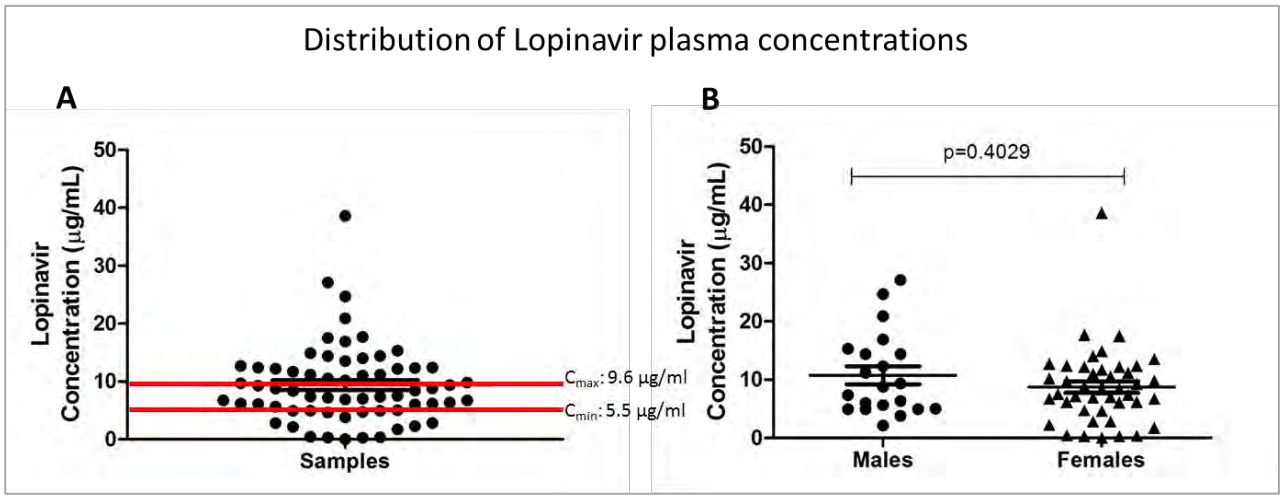

Figure 3.3 Distribution of LPV plasma concentrations. N=10 samples were omitted with LPV levels that were below level of quantification (<0.0195 µg/mL). A: Distribution of LPV levels in the study cohort; B: Gender comparison of distribution of LPV levels.

One Way ANOVA and Mann-Whitney t test were performed to determining any association between LPV plasma levels and the different genotypes observed (Table 3.2). Only *CYP3A4* rs2740574A>G (p=0.0479) showed a significant association with LPV plasma levels (Table 3.2 and Fig. 3.5). *ABCC2* rs7910642G>A showed a trend towards significance (P=0.072) when using an additive genetic model to compare the G/G genotype to the G/A and A/A genotypes. A few of the graphs have been shown

in this section and the remaining graphs can be found in Appendix B. Figures 3.5, 3.6 and 3.7 show a selection of some of the correlations between LPV concentration levels and genotypes. No association was found with any of the *CYP3A5* rs776746A>G or rs10264272C>T genotypes and LPV levels (Fig. 3.6; Table 3.2). For both SNPs, analysis was also conducted using an additive genetic model. For the *ABCC2* rs2273697G>A and rs17222653 SNPs, no patients had the A/A genotype (Table 3.2; Fig. 3.7). For the *ABCC2* rs7910642 (c.-1023G>A) SNP, the combined G/A and A/A genotypes showed a trend towards association with lower LPV levels (Fig. 3.8 B).

The therapeutic range for LPV concentrations was taken into account to evaluate if there were particular genotypes that correlated with particular levels. As observed in Table 3.3, it was observed that the *SLCO1B1* rs4149049A>G genotypes showed a significant difference in their distribution when comparing LPV concentrations below therapeutic range to those within the therapeutic range (P=0.0001). Nearly 87% (n=13/15) of the patients with either the A/G or G/G had LPV levels within the therapeutic range compared to only 24% (n=7/29) of the patients presenting with the A/A genotype. This result is also presented in Figure 3.4. *SLCO1B1* rs4149050A>G, rs4149051A>G, and rs4149052A>G genotypes showed borderline significance also when comparing LPV concentrations below therapeutic range to those within the therapeutic range (p=0.0531).

Table 3.2 The association of variant SNPs with plasma lopinavir levels.

SNP	Genotype	N	Mean plasma LPV level (± SD)	p-value	95% CI
CYP3A4					
rs2740574A>G	A/A	2	12.91 (± 11.30)	0.1011	(-88.61 to 114.4)
	A/G	22	7.287 (±4.559)		(5.27 to 9.31)
	G/G	37	11.12 (±7.246)		(8.70 to 13.53)
	A/A and A/G	24	7.756 (±5.201)	**0.0479***	(5.56 to 9.95)
ABCC2					
rs7910642G>A	G/G	45	10.48 (±6.822)	0.2065	(8.43 to 12.52)
	G/A	16	7.589 (±6.207)		(4.28 to 10.90)
	A/A	1	6.875		N/A
	G/A and A/A	17	7.547 (±6.013)	0.0722	(4.46 to 10.64)
SLCO1B1					
rs2306283A>G	A/A	1	N/A	0.1198	0
	A/G	19	7.140 (±3.917)		(5.25 to 9.028)
	G/G	42	10.71 (±7.436)		(8.39 to 13.02)
	A/A and A/G	20	7.503 (±4.144)	0.1504	(5.56 to 9.443)
rs4149044A>T	A/A	9	9.725 (±7.492)	0.2913	(3.97 to 15.48)
	A/T	31	8.942 (±7.293)		(6.27 to 11.62)
	T/T	22	10.68 (±5.557)		(8.22 to 13.14)
	A/A and A/T	40	9.118 (±7.248)	0.1296	(6.80 to 11.44)
rs4149045G>A	G/G	9	9.725 (±7.492)	0.3895	(3.97 to 15.48)
	G/A	31	8.942 (±7.293)		(6.27 to 11.62)
	A/A	22	10.68 (±5.557)		(8.22 to 13.14)
	G/G and G/A	40	9.118 (±7.248)	0.1296	(6.8 to 11.44)
rs4149057T>C	T/T	52	9.876 (±7.297)	0.3752	(7.85 to 11.91)
	T/C	7	6.626 (±2.111)		(4.68 to 8.58)
	C/C	1	N/A		0
	T/C and C/C	8	6.658 (±1.956)	0.1673	(5.02 to 8.29)

Only SNPs with p≤0.200 have been indicated in this table. See the complete table in Appendix B. LPV=lopinavir; SD=standard deviation.

Table 3.3 The correlation between genotypes and lopinavir levels taking into account lopinavir therapeutic range.

Genotype	<5.5µg/mL	5.5µg/mL≤X≥9.6µg/mL	>9.6µg/mL	Total	Global P-value	P-value [<5.5µg/mL vs. 5.5µg/mL≤X≥9.6µg/mL]	P-value [<5.5µg/mL vs. >9.6µg/mL]
CYP3A4							
rs2740574A>G							
A/A	2 (0.08)	0	1 (0.04)	3	0.2566	0.3135	0.1565
A/G	12 (0.48)	8 (0.4)	7 (0.26)	27			
G/G	11 (0.44)	12 (0.6)	19 (0.7)	42			
Total	25	20	27				
ABCC2							
rs7910642G>A							
G/G	14 (0.58)	16 (0.8)	21 (0.78)	51	0.1325	0.1013	0.4339
G/A	10 (0.42)	3 (0.15)	6 (0.22)	19			
A/A	0	1 (0.05)	0 (0)	1			
Total	24	20	27				
SLCO1B1							
rs4149045G>A							
G/G	2 (0.08)	6 (0.3)	2 (0.07)	10	0.1938	0.1013	0.956
G/A	11 (0.44)	9 (0.45)	13 (0.48)	33			
A/A	12 (0.48)	5 (0.25)	12 (0.44)	29			
Total	25	20	27				
rs4149048A>G							
A/A	4 (0.17)	6 (0.3)	2 (0.07)	12	0.1470	0.1572	0.4516
A/G	8 (0.35)	10 (0.5)	13 (0.48)	31			
G/G	11 (0.48)	4 (0.2)	12 (0.44)	27			
Total	23	20	27				
rs4149049A>G							
A/A	22 (0.92)	7 (0.35)	20 (0.77)	49	**0.00008**	**0.0001**	**0.0686**
A/G	0 (0)	10 (0.5)	5 (0.19)	15			
G/G	2 (0.08)	3 (0.15)	1 (0.04)	6			
Total	24	20	26				

Table 3.3 continued

Genotype	<5.5µg/mL	5.5µg/mL≤X≥9.6µg/mL	>9.6µg/mL	Total	Global P-value	P-value [<5.5µg/mL vs. 5.5µg/mL≤X≥9.6µg/mL]	P-value [<5.5µg/mL vs. >9.6µg/mL]
SLCO1B1							
rs4149050A>G							
A/A	2 (0.09)	6 (0.3)	2 (0.08)	10	0.0946	0.0531	0.995
A/G	9 (0.39)	10 (0.5)	10 (0.4)	29			
G/G	12 (0.52)	4 (0.2)	13 (0.52)	29			
Total	23	20	25				
rs4149051A>G							
A/A	2 (0.09)	6 (0.3)	2 (0.08)	10	0.0946	0.0531	0.995
A/G	9 (0.39)	10 (0.5)	10 (0.4)	29			
G/G	12 (0.52)	4 (0.2)	13 (0.52)	29			
Total	23	20	25				
rs4149052A>G							
A/A	2 (0.09)	6 (0.3)	2 (0.08)	10	0.0946	0.0531	0.995
A/G	9 (0.39)	10 (0.5)	10 (0.4)	29			
G/G	12 (0.52)	4 (0.2)	13 (0.52)	29			
Total	23	20	25				
rs4149053G>T							
G/G	7 (0.32)	7 (0.35)	4 (0.16)	18	0.2005	0.1628	0.2357
G/T	5 (0.23)	9 (0.45)	11 (0.44)	25			
T/T	10 (0.45)	4 (0.2)	10 (0.4)	24			
Total	22	20	25				
rs4149057T>C							
T/T	19 (0.76)	16 (0.8)	23 (0.96)	58	0.1192	0.4232	0.2332
T/C	6 (0.24)	3 (0.15)	1 (0.04)	10			
C/C	0 (0)	1 (0.05)	0 (0)	1			
Total	25	20	24				

Only SNPs with p≤0.200 have been indicated in this table. See the complete table in Appendix B

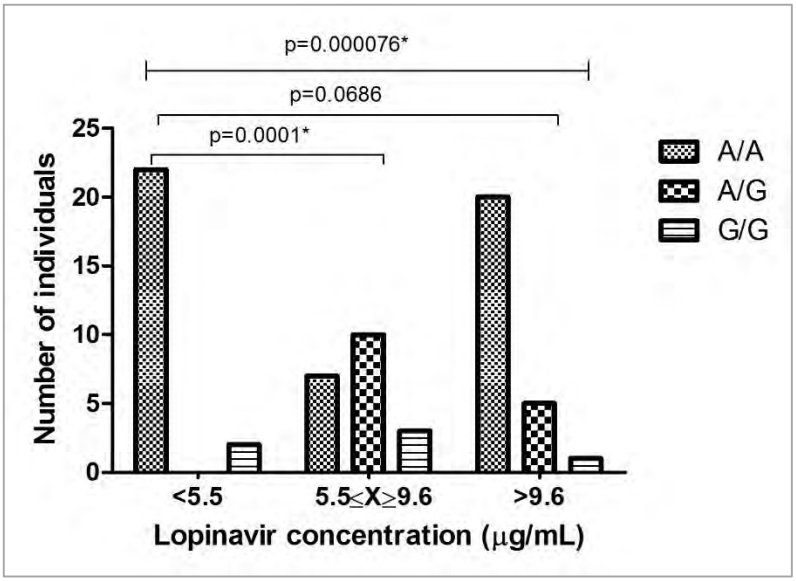

Figure 3.4 Association of rs4149049A>G genotypes and LPV therapeutic range. Fisher's Exact test gave a global p-value=0.000076.

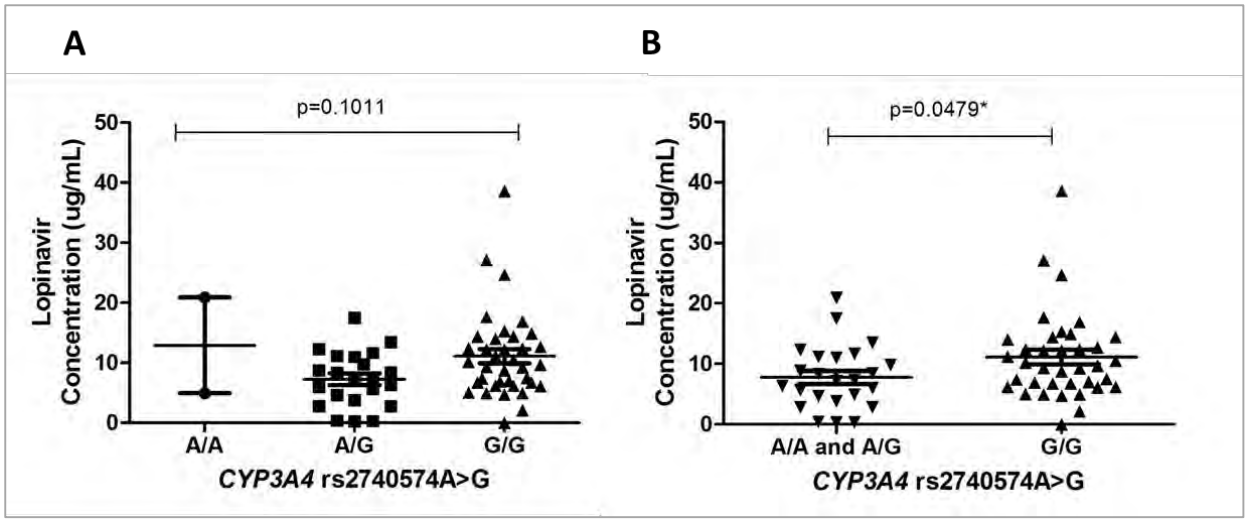

Figure 3.5 Association between LPV plasma levels and *CYP3A4* rs2740574A>G genotypes. A: p=0.1011, Kruskal Wallis One Way ANOVA; B: *p<0.05 when using a recessive genotype model to compare genotypes Mann-Whitney test.

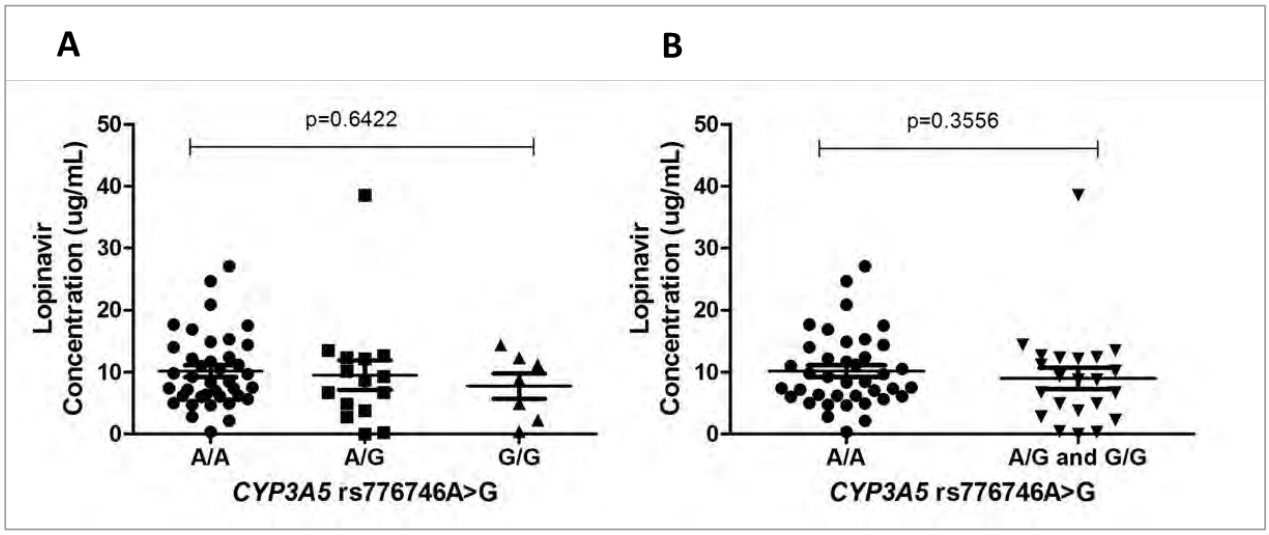

Figure 3.6 Association between LPV plasma levels and *CYP3A5* rs776746A>G genotypes. A: p=0.6422, Kruskal Wallis ANOVA; B: p=0.3556 when using a dominant genetic model to compare genotypes, Mann-Whitney test.

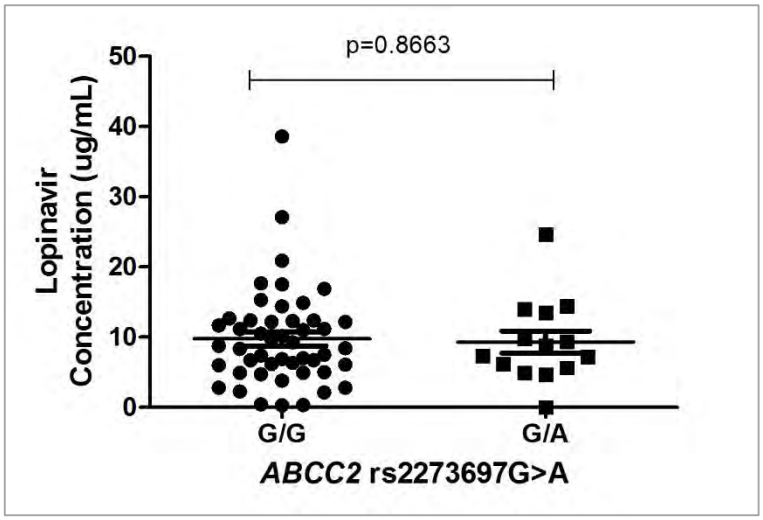

Figure 3.7 Association between LPV plasma levels and *ABCC2* rs2273697 (c.1249G>A) genotypes (p=0.8663, Mann-Whitney test).

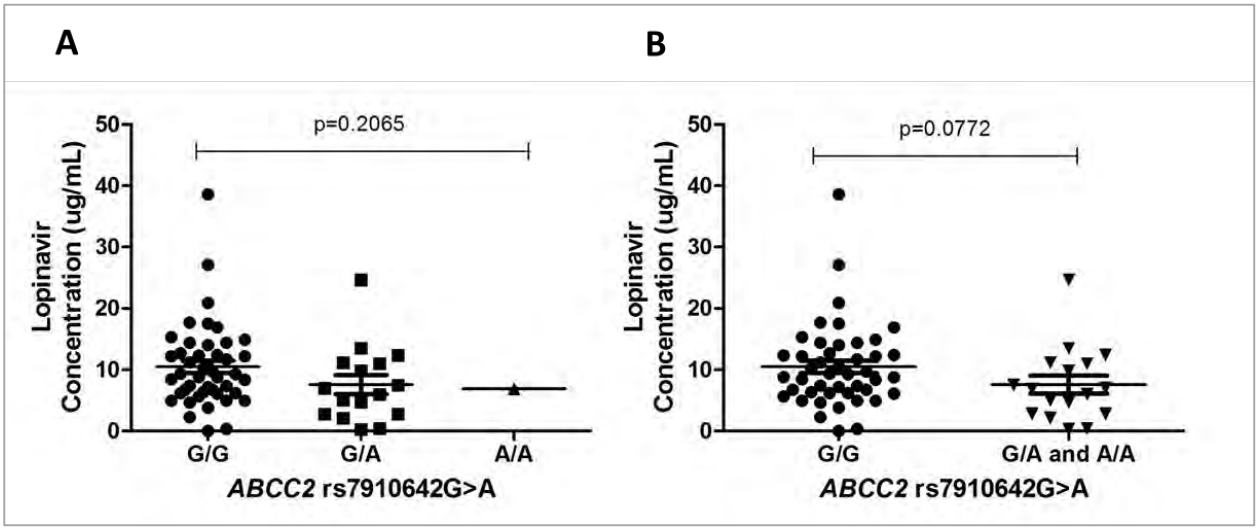

Figure 3.8 Association between LPV plasma levels and *ABCC2* rs7910642 (c.-1023G>A) genotypes (A: p=0.2065, Kruskal-Wallis One Way ANOVA; B: p=0.0722, Mann-Whitney test).

4. Discussion and Conclusion

The treatment of HIV-infected individuals has improved through the introduction and use of combined highly reactive antiretroviral therapy (HAART) instead of using single-drug regimens (21, 179). Different levels of therapy are incorporated in the use of HAART – patients begin treatment on a first-line regimen and then progress on to a second-line and possibly a third-line regimen if treatment failure is experienced. In resource-limited settings, access to treatment becomes more difficult as patients move from one line to the next as drugs become increasingly costly (45). Treatment failure can be due to viral resistance to the drug (virologic failure) which can be due to viral genotypic resistance to the drug, often as a result of poor adherence to treatment. Thus, it is vital to ensure that patients adhere to their treatment. One of the ways to improve adherence is to identify markers of poor response among patients.

Thus, our study evaluated the pharmacogenetics of a second-line protease inhibitor, lopinavir (LPV), to identify genetic markers that were associated with patients presenting with either low LPV (which is associated with development of virus resistance) or high LPV (which may be associated with LPV-associated side-effects. Although the sample cohort used in the study was small, the study is relevant due to the increasing number of HIV-infected individuals moving from first-line to second-line ARV therapy. It is particularly relevant in our context as most studies are conducted in Western or Asian populations, rather than in sub-Saharan Africa where the HIV pandemic has hit the hardest. Indeed, as already shown in the results, some of the genetic variants that are important in LPV response (c.521C>T) are rare or absent in African populations.

Pharmacogenetic factors also play a role in the efficacy and disposition of ARVs in the treatment of HIV. For example, drug metabolising enzyme genes for which certain ARVs are known to be substrates, may harbour genetic variation that influences the metabolism of these drugs. Genes that may be involved in this include those coding for CYP2B6, CYP3A4/5, and CYP1A2 which metabolise some of the common ARV drugs. Other important genes encode for drug transporter proteins such as MDR1, MRP2, and OATP1B1. Within *CYP2B6*, 516G>T has been identified as a marker for variable efavirenz plasma concentrations in HAART patients (180-182).

4.1. Frequency distribution of relevant SNPs

Thirty-two SNPs were genotyped for in four genes which may play a role in LPV pharmacogenetics; namely, *CYP3A4*, *CYP3A5*, *SLCO1B1*, and *ABCC2* (Table 3.1). The SNPs and regions of genes to be sequenced were selected based on their suggested effect on the protein function. In *CYP3A4*, both

CYP3A4 -392G (*1B) and the intronic *CYP3A4*22* alleles have been associated with decreased protein expression as decreased activity. We report a high prevalence of *CYP3A4*1B* (0.80) in our cohort, which is comparable to other African populations (see YRI and LWK, in our study) but much higher than reported in Caucasians (0.017) and Chinese (0.00) (Table 3.1). The *CYP3A4*22* allele which has become an important allele in drug response among Caucasian populations was not found in this population. Already, these differences, at the *CYP3A4* loci, which is important in the metabolism of LPV, are a window, into the possible differences in therapeutic outcome when LPV is administered in different populations.

*CYP3A5*3* and *CPY3A5*6* were selected based on their importance in affecting expression and activity of CYP3A5 enzyme, especially *CYP3A5*3* is thought to exert its effects through the degradation of the mRNA due to a premature stop codon, resulting in low levels of CYP3A5. Both SNPs were observed in our cohort. *CYP3A5*3* was observed at a frequency of 0.21, which is significantly lower than in Caucasians (0.954; $p<0.0001$) and Asians (0.686; $p<0.0001$) when compared to our cohort (0.21) but comparable to other African populations (YRI: 0.159 and LWK: 0.124) (Table 3.1). Together with CYP3A4, CYP3A5 participates in the metabolism of many commonly used therapeutic drugs (114), thus, these observed differences between populations, are bound to present as differences in effectiveness of drugs that are substrates of these enzymes. However, no correlations were observed for *CYP3A5* genotypes and plasma LPV levels in our study, possibly pointing to these enzymes playing a minor role in the metabolism or disposition of LPV.

OATP1B1 is involved in the hepatic uptake of substrates including LPV. Studies have shown a role of this transporter in the disposition of LPV (79, 105, 133). Many SNPs have been reported in SLCO1B1 which form various haplotypes. For example, *SLCO1B1* rs4149056 (c.521T>C) and rs2306283 (c.388A>G) form the haplotypes c.521C/c.388A (*5); and c.521C/c.388G (*15), which have been associated with decreased uptake of substrates by OATP1B1 (134). However, most studies have been carried out in other populations and not much on African populations. *SLCO1B1* is particularly important in southern Africa (given that this is the region hardest hit with HIV/AIDS) because of the protein (OATP1B1)'s involvement in the transport of most ARVs. Thus, two genic regions which included these two SNPs were sequenced to identify any novel variants or other SNPs which may be important. The regions included exon 5 and its intron-exon junctions as well as a region encompassing exon 6 and 7 and their exon-intron junctions. As with *CYP3A4*22*, the *SLCO1B1* c.521T>C was not observed in our cohort. Finally, in *ABCC2*, part of the promoter region was sequenced which included prior-mentioned SNPs of interest, to determine any variation which could influence ABCC2 expression.

Also, the *ABCC2* c.1249G>A SNP was genotyped as it has been reported to be in LD with the c.-24C>T (rs717620) SNP. However, the c.-24C>T SNP was not genotyped for in our study because of its low frequency in sub-Saharan African populations (0.03-0.04) as observed in literature.

Notably, the two SNPs of interest which were absent in our cohort, *SLCO1B1* rs4149056T>C (c.521T>C) and *CYP3A4* rs35599367C>T (*CYP3A4*22* allele), have been reported to influence LPV pharmacokinetics (133) (Table 3.1). Separate studies by Elens *et al*. (183-185) have reported an association of *CYP3A4*22* with greater levels of various drugs, including simvastatin (study in Rotterdam), cyclosporine and taclorimus (studies in Belgium) in respective patients. The majority of the participants for the various studies were white, and very few black participants (between 2 and 4%) were. On the other hand, *SLCO1B1* the influence of c.521T>C on the disposition of statins has been investigated in European (186-188), American (European and African) (189), and Asian (190); and that of LPV in Brazilian (191), American and British cohorts (105), to name a few. The absence of these SNPs in Southern Africans further affirms the need for population-specific pharmacogenetic studies rather than accepting results reported in other populations (e.g. Caucasian) and extrapolating their findings to African populations. There is, in any case huge genetic diversity in African populations relative to non-African populations (192, 193). The observed differences in distribution of genetic variants as well as in their frequencies have implications for personalised medicine and highlight the need, at least, for population-specific if not individualised genetic testing before the prescribing of or adjustment of drug dosages. An example of an application of this is in the use of EFV which is largely metabolised by CYP2B6. Identification of individuals with the slow-metaboliser allele for the *CYP2B6* c.516G>T SNP, 516T, allows for EFV dosing adjustments in those individuals. This allele is found at a higher frequency in African populations (45-47%) compared to European and Asian populations (21.4% and 17.4%, respectively) (194).

The genetic diversity present on the African continent also means that information about a population in one region within (e.g. West Africa) cannot automatically be extrapolated to another region in Africa (e.g. Southern Africa). In our study this was only evident for the *ABCC2* rs2273697 (c.1249A) allele for which there was a significant difference between the frequency of this allele in our cohort (0.12) and in the YRI population (0.25; p=0.0464). Previously, differences in *CYP1A2 -163C>A* allele and genotype frequencies have been reported between Cameroonian (in West Africa) and South African populations (139, 195). In addition, African American populations which have often been used as a proxy for African populations are admixed, mostly from West African and East African populations, and European populations (196). In one study, African Americans were reported to show greater diversity

than either of the two ancestral populations (196). However, as stated earlier, as West African populations may show different genotype and allele frequencies to Southern Africa populations, African Americans are still not a reliable proxy for all African populations. This emphasises the need for studies done on relevant populations and highlights the importance of this current study.

4.2. Influence of genetic variation on Lopinavir disposition

The participants involved in our study had been on treatment including LPV for at least 6 months up to 9 years at the time of recruitment. Participants using LPV as part of their second-line treatment had switched from their first-line regimen due to ART failure. The general side effects that were reported included diarrhoea, persistent vomiting and leg numbness. It has been reported that high efavirenz plasma levels are associated with *CYP2B6* 516G>T SNP in a cohort of Bantu-speaking South Africans (182); and adverse effects to nevirapine treatment have been associated with the *CYP2B6* c.983T>C SNP (197). In our study, we sought to investigate whether similar associations can be observed for genes involved in the metabolism and transport of LPV.

As described earlier, LPV (as a protease inhibitors) acts by binding to the HI-viral protease and preventing the cleavage of gag and gag-pol precursor proteins by this enzyme. The current regimens that include LPV are as follows: AZT+3TC + LPV/r for patients in whom TDF was used in first-line treatment or; TDF+3TC (or FTC) + LPV/r for patients in whom AZT was used in first-line treatment. For twice daily administration, the elimination half-life of LPV is about 4-6 hrs, whilst its oral clearance is 6-7 L/h (73, 74). Common adverse effects associated with LPV use are, hepatotoxicity, dyslipidaemia, diarrhoea and nausea (8, 56-60). Differences in plasma LPV levels may explain variation in response to treatment. LPV concentrations below the C_{min} of 5.5 µg/mL (i.e. the minimum therapeutic concentration of LPV) may indicate plasma LPV levels at which the HI-virus will not be suppressed, while concentrations above the C_{max} of 9.6 µg/mL may lead to adverse side effects. We report on a significant association between *CYP3A4* rs2740574A>G genotypes (p=0.0479; Fig. 3.5 B, Table 3.2) and plasma LPV levels. Higher LPV plasma levels were observed with the G variant when a recessive genetic model was used to compare the combined A/A and A/G genotypes to the G/G genotype. The significance of this *CYP3A4*1B* allele remains contentious (138, 139, 141). The role of this allele in CYP3A4 expression and activity and in drug pharmacogenetics remains the subject of debate (112, 138), as discussed in the introduction. Regarding its role in LPV pharmacogenetics specifically, a pilot study in 20 Caucasian HIV-infected individuals undergoing LPV/r monotherapy identified two patients with the *CYP3A4*1B* allele, one who had virological rebound (>40 copies/mL of the virus) and the other low plasma LPV levels (1.748 µg/mL) (198). However, this was a small cohort of individuals as

their study was a pilot study. There is a need for more studies to be carried out to determine the role *CYP3A4* variation in LPV levels and HI-viral suppression in HIV/AIDS patients, especially in sub-Saharan Africa.

An association was observed with the *SLCO1B1* rs4149049A>G SNP and LPV levels (Fig. 3.4; Table 3.3). This SNP is found in intron 5 of the gene. Although the SNP has been observed in other populations (199), no contribution to the pharmacokinetics of LPV or other drugs transported by OATP1B1 has been reported on.

4.3. Limitations of the study

One of the main limitations of our study was the small sample size. At the time of recruitment, few patients were on a regimen with LPV as the number of individuals on second-line therapy will generally be lower than that of individuals on first-line therapy. The small sample size may explain the allele frequencies which were not in HWE: *CYP3A5* rs776746A>G; *SLCO1B1* rs4149049A>G, and rs4149053G>T.

Although two different methods were used for DNA isolation (see section 2.2.1 and section 2.2.2), we do not think this could have accounted for the differences observed in amplification success of different SNPs because all the DNA was measured using a spectrophotometer to evaluate concentrations and electrophoresed on agarose gel to evaluate integrity and no differences have been observed between the two methods. We do suspect that possibly, subsequent degradation of DNA may have played a role in the observed failures in amplification.

Other genes which play or may play a role in LPV pharmacogenetics were not included in the study; these include *ABCB1* (or *MDR1*, the multidrug resistance transporter gene which encodes p-glycoprotein) and the *ORM* genes which code for the α_1-acid glycoprotein (AGP). P-glycoprotein has been shown to be a transporter of LPV (71). Studies of the association between *ABCB1* and LPV pharmacokinetics have been conducted in European children (108, 200). Bellusci *et al.* (200) reported an association with the *ABCB1* 1236C>T SNP and low LPV levels. This gene may be relevant in this African population.

Sequencing for *SLCO1B1* and *ABCC2* was only carried out on short sections of the genes which may be mutational hotspots. Therefore, SNPs outside of these regions which may have been relevant were not included, such as *SLCO1B1* rs4149032 which has been reported to be highly prevalent in a South

African cohort of black African Xhosa-speaking and mixed ancestry individuals (201). This SNP is located in intron 2, and hence was not covered by our sequencing of exons 5 to 7 including their intron-exon junctions. Sequencing in *ABCC2* was only conducted in parts of the promoter region.
As this was an MSc project, time was a major limiting factor and prevented us from genotyping other SNPs within the selected genes, or other genes of interest.

As complete data was not available for all of the patients, such as CD4 count and viral load, the possible reasons for some of the patient having LPV levels below the limit of detection could include poor adherence to drug treatment (see section 3.3). However, there was no correlation with increased viral load.

4.4. Recommendations for future studies

With a larger sample size, a future study of this nature could further investigate the differences in the pharmacogenetics of LPV within sub-populations. The Malawian and South African cohorts in this study were investigated as one group and not stratified according to ethnicities.

As knowledge and understanding of the pharmacogenetics of LPV grows including the proteins which influence its disposition (the pharmacokinetics) and the proteins which are influenced by LPV (the pharmacodynamics), the selection of genes can be either narrowed down or expanded to those which are most relevant across all populations.

Regions and SNPs selected for genotyping were selected based on available literature. However, as knowledge about genetic variation African populations, especially, is constantly being added to the sequencing of other regions in the genes chosen for our study may allow for a greater understanding of their role, if any, in the pharmacogenetics of LPV.

The focus of our study was LPV, but as LPV is boosted with RTV, it may be worthwhile to investigate the pharmacogenetics of RTV and how (if any) they may be associated with the pharmacogenetics of LPV. RTV concentrations were not measured in our study.

4.5. Conclusion

In our study in which we sought to identify genes, and particular SNPs in those genes which may be associated with the pharmacogenetics of LPV, two variants were identified which showed a significant association. These are *CYP3A4*1B* (c.-392G) found in the promoter region of *CYP3A4*, and *SLCO1B1*

rs4149049A>G, an intronic SNP. These results need to be followed up in a cohort with an appropriate sample size. Our study further highlights the need to genetically characterise as many African population group in order to realise the dream of personalised medicine.

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6. Appendices

Appendix A: Solutions

I. 1 M Tris-HCl

121.1 g Tris

1 L dH2O

Autoclave

II. Sucrose Triton X-100 Lysis Buffer

For 1 L, add:

10 mL 1 M Tris-HCl (pH 8.0)

5 mL 1 M $MgCl_2$

10 mL Triton X-100

109.5 g (add just before use)

Make solution up to 1 L with dH_2O

Autoclave and keep at 4°C

III. T20E5

20 ml 1M Tris-HCl

10 ml 0.5M EDTA (pH 8.0)

Make up to 1 L with dH_2O and autoclave

IV. 10X TBE Buffer

108 g Tris

55 g Boric acid

7.44 g EDTA

Make up to 1 L with dH_2O and autoclave

Appendix B: Additional Graphs and Tables

Table 6.1 The Association of variant SNPs with Plasma Lopinavir Levels (all SNPs included; refer to Table 3.2).

SNP	Genotype	N	Mean Lopinavir level	p-value	Lower 95% CI	Upper 95% CI	Std Dev
CYP3A4							
rs2740574A>G	A/A	2	12.91	0.1011	-88.61	114.4	11.30
	A/G	22	7.287		5.266	9.309	4.559
	G/G	37	11.12		8.700	13.53	7.246
	A/A and A/G	24	7.756	**0.0479***	5.560	9.952	5.201
CYP3A5							
rs776746A>G	A/A	39	10.14	0.6422	8.215	12.07	5.944
	A/G	15	9.533		4.453	14.61	9.172
	G/G	7	7.759		2.842	12.68	5.317
	A/G and G/G	22	8.968	0.3556	5.397	12.54	8.055
rs10264272C>T	C/C	39	9.485	0.3619	7.216	11.75	6.999
	C/T	21	10.39		7.464	13.31	6.427
	T/T	1	N/A		N/A	N/A	0
	C/T and T/T	22	10.13	0.5184	7.300	12.96	6.387
ABCC2							
rs2273697G>A	G/G	48	9.775	0.8663	7.758	11.79	6.947
	G/A	14	9.320		5.878	12.76	5.960
rs17222653G>A	G/G	52	10.29	0.2078	8.389	12.20	6.843
	G/A	2	3.968		-41.04	48.98	5.010
rs1885301A>G	A/A	9	12.03	0.7546	4.083	19.97	10.33
	A/G	24	9.895		7.321	12.47	6.096
	G/G	21	9.405		6.642	12.17	6.070

	A/A and A/G	33	10.48	0.8540	7.862	13.09	7.371
rs7910642G>A	G/G	45	10.48	0.2065	8.426	12.52	6.822
	G/A	16	7.589		4.282	10.90	6.207
	A/A	1	6.875				0
	G/A and A/A	17	7.547	0.0722	4.456	10.64	6.013
rs2804402A>G	A/A	29	9.222	0.6228	7.107	11.34	5.559
	A/G	25	9.178		6.586	11.77	6.280
	G/G	8	12.85		3.760	21.94	10.87
	A/G and G/G	33	10.07	0.6516	7.368	12.77	7.616
SLCO1B1							
rs2306283A>G	A/A	1	N/A	0.1198	0	0	0
	A/G	19	7.140		5.252	9.028	3.917
	G/G	42	10.71		8.388	13.02	7.436
	A/A and A/G	20	7.503	0.1504	5.564	9.443	4.144
rs11045819C>A	C/C	57	9.826	0.3388	8.063	11.59	6.644
	C/A	5	7.922		-1.762	17.61	7.800
rs77271279G>T	G/G	59	9.598	0.4407	7.830	11.37	6.784
	G/T	3	11.14		-2.018	24.30	5.298
rs4149044A>T	A/A	9	9.725	0.2913	3.966	15.48	7.492
	A/T	31	8.942		6.267	11.62	7.293
	T/T	22	10.68		8.216	13.14	5.557
	A/A and A/T	40	9.118	0.1296	6.800	11.44	7.248
rs4149045G>A	G/G	9	9.725	0.3895	3.966	15.48	7.492
	G/A	31	8.942		6.267	11.62	7.293
	A/A	22	10.68		8.216	13.14	5.557
	G/G and G/A	40	9.118	0.1296	6.800	11.44	7.248
rs4149046G>A	G/G	58	9.830	0.4307	8.023	11.64	6.873
	G/A	4	7.380		3.614	11.15	2.367
rs4149048A>G	A/A	10	9.035	0.2352	3.746	14.32	7.393

	A/G	30	9.374		6.688	12.06	7.192
	G/G	22	12.11		8.551	15.67	8.025
rs4149049A>G	A/A	39	10.15	0.8902	7.749	12.55	7.402
	A/G	16	8.751		6.856	10.65	3.556
	G/G	5	10.75		-0.7851	22.28	9.287
	A/G and G/G	21	9.226	0.6419	6.839	11.61	5.243
rs4149050T>C	T/T	9	9.725	0.3662	3.966	15.48	7.492
	T/C	27	9.339		6.367	12.31	7.511
	C/C	22	10.62		8.089	13.14	5.699
rs4149051A>G	A/A	9	9.725	0.3662	3.966	15.48	7.492
	A/G	27	9.339		6.367	12.31	7.511
	G/G	22	10.62		8.089	13.14	5.699
rs4149052A>G	A/A	9	9.725	0.3662	3.966	15.48	7.492
	A/G	27	9.339		6.367	12.31	7.511
	G/G	22	10.62		8.089	13.14	5.699
rs4149053G>T	G/G	16	8.199	0.3746	4.707	11.69	6.554
	G/T	23	10.69		7.428	13.96	7.549
	T/T	19	10.32		7.411	13.23	6.035
rs4149054G>A	G/G	17	9.988	0.4217	4.986	14.99	9.728
	G/A	22	9.080		7.022	11.14	4.641
	A/A	19	10.72		7.881	13.56	5.886
rs141555703G>A	G/G	50	9.642	0.6528	7.704	11.58	6.818
	G/A	7	11.27		4.536	18.01	7.285
rs67496683TACTTG>del	TACTTG/TACTTG	12	9.453	0.6828	4.975	13.89	7.019
	TACTTG/del	26	9.318		6.379	12.26	7.278
	del/del	21	10.51		7.718	13.30	6.130
rs4149057T>C	T/T	52	9.876	0.3752	7.845	11.91	7.297
	T/C	7	6.626		4.675	8.578	2.111
	C/C	1	N/A		0	0	0

	T/C and C/C	8	6.658	0.1673	5.022	8.293	1.956
rs2291075C>T	C/C	13	8.817	0.5891	4.658	12.98	6.884
	C/T	25	9.333		6.242	12.42	7.488
	T/T	21	10.42		7.569	13.27	6.266

Table 6.2 Association of genotypes with Lopinavir therapeutic range (all SNPs included; refer to Table 3.3)

Genotype	<5.5µg/mL	5.5µg/mL≤X≥9.6µg/mL	>9.6µg/mL	Total	Global P-value	P-value [<5.5µg/mL vs. 5.5µg/mL≤X≥9.6µg/mL]	P-value [<5.5µg/mL vs. >9.6µg/mL]
CYP3A4							
rs2740574A>G							
A/A	2 (0.08)	0	1 (0.04)	3	0.2566	0.3135	0.1565
A/G	12 (0.48)	8 (0.4)	7 (0.26)	27			
G/G	11 (0.44)	12 (0.6)	19 (0.7)	42			
Total	25	20	27				
CYP3A5							
rs776746A>G							
A/A	14 (0.56)	16 (0.76)	17 (0.68)	47	0.4930	0.3166	0.3042
A/G	5 (0.2)	3 (0.14)	6 (0.24)	14			
G/G	6 (0.24)	2 (0.1)	2 (0.08)	10			
Total	25	21	25				
rs10264272C>T							
C/C	19 (0.76)	11 (0.58)	17 (0.63)	47	0.2848	0.2165	0.2592
C/T	5 (0.2)	8 (0.42)	10 (0.37)	23			
T/T	1 (0.04)	0	0	1			
Total	25	19	27				
ABCC2							
rs2273697G>A							
G/G	19 (0.79)	15 (0.75)	22 (0.81)	56	0.9334	1.00	0.7230
G/A	5 (0.21)	5 (0.25)	5 (0.19)	15			
Total	24	20	27				
rs7910642G>A							
G/G	14 (0.58)	16 (0.8)	21 (0.78)	51	0.1325	0.1013	0.4339
G/A	10 (0.42)	3 (0.15)	6 (0.22)	19			
A/A	0	1 (0.05)	0 (0)	1			
Total	24	20	27				
rs2804402A>G							

A/A	14 (0.58)	11 (0.55)	11 (0.41)	36	0.5358	0.7483	0.2165
A/G	9 (0.38)	7 (0.35)	11 (0.41)	27			
G/G	1 (0.04)	2 (0.1)	5 (0.19)	8			
Total	24	20	27				
rs17222653G>A							
G/G	18 (0.95)	18 (0.95)	23 (1)	59	0.5224	1.00	0.4524
G/A	1 (0.05)	1 (0.05)	0 (0)	2			
Total	19	19	23				
rs1885301A>G							
A/A	1 (0.05)	4 (0.26)	4 (0.17)	9	0.5519	0.3517	0.3214
A/G	8 (0.42)	7 (0.37)	12 (0.5)	27			
G/G	10 (0.53)	8 (0.42)	8 (0.33)	26			
Total	19	19	24				
SLCO1B1							
rs2306283A>G							
A/A	0 (0)	0 (0)	1 (0.04)	1	0.7286	0.5151	0.6219
A/G	6 (0.24)	7 (0.35)	6 (0.22)	19			
G/G	19 (0.76)	13 (0.65)	20 (0.74)	52			
Total	25	20	27				
rs11045819C>A							
C/C	22 (0.88)	18 (0.9)	26 (0.96)	66	0.5673	1.00	0.5671
C/A	3 (0.12)	2 (0.1)	1 (0.04)	6			
Total	25	20	27				
rs77271279G>T							
G/G	24 (0.92)	20 (1)	25 (0.92)	69	0.7736	0.2507	0.5005
G/T	1 (0.08)	0 (0)	2 (0.08)	3			
Total	25	20	27				
rs4149044A>T							
A/A	4 (0.16)	6 (0.3)	2 (0.07)	12	0.2914	0.3379	0.5975
A/T	10 (0.4)	9 (0.45)	13 (0.48)	32			
T/T	11 (0.44)	5 (0.25)	12 (0.44)	28			
Total	25	20	27				
rs4149045G>A							
G/G	2 (0.08)	6 (0.3)	2 (0.07)	10	0.1938	0.1013	0.956
G/A	11 (0.44)	9 (0.45)	13 (0.48)	33			

A/A	12 (0.48)	5 (0.25)	12 (0.44)	29			
Total	25	20	27				
rs4149046G>A							
G/G	24 (0.96)	18 (0.9)	26 (0.96)	68	0.6719	0.5772	0.5671
G/A	1 (0.04)	2 (0.1)	1 (0.04)	4			
Total	25	20	27				
rs4149048A>G							
A/A	4 (0.17)	6 (0.3)	2 (0.07)	12	0.1470	0.1572	0.4516
A/G	8 (0.35)	10 (0.5)	13 (0.48)	31			
G/G	11 (0.48)	4 (0.2)	12 (0.44)	27			
Total	23	20	27				
rs4149049A>G							
A/A	22 (0.92)	7 (0.35)	20 (0.77)	49	**0.000076**	**0.0001**	**0.0686**
A/G	0 (0)	10 (0.5)	5 (0.19)	15			
G/G	2 (0.08)	3 (0.15)	1 (0.04)	6			
Total	24	20	26				
rs4149050A>G							
A/A	2 (0.09)	6 (0.3)	2 (0.08)	10	0.0946	0.0531	0.995
A/G	9 (0.39)	10 (0.5)	10 (0.4)	29			
G/G	12 (0.52)	4 (0.2)	13 (0.52)	29			
Total	23	20	25				
rs4149051A>G							
A/A	2 (0.09)	6 (0.3)	2 (0.08)	10	0.0946	0.0531	0.995
A/G	9 (0.39)	10 (0.5)	10 (0.4)	29			
G/G	12 (0.52)	4 (0.2)	13 (0.52)	29			
Total	23	20	25				
rs4149052A>G							
A/A	2 (0.09)	6 (0.3)	2 (0.08)	10	0.0946	0.0531	0.995
A/G	9 (0.39)	10 (0.5)	10 (0.4)	29			
G/G	12 (0.52)	4 (0.2)	13 (0.52)	29			
Total	23	20	25				
rs4149053G>T							
G/G	7 (0.32)	7 (0.35)	4 (0.16)	18	0.2005	0.1628	0.2357
G/T	5 (0.23)	9 (0.45)	11 (0.44)	25			
T/T	10 (0.45)	4 (0.2)	10 (0.4)	24			

Total	22	20	25				
rs4149054G>A							
G/G	7 (0.3)	7 (0.35)	5 (0.25)	19	0.5288	0.5516	0.6771
G/A	8 (0.35)	9 (0.45)	9 (0.36)	26			
A/A	8 (0.35)	4 (0.2)	11 (0.44)	23			
Total	23	20	25				
rs141555703G>A							
G/G	18 (0.78)	17 (0.85)	21 (0.875)	56	0.7112	0.7041	1.00
G/A	5 (0.22)	3 (0.15)	3 (0.125)	11			
Total	23	20	24				
rs67496683TACTTG>-							
TACTTG/TACTTG	5 (0.21)	5 (0.25)	4 (0.16)	14	0.8818	0.7672	0.7371
TACTTG/-	8 (0.33)	8 (0.4)	11 (0.44)	27			
-/-	11 (0.46)	7 (0.35)	10 (0.4)	28			
Total	24	20	25				
rs4149057T>C							
T/T	19 (0.76)	16 (0.8)	23 (0.96)	58	0.1192	0.4232	0.2332
T/C	6 (0.24)	3 (0.15)	1 (0.04)	10			
C/C	0 (0)	1 (0.05)	0 (0)	1			
Total	25	20	24				
rs2291075C>T							
C/C	5 (0.2)	6 (0.29)	3 (0.13)	14	0.8004	0.7906	0.7711
C/T	11 (0.44)	8 (0.38)	10 (0.43)	29			
T/T	9 (0.36)	7 (0.33)	10 (0.33)	26			
Total	25	21	23				

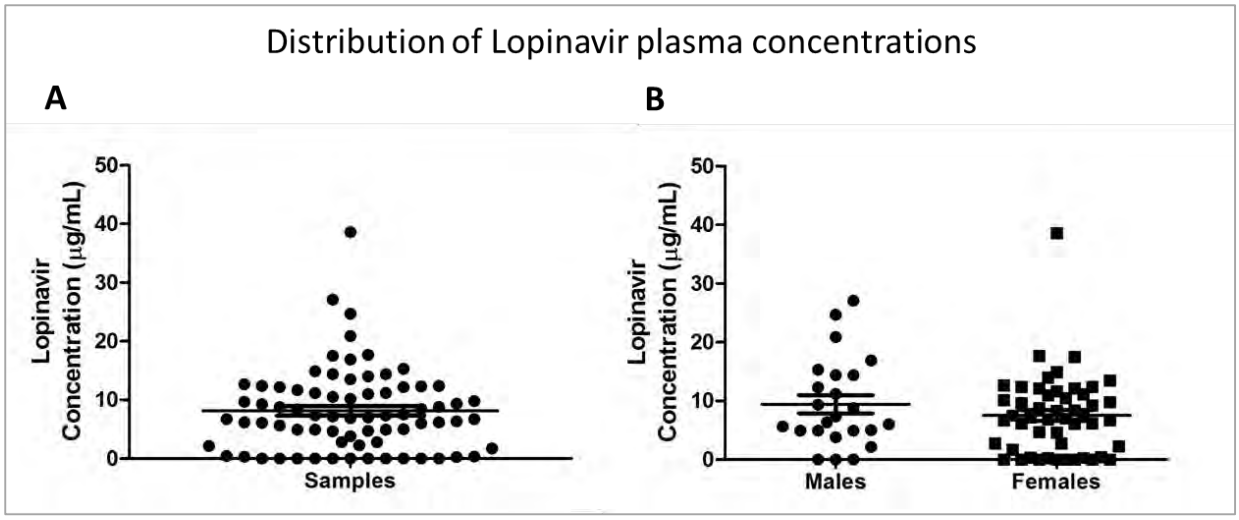

Figure 6.1 Distribution of lopinavir plasma concentrations. Samples with BLQ levels (<0.0195 µg/mL) have been included.

V. CYP3A4

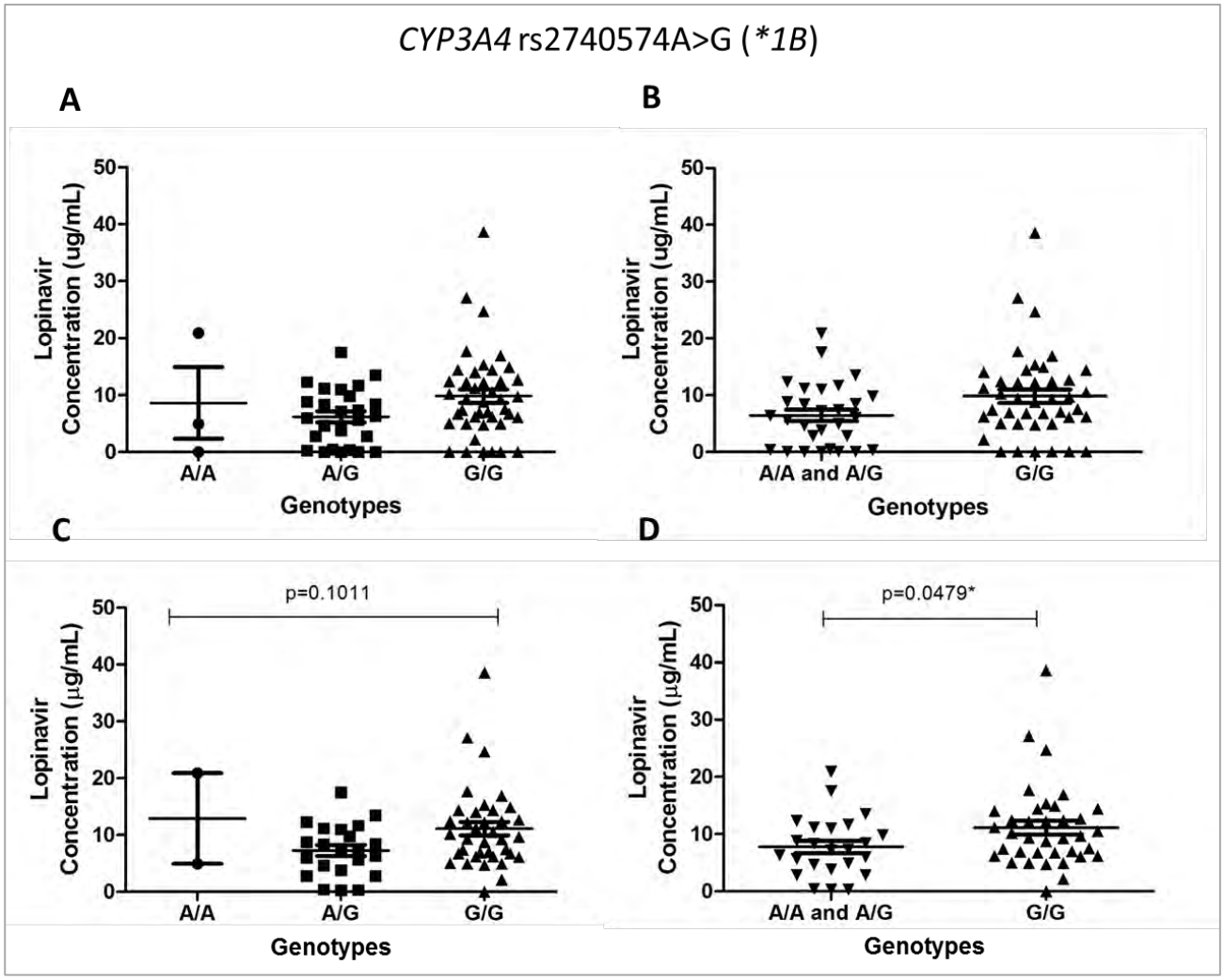

Figure 6.2 *CYP3A4*1B* genotypes vs lopinavir levels. A: with BLQ samples (p-value: 0.1461, Kruskal Wallis One Way ANOVA); B: Recessive model with BLQ samples (p-value: 0.0511, Mann-Whitney test); C: without BLQ samples (p-value: 0.1011, Kruskal Wallis One Way ANOVA); D: Recessive model without BLQ samples (p-value: 0.0479, Mann-Whitney test).

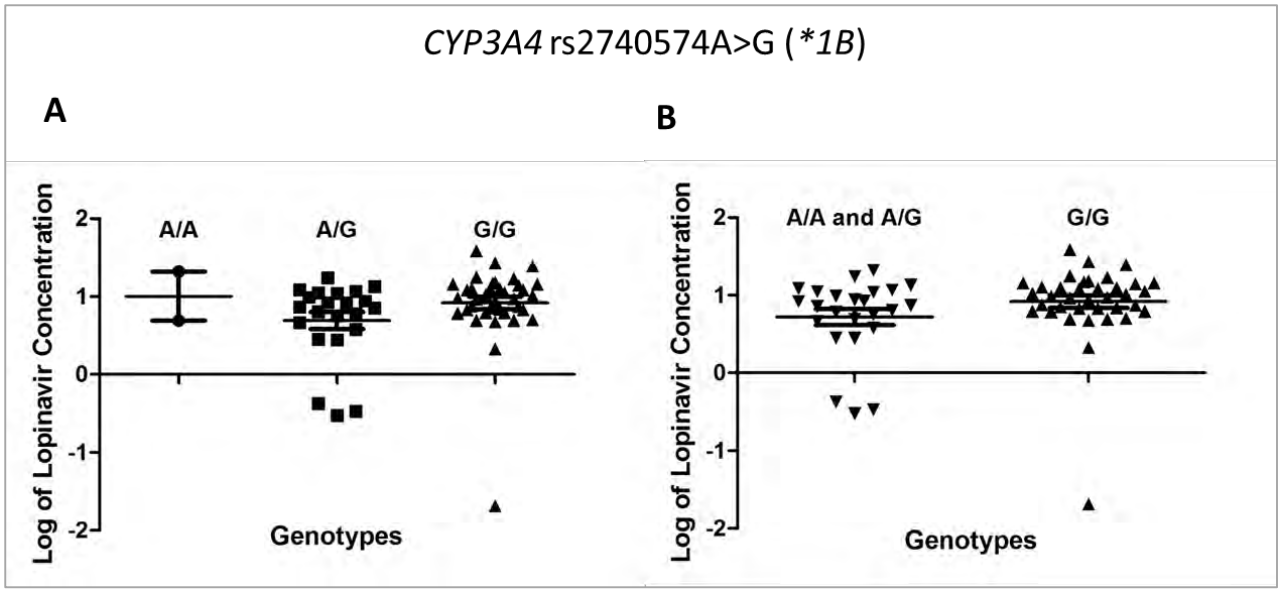

Figure 6.3 Log of *CYP3A4* rs2740574A>G (1B*) genotypes vs lopinavir levels without BLQ samples.** A: p-value: 0.1011, Kruskal Wallis One Way ANOVA; B: Recessive model (p-value: 0.0479, Mann-Whitney test).

VI. CYP3A5

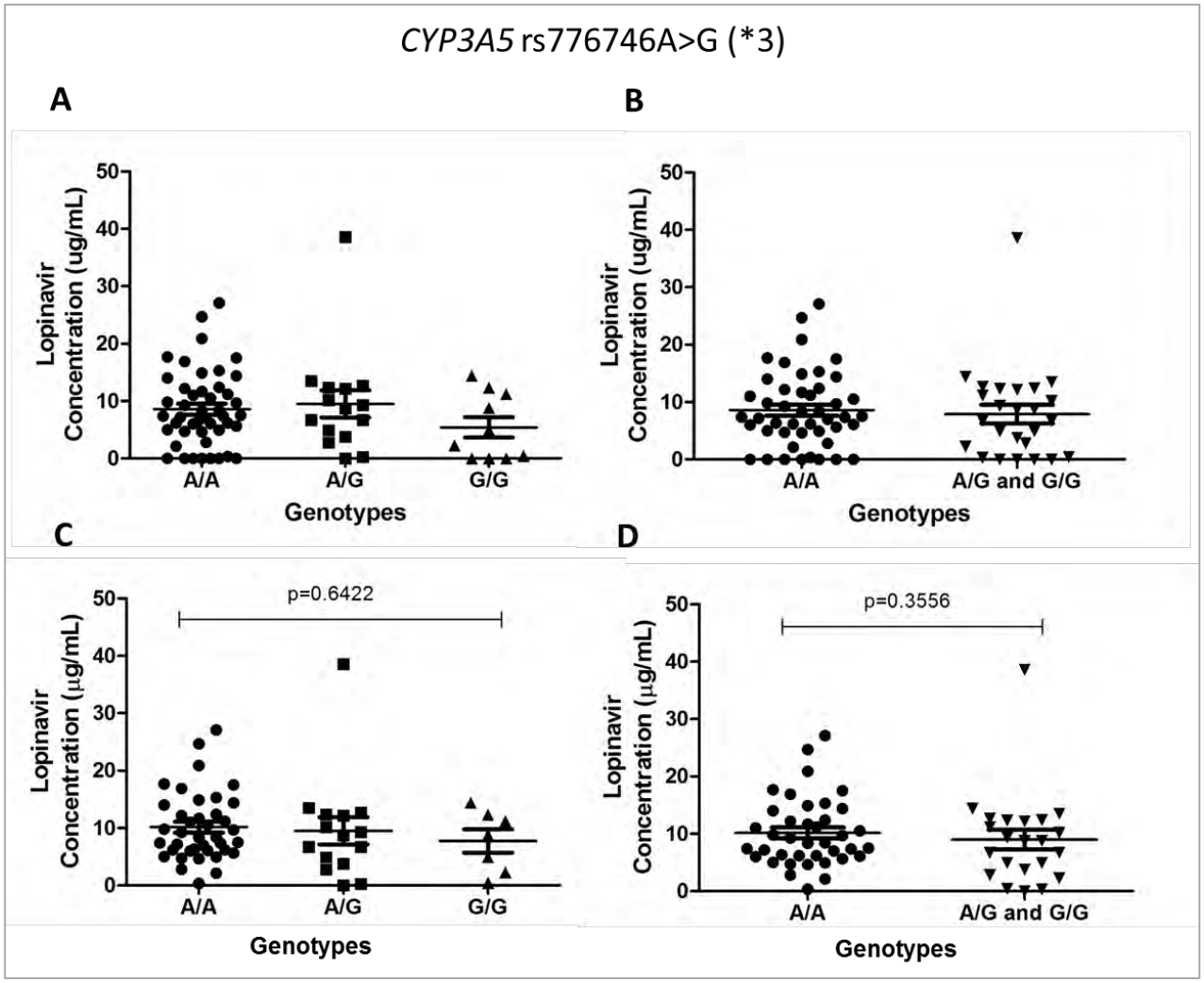

Figure 6.4 *CYP3A5* rs776746A>G (3*) genotypes vs lopinavir levels.** A: with BLQ samples (p-value: 0.3892, Kruskal Wallis ANOVA); B: Dominant model with BLQ samples (p-value: 0.6042, Mann-Whitney test); C: without BLQ samples (p-value: 0.6422, Kruskal Wallis ANOVA); D: Dominant model without BLQ samples (p-value: 0.3556, Mann-Whitney test).

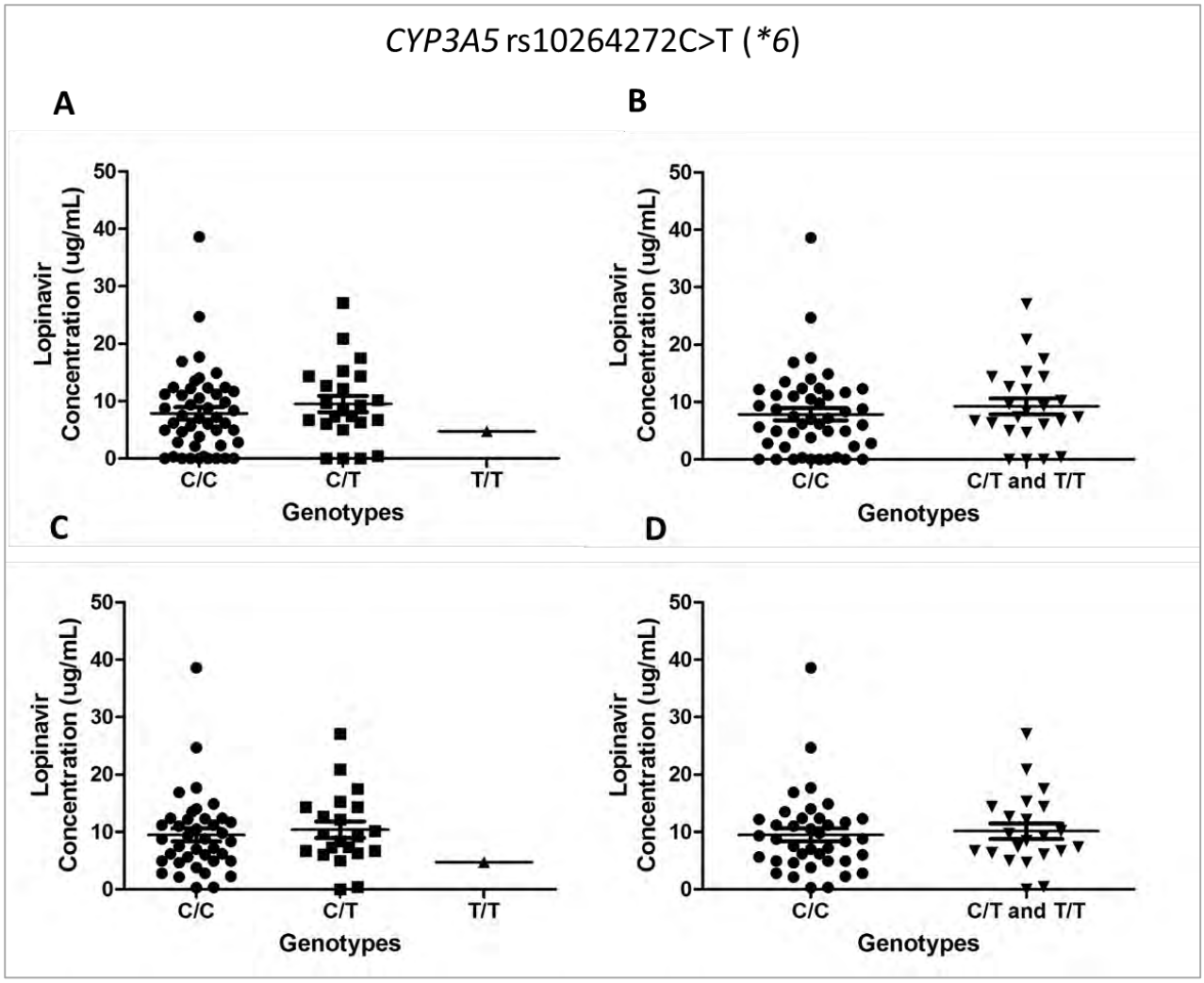

Figure 6.5 *CYP3A5* rs10264272C>T (6*) genotypes vs lopinavir levels.** A: with BLQ samples (p-value: 0.3428, Kruskal Wallis ANOVA); B: Dominant model with BLQ samples (p-value: 0.2628, Mann-Whitney test); C: without BLQ samples (p-value: 0.3619, Kruskal Wallis ANOVA); D: Dominant model without BLQ samples (p-value: 0.5184, Mann-Whitney test).

VII. ABCC2

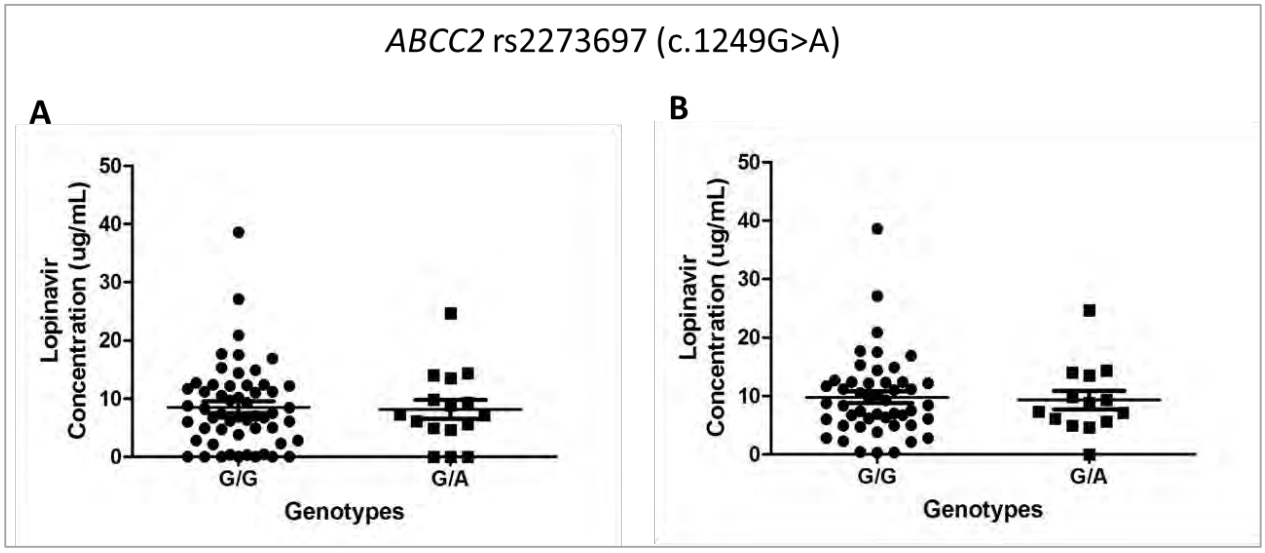

Figure 6.6 *ABCC2* rs2273697 (c.1249G>A) genotypes vs lopinavir levels. A: with BLQ samples (p-value: 0.9013, Mann-Whitney test); B: without BLQ levels (p-value: 0.8663, Mann-Whitney test)

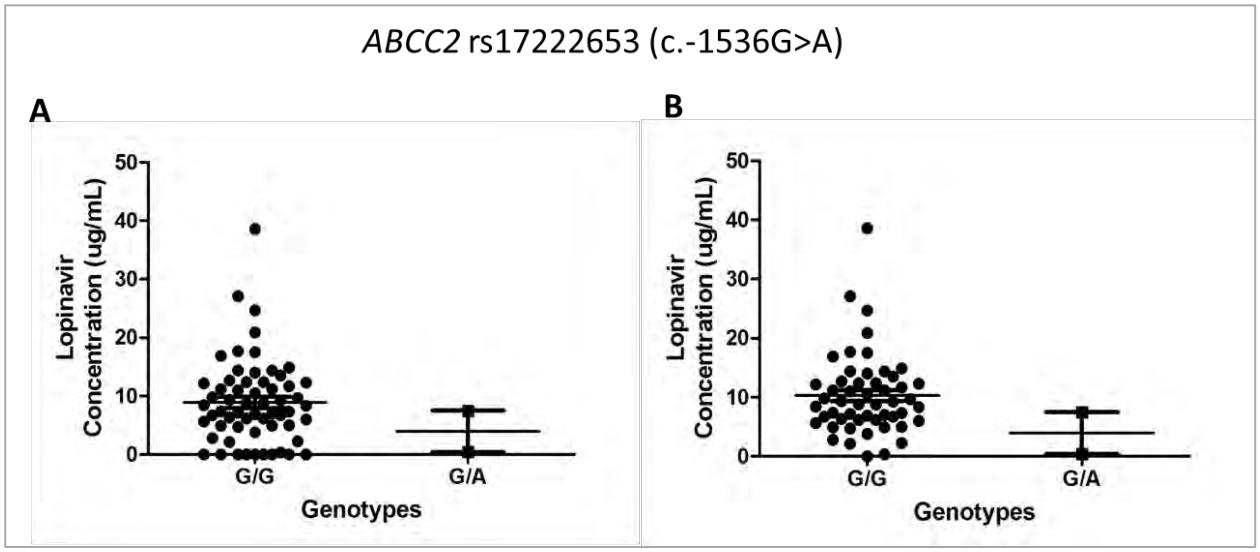

Figure 6.7 *ABCC2* rs17222653 (c.-1536G>A) genotypes vs lopinavir levels. A: with BLQ levels (p-value: 0.4367, Mann-Whitney test); B: without BLQ levels (p-value: 0.2078, Mann-Whitney test).

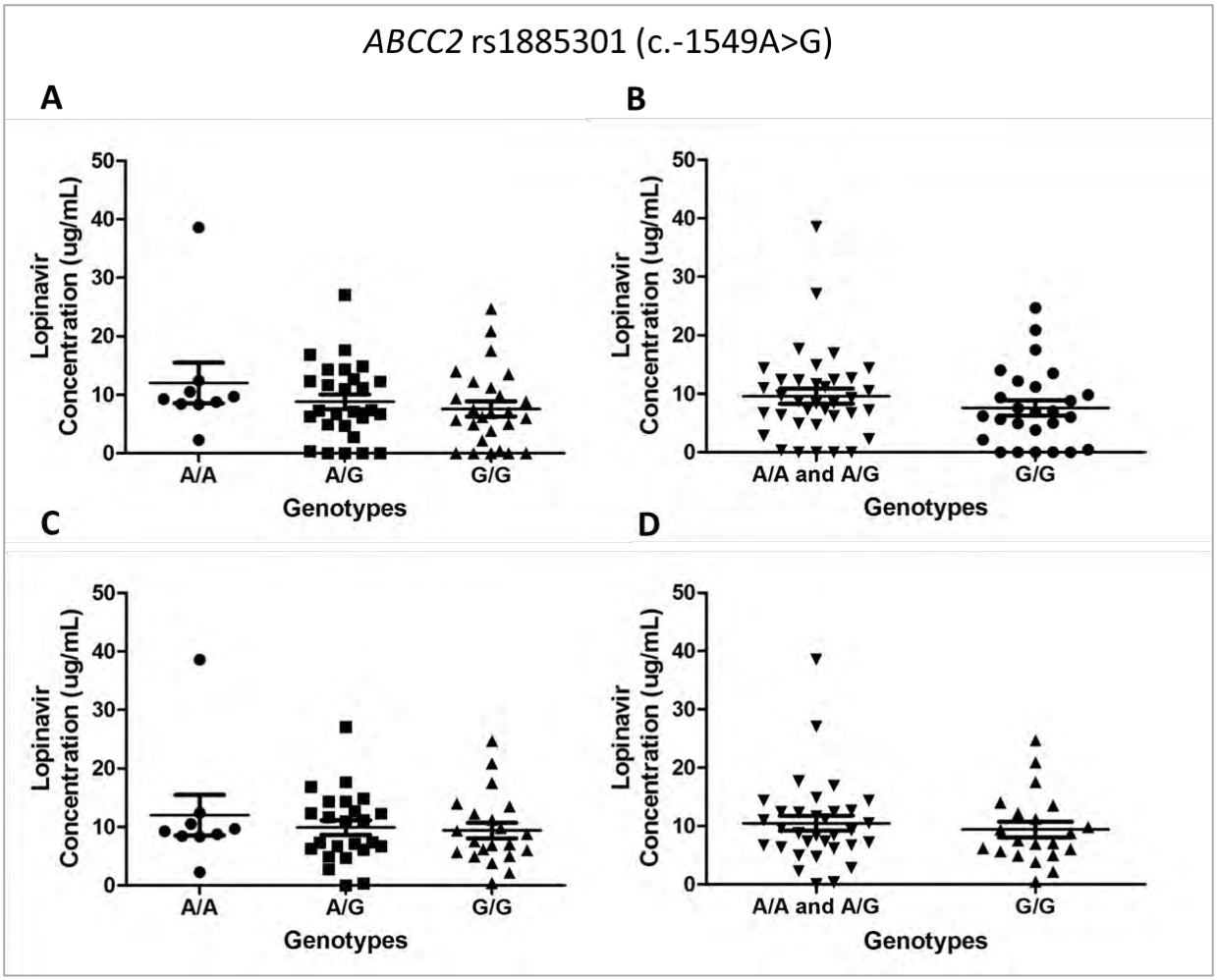

Figure 6.8 *ABCC2* rs1885301 (c.-1549A>G) genotypes vs lopinavir levels. A: with BLQ levels (p-value: 0.3254, Kruskal-Wallis One Way ANOVA); B: Additive recessive model with BLQ levels (p-value: 0.5799, Mann-Whitney test); C: without BLQ levels (p-value: 0.7546, Kruskal-Wallis One Way ANOVA); D: Additive recessive model without BLQ levels (p-value: 0.854, Mann-Whitney test).

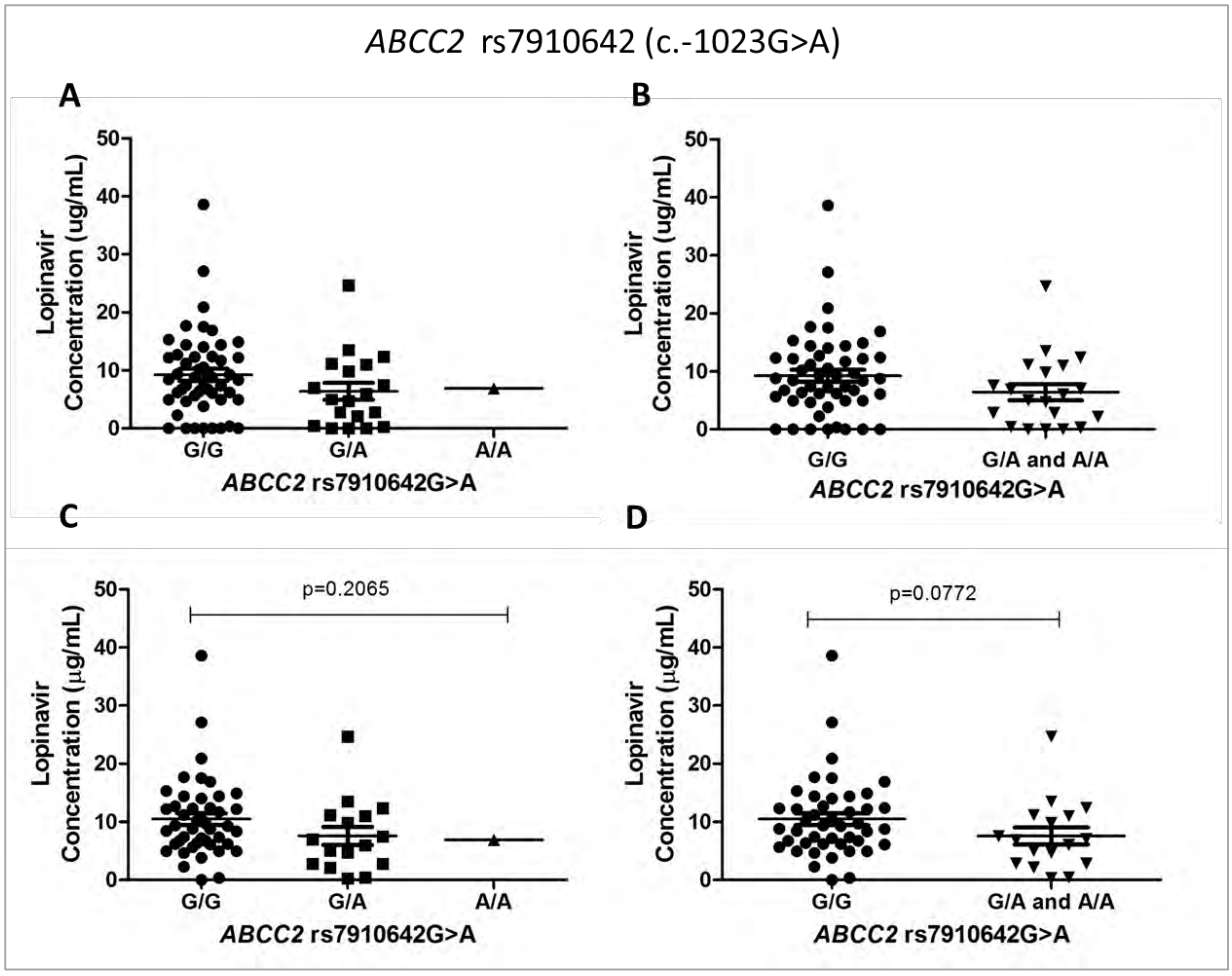

Figure 6.9 *ABCC2* rs7910642 (c.-1023G>A) genotypes vs lopinavir levels. A: with BLQ levels (p-value: 0.2523, Kruskal-Wallis One Way ANOVA); B: Dominant model with BLQ levels (p-value: 0.1001, Mann-Whitney test); C: without BLQ levels (p-value: 0.2065, Kruskal-Wallis One Way ANOVA); D: Dominant model without BLQ levels (p-value: 0.0722, Mann-Whitney test).

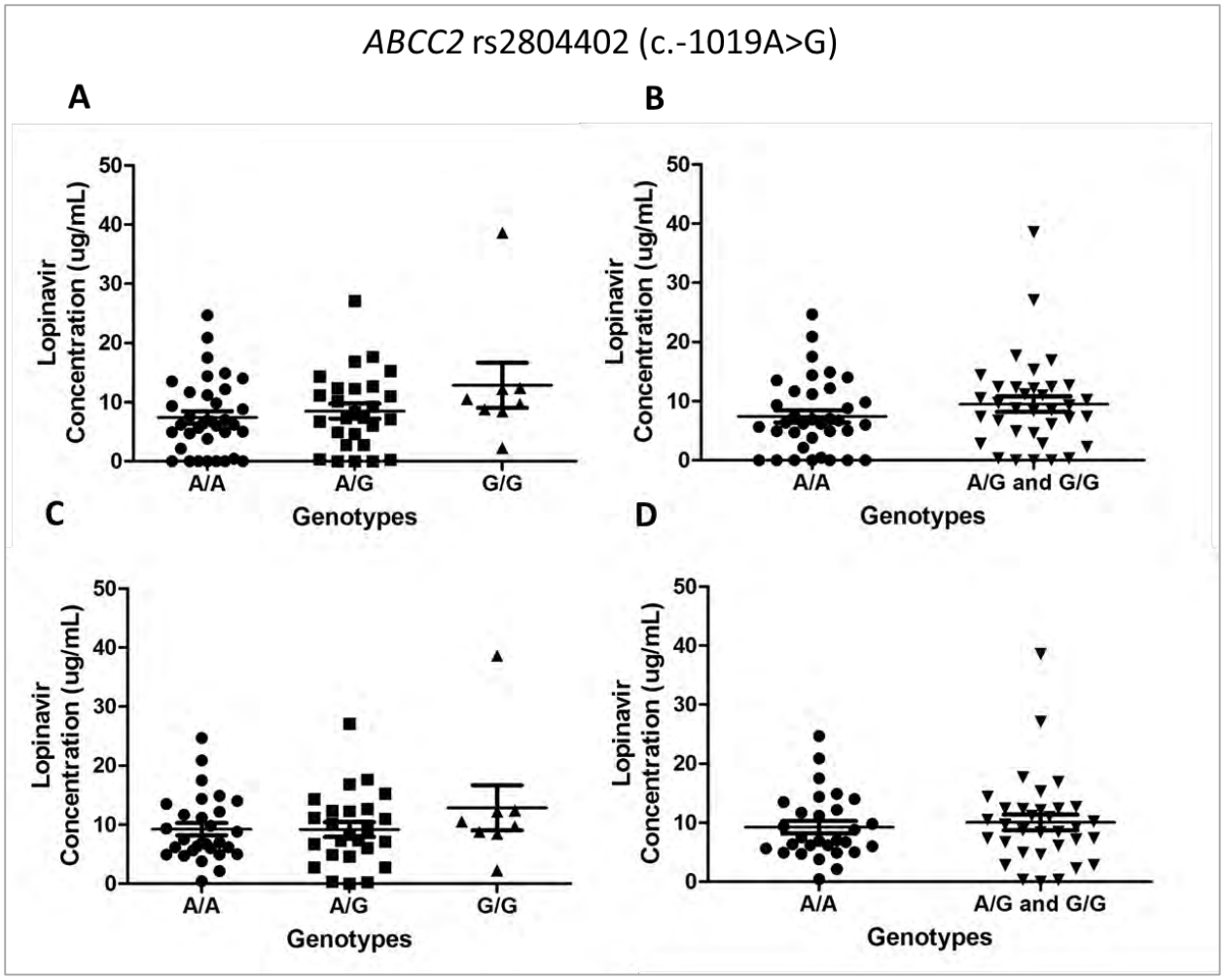

Figure 6.10 *ABCC2* rs2804402 (c.-1019A>G) genotypes vs lopinavir levels. A: with BLQ levels (p-value: 0.2207, Kruskal-Wallis One Way ANOVA); B Dominant model with BLQ levels (p-value: 0.1725, Mann-Whitney test); C: without BLQ levels (p-value: 0.6228, Kruskal-Wallis test); D: Dominant model without BLQ levels (p-value: 0.6516, Mann-Whitney test).

VIII. SLCO1B1

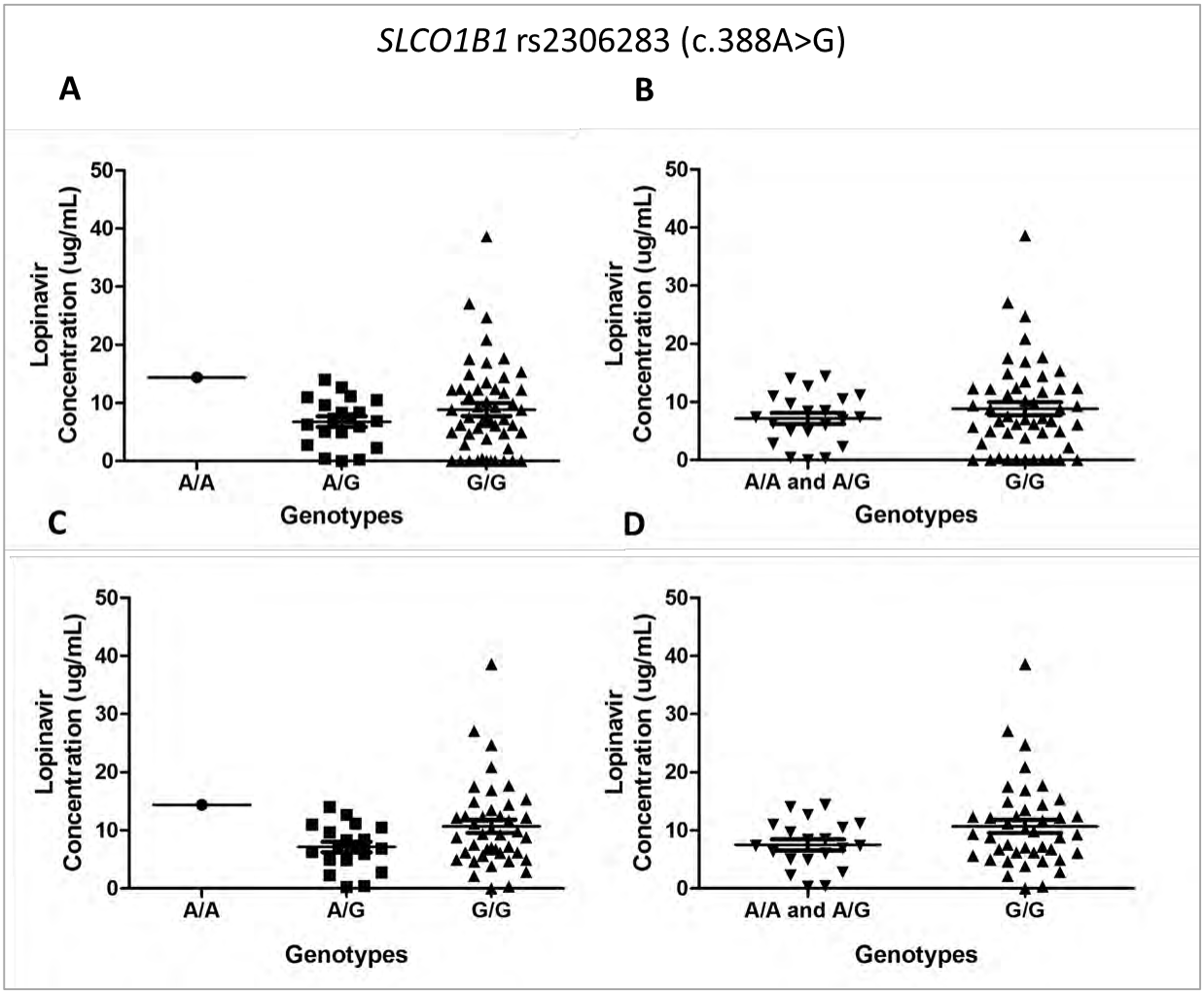

Figure 6.11 *SLCO1B1* rs2306283 (c.388A>G) genotypes vs lopinavir levels. A: with BLQ samples (p-value: 0.3864, Kruskal-Wallis One Way ANOVA); B: Recessive model with BLQ samples (p-value: 0.7423, Mann-Whitney test); C: without BLQ samples (p-value: 0.1198, Kruskal-Wallis One Way ANOVA); D: Recessive model without BLQ samples (p-value: 0.1504, Mann-Whitney test).

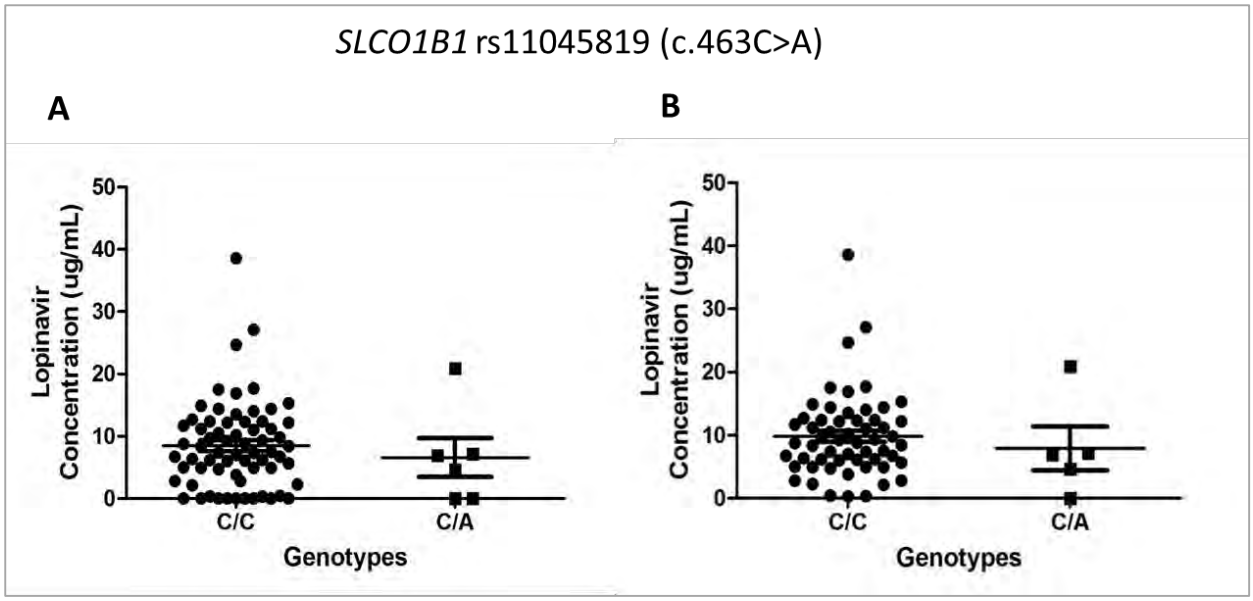

Figure 6.12 *SLCO1B1* rs11045819 (c.463C>A) genotypes vs lopinavir levels. A: with BLQ samples (p-value: 0.3803, Mann-Whitney test); B: without BLQ samples (p-value: 0.3388, Mann-Whitney test).

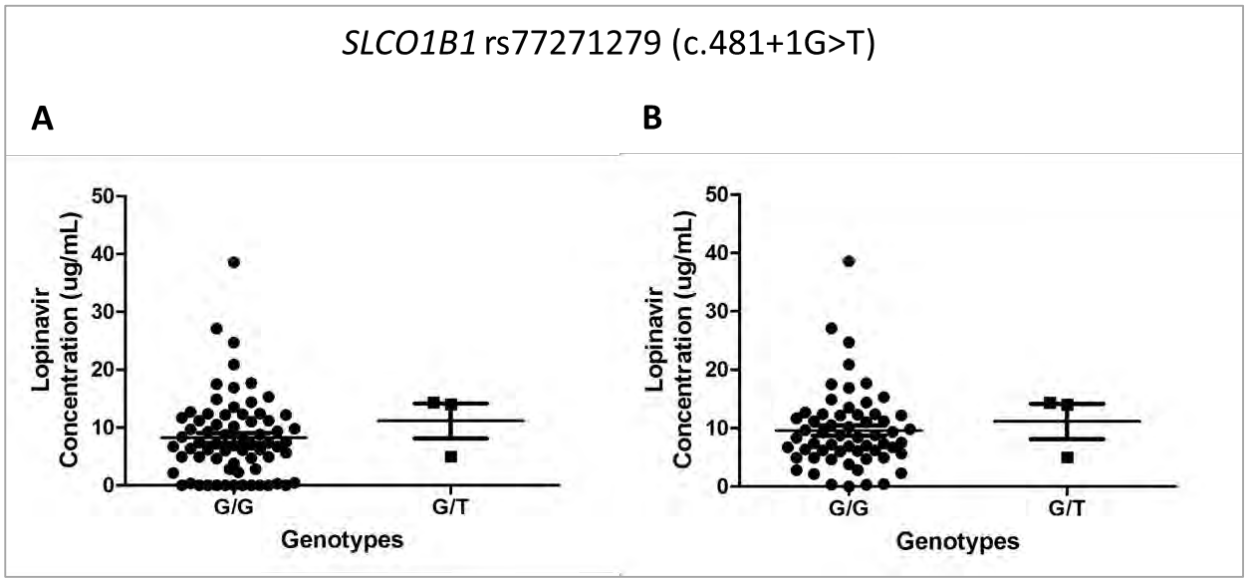

Figure 6.13 *SLCO1B1* rs77271279 (c.481+1G>T) genotypes vs lopinavir levels. A: with BLQ samples (p-value: 0.2773, Mann-Whitney test); B: without BLQ samples (p-value: 0.4407, Mann-Whitney test).

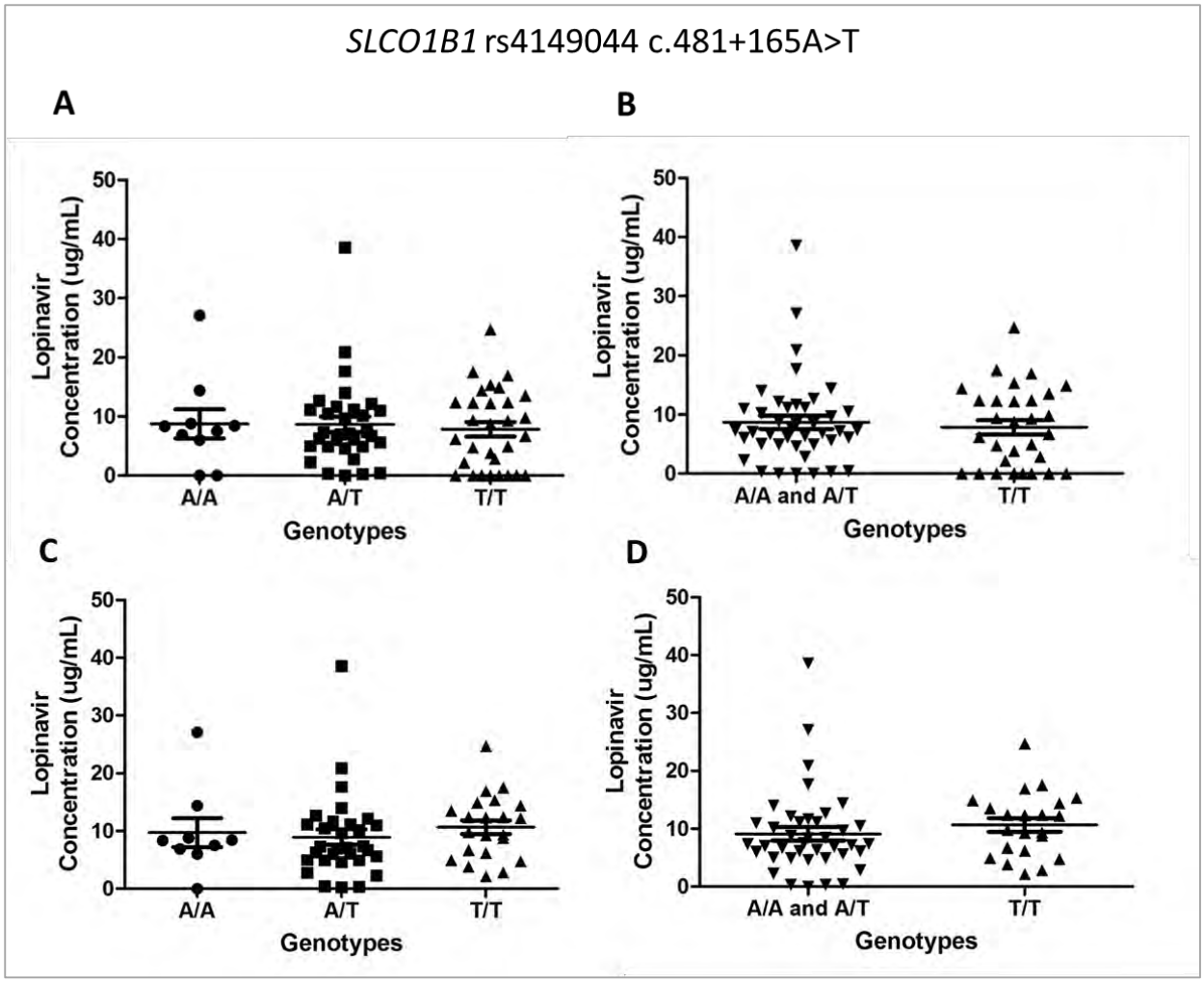

Figure 6.14 *SLCO1B1* rs4149044 c.481+165A>T genotypes vs lopinavir levels. A: with BLQ samples (p-value: 0.9250, Kruskal-Wallis One Way ANOVA); B: Recessive model with BLQ samples (p-value: 0.6974, Mann-Whitney test); C: without BLQ samples (p-value: 0.2913, Kruskal-Wallis One Way ANOVA); D: Recessive model without BLQ samples (p-value: 0.1296, Mann-Whitney test).

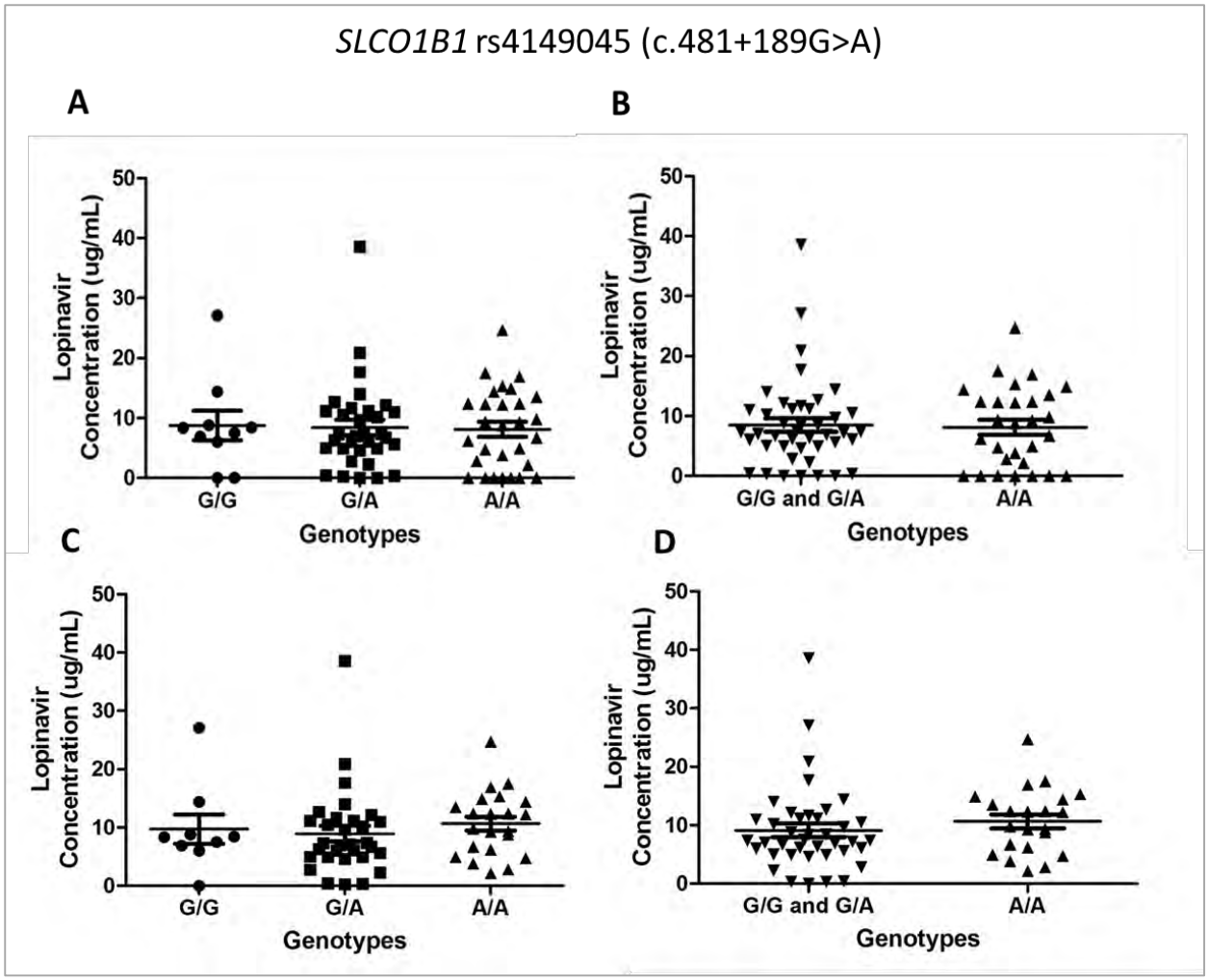

Figure 6.15 *SLCO1B1* rs4149045 (c.481+189G>A) genotypes vs lopinavir levels. A: with BLQ samples (p-value: 0.9984, Kruskal-Wallis One Way ANOVA); B: Recessive model with BLQ samples (p-value: 0.9725, Mann-Whitney test); C: without BLQ samples (p-value: 0.3895, Kruskal-Wallis One Way ANOVA); D: Recessive model without BLQ samples (p-value: 0.1296, Mann-Whitney test).

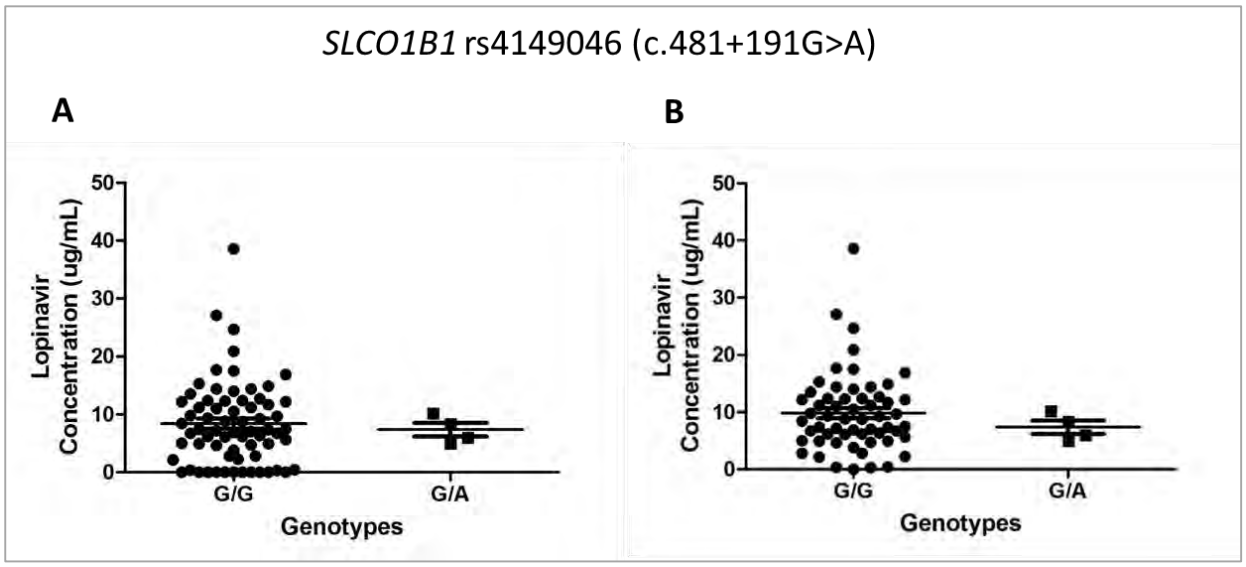

Figure 6.16 *SLCO1B1* rs4149046 (c.481+191G>A) genotypes vs lopinavir levels A: with BLQ samples (p-value: 0.8535, Mann-Whitney test); B: without BLQ samples (p-value: 0.4307, Mann-Whitney test).

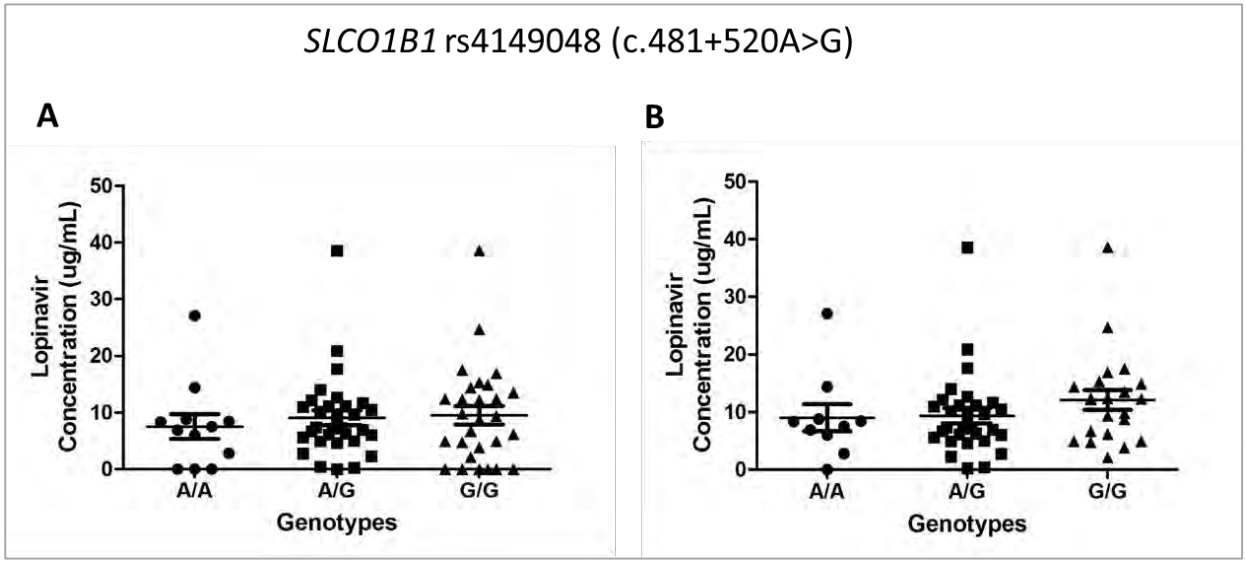

Figure 6.17 *SLCO1B1* rs4149048 (c.481+520A>G) genotypes vs lopinavir levels. A: with BLQ samples (p-value: 0.7042, Kruskal-Wallis One Way ANOVA); B: without BLQ samples (p-value: 0.2352, Kruskal-Wallis One Way ANOVA).

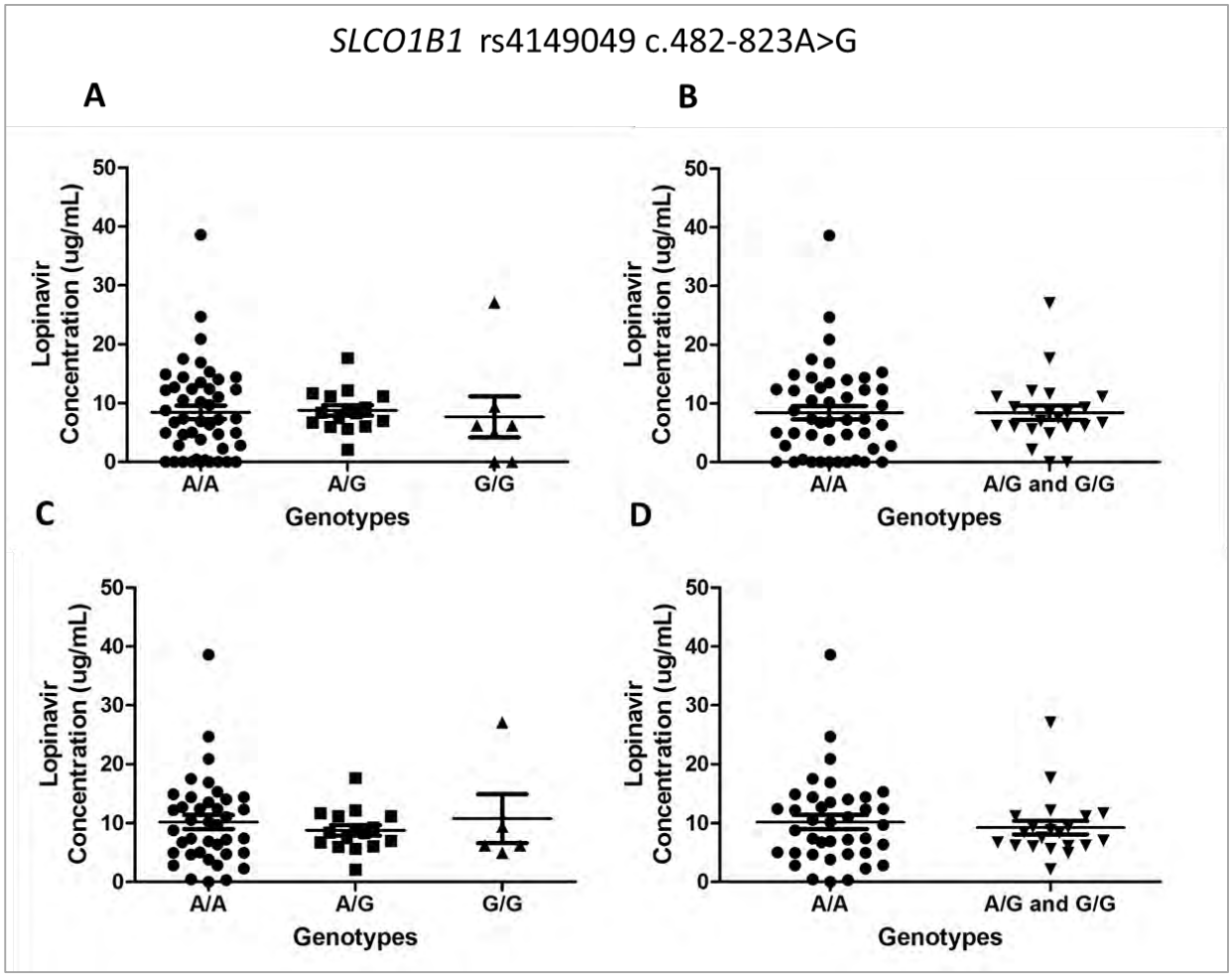

Figure 6.18 *SLCO1B1* rs4149049 (c.482-823A>G) genotypes vs lopinavir levels. A: with BLQ samples (p-value: 0.5294, Kruskal-Wallis One Way ANOVA); B: Dominant model without BLQ samples (p-value: 0.8608, Mann-Whitney test); C: without BLQ samples (p-value: 0.8902, Kruskal-Wallis One Way ANOVA); D: Dominant model without BLQ samples (p-value: 0.6419, Mann-Whitney test).

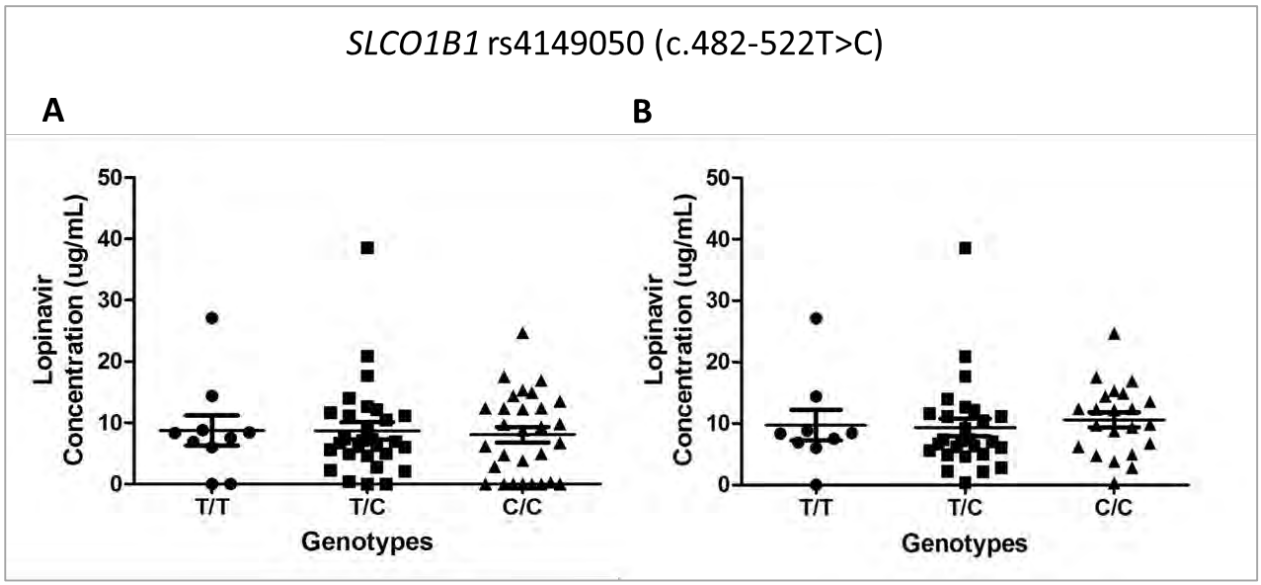

Figure 6.19 *SLCO1B1* rs4149050 (c.482-522T>C) genotypes vs lopinavir levels. A: with BLQ samples (p-value: 0.9873, Kruskal-Wallis One Way ANOVA); B: without BLQ samples (p-value: 0.3662, Kruskal-Wallis One Way ANOVA).

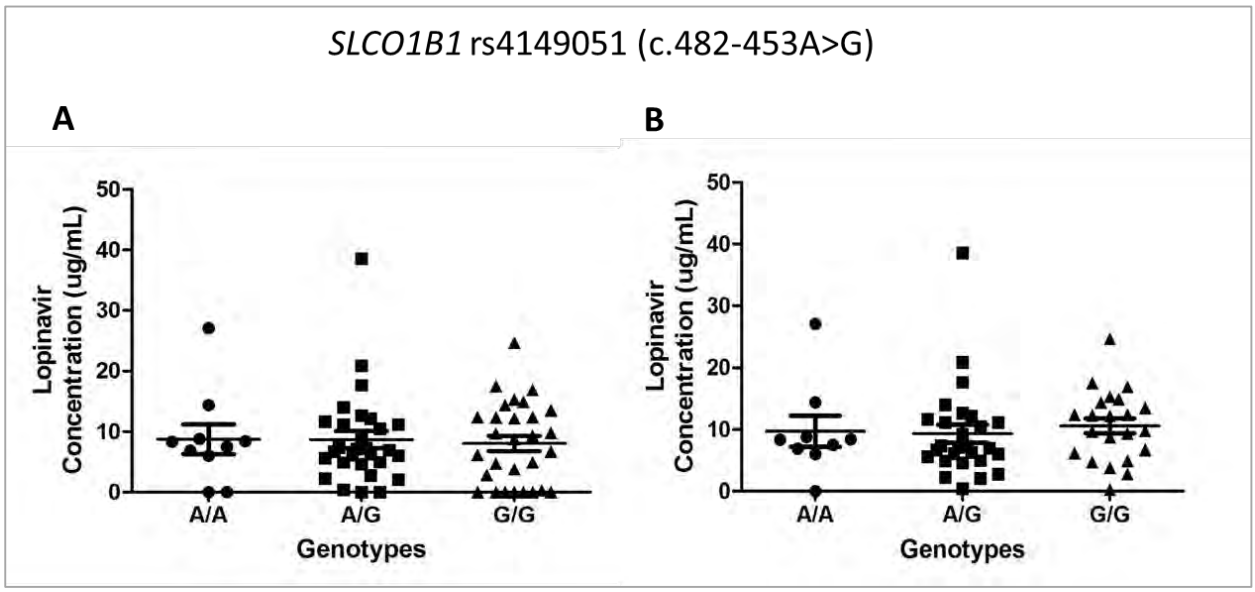

Figure 6.20 *SLCO1B1* rs4149051 (c.482-453A>G) genotypes vs lopinavir levels. A: with BLQ samples (p-value: 0.9873, Kruskal-Wallis One Way ANOVA); B: without BLQ samples (p-value: 0.3662, Kruskal-Wallis One Way ANOVA).

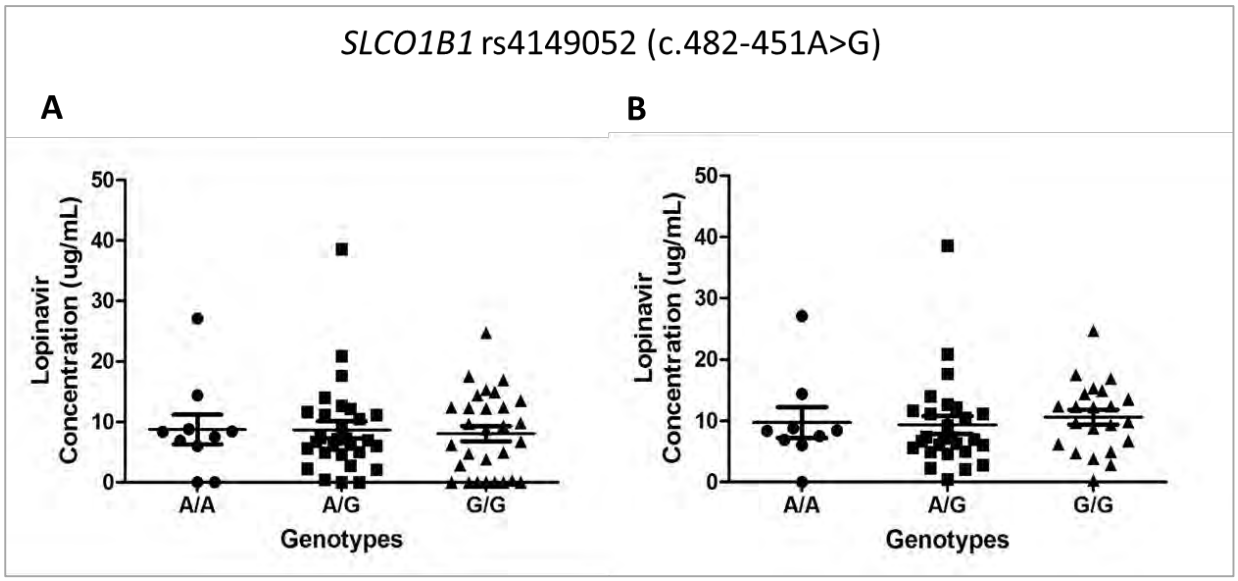

Figure 6.21 *SLCO1B1* rs4149052 (c.482-451A>G) genotypes vs lopinavir levels. A: with BLQ samples (p-value: 0.9873, Kruskal-Wallis One Way ANOVA); B: without BLQ samples (p-value: 0.3662, Kruskal-Wallis One Way ANOVA).

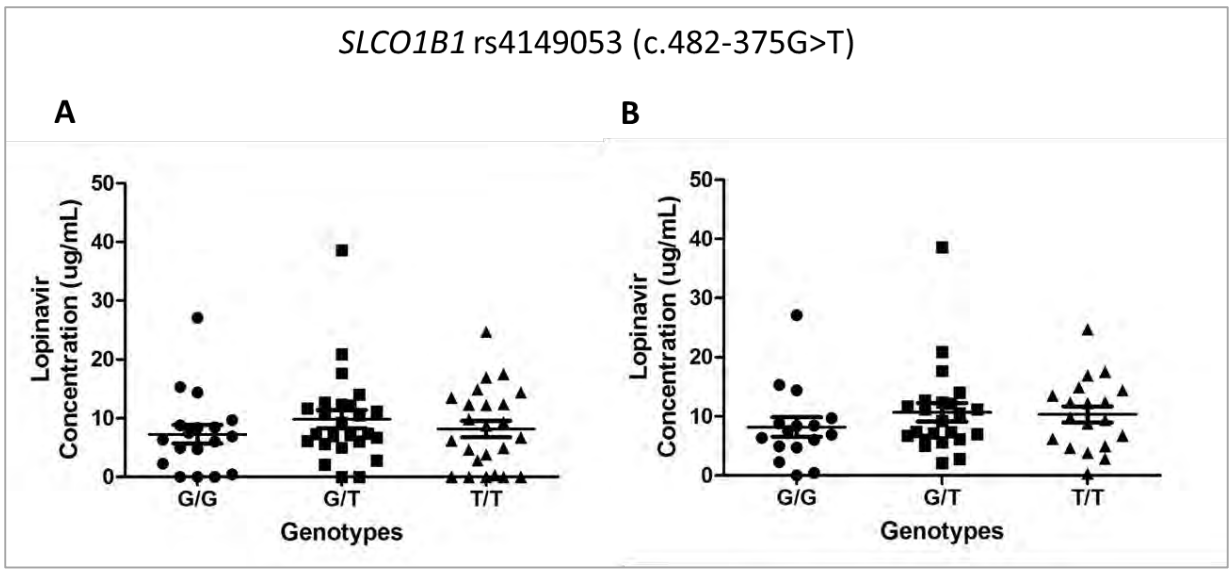

Figure 6.22 *SLCO1B1* rs4149053 (c.482-375G>T) genotypes vs lopinavir levels. A: with BLQ samples (p-value: 0.51, Kruskal-Wallis One Way ANOVA); B: without BLQ samples (p-value: 0.3746, Kruskal-Wallis One Way ANOVA).

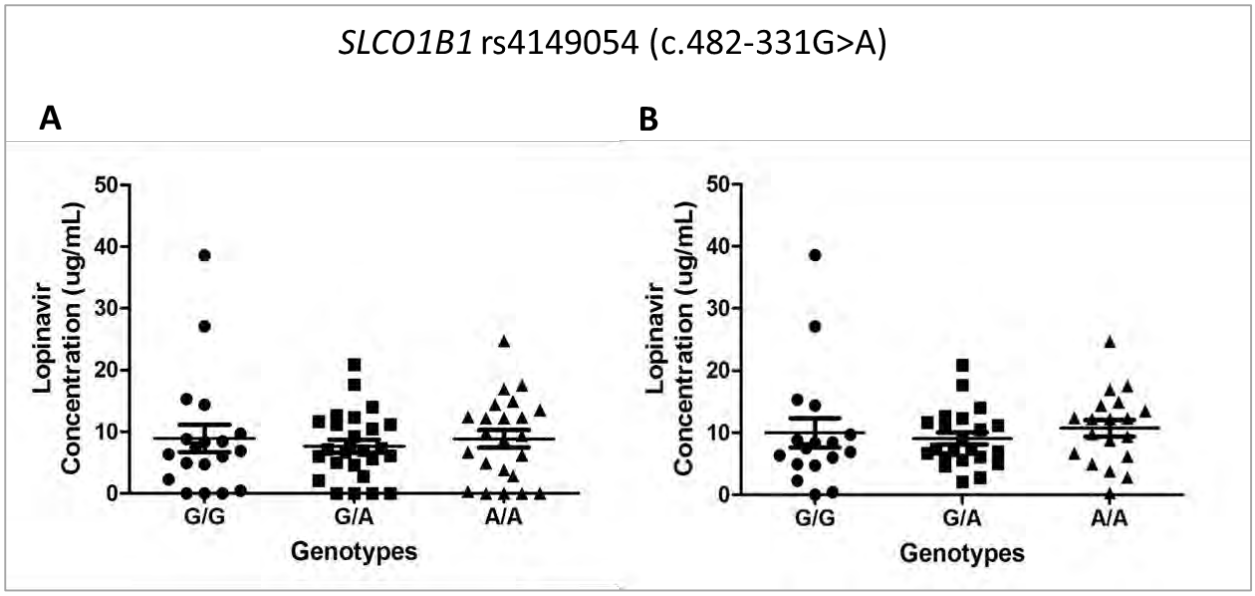

Figure 6.23 *SLCO1B1* rs4149054 (c.482-331G>A) genotypes vs lopinavir levels. A: with BLQ samples (p-value: 0.7712, Kruskal-Wallis One Way ANOVA); B: without BLQ samples (p-value: 0.4217, Kruskal-Wallis One Way ANOVA).

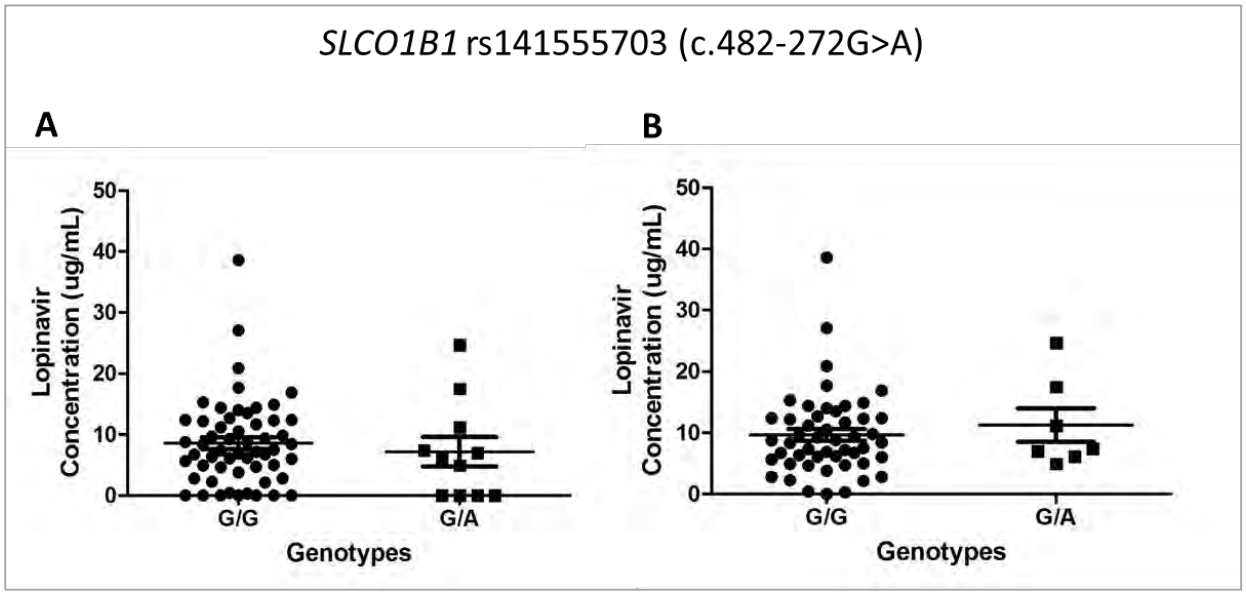

Figure 6.24 *SLCO1B1* rs141555703 (c.482-272G>A) genotypes vs lopinavir levels. A: with BLQ samples (p-value: 0.3131, Kruskal-Wallis One Way ANOVA); B: without BLQ samples (p-value: 0.6528, Kruskal-Wallis One Way ANOVA).

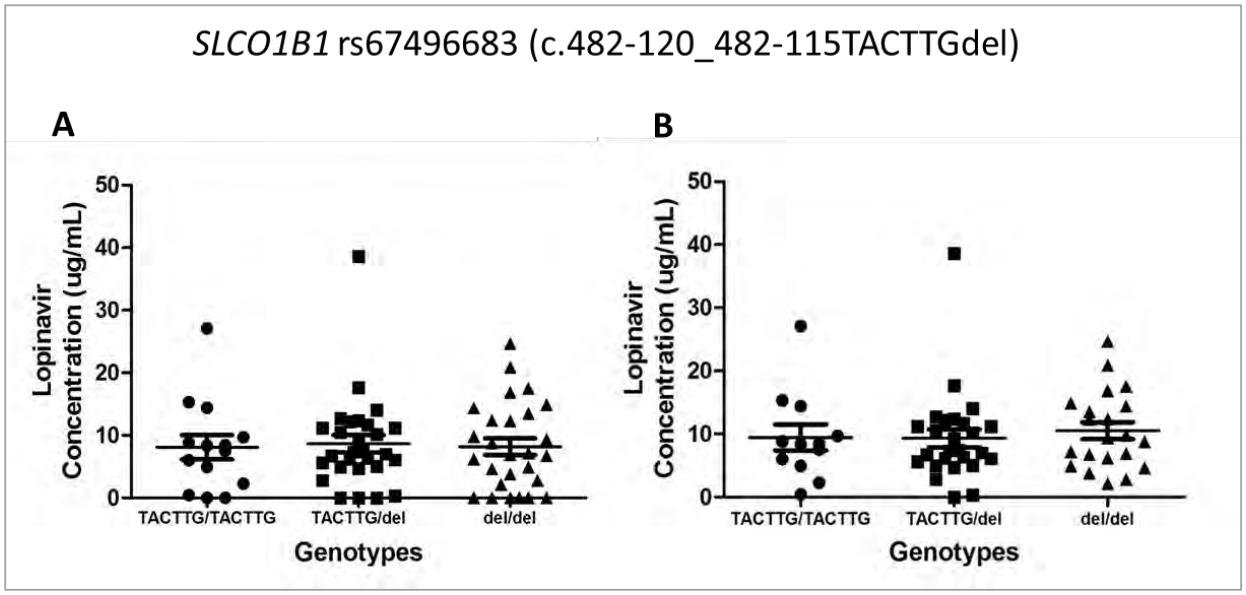

Figure 6.25 *SLCO1B1* rs67496683 (c.482-120_482-115TACTTGdel) genotypes vs lopinavir levels. A: with BLQ samples (p-value: 0.9469, Kruskal-Wallis One Way ANOVA); B: without BLQ samples (p-value: 0.6828, Kruskal-Wallis One Way ANOVA).

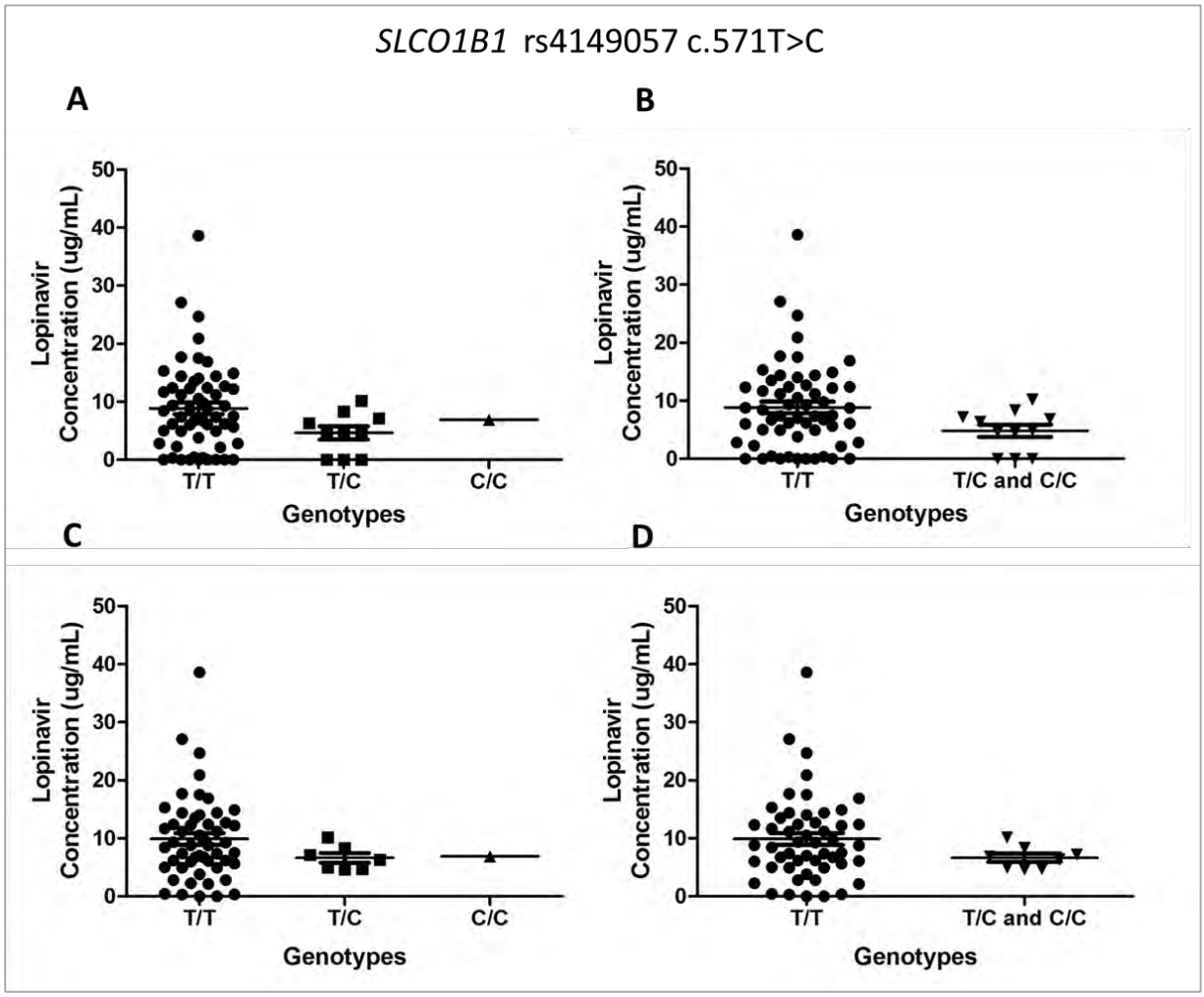

Figure 6.26 *SLCO1B1* rs4149057 (c.571T>C) genotypes vs lopinavir levels. A: with BLQ samples (p-value: 0.1415, Kruskal-Wallis One Way ANOVA); B: Dominant model with BLQ samples (p-value: 0.0569, Mann-Whitney test); C: without BLQ samples (p-value: 0.3752, Kruskal-Wallis One Way ANOVA); D: Dominant model without BLQ samples (p-value: 0.1673, Mann-Whitney test).

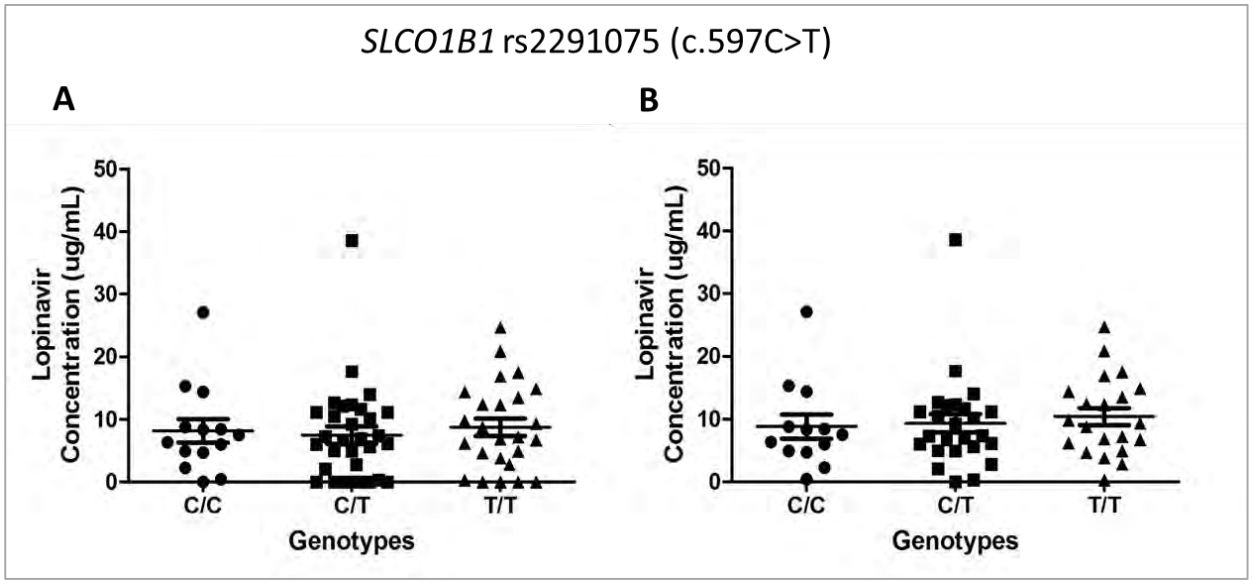

Figure 6.27 *SLCO1B1* rs2291075 (c.597C>T) genotypes vs lopinavir levels. A: with BLQ samples (p-value: 0.6863, Kruskal-Wallis One Way ANOVA); B: without BLQ samples (p-value: 0.5891, Kruskal-Wallis One Way ANOVA).

Appendix C: Genotyping Results

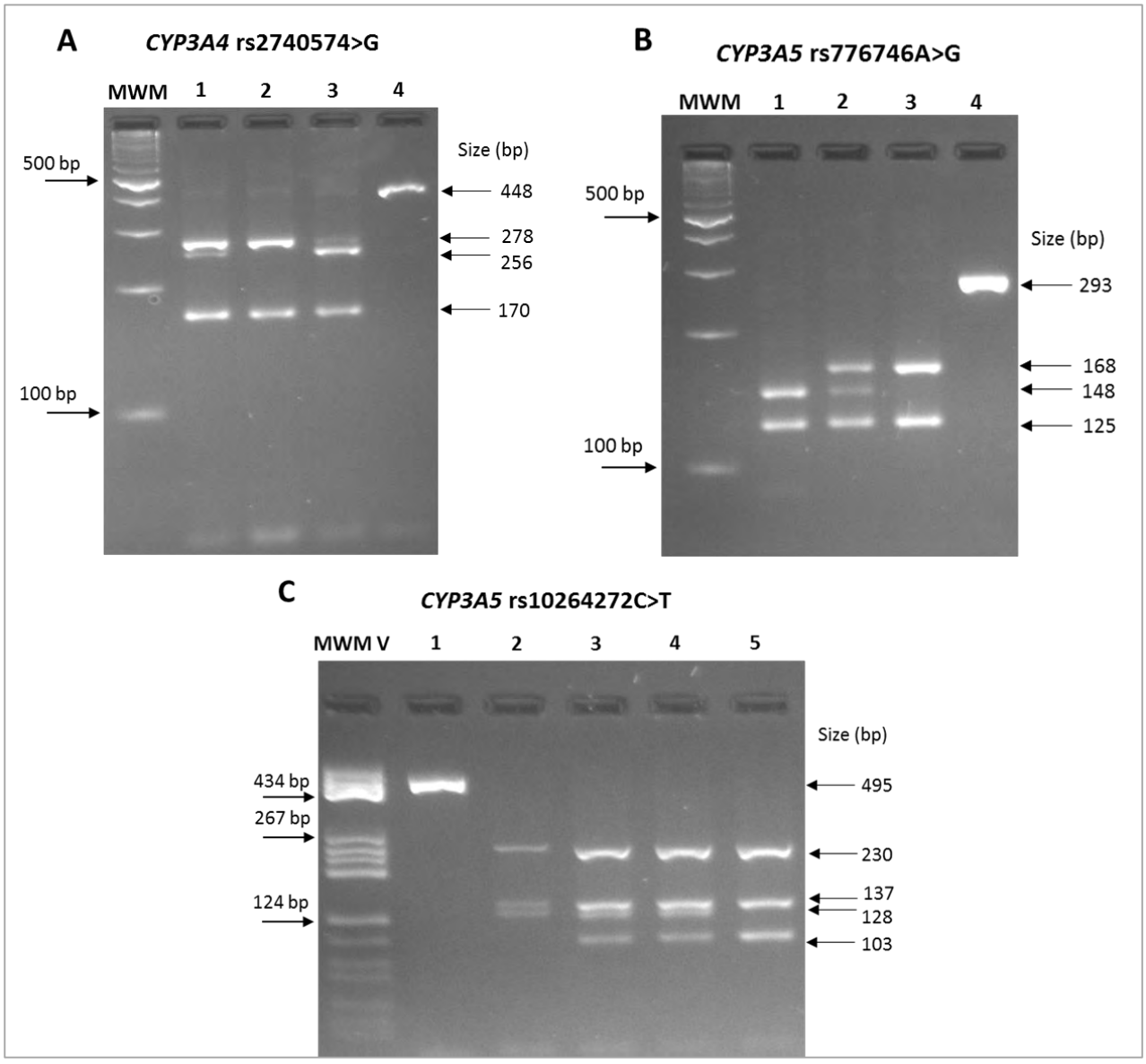

Figure 6.28 PCR-RFLP for genotyping of *CYP3A4* rs2740574A>G, *CYP3A5* rs776746A>G and *CYP3A5* rs10264272C>T. A: *CYP3A4* rs2740574A>G - Generuler 500 bp plus DNA ladder was used (MWM), lanes 1 to 3 represent samples with different genotypes (A/G; G/G; and A/A respectively); and lane 4 contained undigested PCR product. **B:** *CYP3A5* rs776746A>G - Lanes 1 to 3 represent samples with A/A, A/G and G/G genotypes respectively, lane 4 contained undigested PCR product. **C:** *CYP3A5* rs10264272C>T – Molecular Weight Marker V (MWM V) was used; lane 1 contains undigested PCR product; and in lanes 2 to 5 are samples with genotypes C/C, C/T, C/T and T/T.

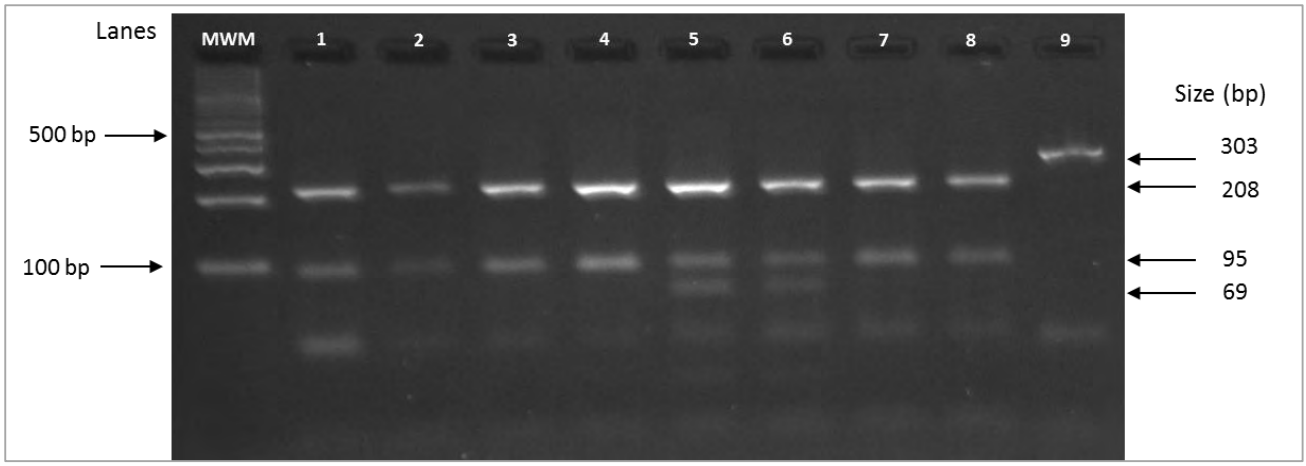

Figure 6.29 PCR-RFLP for *ABCC2* rs2273697 (c.1249G>A). A 500 bp plus DNA ladder was used (MWM); in lanes 1 to 4, 7 and 8 are samples with G/G genotype; and in lane 5 and 6 are samples with A/G genotype. Lane 9 contained undigested PCR product.

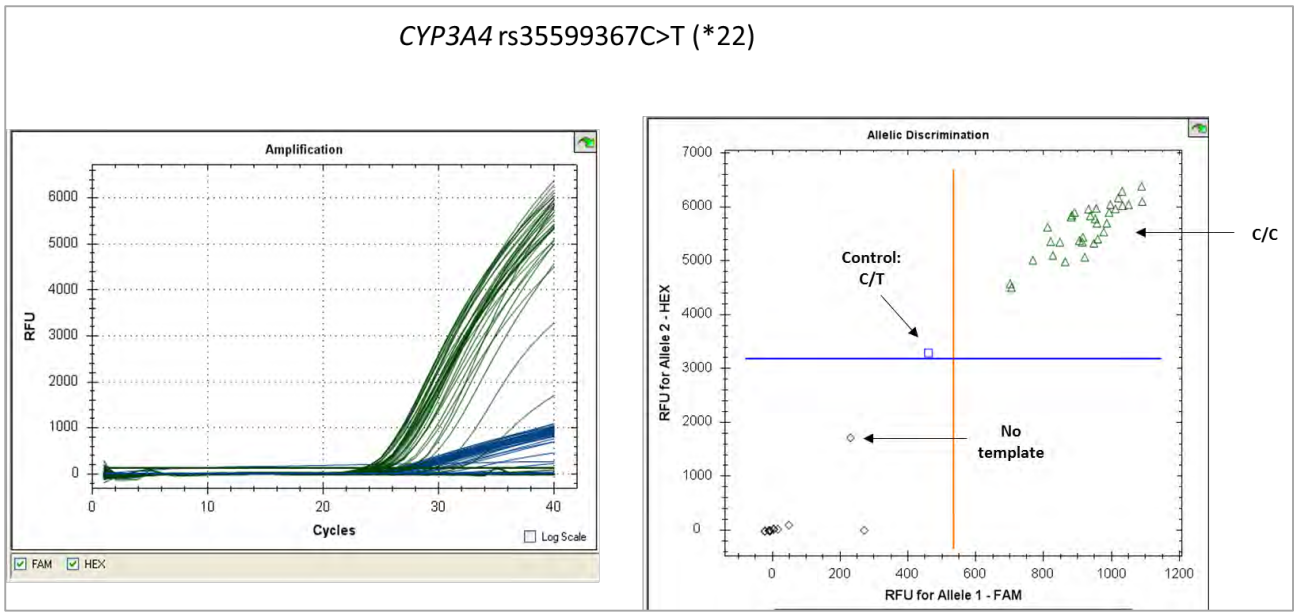

Figure 6.30 TaqMan assay results for *CYP3A4* rs35599367C>T. Blue represents the 6-FAM label for the T allele; Green represents the VIC (HEX) label for the C allele. A heterozygote sample from a separate population was included as a positive control.

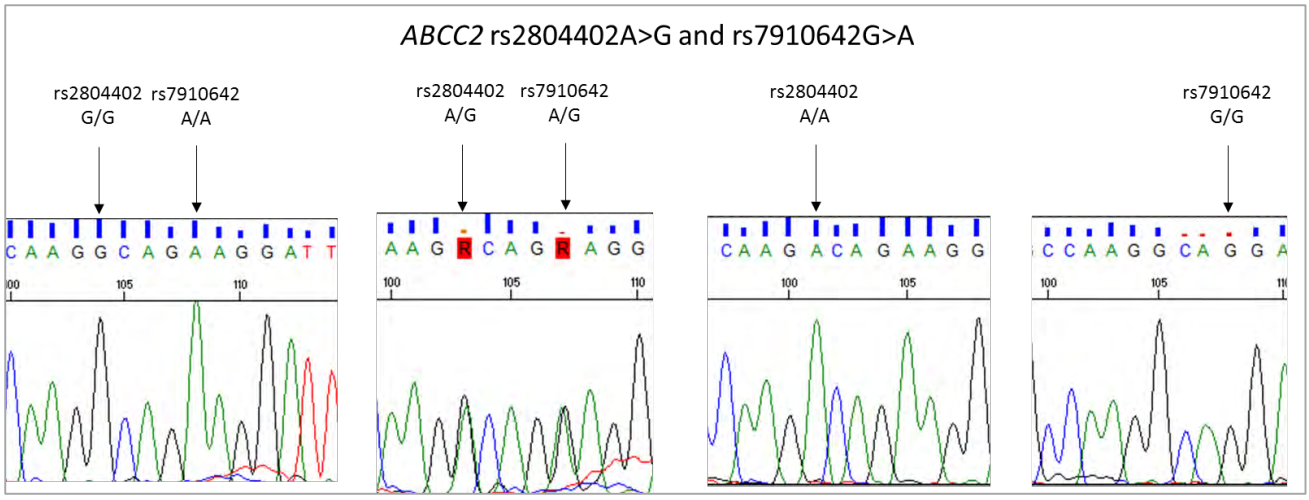

Figure 6.31 Sequencing results for *ABCC2* rs2804402 (c-1019A>G) and rs7910642 (c.-1023G>A).

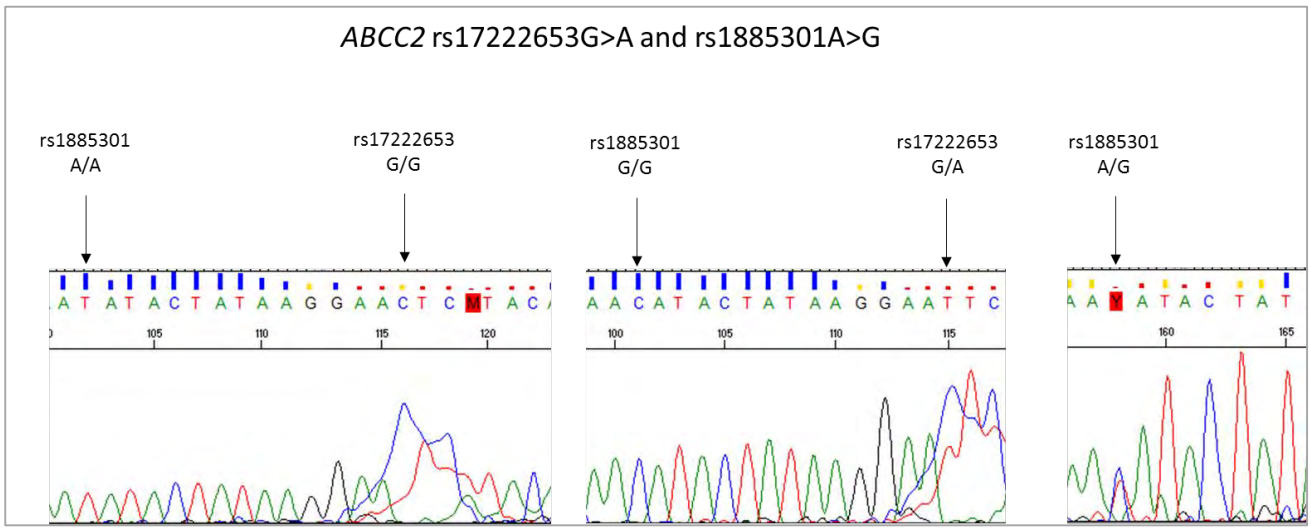

Figure 6.32 Sequencing results for *ABCC2* rs17222653 (c-1563G>A) and rs1885301 (c.-1549A>G). The inverted trace diagram is shown.

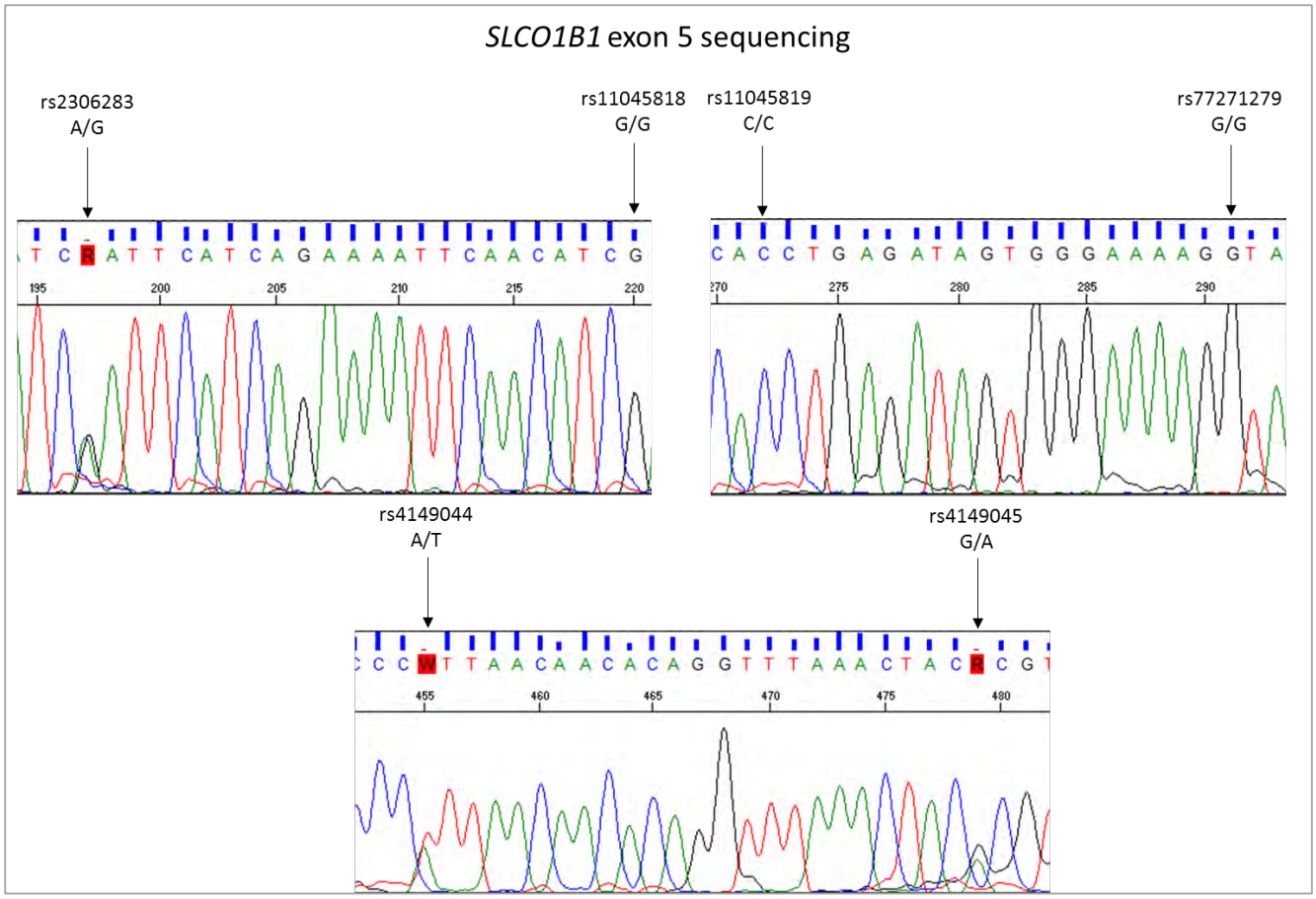

Figure 6.33 Sample of sequencing results for *SLCO1B1* exon 5 showing a few SNPs which were identified.

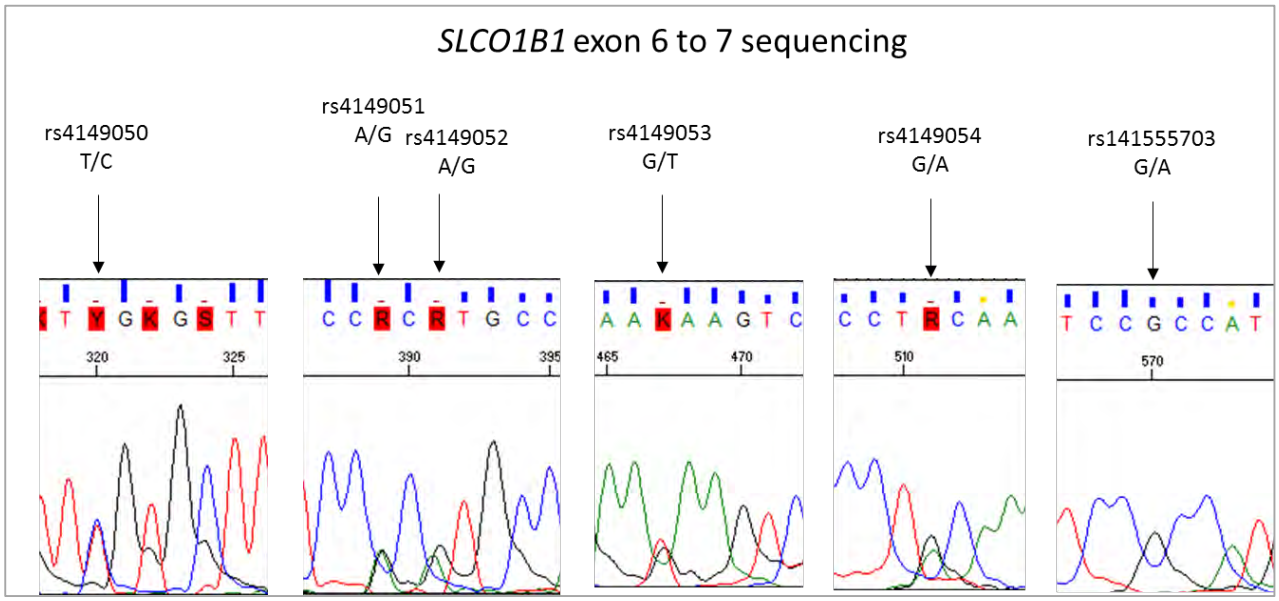

Figure 6.34 Sample of sequencing results for *SLCO1B1* exon 6 to 7 showing a few SNPs which were identified.

Appendix D: Ethics Approval Letter

UNIVERSITY OF CAPE TOWN

Faculty of Health Sciences
Faculty of Health Sciences Human Research Ethics Committee
Room E52-24 Groote Schuur Hospital Old Main Building
Observatory 7925
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e-mail: sumayah.ariefdien@uct.ac.za
www.health.uct.ac.za/research/humanethics/forms

22 July 2013

HREC REF: 439/2013

A/Prof C Dandara
Human Genetics
Falmouth building
FHS

Dear A/Prof Dandara

PROJECT TITLE: THE PHARMACOGENETICS OF LOPINAVIR (A SUB-PROTOCOL 103-2009 TO: THE ROLE OF PHARMACOGENETICS ON THE RESPONSE TO TREATMENT USING ANTIRETROVIRALDRUGS

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee for review.

It is a pleasure to inform you that the HREC has **formally approved** the above mentioned study.

Approval is granted for one year till the 28 July 2014.

Please submit a progress form, using the standardised Annual Report Form, if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.

Please note that the on-going ethical conduct of the study remains the responsibility of the principal investigator.

Please quote the REC. REF in all your correspondence.

Yours sincerely

signature removed

pp

PROFESSOR M BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS

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