

**Genetic Changes in HIV-1 *Gag* and *Pol* genes associated
with Protease Inhibitor-Based Therapy Failure in
Paediatric Patients**

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Abstract

HIV-1 infection in paediatric patients is a major public health issue with an estimated 740 000 children living with HIV-1 globally, and South Africa accounting for almost half of these infections. In South Africa, children <3 years of age are initiated on a protease inhibitor (PI)-based regimen on diagnosis. For those who fail therapy, there are limited second-line treatment options available. While virological failure is often associated with HIV-1 drug resistance, a number of studies have shown infrequent selection of *protease* mutations in adults and children. The protease enzyme recognises and cleaves the *gag* substrate at specific sites to release structural components and enzymes essential for viral replication. Thus, changes occurring at the *gag* cleavage sites (CS) represent an alternate route of PI resistance. We therefore investigated the genotypic changes in the *gag* substrate in paediatric patients failing PI-based therapy to determine if changes in *gag* could contribute to PI failure. The phenotypic effects of these changes on PI susceptibility both in the presence and absence of PI mutations were also examined. In addition, the impact of key *protease* and *gag* mutations were assessed by site-directed mutagenesis on phenotypic resistance. Furthermore, cross-resistance to other PIs were tested to identify PIs suitable for subsequent use.

Plasma samples from 20 HIV-1 subtype C-infected children <2 years of age at pre-treatment and failing PI-based therapy were genotyped to identify changes in *gag* and *protease* associated with regimen failure. Fourteen patients received RTV as a single PI of which 7 were later switched to LPV/r. Six patients received LPV/r only. Following laboratory confirmed virologic failure, genotyping was performed on the first available sample (mean time to failure of 285 days). Major PI mutations (M46I, I54V and V82A) were found in 8 (40%) patients, all of whom had received RTV as a single PI at some

point. *Gag* CS mutations were observed in 5 of these 8 patients (at codons 374, 378, 428, 431 and 451). In addition, amino acid changes at *gag* non-CS were noted, some of which were predicted to be under HLA/KIR immune-mediated pressure and/or drug selection pressure. These changes in *gag* following PI therapy failure were further explored by phenotypic analysis to assess whether they contributed to resistance.

The *gag-protease* genes from pre-treatment and post-failure specimens from all 20 patients were cloned into an HIV-1 expression vector and tested for their PI susceptibility using an *in vitro* single-cycle phenotypic assay. Phenotypic resistance to LPV and RTV (up to >100-fold) was seen in 7 of the 8 patients with major PI mutations. Of the 12 patients with genotypically wild-type *protease*, 3 showed reduced susceptibility to RTV, 1 of which also had reduced susceptibility to LPV. These patients had changes in *gag* CS (L449P and P453L) or non-CS (codons 62 and 69 also under positive selection pressure) causing up to a 7-fold increase in resistance. Investigation of cross-resistance to other PIs, revealed that of the 8 patients with PI major mutations, 6 showed reduced susceptibility to atazanavir (ATV), 4 to amprenavir (APV), 1 to darunavir (DRV) and 4 to saquinavir (SQV). In addition, 3 patients with genotypically wild-type *protease* who showed reduced susceptibility to ATV (up to 11-fold) and in one case also to DRV (5-fold), had *gag* CS (S373A, L449P and P453L) and/or *gag* non-CS mutations (T62A and K69R).

To investigate the effects of individual *gag* and *protease* mutations on PI susceptibility, single and multiple mutants were created in a subtype C reference vector by site-directed mutagenesis and tested in the single-cycle phenotypic assay. Analysis of single and multiple *gag* and *protease* site-directed mutants showed that individual changes were not sufficient to cause resistance in this assay system. However, in combination with major *protease* mutations, *gag* mutations were shown to contribute to

PI resistance by reducing susceptibility to PIs by up to 4-fold. This suggests a minimal but significant impact and is consistent with findings using clinical samples.

Overall, these results show that RTV therapy selects for genotypic and phenotypic resistance that caused cross-resistance to other PIs. This rarely occurred following LPV/r-based therapy which is the standard first-line treatment for infants. PI resistance was predominantly explained by the presence of major *protease* mutations. In some patients, *gag* mutations reduced PI susceptibility but their effect was more pronounced when they were present in combination with major *protease* mutations. Thus, incorporating *gag* in drug resistance algorithms could improve interpretation of genotyping data and drug resistance prediction. Furthermore, these results support the use of genotypic testing of children failing first-line treatment to guide future PI therapy options. However, eight children failed PI therapy in the absence of *gag* and *protease* mutations, highlighting the importance of further investigation into the reasons for paediatric failure, including reliable measures of adherence.

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The following articles and conference presentations arose from work presented in this thesis.

Publications

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List of Abbreviations

3TC	Lamivudine
ABC	Abacavir
AIDS	Acquired Immunodeficiency Syndrome
APOBEC3G	Apolipoprotein G
APV	Amprenavir
ART	Antiretroviral therapy
ARV	Antiretroviral
ATV	Atazanavir
AZT	Zidovudine
BCG	Bacille Calmette-Guérin
CA	Capsid
CCR5	Chemokine receptor 5
cDNA	Copy Deoxy-ribonucleic Acid
CS	Cleavage Site
CXCR4	Chemokine receptor 4
CypA	Cyclophilin A
D4T	Stavudine
DDI	Didanosine
dH ₂ O	Distilled water
DMEM	Dulbecco's Modified Eagle's Medium
dN/dS	The ratio of divergence at nonsynonymous and synonymous sites

DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
DTT	Dithiothreitol
DRV	Darunavir
DTG	Dolutegravir
<i>E.coli</i>	<i>Escherichia coli</i>
EFV	Efavirenz
Env	Envelope
ETR	Etravirine
EVG	Elvitegravir
FBS	Fetal Bovine Serum
FC	Fold Change
FDA	Food and Drug Administration
FEL	Fixed Effects Likelihood
FPV	Fosamprenavir
FTC	Emtricitabine
gp120	Glycoprotein 120
gp41	Glycoprotein 41
HAART	Highly Active Antiretroviral Therapy
HIV-1	Human Immunodeficiency Virus Type – 1
HIV-2	Human Immunodeficiency Virus Type – 2
HIVdb	HIV Drug Resistance Database
HLA	Human Leukocyte Antigen

IC ₅₀	Concentration of inhibitor that shows 50% inhibition
IDV	Indinavir
IN	Integrase
KIR	Killer Immunoglobulin-like Receptor
LPV	Lopinavir
LPV/r	Ritonavir-boosted Lopinavir
MA	Matrix
MEGA	Molecular Evolutionary Genetics Analysis
MHC	Major Histocompatibility Complex
MSW	Mutation Selection Window
MVC	Maraviroc
NC	Nucleocapsid
NFV	Nelfinavir
NK	Natural Killer
NNRTI	Non-nucleoside Reverse Transcriptase Inhibitor
NRTI	Nucleoside Reverse Transcriptase Inhibitor
NVP	Nevirapine
PCR	Polymerase Chain Reaction
PEI	Polyethylenimine
PI	Protease Inhibitor
PMTCT	Prevention of Mother-to-Child Transmission
PR	Protease
RAL	Raltegravir

RNA	Ribonucleic Acid
RNase H	Ribonuclease H
RT	Reverse Transcriptase
RTV	Ritonavir
RVP	Rilpivirine
SDM	Site Directed Mutagenesis
sdNVP	Single-Dose Nevirapine
SIV	Simian Immunodeficiency Virus
SQV	Saquinavir
T-20	Enfuvirtide
TB	Tuberculosis
TDF	Tenofovir Disoproxil Fumarate
TFP	Transframe Protein
TRIM5 α	Tripartite Interaction Motif 5 α
TPV	Tipranavir
UV	Ultraviolet
WHO	World Health Organisation

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Chapter 1

Introduction

1.1. The Epidemiology of HIV

In 1981, acquired immunodeficiency syndrome (AIDS) was first identified in a group of homosexual men presenting with symptoms of rare opportunistic infections (Gottlieb et al., 1981). The causative agent of AIDS was discovered in 1983 to be the human immunodeficiency virus (HIV) (Barre-Sinoussi et al., 1983, Gallo et al., 1984). HIV is a member of the family of Retroviruses, in the genus Lentiviruses and was initially known as human T-lymphotropic virus type III (HTLV-III) (Sharp, 2011).

There are two types of human immunodeficiency virus (HIV) which cause infection in humans i.e. HIV type-1 (HIV-1) and HIV type-2 (HIV-2) (Cohen et al., 2008). HIV-1 is believed to have originated from the cross-species transmission of simian immunodeficiency virus (SIV) infecting chimpanzees to humans in West-central Africa and HIV-2 from a cross-species transmission of the Sooty mangabey virus (Lihana RW, 2012, Cohen et al., 2008). HIV-1 and HIV-2 differ by up to 40% at the sequence level (Cohen et al., 2008). Globally, HIV-1 is more prevalent and is associated with a faster progression to immune deficiency (Cohen et al., 2008). Both viruses are transmitted through contaminated blood, sexual intercourse and from an infected mother to her baby (Cohen et al., 2008).

There was an estimated 35 million individuals living with HIV/AIDS globally by the end of 2013 (UNAIDS, 2014). Regionally, Africa accounts for the greatest burden of HIV infections (Figure 1.1.), with an estimated 5.6 million infected adults and 360 000 infected children, in South Africa (UNAIDS, 2013).

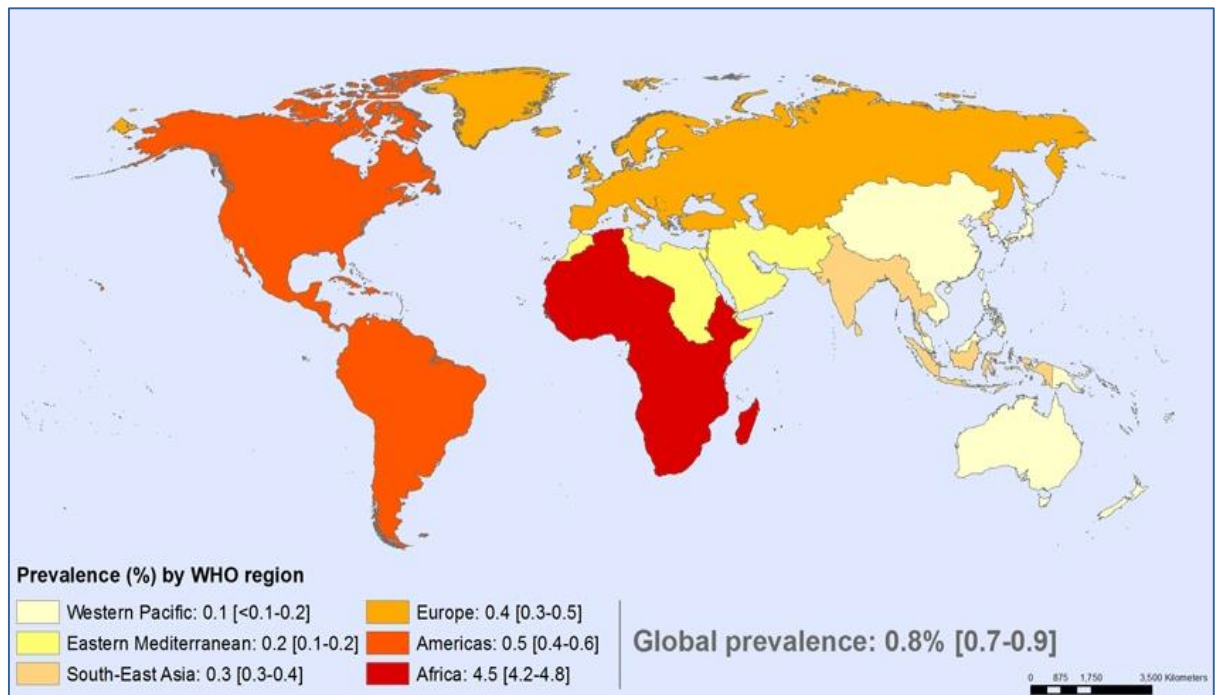


Figure 1.1. The global prevalence of HIV-1

An estimated 0.8% of adults aged between 15–49 years worldwide are living with HIV, although the burden of the epidemic continues to vary considerably between countries and regions. South Africa remains most severely affected with a prevalence of 17.9%. Picture taken from the World Health Organisation (WHO) Map production: Health Statistics and Information Systems 2012, WHO (2014)

1.2. The Classification of HIV-1

HIV-1 is characterised by extensive genetic diversity and comprises of four distinct lineages, groups M (Major), N (non-M and non-O), O (Outlier) and P (pending the identification of further human cases), of which group M is responsible for the HIV-1 pandemic (Sharp, 2011). Group M can be further subdivided into nine different subtypes, namely A-D, F-H, J and K, which differ in their protein sequence of the major structural proteins Gag, Pol and Env by 15%, 10% and 24%, respectively (Sampathkumar et al., 2012, Lihana RW, 2012). The HIV-1 subtypes show distinct regional prevalence of variants around the world (Santos and Soares, 2010) with subtype C predominating in sub-Saharan Africa and accounting for 48% of HIV-1 infections worldwide (Lihana RW, 2012). Subtype A is prevalent in Central Africa, Iran and Eastern Europe, and in Central Asia while subtype B predominates in developed countries, such as the United States,

countries of Western Europe, Japan and Australia (Santos and Soares, 2010). The remaining six HIV-1 subtypes (D, F, G, H, J and K) together represent a small percentage of infections (Hemelaar et al., 2006). Circulating recombinant forms (CRFs) are recombinant viruses from different subtypes of HIV-1 that became established within the pandemic, with A/G recombinant strains predominating in West Africa and B/C recombinant strains predominating in China (Cohen et al., 2008).

1.3. The HIV-1 Genome

The HIV-1 genome is 9.8 kb in size and consists of nine genes which are classified into three groups: structural genes, regulatory genes, and accessory genes (Costin, 2007) (Figure 1.2). The group-specific antigen (Gag) and gag-pol (Polymerase) polyproteins from the structural group are cleaved by the viral protease to release matrix (MA), capsid (CA), p2, nucleocapsid (NC), p1 and p6, protease (PR), reverse transcriptase (RT which contains RNase H) and integrase (IN) (Peterlin and Trono, 2003). The structural protein envelope (Env) is cleaved by cellular proteins into surface glycoprotein-120 (gp120) and transmembrane glycoprotein-41 (gp41). The viral regulatory proteins comprise of transcriptional transactivator (Tat) and regulator of virion gene expression (Rev) which are localised in the nucleus. Tat is the main transcriptional regulator of the long term repeat (LTR) and acts by binding to the transactivation response (TAR) element and activating transcription and elongation from the 5' LTR promoter (Rhee et al., 2003). The main nuclear-export protein, Rev, is responsible for regulating early and late viral gene expression (Peterlin and Trono, 2003). The accessory proteins consist of the viral-infectivity factor (Vif), viral protein r (Vpr), viral protein u (Vpu) and negative effector (Nef). Vif promotes the infectivity of viruses, while Vpr is involved in the nuclear import of pre-integration complexes, cell growth arrest and induction of cellular differentiation

(Rhee et al., 2003). Vpu which is unique to HIV-1 is responsible for the degradation of CD4 in the endoplasmic reticulum and the release of virions from infected cells (Rhee et al., 2003). The Nef protein is the most immunogenic of the accessory proteins and is responsible for downregulating CD4 (the primary viral receptor) and the major histocompatibility (MHC) class I molecules (Rhee et al., 2003).

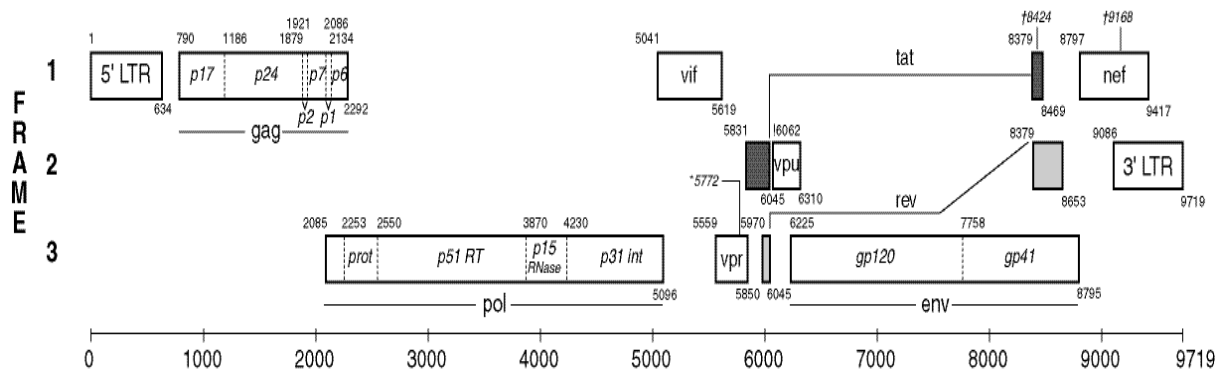


Figure 1.2. Landmarks of the HIV-1 genome

The HIV-1 genome consists of three main genes i.e. *gag*, *pol* and *env*. These genes code for structural proteins and essential enzymes. The HIV genome also encodes regulatory proteins (tat and rev) and accessory proteins (vif, vpr and nef). Picture taken from Rhee et al. (2003).

1.4. The Structure of HIV-1

The HIV-1 virion is made up of a viral envelope comprising of a surface envelope protein (gp120, SU) and a transmembrane envelope protein (gp41, TM) (Figure 1.3) embedded in a lipid bilayer membrane. These two proteins are responsible for attachment of the virus to the host cell. The matrix (p17/MA) is associated with the inner compartment of the membrane while the capsid (p24/CA) forms a shell around the viral RNA/NC complex (Freed, 1998). Contained within the capsid is the virus's genetic material which is made up of two identical strands of single stranded RNA.

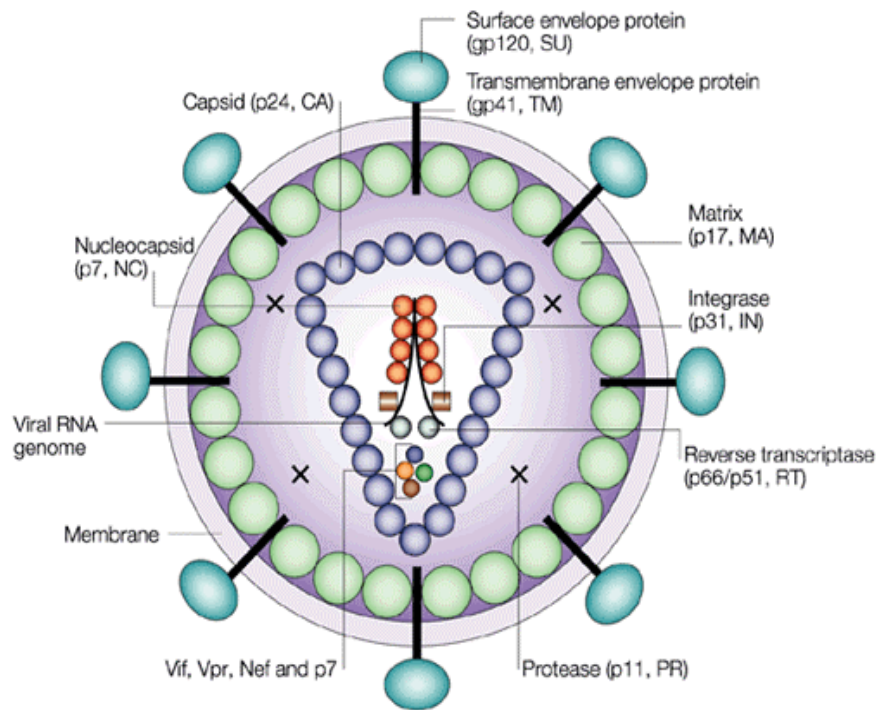


Figure 1.3. An illustration of the HIV-1 virion

The location of the gag proteins, envelope glycoproteins and the pol enzymes are shown. Picture taken and modified from Robinson (2002).

1.5. The Pathogenesis of HIV-1 Infection

Infection with HIV-1 is characterised by 3 stages which consist of initial infection, latency and clinical AIDS (Figure 1.4). During the acute stage of infection, the virus proliferates in the patient's periphery, until approximately after the first 4 weeks. HIV-1 specific antibody-mediated immune response begins (Cohen et al., 2008). During the latency phase the CD4 T cell count starts to decline and the viral load steadily increases over time (Costin, 2007). The onset of AIDS is defined as a CD4 T cell count of <200 cells/mm³ and/or the presence of an opportunistic infection (Costin, 2007).

After HIV-1 transmission, the interaction between the virus and the host determines the natural history of infection in that specific individual (Cohen et al., 2008). A small proportion of HIV-1 infected patients (known as long term non-progressors (LTNPs)) can remain asymptomatic for a prolonged period in the absence of antiretroviral therapy, with

a high CD4 cell count and detectable viral load (Sabin and Lundgren, 2013). Elite controllers are a subset of LTNP and can further suppress viral load levels to remain undetectable in the absence of antiretroviral therapy (Sabin and Lundgren, 2013). Studies on these unique individuals are ongoing to understand how the host immune response is able to control HIV infection providing insight for the development of vaccines and new antiretroviral agents (Sabin and Lundgren, 2013).

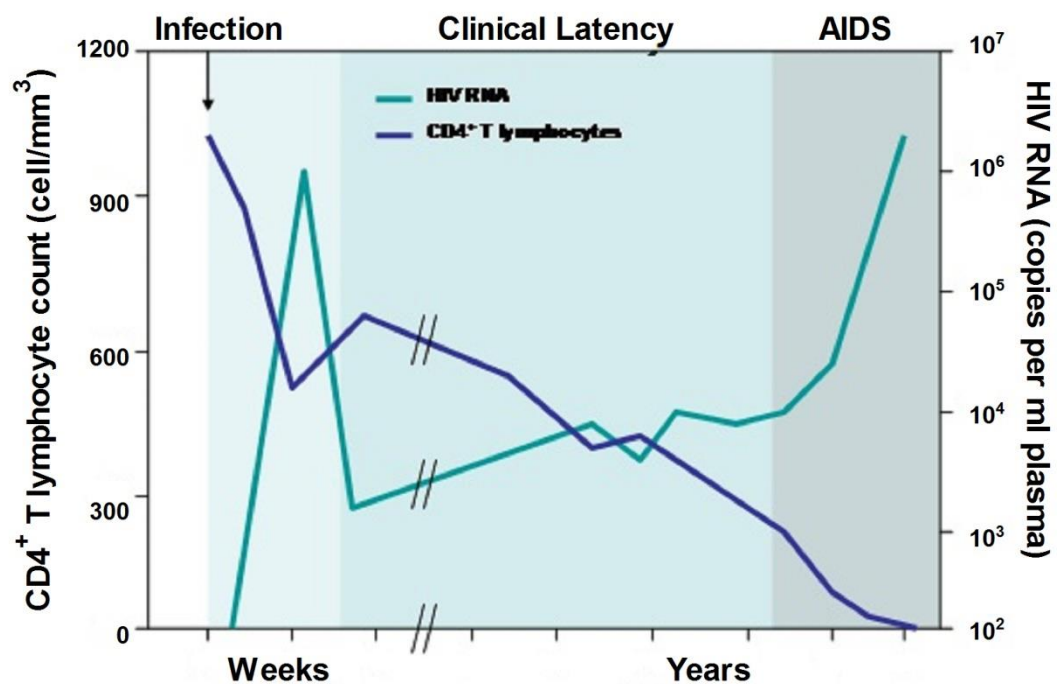


Figure 1.4. Time course of HIV-1 infection

The three stages of HIV-1 infection are shown i.e. initial infection, latency phase and clinical AIDS. The initial infection is characterised by a rapid increase in viral load and decline in CD4 T cell count till four weeks. During the clinical latency phase, the viral load remains low and stable. Over time the clinical AIDS phase develops and is characterised by a continuous decline of CD4 T cells and increase in viral load. Picture modified from Costin (2007).

1.6. The HIV-1 Life Cycle

The HIV-1 life cycle comprises a series of steps to allow the virus to enter the host cell, efficiently replicate its DNA and release mature infectious virions to infect more target cells (Figure 1.5) (Arts and Hazuda, 2012, Pomerantz and Horn, 2003). Virus entry is mediated by binding of the gp120 envelope protein to a CD4 receptor and co-receptor (either CXCR4 or CCR5) found on the surface of immune cells. This results in gp41

inducing a membrane fusion reaction between the lipid bilayer of the virion and the plasma membrane of the host cell and consequently causing the release of the viral core into the cytoplasm (Freed, 1998). Once the virus contents enter the cell, the nucleocapsid undergoes a process of uncoating where its capsid is lost and through the process of reverse transcription, mediated by the reverse transcriptase (RT) enzyme, the two single stranded viral RNAs are converted to double stranded DNA (Campbell and Hope).

The viral DNA is transported into the nucleus where it is integrated with the host cell's DNA by the enzyme integrase (IN). When the cells become activated, the integrated DNA which is known as the provirus serves as a template for the synthesis of viral RNA using host cell's machinery (Freed, 1998). The Env glycoproteins, Gag and Gag-Pol polyproteins are expressed and transported to the plasma membrane where the Gag precursor deploys two copies of single stranded RNA and assembles close to the inner plasma membrane (Freed, 1998). As the immature viruses bud from the cell, the viral Env glycoproteins are incorporated into the virus membrane. After budding, the Gag and Gag-Pol polypeptide chains are cleaved by HIV-1 Protease into smaller proteins resulting in the formation of a mature infectious virus which can now initiate a new round of infection (Arts and Hazuda, 2012). HIV-1 protease is initially processed as part of the Gag-Pol polyprotein, however due to the regulated cleavage reactions it undergoes a process called auto-processing whereby it is released (Lee et al., 2012).

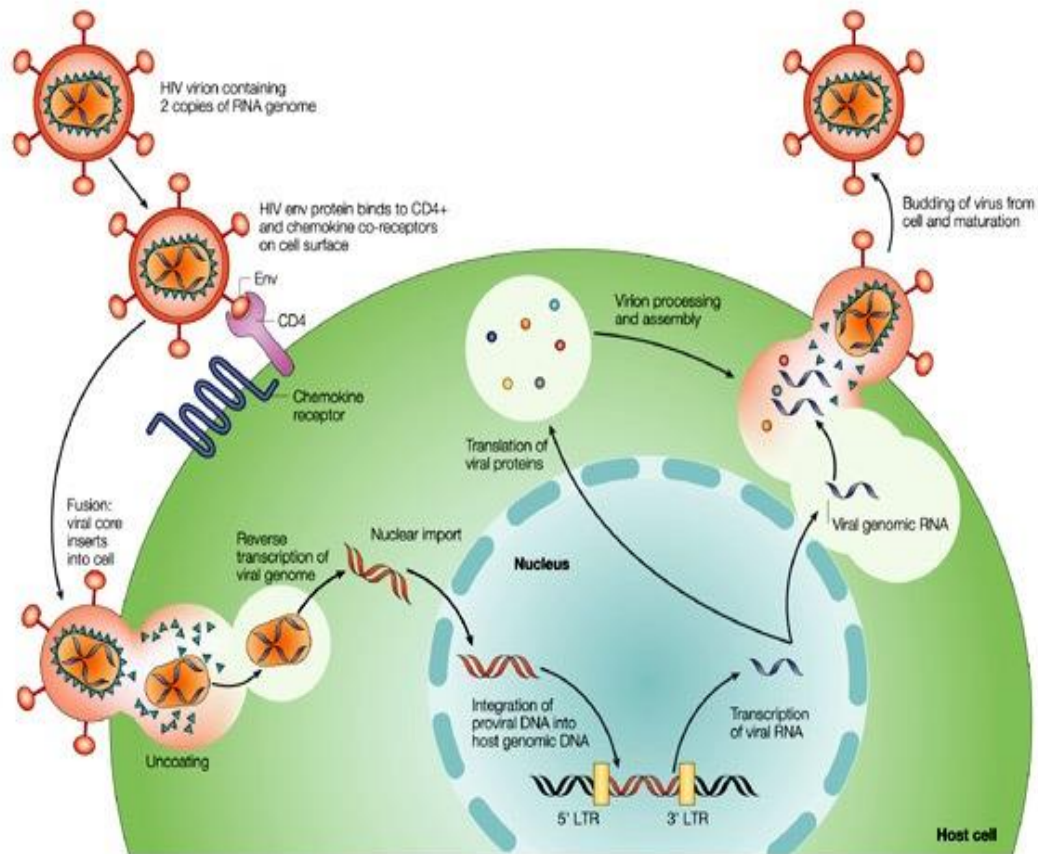


Figure 1.5. The life cycle of HIV-1

The different stages of the viral life cycle are shown. After the virus binds to the CD4 T cell, it enters the cell and undergoes uncoating to release its contents into the cell. The viral single-stranded DNA is reverse transcribed to double-stranded DNA which is integrated with the host cell DNA forming a provirus. The viral proteins are expressed and assembled close to the viral membrane. An immature virion buds off. The poly-proteins are cleaved to release a mature and infectious virus. Picture taken from Rambaut et al. (2004).

1.7. Intrinsic Factors Influencing HIV-1 Evolution of *gag* and *protease*

HIV is subjected to selection pressures from the host immune system which results in viral evolutionary measures to adapt to these pressures (Sampathkumar et al., 2012). The rapid evolution of HIV-1 is primarily as a result of the highly error prone RT enzyme, resulting in extensive genetic variability. The RT enzyme lacks a 3' – 5' proof-reading function with rates of misincorporation estimated to be 1 in 6900 and 1 in 5900 nucleotides on the RNA and DNA templates, respectively (Sampathkumar et al., 2012). This results in the generation of multiple variants or quasispecies, some of which render the virus resistant to ARVs or afford an evolutionary advantage. In addition to

antiretroviral drug pressure, cell-mediated immune responses can exert significant pressure on the virus thereby selecting for changes in the viral genome.

1.7.1. Cyclophilin A

Cyclophilin A (CypA) is a host factor responsible for regulating protein folding and trafficking (Nigro et al., 2013). It is recruited by the Gag polyprotein during infection, and is essential for viral infectivity (Lama and Planelles, 2007). Many single nucleotide polymorphisms (SNPs) have been identified in the CypA gene (*PP1A*). One SNP (1650A/G) was found to be associated with lower *ex vivo* virus replication in cells derived from *PP1A* homozygous individuals (AA), and slower disease progression *in vivo* (Bleiber et al., 2005).

1.7.2. Tripartite Interaction Motif 5 α (TRIM5 α)

TRIM5 α is an intrinsic immune factor important for the innate immune defence in humans. It is involved in the early steps of viral replication and acts by restricting the replication of HIV-1 through its interaction with the viral capsid leading to unsuccessful infection (Restrepo et al., 2011). To avoid restriction by TRIM5 α , escape mutations may arise in the capsid region of the virus as proof of the existence of selective pressure on the virus (Lama and Planelles, 2007). Studies have shown that genetic changes in the TRIM5 α gene may influence susceptibility to HIV-1 infection as was shown with the R136Q SNP which occurs at a higher frequency in HIV-1 infected individuals (Lama and Planelles, 2007).

1.7.3. Apolipoprotein B (APOBEC3G)

APOBEC3G is a protein that plays a role in innate anti-viral immunity (Restrepo et al., 2011). During reverse transcription, APOBEC3G deaminates cytosine (C) to guanine

(G) in one DNA strand causing a G→A hypermutation in the complementary strand (Restrepo et al., 2011). Integration of the cDNA carrying hypermutations with the host DNA leads to the formation of aberrant viruses (Lama and Planelles, 2007). High expression of the APOBEC3G gene has been associated with reduced *in vitro* susceptibility to HIV-1 (Biasin et al., 2007). The HIV-1 Vif protein targets APOBEC3G for degradation thereby preventing its incorporation into the virions (Lama and Planelles, 2007). In the absence of HIV-1 Vif, APOBEC3G is incorporated into the HIV-1 particles.

1.7.4. The Human Leukocyte Antigen (HLA) System

The HLA system, which is located within the major histocompatibility complex (MHC) on chromosome 6, is highly polymorphic due to its exposure to millions of pathogens throughout the existence of the human species (Klein and Sato, 2000). The HLA class I and class II molecules function by recognising and presenting specific antigenic peptides to CD8 and CD4 T cells respectively to trigger an adaptive immune response. HLA class I consists of HLA-A, B and C which are expressed by all nucleated cells, while HLA class II consists of HLA-DP, DQ and DR, which are expressed on the surface of antigen presenting cells and memory cells (Bashirova et al., 2011). During HIV-1 infection, pressure from the HLA system leads to the selection of immune escape mutations most of which are well described in the Gag, Pol and Nef proteins (Sampathkumar et al., 2012, Bashirova et al., 2011, Brumme et al., 2009). Immune escape mutations disrupt binding of the viral peptide to HLA alleles and allow the virus to escape immune recognition (Brumme et al., 2009). The different HLA alleles have varied effects on the rates of HIV-1 progression with some alleles associated with a low viral load and slow progression to AIDS and some associated with a more rapid progression to AIDS (Bashirova et al., 2011).

1.7.5. Killer-cell Immunoglobulin-like Receptors (KIRs)

Natural killer (NK) cells are important cells of the innate immune system and function by interacting directly with infectious agents or infected cells (Jamil and Khakoo, 2011). This interaction is facilitated by the direct binding of the surface protein killer-cell immunoglobulin-like receptors (KIRs) to MHC class I molecules, expressed on all cell types (Jamil and Khakoo, 2011). The highly polymorphic KIR genes are located on chromosome 19, and function by activating or inhibiting NK cell activity (Sampathkumar et al., 2012). A minimum of 17 highly diverse KIR genes exist and their diversity is thought to be driven by selective pressure from exposure to pathogens (Parham et al., 2010). A study by Alter et al. (2011) identified polymorphisms in HIV-1 located in Vpu, Env, Gag and Nef that enhanced the binding of inhibitory KIRs to infected cells, resulting in inhibition of NK cell function and thus allowing HIV-1 to escape the immune response.

1.8. Antiretroviral Therapy

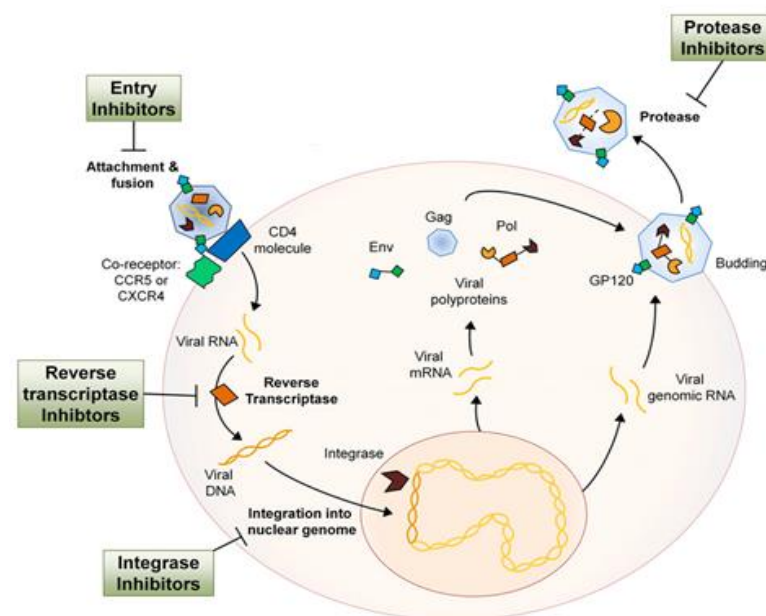


Figure 1.6. Antiretroviral drugs targeting specific steps in the viral life cycle

Entry inhibitors target entry of the virus into the infected thereby preventing attachment and fusion. Reverse transcription of the single stranded RNA is inhibited by reverse transcriptase inhibitors and integration of viral DNA with the host cell's DNA is inhibited by integrase inhibitors. Protease inhibitors prevent cleavage of the Gag and Gag-Pol polyproteins. The maturation of the immature virus is inhibited by maturation inhibitors. Picture taken from Smith et al. (2013).

In March 1987, the first drug (Zidovudine) was approved for antiretroviral treatment (ART) of HIV-1 by the U.S. Food and Drug Administration (FDA) (Santos and Soares, 2010). Since then, 26 drugs have been developed, targeting different stages of the virus life cycle, including viral entry, reverse transcription, integration, polyprotein cleavage and maturation (Figure 1.6). Newer ARVs, with improved pharmacological properties and potency, and a higher barrier to resistance, are continually being developed. To date, the US FDA have approved seven nucleotide reverse transcriptase inhibitors (NRTIs), five non-nucleotide reverse transcriptase inhibitors (NNRTIs), nine protease inhibitors (PIs), a fusion and an entry inhibitor, and three integrase inhibitors (INs) (Table 1.1) (National Institutes of Health, 2014).

Table 1.1. FDA approved antiretroviral drugs (National Institutes of Health, 2014)

Drug Class	Drug Name	FDA Approval Date
Nucleoside Reverse Transcriptase Inhibitors (NRTIs)		
	Abacavir (ABC)	December 17, 1998
	Didanosine (ddI)	October 9, 1991
	Emtricitabine (FTC)	July 2, 2003
	Lamivudine (3TC)	November 17, 1995
	Stavudine (d4T)	June 24, 1994
	Tenofovir (TDF)	October 26, 2001
	Zidovudine (AZT)	March 19, 1987
Non-nucleoside Reverse Transcriptase Inhiitors		
	Delavirdine (DLV)	April 4, 1997
	Efavirenz (EFV)	September 17, 1998
	Etravirine (ETR)	January 18, 2008
	Nevirapine (NVP)	June 21, 1996
	Rilpivirine (RPV)	May 20, 2011
Protease Inhibitors		
	Atazanavir (ATV)	June 20, 2003
	Darunavir (DRV)	June 23, 2006
	Fosamprenavir (FPV)	October 20, 2003
	Indinavir (IDV)	March 13, 1996
	Lopinavir (LPV)	September 15, 2000
	Nelfinavir (NFV)	March 14, 1997
	Ritonavir (RTV)	March 1, 1996
	Saquinavir (SQV)	December 6, 1995
	Tipranavir (TPV)	June 22, 2005
Fusion Inhibitors		
	Enfuvirtide (T-20)	March 13, 2003
Entry Inhibitors		
	Maraviroc (MVC)	August 6, 2007
Integrase Inhibitors		
	Dolutegravir (DTG)	August 13, 2013
	Elvitegravir (EVG)	August 27, 2012
	Raltegravir (RAL)	October 12, 2007

1.8.1. Mechanism of Action of the Different Drug Classes

1.8.1.1. Nucleoside Reverse Transcriptase Inhibitors (NRTIs)

NRTIs target the reverse transcriptase enzyme and have been instrumental in the treatment of HIV-1 (Iyidogan and Anderson, 2014). NRTIs are competitive inhibitors which are structurally similar to the deoxy-nucleotide triphosphates (dNTPs) but lack the 3'-hydroxyl group needed for DNA elongation (Ammaranond and Sanguansittianan, 2012). NRTIs are administered as prodrugs and are converted to the triphosphate form by

host cellular enzymes (Tang and Shafer, 2012). By out-competing endogenous dNTPs for incorporation into the growing DNA chain, NRTIs cause chain termination and viral replication is unable to proceed (Tang and Shafer, 2012).

1.8.1.2. Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)

The NNRTIs are non-competitive inhibitors that act by binding to a hydrophobic pocket close to the polymerase active site unique to the HIV-1 reverse transcriptase enzyme (Iyidogan and Anderson, 2014). Once bound to the hydrophobic pocket, NNRTIs inhibit dNTP binding by inducing a conformational change in the dNTP-binding pocket p66 (Ammaranond and Sanguansittianan, 2012). This change blocks the required alignment of substrates for the phosphodiester bond formation and prevents further replication (Iyidogan and Anderson, 2014)

1.8.1.3. Protease Inhibitors (PIs)

HIV-1 Protease is responsible for recognising and cleaving 10 sites in the Gag and Gag-Pol polyproteins to form infectious virions (Huang et al., 2011). HIV-1 Protease is a homodimer consisting of 2 identical proteins each with 99 amino acids in length (Figure 1.7) (Yang et al., 2012). The two flaps contain flexible β -hairpins that allow access to the active site (Huang et al., 2014). PIs are competitive active site inhibitors of HIV-1 protease (Tang and Shafer, 2012), and prevent cleavage of the Gag and Gag-Pol polyproteins into mature infectious viruses (Arts and Hazuda, 2012).

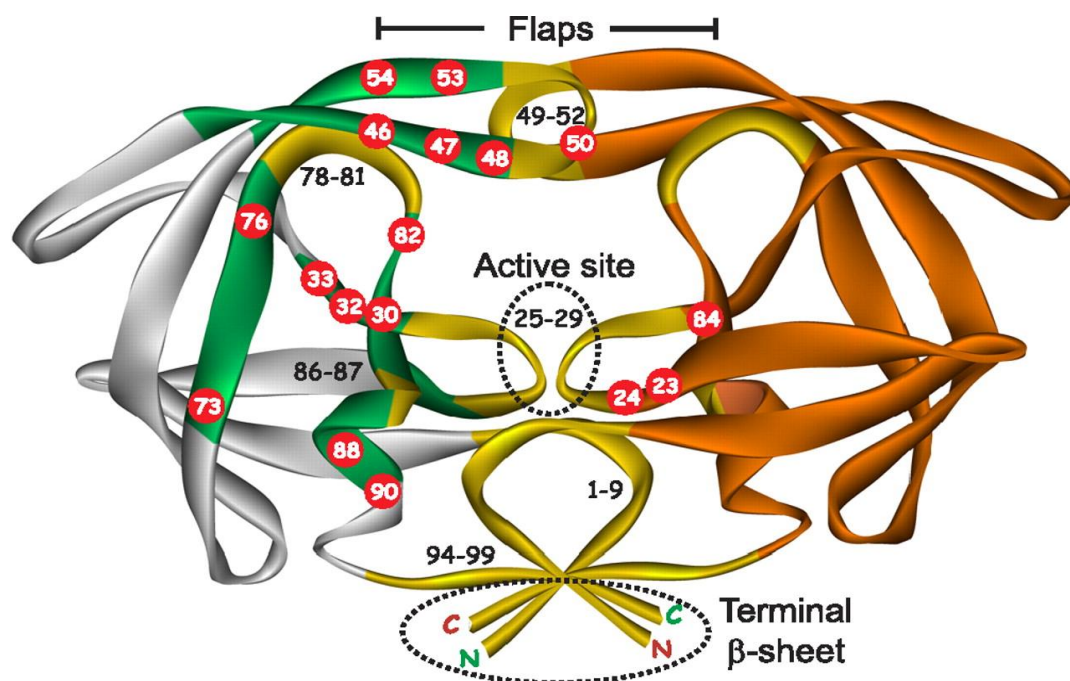


Figure 1.7. Structure of HIV-1 Protease

Ribbon representation of the homodimeric structure of HIV-1 Protease, indicating the flap and active site regions. The numbers within the spheres represent amino acid positions associated with drug resistance. Picture taken from Louis et al. (2011).

1.8.1.4. Integrase Inhibitors (INs)

The HIV-1 integrase enzyme is required to catalyse the integration of viral DNA into the host cell chromosomal DNA during viral replication (Ammaranond and Sanguansittianan, 2012). It consists of three domains i.e. the N- and C-terminal domains and a catalytic core (Iyidogan and Anderson, 2014). Two main residues in the active site, Asp64 and Asp116 are critical for viral integration, such that mutations arising at these residues result in complete inhibition of viral integration (Ammaranond and Sanguansittianan, 2012). INs binds to the specific complex between integrase and the viral DNA and interact with the magnesium metal ion cofactors in the integrase active site and the DNA thereby interfering with the integration process during strand transfer (Arts and Hazuda, 2012).

1.8.1.5. Fusion Inhibitors (FIs)

HIV-1 gp41 is responsible for anchoring the envelope complex within the cell membrane. After binding of gp120 to CD4⁺ coreceptors, the heptad repeat regions of gp41 fold

shortening the protein and bringing the viral and host cells membranes into contact (Iyidogan and Anderson, 2014). The FI Enfuvirtide acts by binding to the first heptad repeat region of gp41, thereby preventing the virus from infecting the host cell (Iyidogan and Anderson, 2014).

1.8.1.6. Entry Inhibitors

Maraviroc (MVC) inhibits the binding of HIV-1 gp120 to the host CCR5 co-receptor (Tang and Shafer, 2012), by binding to a hydrophobic transmembrane pocket of CCR5, altering the conformation of the extracellular loop of the receptor, and preventing interaction with the V3 stem loop of gp120 (Iyidogan and Anderson, 2014). Prior to initiation of MVC treatment, a tropism assay is required to identify the co-receptor of the virus that the patient is infected with. The Trofile test (Monogram) assesses the tropism of *envelope* genes from patient samples using reporter cell lines expressing CCR5 or CXCR4 (Tang and Shafer, 2012)

1.8.2. Highly Active Antiretroviral Therapy (HAART) and Treatment Regimens

Currently, the standard of care for treatment of HIV-1 patients is combined antiretroviral therapy (cART) consisting of at least three different drugs, from 2 or more drug classes (Pomerantz and Horn, 2003). The use of cART ensures targeting of more than one viral replication step thereby maximising the ability to inhibit HIV-1 replication. The South African treatment guidelines follow the WHO guidelines and recommend the use of ritonavir (RTV)-boosted lopinavir (LPV/r) as a second-line regimen for adults and children over 3 years of age, and a first-line regimen for paediatric patients <3 years of age (Department of Health, 2013). A summary of the national ART regimen for adults and children are shown in Table 1.2 and 1.3 respectively.

Table 1.2. Standardised South African national treatment regimens for adults and adolescents (Department of Health, 2013)

First Line	
All new patients initiating treatment	TDF+FTC (or 3TC) +EFV
Adolescents	ABC+3TC+EFV
Contraindications to EFV	TDF+(FTC or 3TC)+NVP
Contraindications to TDF	AZT+3TC+EFV (or NVP)
Contraindication to TDF and AZT	d4T+3TC+EFV (or NVP)
Contraindication to TDF, AZT and D4T	ABC+3TC+EFV (or NVP)
Second Line	
Failing on a TDF-based 1 st line regimen	AZT+3TC+LPV/r
Failing on a d4T-based 1 st line regimen	TDF+3TC (or FTC) and LPV/r
Third Line	
Failing 2 nd line regimen	Specialist referral- Most likely regimen would be RAL/DRV/ETR (according to genotype)

Table 1.3. Standardised South African national treatment regimens for infants and children (Department of Health, 2013)

First Line	
< 3 years (or < 10kg)	ABC+3TC+LPV/r
> 3 years (and > 10 kg)	ABC+3TC+EFV
Second Line	
Failed first line PI-based regimen: ABC+3TC+LPV/r d4T+3TC+LPV/r	Under expert advice: AZT+3TC+EFV+LPV/r AZT+ABC+EFV+LPV/r
Failed first line NNRTI-based regimen ABC+3TC+EFV (or NVP) d4T+3TC+EFV (or NVP)	AZT+3TC+LPV/r AZT+ABC+LPV/r
Third Line	
Failing 2 nd line regimen	Specialist referral- Based on genotype.

Although antiretroviral therapy has significantly reduced morbidity and mortality, the emergence of drug resistance complicates life-long effective therapy (Ammaranond and Sanguansittianan, 2012). Drug resistance has been associated with each class of drugs, with some patients even showing multi-class drug resistance.

1.8.3. Antiretroviral Therapy in Paediatric Patients

Earlier prevention of mother-to-child transmission (PMTCT) programs were predominantly NNRTI-based (Zanoni et al., 2012) rapidly selecting for high rates of NNRTI drug resistance in infants and young children infected with HIV-1 and compromising the effectiveness of NNRTIs in these children (Coovadia et al., 2009).

Therefore, PI-based therapy is preferred as a first-line regimen for infants. Results from the Children with HIV Early Antiretroviral Therapy (CHER) trial showed that early diagnosis and treatment reduced early infant mortality and HIV progression (Palumbo et al., 2010, Chadwick et al., 2009). The recommendations from the trial have been adopted by the South African government and children now start therapy at diagnosis regardless of CD4 percentage. South Africa has an estimated 410 000 children infected with HIV, of whom just over half are eligible and receiving antiretroviral treatment (UNAIDS, 2013). The continued use of LPV/r in paediatric patients is limited by its poor palatability, drug-drug interactions and development of resistance (Gupta et al., 2009). In patients with concomitant tuberculosis, therapy with LPV/r and rifampicin can drastically result in sub-therapeutic levels of LPV/r (McIlleron et al., 2011). Currently children co-infected with HIV-1 and TB are given double doses of lopinavir/ritonavir during rifampicin-based therapy (McIlleron et al., 2011). However, further studies are required to determine the optimal dose to be used. Most PIs have not yet been approved for use in children, since adequate formulation and pharmacokinetic studies are still required (Hazra et al., 2010). Therefore it is necessary to identify more ARVs which are effective and easily accessible in children failing PI-based therapy.

Prior to 2008, RTV monotherapy was used to treat paediatric patients receiving concomitant treatment for tuberculosis (TB) and/or patients <6 months of age due to drug-drug interactions with LPV/r (van Zyl et al., 2011, Reitz et al., 2010). HAART has significantly reduced disease progression and mortality in children (Ramos et al., 2011). However, due to their high viral loads and challenges to accurate dosing, those under 2 years have poorer virological outcomes and greater chances of developing drug resistance compared to older children and adults (Hazra et al., 2010, Gupta et al., 2009).

1.9. Antiretroviral Drug Resistance

1.9.1. Drug Resistance to the Different Classes of Drugs

1.9.1.1. Nucleoside reverse transcriptase inhibitors

Two biochemical mechanisms of NRTI resistance can occur. The first mechanism involves selecting for single mutations which allow the reverse transcriptase enzyme to discriminate between dideoxy-NRTI chain terminators and the cells natural dNTPs thereby disrupting incorporation of the NRTIs into the growing DNA chain (Tang and Shafer, 2012). The K65R, L74V, Q151M and M184V mutations cause resistance to NRTIs in this manner, and are also known as discriminatory mutations. The second mechanism is mediated by mutations (known as excision mutations) that facilitate the phosphorylytic excision of the chain terminating NRTI residue from the 3' end of the growing viral DNA chain, which in turn permits continued DNA synthesis (Ammaranond and Sanguansittianan, 2012). These mutations are also referred to as thymidine analogue mutations (TAMs) since they are selected by thymidine analogue drugs (Tang and Shafer, 2012), and include the M41L, D67N, K70R, L210W, T215YF and K219QE mutations. TAMs can cause broad cross-resistance to NRTIs (Rhee et al., 2003). The T69 insertion complex is also associated with other NRTI mutations that can cause multi-NRTI resistance (Iyidogan and Anderson, 2014). Therefore, with the development of TAMs, genotypic resistance testing should be performed prior to the use of another thymidine analogue.

1.9.1.2. Non-nucleoside reverse transcriptase inhibitors

NNRTIs, such as EFV and NVP, have a low genetic barrier to resistance, in that a single mutation may cause high-level resistance and even cross-resistance among the NNRTI drug classes (Ammaranond and Sanguansittianan, 2012). The mutations responsible for

NNRTI resistance are located close to the hydrophobic pocket of reverse transcriptase and these the binding affinity of the inhibitors (Ammaranond and Sanguansittianan, 2012). Mutations K103N, V106M, Y181C and G190A are the most common single mutations which confer cross-resistance to NVP and EFV (Iyidogan and Anderson, 2014). The second-generation NNRTIs ETR and RVP show promise following use of NVP or EFV since most of the mutations acquired with NVP or EFV use do not cause cross-resistance to the newer NNRTIs (Basson et al., 2014).

1.9.1.3. Protease Inhibitors

Resistance to PIs is mediated by the development and accumulation of patterns of mutations primarily located in the active site of the *protease* gene (Figure 1.7) (Ammaranond and Sanguansittianan, 2012, Molla et al., 1996). Of the 99 amino acids in *protease*, 46 have been shown to be associated with selection by PIs, with 26 positions identified as those commonly involved in PI resistance (Rhee et al., 2010). *Protease* mutations are classified as either major or minor mutations: major *protease* mutations are those which by themselves cause reduced susceptibility to one or more PIs, whereas minor mutations are those which have little or no effect on PI susceptibility on their own but may further reduce susceptibility in combination with major mutations (Rhee et al., 2003). In the presence of a mutant *Protease*, the natural substrate (i.e gag and gag-pol) can still bind to the enzyme and be cleaved while the inhibitor is no longer effective against the enzyme (Figure 1.8).

Cross-resistance refers to the ability of PI-selected mutations to cause resistance to other PIs. This presents a predicament when choosing new PI-based regimens in PI-experienced individuals. Major mutations arising due to LPV/r-based therapy include: I47V/A, I50V, I54V/T/A/L/M, L76V, V82A/F/T/S and I84V. Some of which cause high

level resistance to other PIs such as I47A (to FPV/r), L76V (to FPV/r and IDV/r), V82A (to IDV/r and NFV), I84V (to ATV/r, FPV/r, IDV/r, NFV and SQV/r) and L90M (to NFV and SQV/r) (Rhee et al., 2003).

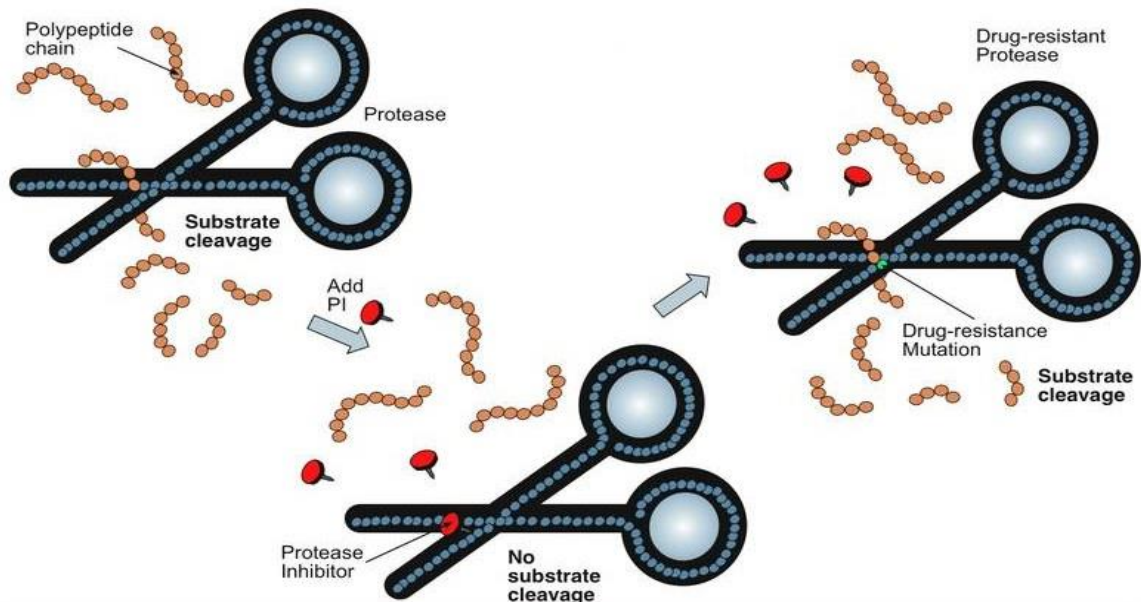


Figure 1.8. Mechanism of action of protease inhibitors

The natural substrate (Gag) binds to the active site of Protease, thereby allowing Protease to cleave Gag into smaller polypeptides rendering the virus mature and infectious. The scissor is an analogy used to show the function of the flap region of Protease. Protease inhibitors act by recognising and binding to the active site of the enzyme and preventing substrate cleavage. In the presence of drug resistant protease inhibitors do not bind to the active site, but the substrate still has the ability to recognise and bind to mutant *protease*. Picture taken from Immunopaedia (2010).

Although the presence of PI mutations predicts failure on a PI-based regimen, a number of studies have reported a low frequency of *protease* mutations in adult and paediatric patients failing PI-based therapy (Wallis et al., 2011, van Zyl et al., 2009, Levison et al., 2012). Although the lack of PI mutations is often associated with non-adherence, some patients still show resistance to other classes of drugs in the regimen suggesting that patients are being exposed to drug levels (Siliciano and Siliciano, 2013). These findings suggest that there may be alternate pathways of PI resistance (Bartlett, 2013, Siliciano and Siliciano, 2013). Many studies have shown mutations at *gag* cleavage sites (CS) associated with PI resistance, either in the presence and absence of major PI mutations (Dam et al., 2009, Nijhuis M, 2007, Malet et al., 2007). A study by Rabi et al. (2013)

showed that the presence of PIs inhibit the entry, reverse transcription and post-reverse transcription steps of the virus life cycle and that inhibition was greatest at the entry step. The *envelope* gene was suggested as a possible site of PI resistance following the selection of mutations in *envelope*. The second study by Rosenbloom et al. (2012) found that the pharmacodynamic properties of PIs restrict the evolution of resistance. PIs have a very short half-life and a limited mutation selection window (MSW), a window during non-adherence where the mutant virus has a selective advantage over wild-type virus. In these instances, wild-type virus will emerge rapidly upon therapy interruption.

1.9.1.3.1. The Role of HIV-1 Gag in Protease Inhibitor Drug Resistance

HIV-1 Protease recognises and cleaves the Gag and Gag-Pol polyproteins at 10 different cleavage sites to yield the structural components of matrix (MA), capsid (CA), nucleocapsid (NC), spacer peptides 1 and 2, trans-frame protein (TFP) and essential enzymes protease (PR), reverse transcriptase (RT), RNase H and integrase (IN). Cleavage sites comprise of 5 amino acids flanking either side of the scissile bond with position 1 (P1) located immediately upstream and position 1 prime (P1') located immediately downstream of the cleavage point (Figure 1.9.) (de Oliveira et al., 2003, Pettit et al., 1994).

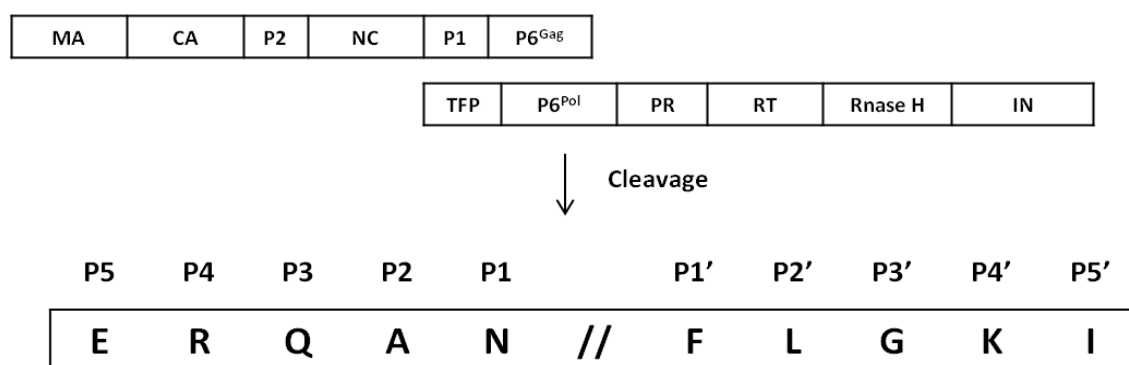


Figure 1.9. Illustration of the *gag* NC/p1 CS and the nomenclature used for each position within the cleavage site. *Gag* CS span 10 amino acids with 5 amino acids on either side of the scissile bond.

Earlier studies showed that *gag* CS mutations compensate for amino acid changes (i.e. drug resistance mutations) arising in *protease* under PI pressure to maintain viral fitness (Mammano et al., 1998, Maguire et al., 2002). However, mutations at CS NC/p1 and p1/p6^{Gag} have been associated with PI resistance even in the absence of drug resistance mutations in *protease* (Dam et al., 2009, Nijhuis M, 2007). Dam et al., (2009) found that the carboxyl terminus of *gag* was responsible for directly contributing to PI resistance mediated primarily by mutations A431V and I437V at NC/p1 in the absence of mutations in *protease*. Studies have also shown that mutations occurring at *gag* CS co-evolve with *protease* mutations to contribute to drug resistance (Kolli et al., 2014, Maguire et al., 2002, Sutherland et al., 2013, Gupta et al., 2010).

Few studies have looked at the role of non-CS mutations in drug resistance. An early study found that *gag* non-CS mutations H219Q and R409K were responsible for conferring PI resistance and were essential for viral replication in the presence of PIs (Gatanaga et al., 2002). The authors suggested that changes at *gag* non-CS may render the cleavage site more accessible to mutant *protease* thereby making the replication process more efficient. Non-CS mutations occurring at the amino terminus of *gag* have been shown to confer PI resistance in the presence of mutant *protease* and absence of *gag* CS mutations (Parry et al., 2009).

Since changes at *gag* CS and non-CS have been shown to contribute to PI resistance, recent studies advocate for the inclusion of full length *gag* in addition to *protease* when studying PI resistance (Gupta et al., 2010, Sutherland et al., 2013, Sutherland et al., 2014).

1.9.1.4. Integrase inhibitors

Resistance to RAL, the first approved integrase inhibitor, can occur by three main pathways which occasionally overlap: N155H followed by E92Q; Q148HRK+G140SA; and Y143CR+T97A (Tang and Shafer, 2012). Most RAL mutations confer cross-resistance to EVG with the exception of Y143CR. However, DTG has a higher genetic barrier compared to RAL and EVG since only G148+G140 can confer resistance to DTG (Tang and Shafer, 2012). Further studies with patients on treatment with INs for a long period are necessary to determine additional resistance patterns.

1.9.1.5. Fusion inhibitors

Resistance to the currently available FI, Enfuvirtide (T-20), has been shown to occur by the development of mutations in the HR1 region in gp41 at codons 36-45 (Tang and Shafer, 2012). T-20 has a low genetic barrier resulting in rapid development of resistance, therefore its use in salvage therapy will require a sufficient number of additional active drugs (Tang and Shafer, 2012). Major mutations conferring resistance to T-20 include G36DS, I37V, V38AME, Q39R, Q40H, N42T and N43D (Rhee et al., 2003), which occur in most subtypes (Iyidogan and Anderson, 2014).

1.9.1.6. Entry Inhibitors

The entry inhibitor MVC is a negative allosteric modulator of CCR5 and blocks gp120 binding to the coreceptor (Iyidogan and Anderson, 2014). The primary mechanism by which HIV-1 develops resistance to MVC is through tropism switching by using the CXCR4 co-receptor instead of CXCR5 for entry (Moore and Kuritzkes, 2009). In addition MVC resistance can occur through the development of mutations in the *envelope* gene which allow gp120 to bind an MVC-bound CXCR5 receptor (Westby et al., 2007).

Most of these mutations are located in the V3 loop of *envelope* gene (Iyidogan and Anderson, 2014).

1.9.2. The South African Antiretroviral Treatment Program

South Africa has the largest ARV programme worldwide and combined with the ART scale-up, there has been significant population-level reduction in HIV-related mortality in many populations (Bor et al., 2013). A study by the HIV Prevention Trials Network (HPTN) 052 trial was conducted in HIV-1 discordant couples showed that treatment with ART significantly lowered the transmission of the virus to the uninfected partner (Cohen et al., 2011). This suggests that increased ART coverage could reduce the rate of ARV infections at a population level (Tanser et al., 2013). A recent study by the Africa Centre in South Africa followed up a total of 16 667 individuals who were uninfected at baseline and their seroconversions were monitored from 2004 to 2011 (Tanser et al., 2013). This study found that the risk of acquiring HIV-1 significantly declined with increasing ART coverage in the surrounding local community.

1.9.3. Monitoring of ARV Drug Resistance

ARV therapy has been instrumental in improving the health of HIV-1 infected patients (Cohen et al., 2008). However, the development of drug resistance most often leads to viral load rebound in the infected individual. The monitoring and prevention of drug resistance development is imperative for a successful treatment outcome. Infrequent viral load monitoring, as is often observed in resource-limited settings, resulting in patients that present with more drug resistance mutations and cross-resistance compared to those receiving regular viral load monitoring in well-resourced countries (Gupta et al., 2008). Regular viral load monitoring and viral genotyping is important to identify non-adherence

and ineffective drug regimens. These are valuable tools to guide clinicians in choosing appropriate drug regimens (Iyidogan and Anderson, 2014).

1.9.3.1. Viral Load Monitoring

Viral load monitoring technologies can be characterised as nucleic acid based tests (NAT) and non-NAT based tests (Haleyur et al., 2014). NAT testing involves the direct detection and quantification of viral RNA while non-NAT technology involves the detection and quantification of viral enzymes which are used to determine the amount of viral RNA present (Haleyur et al., 2014). The NAT-based assays have wider availability and are easily interpreted by clinicians (Haleyur et al., 2014). In addition to the in-house tests available, four commercial assays for viral load tests are available, including the Real-Time HIV-1 assay (Abbott), VERSANT HIV-1 RNA 1.0 assay (Siemens), COBAS AMPLICOR HIV-1 MONITOR v1.5 assay (Roche Molecular Systems), COBAS AmpliPrep/COBAS TaqMan v2.0 assay (Roche Molecular Systems) (Haleyur et al., 2014). Viral load monitoring is used to guide regimen switch, as 2 consecutive viral loads >1000 copies/mL more than 3 months apart is considered as a sign of possible drug resistance (Jordan et al., 2008). A study was done to determine the cost-effectiveness of using viral load as an alternative to CD4 monitoring following ART initiation. It was found that viral load monitoring alone could save costs since it led to earlier identification of patients that required switching to second-line therapy (Hamers et al., 2012). The Development of AntiRetroviral Therapy in Africa (DART) trial in Uganda and Zimbabwe showed that routine monitoring of CD4 cell counts had a small but significant benefit in terms of disease progression and mortality (DART, 2010).

1.9.3.2. Drug Resistance Testing

Drug resistance is measured by genotypic or phenotypic testing: Genotypic testing involves sequencing of the HIV-1 gene to detect resistance-associated amino acid substitutions (i.e. mutations) while phenotypic testing directly quantifies the concentration of drug required to inhibit viral replication (Dunne et al., 2001). Although genotypic tests are a powerful tool in predicting drug resistance, phenotypic tests are a more direct measure of drug resistance. However, both methods require specialisation. Genotypic tests require skilled individuals to perform the laboratory tests and interpretation of results and phenotypic tests require special facilities (biosafety level 3 laboratory) and experienced personnel.

1.9.3.2.1. Genotyping

Genotypic tests are more widely used since it is less expensive and has a faster turnaround time compared to phenotypic tests (one week vs 6 weeks). Standard genotypic tests involves PCR amplification and Sanger sequencing (population-based) of the HIV-1 *protease*, *reverse transcriptase* and *integrase* genes to detect mutations in the majority of circulating viruses (Tang and Shafer, 2012). The use of plasma for genotyping is considered to be the most appropriate; however dried blood spots (DBS) are an alternate specimen for genotyping in areas where preparation and storage requirements are not optimal (Bertagnolio et al., 2010). DBS can be stored at room temperature for ≤ 2 weeks and can be easily transported as non-hazardous material (Bertagnolio et al., 2010)

The length of sequences obtained for protease is up to 99 amino acids, for reverse transcriptase is up to 450 amino acids and for integrase is up to 288 amino acids. The sequencing process produces an amino acid sequence which is compared to a reference subtype B sequence using a resistance interpretation algorithm. There are currently 8 different resistance interpretation algorithms available for genotypic testing with few

differences usually involving relatively infrequent mutations (Liu et al., 2008). These algorithms include HIVdb and CPR (Stanford University Medical Center, CA, USA), REGA (Rega Institute for Medical Research and University Hospitals Leuven, Katholieke Universiteit Leuven, Belgium), ANRS (Agence Nationale de Recherches sur le SIDA), Celera Viroseq Genotyping System (Abbott), TRUGENE HIV-1 Genotyping Assay (Bayer), HIV-Grade (developed by a German standardisation initiative) and RetroGram Guidelines (Virology Networks BV, Utrecht, the Netherlands) (Sing, 2006). The algorithm highlights differences between the reference sequence and the clinical virus and provides a predicted phenotype to each ARV drug based on the mutations present, which in turn assists clinicians in identifying suitable drugs for future use.

There are two clinically available genotypic resistance tests i.e. Celera Viroseq Genotyping System and TRUGENE HIV-1 Genotyping Assay. However, many in-house resistance genotyping methods have been developed to make genotyping more cost-effective in different settings. In addition to population-based sequencing, allele-specific PCR (AS-PCR) and ultra-deep pyrosequencing technology has also been used to detect resistant mutations in minority variants within a sample. AS-PCR is based on the detection of specific point mutations (Johnson et al., 2007). A study reported on the development of a multiplex allele-specific (MAS) drug resistance detection assay for high-throughput and sensitive detection of multiple HIV-1 drug resistance mutations (Zhang et al., 2013). This assay was designed to detect wild-type and mutant alleles at the 20 loci of HIV-1 subtype C in the *protease* and *reverse transcriptase* genes. The limits of detection for the different mutations differed from 1.56% to 12%. AS-PCR may be a feasible genotyping tool for large-scale drug resistance surveillance and monitoring in resource-limited settings. Ultra-deep pyrosequencing sequencing (UDPS) technology has been used by many studies to detect minority variants in as little as 1% of the viral population (Fisher et al., 2012). A recent study compared the use of UDPS to Sanger

sequencing for detecting HIV-1 drug resistant minority variants (Mohamed et al., 2014). There was a significant difference in the mutations picked up by UDPS compared to Sanger sequencing for 19 ARV drugs where UPS picked up 60% more clinically relevant mutations. This suggests that a cut-off of 1% allows for improved characterization of the viral population by identifying additional resistance mutations (Mohamed et al., 2014).

1.9.3.2.2. Phenotyping

Phenotypic susceptibility assays measure the ability of a virus to replicate in cell culture in serial dilutions of ARV drugs (Tang and Shafer, 2012). Traditionally, phenotyping involved the growing of whole patient viruses in culture in the presence of varying amounts of drug. However, modern assays use cloning techniques where the HIV-1 genes of interest are cloned into an expression vector after which virus-like particles are produced and tested for drug susceptibility. Phenotypic assay can be limited to a single cycle of viral replication or allow for multiple rounds of replication. Results are interpreted by plotting a sigmoidal dose-response curve of viral growth over the various drug concentrations. The inhibitory concentration-50 (IC_{50}) of the clinical virus is compared to that of a reference wild-type virus to calculate the fold change (FC) in difference in susceptibility. Two commercial phenotypic tests are available, and include the Phenosense assay (Monogram Biosciences) and Antivirogram assay (Virco) (Tang and Shafer, 2012). Since phenotyping is expensive and labour intensive, is it not considered for testing all patients but recommended for patients with complex mutational patterns, especially for those selecting salvage options (Chen and Aldrovandi, 2008). Phenotyping is also recommended for patients when starting MVC to determine which co-receptor the virus is using since MVC is effective only against CCR5-tropic HIV-1.

Approved methods for genotypic and phenotypic tests only include the HIV-1 enzymes (RT, PR, IN) and mutations outside of these regions are not considered. In the case of *protease*, the impact of *gag* mutations is not considered which may underestimate PI susceptibility to the drugs tested.

1.9.4. Transmitted Drug Resistance

Current WHO guidelines recommend early ART initiation at CD4 cell counts <500 in an attempt to reduce the number of people acquiring infection. With early ART initiation, there may be an increased risk of TDR however, this is outweighed by a reduction in new infections (Hamers et al., 2013). Currently, an estimated 10 million individuals are on ART globally with the majority receiving first-line NNRTI-based therapy (Phillips et al., 2014). Virological failure on ART is often accompanied by drug resistant mutations which can lead to transmission of drug resistant variants (TDR) to treatment naive individuals (Phillips et al., 2014). Global surveys have indicated an increase of TDR in recently infected individuals in African countries (such as South Africa, Burkina Faso, Uganda and Kenya) owing primarily to NNRTI resistance (WHO, 2012). This raises concern especially since NNRTIs are used in most first-line regimens and for PMTCT (Hamers et al., 2013). K103N is associated with TDR and is found in patients failing first-line therapies in Gauteng and Kwa-Zulu Natal provinces in South Africa (Hunt et al., 2012). The PharmAccess African Studies to Evaluate Resistance Monitoring (PASER-M) cohort study conducted in ART naive individuals in South Africa, Kenya, Nigeria, Uganda, Zambia and Zimbabwe reported a 5.6% prevalence of drug resistant mutations (Hamers et al., 2011).

A study by Phillips et al. (2014) investigated the effectiveness and cost-effectiveness of suggested public health responses to TDR. Due to the high potency of PIs, mutations accumulate slowly during poor adherence, therefore, the use of PIs in first-line was the most cost effective option (Phillips et al., 2014). The study also found that if the prevalence of NNRTI resistance mutations in treatment naive individuals should exceed >10%, a policy to measure viral load 6 months after ART initiation was cost effective while individualised pre-treatment resistance testing was not cost-effective. With an increasing number of patients initiating ART, there will inevitably be an increased prevalence of TDR, however, maintaining adherence and retention on ART will help reduce HIV-1 transmission (Phillips et al., 2014).

1.9.5. Acquired Drug Resistance

1.9.5.1. Acquired Drug Resistance After First-Line ARV Therapy

Following exposure to NRTIs in first-line therapy, many individuals develop NRTI resistance mutations. Because of high rates of 3TC usage, M184V is very common following NRTI treatment but results in reduced viral fitness (Lazarus et al., 2013). A study has also shown that patients who are failing therapy due to adherence or drug resistance and for whom new drugs are currently unavailable, 3TC monotherapy may be used as a holding strategy since it selects for M184V thereby maintaining viral load suppression (Lazarus et al., 2013). WHO surveys on acquired drug resistance in adults have shown that 12 months after therapy initiation, 91% of patients achieved viral suppression, and of the patients failing therapy, 72% had resistance mutations (WHO, 2012). The drug resistance mutations observed at 12 months suggested that the current NRTIs used in second-line combined with a boosted PI would be effective for most patients (WHO, 2012).

Most patients treated with NVP in first-line therapy develop mutations which compromise the effectiveness of future NNRTIs used (Hamers et al., 2013). Mutations selected by NVP such as L100I, K101P and Y181IV cause cross resistance to new NNRTIs ETR and RPV (Rhee et al., 2003). In addition to these mutations, K103NS, V106AM, Y188LC, G190SE and M230L also selected by use of NVP, confer resistance to EFV (Rhee et al., 2003).

1.9.5.2. Acquired Drug Resistance After Second-Line ARV Therapy

Protease inhibitors are commonly used in second line regimens. It has been shown that boosted PI-based regimens can re-suppress HIV-1 replication even in the presence of numerous NRTI mutations (Hosseinipour et al., 2010). However, the use of boosted PI monotherapy has been investigated by a few studies and is currently not recommended. The ACTG5230 trial found that a third of patients receiving PI monotherapy did not achieve complete viral suppression after 24 weeks (Bartlett et al., 2012). Protease inhibitor monotherapy was also found to be inferior to triple therapy with NNRTIs in the HIV Star Study (Bunupuradah et al., 2012). In resource-limited settings, rates of virologic failure on PI-based therapy have been shown to be quite high. However, virological failure was associated with poor adherence rather than drug resistance. Therefore maintenance of adherence is crucial to avoid unnecessary switching to more expensive regimens (AJose et al., 2012).

1.9.5.3. Acquired Drug Resistance After Prevention of Mother-to-Child Transmission (PMTCT)

The use of single dose NVP (sdNVP) for PMTCT has shown to rapidly select for NNRTI mutations thereby compromising subsequent NNRTI-based regimens (Jourdain et al.,

2004). Following revision of the WHO PMTCT guidelines in 2010, the use of combined ART is advocated either as dual therapy (Option A) or triple therapy (Option B) (Bateman, 2013). The use of sdNVP together with AZT in women from Mozambique and Kenya showed reduced postpartum NVP resistance (Micek et al., 2012, Kesho Bora Study and de Vincenzi, 2011). In addition, women who received triple ART in Botswana had no resistance after one month of PMTCT discontinuation (Souda et al., 2013). A further recommendation by the WHO in 2013 suggested that all HIV-1 positive pregnant women be put on triple therapy for life (Option B+) regardless of CD4+ cell count (Bateman, 2013). This is expected to reduce the HIV-1 mother-to-child transmission rate especially in subsequent pregnancies (Thyssen et al., 2013)

1.9.5.4. Acquired Drug Resistance After Pre-Exposure Prophylaxis (PrEP)

PrEP is used to protect HIV-uninfected individuals before sexual exposure from an HIV-infected partner (FDA, 2012). The results of different trials investigating the effectiveness of PrEP have produced conflicting results primarily due to differences in regimen acceptance and adherence with different population groups studied. The Centre for the AIDS Program of Research in South Africa (CAPRISA) 004 trial assessed the effectiveness and safety of a 1% vaginal gel formulation of TDF, for the prevention of HIV acquisition in women (Abdool Karim et al., 2010). It was found that the TDF gel reduced HIV acquisition by an estimated 39% overall, and by 54% in women with high gel adherence. There was an absence of mutations suggesting little immediate risk of TDF-resistant HIV-1 emergence in the seroconverters (Wei et al., 2014). Although further studies are needed to evaluate drug resistance acquired from PrEP, a modelling study showed that drug resistance from ART is much greater than that from PrEP (Parikh and Mellors, 2012).

1.9.6. WHO Paediatric Drug Resistance Surveillance

There was an estimated 740 000 children living with HIV-1 globally by the end of 2013, and of these 23% were receiving ART (WHO/UNAIDS, 2013). Monitoring and collecting data on HIV-1 infected children is important as these children require life-long care and treatment. Currently there is limited surveillance data on infants and children.

Although the use of NNRTIs in pregnant and breastfeeding women results in a much lower proportion of children being infected with HIV-1, some children are infected with NNRTI resistant variants (Bertagnolio et al., 2012). This compromises the use of NNRTI-based regimens as first-line treatment in these children. The WHO, HIV Resistance Network (HIVResNet) and the Centers for Disease Control (CDC) developed a surveillance protocol to assess drug resistance among children <18 months of age and newly diagnosed with HIV-1 using dried blood spots (Bertagnolio et al., 2012). This surveillance programme was designed to be repeated over time so that evolving drug resistance patterns could be monitored as PMTCT regimens change and coverage increases (Bertagnolio et al., 2012). The programme requires minimal infrastructure and resources, and can be easily implemented therefore encouraging countries to monitor paediatric HIV-1 drug resistance over time (Bertagnolio et al., 2012).

It is important to monitor the PMTCT program and HIV-1 drug resistance in order to support decision making about paediatric ART guidelines.

1.10. Project Aim and Objectives

Due to the limited knowledge of drug resistance in subtype C paediatric patients failing PI-based therapy, this study aimed to investigate the effect of *gag* on PI resistance in paediatric patients at baseline and failing a PI-based regimen. The objectives of this study were as follows:

1. To genotype the HIV-1 *gag-pol* region of paediatric patients failing a PI-based regimen and to compare these to the baseline sequences.
2. To phenotypically assess the effect of patient-derived *gag-protease* on susceptibility to lopinavir and ritonavir.
3. To determine the levels of cross-resistance to other PIs conferred by patient-derived *gag-protease*.
4. To assess the impact of key *protease* and *gag* mutations introduced by site-directed mutagenesis on phenotypic resistance.

Chapter Two

Materials and Methods

2.1. Summary

Plasma samples from 20 matched paediatric patients at baseline (pre-treatment) and failing a protease inhibitor (PI)-based therapy were genotyped to identify changes in HIV-1 subtype C *gag* and *protease*. In addition, amino acid changes at *gag* cleavage sites (CS) and non-CS sites were analysed to determine if they were associated with the development of PI resistance, and/or under HLA and/or KIR immune-mediated pressure. The *gag-protease* products from both the baseline and failing samples were cloned into an expression vector. The resulting clones were tested *in vitro* using a single-cycle assay to identify the susceptibility of the patient-derived *gag-proteases* to different protease inhibitors. The IC₅₀ of the failing samples were compared to that of the baseline samples to determine the fold change in susceptibility. Site-directed mutants were generated based on specific *gag* and *protease* changes found in the patient samples. These mutants were phenotyped using the single cycle assay to determine their individual contribution to PI susceptibility.

2.2. Cohort Details

A retrospective cohort study was undertaken on 20 matched baseline and post-treatment plasma samples from children less than 24 months of age and failing PI-based therapy. These children were initiated on treatment and followed as part of the pre-randomization screening phase of a randomized clinical trial between 2005 and 2009 (Coovadia et al., 2010, Reitz et al., 2010). Children over 6 months of age were treated with lamivudine (3TC), stavudine (d4T) and ritonavir-boosted lopinavir (LPV/r) as per standard clinical practice at the time. Those younger than 6 months of age and those receiving rifampicin-based regimens for treatment of tuberculosis or Bacille Calmette Guerin (BCG) disease

received ritonavir (RTV) until reaching 6 months of age and/or completing TB treatment at which point they were started on or returned to a LPV/r-based regimen. For children who suppressed and then rebounded, genotyping was done on the first sample where an elevated viral load was observed. For children who did not suppress, a sample within the first 3 to 6 months of treatment was selected. This study was approved by the institutional review boards of Columbia University (NEVEREST II M040912) and the University of the Witwatersrand (M110971).

2.3. Genotypic Analysis of HIV-1 *gag* and *protease* Genes

The HIV-1 *gag* and *protease* genes from paediatric patients were genotyped to identify genetic changes at baseline and PI failure. The sequences and positions relative to the HXB2 viral genome of the primers used for the sequencing of HIV-1 *gag-protease* are shown in Table 2.1. Components used in the cDNA synthesis mixture are shown in Table 2.2. An alignment of the primers relative to the HIV-1 *gag-protease* genes are shown in Figure 2.2.

2.3.1. RNA Extraction

Extraction of RNA was performed using the QIAamp Viral RNA Mini Kit (QIAGEN, Belgium) according to the manufacturer's protocol. Briefly, RNA was extracted from 140 µl of plasma using a spin-column procedure carried out in an Eppendorf Centrifuge 5415D (Eppendorf, Germany). The viral RNA was eluted in 50 µl elution buffer (RNase-free).

2.3.2. cDNA Synthesis

The Thermoscript RT-PCR kit (Invitrogen, CA, USA) was used for cDNA synthesis. The HXB2 position and sequence of the gene specific primer (3'InProt) used for cDNA synthesis is shown in Table 2.1. In a 0.2 µl tube, a reaction mixture (Table 2.2) containing 10 µl of the patient specific RNA was denatured on a GeneAmp 9700 thermal cycler (Applied Biosystems, Carlsbad, CA) at 65°C for 5 minutes. Following denaturation, 7 µl of the cDNA mastermix (Table 2.3) was added to the RNA and run on the thermal cycler for 1 hour at 53°C, followed by 5 minutes at 85°C and a final hold at 4°C. One microlitre of RNase H was added to each reaction to ensure degradation of RNA still bound to cDNA and incubated at 37°C for 20 minutes. A negative control (distilled water) was used for all reactions set up. Reactions were either used immediately for 1st round PCR or stored at -20°C.

Table 2.1. Primers used for cDNA synthesis and amplification of the *gag-protease* region

	Primer Name	HXB2 Positions	Primer Sequence (5' – 3')
cDNA Synthesis	3'InProt	2569 to 2593	CCTGGCTTTAATTTTACTGGTACAG
1 st round PCR	BKT03	690 to 706	CGCAGGACTCGGCTTGC
	ProOutR	2589 to 2607	TTGGGCCATCCATTCCTGG
2 nd Round PCR	GagNot	769 to 798	GCGGCGGCCGCAAGGAGAGAGATGGGTGCG
	ProXhoR2	2550 to 2570	CTGGTACAGTCTCGAGRGGACTRATKGG

First- and second-round PCR primers were previously described by Sutherland et al. (2013). Primer 3'InProt was designed by Dr Michelle Gordon.

Table 2.2. Components of the cDNA synthesis mixture

	Final Concentration	µl / reaction
Primer 3' InProt	10µM	1
dNTP	10µM	2
Water		1
RNA		10
Final Reaction Volume		14

Table 2.3. cDNA Mastermix

	Final Concentration	µl / reaction
Thermoscript buffer	5x	4
DTT	0.1 M	1
RNase OUT	40 U/µl	1
Thermoscript RT	15 U/µl	1
Final Reaction Volume		7

2.3.3. Amplification of HIV-1 *gag-protease*

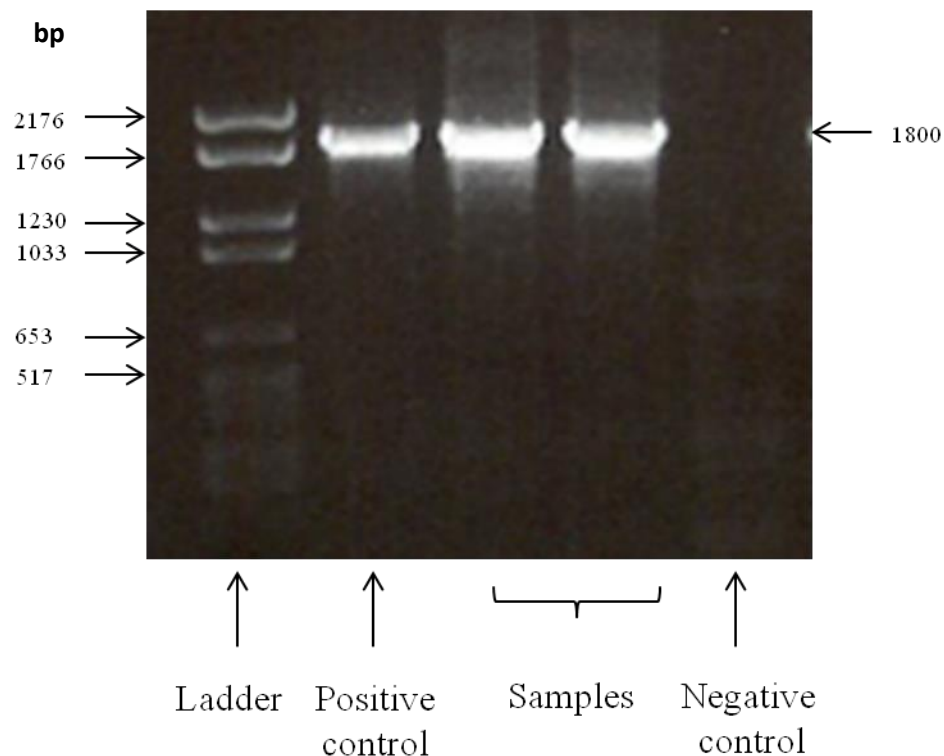
The Expand High Fidelity PCR System (Roche Applied Science, Basel, Switzerland) was used for first and second round nested PCR (Tables 2.4 and Table 2.5). Two microlitres of cDNA was used as a template for first round amplification. For the first-round PCR, the thermal cycler was programmed for 2 minutes at 94°C for initial denaturation, followed by 35 cycles of 30s at 94°C for denaturation, 30s at 53°C for annealing, 2 minutes at 72°C for extension, and 7 minutes at 72°C for the final extension. For the second-round PCR it was the same as the first-round except for the annealing step that was reduced to 51°C. To confirm the presence of positive PCR products after nested (second-round) PCR four microlitres of PCR product was loaded with 1 µl of GelPilot DNA 5x loading dye (QIAGEN, Belgium), on a 0.7% agarose gel and run at 100V for 45 minutes. An example of the correct *gag-protease* PCR product on a gel is shown in Figure 2.1. The gel was viewed on a Bio-Rad ChemiDoc XRS Imaging system (Bio-Rad, CA, USA).

Table 2.4. First-round PCR mastermix

	Final Concentration	µl / reaction
dH2O		17.9
Expand HiFi Buffer/MgCl ₂	1x	6
dNTP	0.2 mM	0.6
Primer BKT03	0.5 µM	1.5
Primer ProOutR	0.5 µM	1.5
Expand HiFi Enzyme	0.05 U/µl	0.5
Final Reaction Volume		28

Table 2.5. Second-round PCR mastermix

	Final Concentration	µl / reaction
dH ₂ O	X	17.9
Expand HiFi Buffer/MgCl ₂	1x	6
dNTP	0.2 mM	0.6
Primer GagNot	0.5 µM	1.5
Primer ProXhoR	0.5 µM	1.5
Expand HiFi Enzyme	0.05 U/µl	0.5
Final Reaction Volume		28

**Figure 2.1. Picture of a PCR gel showing the presence of 1.8 kb *gag-protease* fragments**

A DNA molecular weight marker VI (0.15-2.1 kbp) (Roche Applied Sciences, Mannheim, Germany) was used to identify the 1.8 kb fragment. A negative control was included in each assay which consisted of distilled water instead of template DNA. A positive control was a sample which had previously amplified.

2.3.3.1. Purification of PCR Products

Amplified products were purified using the QIAquick PCR Purification Kit (QIAGEN, Belgium). Binding buffer was added to the PCR product and this mixture was applied to the QIAquick spin column which has a silica membrane to allow the binding of DNA in high salt conditions provided by the buffer. The column was spun down in a Heraeus Biofuge fresco centrifuge (Thermo Electron Corporation, Germany) at 13 000 x g for 1

minute. The column was washed with wash buffer at 13 000 x *g* for 1 minute twice, to remove all impurities from the DNA samples. Pure DNA was eluted with 55 µl in a low salt elution buffer.

2.3.4. Sequencing of HIV-1 *gag-protease*

The sequencing reaction was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA) (Table 2.10). Primers used for sequencing the HIV-1 *gag* and *protease* genes from the baseline and failing samples are shown in Table 2.6. and Figure 2.2. The sequencing reaction was plated in a MicroAmp Optical 96-well Reaction Plate (Applied Biosystems, China) and sealed with Microseal plate covers (Bio-Rad, UK). The reaction was performed on GeneAmp 9700 thermal cycler (Applied Biosystems, Carlsbad, CA). The thermal cycler was programmed for 1 minute at 96°C for initial denaturation, followed by 35 cycles of 10s at 96°C for denaturation, 5s at 50°C for annealing, 4 minutes at 60°C for extension and a final hold at 4°C.

Table 2.6. Primers used for sequencing the *gag-protease* region

Primer Name	HXB2 Positions	Primer Sequence (5' – 3')
GagNot	769 to 798	GCGGCGGCCGCAAGGAGAGAGATGGGTGCG
Gag1F*	1090 to 1109	TTAGACAAGATAGAGGAAGA
Gag1.5R*	1414 to 1431	TCTATCCCATTCTGCAGC
Gag2F	1825 to 1844	ATGATGACAGCATGTCAGGG
DuGagIn2	1282 to 1303	ACATGGGTATTAGCTCTGGGCT
ProXhoR2	2550 to 2570	CTGGTACAGTCTCGAGRGGACTRATKGG
Gag5F*	1697 to 1716	CTTTAAGAGCTGAACAAGCT
Gag6R*	1771 to 1790	AAAATGGTCTTACAATCTGG

Primers marked with an asterisk were designed by Dr Michelle Gordon. Primer DuGagIn2 was designed by Dr Jaclyn Wright. Primers GagNot, Gag2F and ProXhoR2 were previously described by Sutherland et al. (2013).

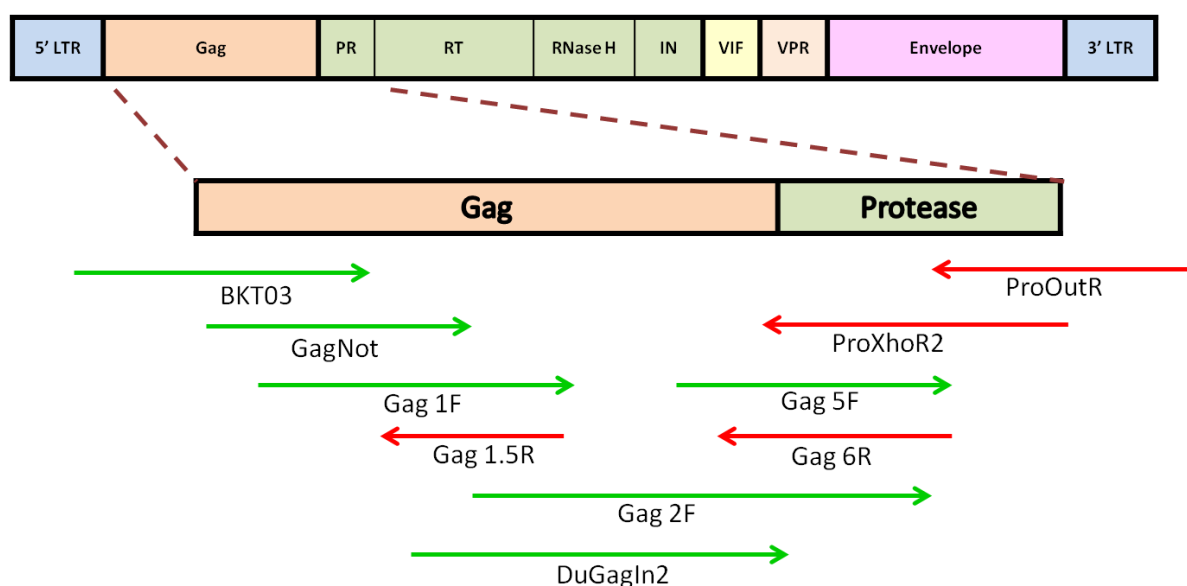


Figure 2.2. Diagram illustrating the positions of the primers used for PCR and sequencing Positions of forward primers (green) and reverse primers (red) which were used for PCR amplification and sequencing of *gag-protease* are shown.

Table 2.7. Sequencing mastermix

	Final Concentration	µl / reaction
dH ₂ O		5
5x Buffer	0.75x	1.5
BigDye		1
Primer	0.48 µM	1.5
DNA	~ 50 -100 ng	1
Final Reaction Volume		10

2.3.4.1. Sequencing Cleanup

Prior to running the sequencing reaction on the ABI 3130xl Genetic Analyser (Applied Biosystems, Foster City, CA), the reaction was cleaned up by sodium acetate precipitation. A mixture containing 3.0 M sodium acetate (pH 4.6) and 100% ethanol was prepared. The amount required was calculated according to the number of samples being sequenced: 1 µl/well of 3.0 M sodium acetate + 25 µl/well of 100% ethanol. Twenty-six microlitres of the sodium acetate and ethanol mix was added to each well and mixed using a multi-channel pipette. The plate was sealed with Microseal plate covers (Bio-Rad, UK) and centrifuged in a Jouan B4i multifunction centrifuge (Thermo Electron

Corporation, Germany) at 2000 x *g* for 30 minutes to allow the DNA to be precipitated. The plate seal was then removed and the plate was inverted and centrifuged at 150 x *g* for 1 minute to remove all sodium acetate and ethanol. To wash the DNA pellet, 35 µl of 70% ethanol was added to each well, the plate was sealed and centrifuged at 2000 x *g* for 5 minutes. The plate seal was removed and the plate was inverted and centrifuged at 150 x *g* for 1 minute to remove remaining ethanol. The pellet was then dried on a thermal cycler at 65°C for 5 minutes. Prior to sequencing, the dried pellet was resuspended with 10 µL formamide and denatured in a thermocycler at 95°C for 3 minutes. The samples were loaded and run on the ABI 3130xl Genetic Analyser which is a 16-capillary electrophoresis system which allows for increased sequencing throughput.

2.3.4.2. Sequence Analysis

Sequences were analysed on Sequencher 4.10.1 sequence analysis software (Gene Codes Corporation, Ann Arbor, MI USA). Each sequence read was assembled to form a contig. The ends of each contig were manually trimmed off to remove regions which were ambiguous. Each contig was exported in a fasta file format for analysis with other sequencing analysis programs. Patient sequences were imported into and aligned using ClustalW in BioEdit version 7.0 (Hall, 1999). Sequences were aligned with HXB2 and consensus C.

Major and minor PI mutations were identified using the Stanford Drug Resistance Database algorithm (<http://hivdb.stanford.edu/>). Changes in *gag* at baseline were identified by comparing each sequence to consensus C. Subtype C consensus sequence was downloaded from the Los Alamos Sequence Database (<http://www.hiv.lanl.gov/content/sequence/NEWALIGN/align.html>). Changes at failure

were defined as those developing following PI therapy compared to baseline sequences. Changes at *gag* CS and non-CS which have been associated with PI resistance from the literature were noted. All sequences were aligned to HXB2 to determine the appropriate numbering of amino acids and also aligned to consensus C to compare the baseline samples to. A phylogenetic tree was constructed using MEGA version 5.05 (Tamura et al., 2011) for quality control purposes and to ensure clustering of the baseline and failing sample sequence. Bootstrap was set at 1000 replicates.

2.4. Prediction of HLA and KIR Associated Mutations

Genetic changes in *gag* which have not been previously associated with PI resistance were investigated for their association with immune escape via HLA and KIR-mediated immune pressure. A published immune escape map was used to identify HLA-associated immune escape mutations selected in the patients *gag* gene (Figure 2.3). Briefly, the q-value threshold used for constructing the immune escape maps was $q \leq 0.05$, meaning that only a 5% false-positive proportion was expected among associations displayed on the maps. All changes in *gag* associated with HLA-mediated immune pressure on the escape map and present in the patient sample was noted. KIR-associated sequence polymorphisms in *gag* were identified in a study (Alter et al., 2011) at 5 different codons (Table 2.8) and these codons were compared to changes in the patient's *gag* sequences.

Table 2.8. KIR footprints in HIV-1 *gag* sequence

	Amino-acid Position	KIR association	Consensus amino acid
1	93	KIR2DS3	E
2	138	KIR2DL2	L
3	138	KIR2DS2	L
4	371	KIR2DS5	T
5	389	KIR3DS1	T
6	479	KIR2DS1	I

2.5. Positive Selection in Patient *gag* Genes

The fixed effects likelihood (FEL) test was performed using Datamonkey (<http://www.datamonkey.org/>) (Kosakovsky Pond and Frost, 2005) to test for selection pressure and estimate the ratio of synonymous to non-synonymous (dN/dS) evolutionary changes. Calculations were performed for the entire dataset containing 20 *gag* baseline and failure nucleotide sequences. Fasta files containing aligned sequences were submitted to the database. The program FEL estimates the probability of obtaining the dN and dS values under a neutral mode, with lower P –values indicating rejection of neutrality in favour of positive selection. Positions with a dN/dS ratio >1 were considered to be under positive selection pressure.

2.6. Phenotypic Analysis of HIV-1 *gag* and *protease* Genes from Patient Samples

Phenotypic analysis of the patient-derived *gag-protease* genes was done to determine the effect of *gag* and *protease* on PI susceptibility. For the expression of HIV-1 *gag-pol*, a HIV-1 expression vector, p8.9NSX+, was used. Virus-like particles or pseudoviruses that are produced with this expression vector are capable of a single infectious event (Parry et

al., 2009, Sutherland et al., 2013). The p8.9NSX+ vector contains a *NotI* restriction site at the beginning of *gag* (HXB2 position 772) and a *XhoI* restriction site at the end of *protease* (HXB2 position 2563). This allows for the cloning of a 1.8kb *gag-protease* fragment from patient samples (Fig 2.4). *Gag-protease* amplicons from the patient samples contained *NotI* and *XhoI* restriction sites which were introduced by the second round primers (Chapter 2.3.3) to allow cloning into p8.9NSX+. The clones with inserts of the correct size, assessed by *NotI/XhoI* restriction were sequenced to confirm the presence of the relevant mutations and polymorphisms. Polymorphisms in the *protease* and *gag* genes were defined as changes in amino acid sequence between the failing and baseline sequences. The sequences were submitted to the Stanford drug resistance database (<http://hivdb.stanford.edu/>) to determine the phenotypic levels of resistance based on mutation penalty scores which have been based on previously published phenotypic data. The mutation penalty scores are based on three considerations: studies linking mutations to ARV therapy; studies linking mutations to decreased ARV susceptibility; and studies linking pre-therapy mutations with the virological response to a new ARV treatment regimen

2.6.1. Cloning of Patient-derived *gag-protease* Genes

The purified *gag-protease* PCR products (see Chapter 2.3.3.1.) were first cloned into a commercial vector (pCR-2.1 TOPO) to allow for more efficient cloning into the expression vector. This process was also found to reduce the number of false positive clones.

2.6.1.1. Cloning into pCR 4-TOPO Cloning Vector

Purified second-round PCR products were cloned into a pCR-2.1 TOPO cloning vector (TOPO TA Cloning Kit, Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The TOPO TA cloning kit consists of a plasmid vector (pCR 4-TOPO) that has a 3' thymidine overhang to allow it to ligate to the deoxy-adenosine at the 3' end of PCR products which was inserted by *Taq* polymerase.

To clone the PCR product into the pCR-2.1 TOPO vector, 40 ng of the PCR product was used. The reaction mixture for the TOPO cloning is shown in Table 2.9. The reaction was incubated at room temperature for 5 minutes to allow the vector and PCR product to ligate.

2.6.1.1.1. Transformation into One Shot TOP 10 Competent Cells

The pCR 4-TOPO construct was then transformed into One Shot TOP 10 chemically competent *E. coli* cells. Two microlitres of the cloning reaction was added to a vial of competent cells and mixed by flicking the tube gently. The reaction was incubated on ice for 30 minutes. The cells were then heat-shocked for 30 seconds at 42°C and placed on ice to stop the reaction. Two hundred and fifty microlitres of S.O.C medium was added to tube, and placed in the shaking incubator (225 rpm) for 1 hour at 37°C. Fifty microlitres of the reaction was plated on pre-warmed agar plates and incubated at 37°C for 16 hours. Clones carrying the correct inserts were confirmed by colony PCR and sequencing.

Table 2.9. TOPO cloning reaction mastermix

Reagent	Volume (μl)
Fresh PCR product (~ 80 ng)	0.5 – 4
Salt solution	1
Water	Add to a total volume of 5 μl
TOPO vector (10 ng/μl)	1
Final Volume	6

2.6.1.1.2. Colony PCR

To screen for positive colonies, PCR was performed using primers GagNot and Gag 1.5R and a thermostable DNA polymerase SuperTherm (Table 2.10). The master mix was added to a 96-well plate. A masterplate was created by spotting colonies on a specific area of the LB agar plate using a pipette tip and then touching that tip in a corresponding well containing PCR mastermix. The DNA and mastermix were placed in the thermal cycler which was programmed for 2 minutes at 94°C for initial denaturation, followed by 30 cycles of 30s at 94°C for denaturation, 30s at 53°C for annealing, 45s at 72°C for extension, and 2 minutes at 72°C for the final extension.

To identify positive PCRs containing positive colonies a 0.7% agarose gel was run at 100 V for 45 minutes. The gel was visualized under UV-light and bands of the correct size identified. Corresponding bacterial colonies on the master plate were grown up overnight in 5 ml LB-ampicillin (100 μg/ml) broth in a shaking incubator at 225 rpm.

Table 2.10. Colony PCR mastermix

	Final Concentration	μl / reaction
10X Buffer	1x	1
MgCl ₂	1.5mM	0.5
GagNot	0.2μM	0.2
Gag 1.5R	0.2μM	0.2
dNTP	0.08mM	0.8
SuperTherm	1.25U	0.03
Water		7.3
Final Reaction Volume		10.00

2.6.1.1.3. Plasmid Extraction

The following day, DNA from each clone was extracted from bacterial cultures using the QIAprep Spin miniprep kit (QIAGEN, Belgium) according to the manufacturer's protocol. The QIAprep kit comes with spin columns which have a silica membrane that binds up to 20 µg plasmid DNA. The bacterial cultures were spun down in a Hettich centrifuge (Hettich, Germany) at 5000 rpm for 10 minutes to pellet the bacteria. The supernatant was discarded and the bacteria were lysed with lysis buffer. This reaction was neutralized with neutralization buffer and centrifuged at 13 000 x *g* for 10 minutes to separate the supernatant containing bacterial DNA from the waste products. Lysed bacteria was added to the silica membrane of the QIAprep spin columns and centrifuged at 13 000 x *g* for 1 minute to allow for the DNA to be adsorbed onto the membrane. Wash buffer was added to the membrane and centrifuged at 13 000 x *g* to wash away impurities from the membrane. Pure DNA was eluted in 55 µl of elution buffer. The extracted plasmid DNA was quantified on the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, DE, USA). The clones were sequenced (see Chapter 2.3.4.) to identify those with the correct insert. Sequences generated were analysed on Sequencher 4.10.1 sequence analysis software (Gene Codes Corporation, Ann Arbor, MI USA). Clustal W in BioEdit sequence alignment editor was used for alignment of sequences. Phylogenetic trees were generated using Molecular Evolutionary Genetics Analysis (MEGA) 5.0 as a quality control to identify possible DNA contamination.

2.6.1.2. Cloning into p8.9NSX+ Expression Vector

2.6.1.2.1. Digestion of TOPO Clones and p8.9NSX+ Expression Vector

Clones were digested using restriction enzymes *Not* I and *Xho* I as shown in Figure 2.4. and Table 2.11. The reaction mixture was incubated at 37°C for 2hrs and heat inactivated

at 65°C for 15 minutes. To confirm successful restriction of products, all sample digests were run on a 0.7% agarose gel at 100V for 1.5hrs as shown in Figure 2.5. As a control an unrestricted plasmid (p8.9NSX+) was included.

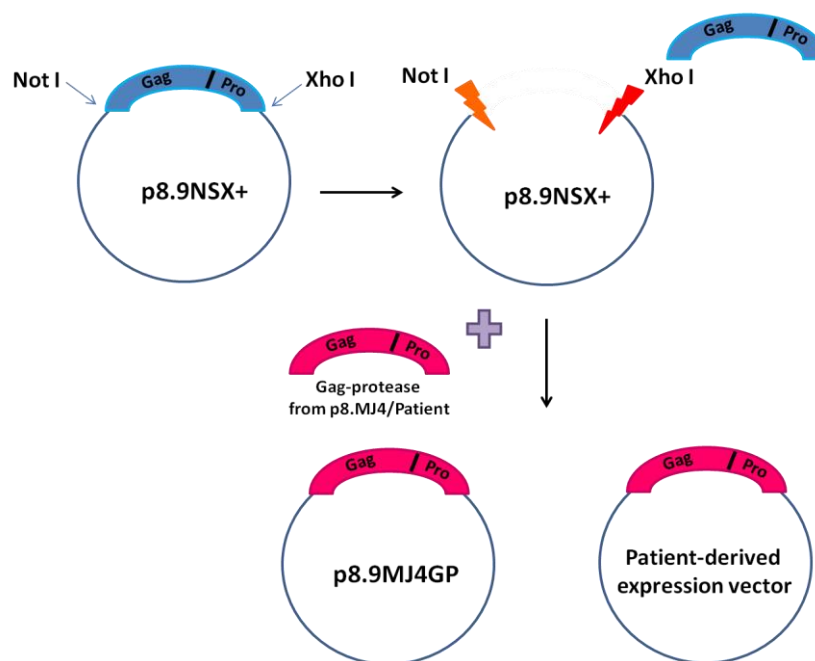


Figure 2.4. Cloning of patient-derived and p8.MJ4-derived *gag-protease* into the p8.9NSX+ expression vector

Restriction enzymes (Not I and Xho I) were used to digest p8.9NSX+ and allow for cloning of patient-derived (baseline and failure) and p8.MJ4 derived *gag-protease* resulting in a patient-derived expression vector and p8.9MJ4GP respectively. The p8.9MJ4GP vector was used as a control in each assay containing patient samples and was also used to calculate the assay cut-offs for the PIs tested.

Table 2.11. Digestion of TOPO clones and p8.9NSX+ expression vector

	Final Concentration	µl / reaction
TOPO patient clone	1 µg	X
p8.9NSX+ plasmid vector	1 µg	X
Buffer A		2
Not I	0.02	0.5
Xho I	0.02	0.5
Total		19

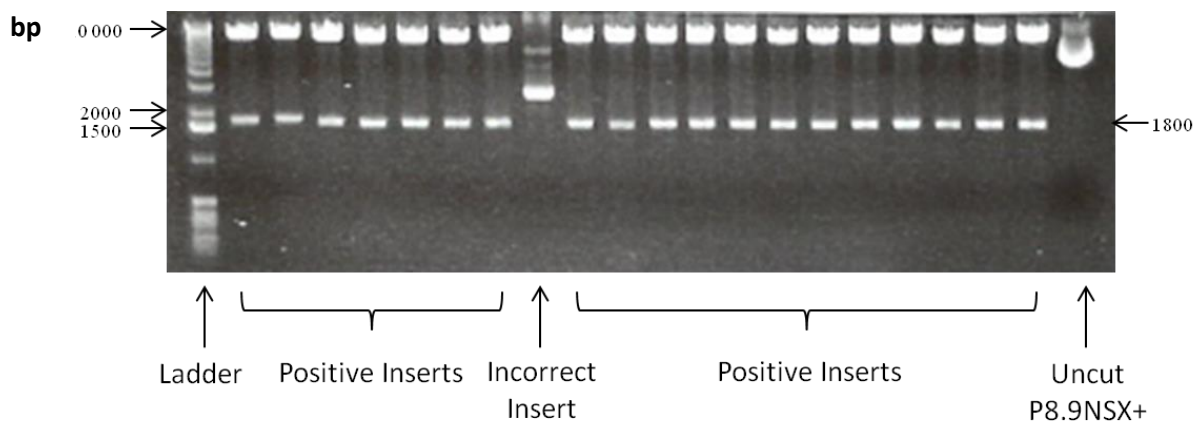


Figure 2.5. Picture of a gel showing the digested products from the TOPO clones

A DNA molecular weight marker X (0.07-12.2kbp) (Roche, Mannheim, Germany) was used to identify clones with the correct sized inserts (1.8 kb).

2.6.1.2.2. Gel purification of Insert from TOPO Vector

The insert bands of the patient-derived *gag-protease* and the vector band were cut out of the gel with brief exposure to ultraviolet (UV) light as to minimize DNA damage through thymine dimerization. The DNA products were gel purified using the PureLink Quick Gel Extraction kit (Life Technologies, Germany) according to the manufacturer's protocol. The kit allows for the removal of primers, dNTPs, enzymes and salts from the PCR product. Binding buffer and was added to the gel slice and heated on a heating block at 42 °C for 15 mins and vortexing every 5 minutes. This was to ensure complete dissolution of the DNA in the binding buffer. This sample was added to a spin column and centrifuged at 10 000 x *g* for 1 minute to allow the DNA to bind to the silica-based membrane. The membrane was then washed with Wash buffer at 10 000 x *g* for 1 minute. DNA products were eluted in 35 µl Elution buffer and quantified on the NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA).

2.6.1.2.3. Dephosphorylation of p8.9NSX+ Expression Vector

The Rapid DNA Dephos and Ligation kit (Roche, Basel, Switzerland) was used for dephosphorylation of the vector and ligation step. The rAPid Alkaline Phosphatase included in the kit, removes phosphate groups from the DNA thereby preventing vector self-ligation and reducing vector background when cloning. To dephosphorylate the p8.9NSX+ vector, 3µl Rapid Dephos (10X) Buffer and 1µl Rapid Dephos Enzyme (1 U/µl) was added to the digested vector. This mixture was incubated at 37°C for 30 minutes and heat-inactivated at 75°C for 2 minutes.

2.6.1.2.4. Ligation of Patient-derived *gag-protease* with p8.9NSX+ Expression Vector

For ligation of the 1.8kb *gag-protease* insert with the restricted 10.2kb p8.9NSX+ expression vector the ratio of vector-to-insert was 1 copy (114 ng): 3 copies (86 ng). This ratio was to increase the chances of the insert ligating with the vector and to prevent the vector from self-ligating. For efficient cloning, the total DNA amount did not exceed 200 ng. The Rapid DNA Dephos and Ligation kit (Roche, Basel, Switzerland) was used for ligation. DNA ligation involves creating a phosphodiester bond between the 3' hydroxyl group of one nucleotide and the 5' phosphate of another. The reagents used to make the ligation mastermix are shown in Table 2.12. The amount of vector and insert differed for each reaction according to their concentrations. T4 DNA Ligase which is included in the kit is used to ligate DNA fragments which have blunt ends. To each reaction 10µl T4 DNA ligation buffer and 1µl T4 DNA ligase was added. The ligation mixture was incubated at 25°C for 30 minutes and used directly for transformation or stored at -20°C.

Table 2.12. Ligation mastermix

	Final Concentration	µl / reaction
DNA dilution buffer	1x	2
Plasmid vector		<i>X</i>
<i>Gag-protease</i> insert		<i>X</i>
Water		3.7
Final Reaction Volume		10

The final concentration of the plasmid vector and insert was different for each patient but had a ratio of 1: 3 and did not exceed a total concentration of 200 ng.

2.6.1.2.5. Transformation into XL-Gold Ultracompetent *E.coli* Cells

For efficient transformation of the 12kb vector+insert XL10-Gold Ultracompetent cells *E.coli* cells (Stratagene, California) were used. The competent cells were thawed on ice till the last ice crystals disappeared. Two microlitres of β -Mercaptoethanol was added to 45µl of competent cells and incubated for 10 min on ice while flicking every 2 minutes to mix cells and β -Mercaptoethanol. Two microlitres of ligation mixture was added to the cells and left on ice for 30 min. The cells were heat shocked in a 42°C water bath for 30 seconds without shaking and then placed on ice for 2 minutes. Five hundred microlitres of pre-warmed S.O.C medium was added to the tubes and incubated in a 37°C shaker for 1hr at 225 rpm. Transformed bacterial cells were then pelleted at 4000 x *g* for 3 minutes. Four hundred and fifty microlitres of supernatant was removed and the pellet was resuspended in 100 µL of the remaining supernatant and plated on pre-warmed LB-ampicilin (100 µg/ml) agar plates. Plates were inverted and incubated at 37°C for ~16hrs. Clones carrying the correct inserts were confirmed by colony PCR, extraction of plasmid DNA and sequencing as described in Chapter 2.6.1.1.2.

2.6.2. Transfection of Patient-derived *gag-protease* Clones

Susceptibility of the patient-derived *gag-protease* genes to the different PIs was determined using a vector-based non-replicative phenotypic assay (Figure 2.6) was used as previously described (Parry et al., 2009, Gupta et al., 2010), with minor modifications. The expression vector p8.9NSX+ in which the *gag-protease* genes were inserted only contained HIV-1 *gag-pol* and none of the other HIV-1 proteins. Baseline and failure clones were transfected into HEK293T cells in 6-well plates. DNA transfection mixes were prepared with 300 ng of plasmid pMDG, 500 ng of plasmid pCSFLW and 300 ng of patient derived expression vector. The pMDG vector encodes the vesicular stomatitis virus G protein which associates with envelope-deficient plasmid DNA to enhance DNA transfection. The pCSFLW plasmid encodes the firefly luciferase reporter gene needed for measuring luciferase activity in the target cells.

To enhance transfection, 3.3 ug polyethylenimine (PEI) (Polyscience, Inc., Warrington, Pennsylvania, USA) was added to Dulbecco's Modified Eagles Medium (DMEM) (Gibco, New York) containing fetal bovine serum (FBS) and antibiotics (see Appendix I), gently swirled to mix and incubated for 2 minutes at room temperature. The DNA mix containing the vectors was added to the media drop by drop, swirled to mix and incubated at room temperature for 10 minutes. The transfection mix was then added to the 6-well plates at incubated at 37°C for 18 hours. Transfected cells were harvested and seeded in the presence of three-fold serial dilutions of protease inhibitors (drug plate). Fresh HEK293T cells were plated (1×10^6 cells/ 96-well plate) and incubated for 24 hours. Supernatants containing infectious virus-like particles were transferred from the drug plate to the corresponding wells of a 96-well cell culture plate that contained fresh uninfected 293T cells in complete DMEM medium.

The final concentration of PIs used is shown in Table 2.13. The degree of infection was determined by the expression of firefly luciferase in infected target cells at

48 hours post-infection. Bright-Glo Luciferase (Promega, CA, USA) was added to each well and incubated for 2 minutes prior to reading the luminescence on the Victor 3 Luminometer (Perkin Elmer, Massachusetts).

For each drug-virus combination, the 50% inhibitory concentration (IC_{50}) was calculated. The phenotypic susceptibility of patient samples at PI failure was expressed as a fold-change (FC) in IC_{50} , relative to that of the baseline virus. For the site-directed mutants, the phenotypic susceptibility was expressed as a FC in IC_{50} , relative to that of the control, p8.MJ4. Assay cut-offs are shown in Table 2.15. The lower FC cut-off value for each drug was determined using the 99th percentile of the average IC_{50} for p8.MJ4GP (control for patient samples) assessed in multiple repeat screens of each drug (Table 2.15). The cut-off values used in this study were not linked to clinical correlates or outcomes. The classifications susceptible, reduced susceptibility and resistant serve to rank the response of the PIs used in our assay. Examples of the different phenotypic classifications for patients are shown in Figure 2.10. The curve for a susceptible patient virus is characterised by a little or no change in IC_{50} compared to the baseline sample. The curves of the patient virus at failure are close to the baseline controls. Reduced susceptibility and resistant is characterised by the curve of the patient sample shifting to the right of the control indicating that a higher concentration of drug is needed to inhibit the virus. Fold changes above the upper cut-off distinguishes resistant virus from those showing reduced susceptibility. The PIs (amprenavir, atazanavir, darunavir, lopinavir, ritonavir and saquinavir) used in this study were obtained from the AIDS Reagent Program, Division of AIDS, NIAID, NIH (<https://www.aidsreagent.org/>).

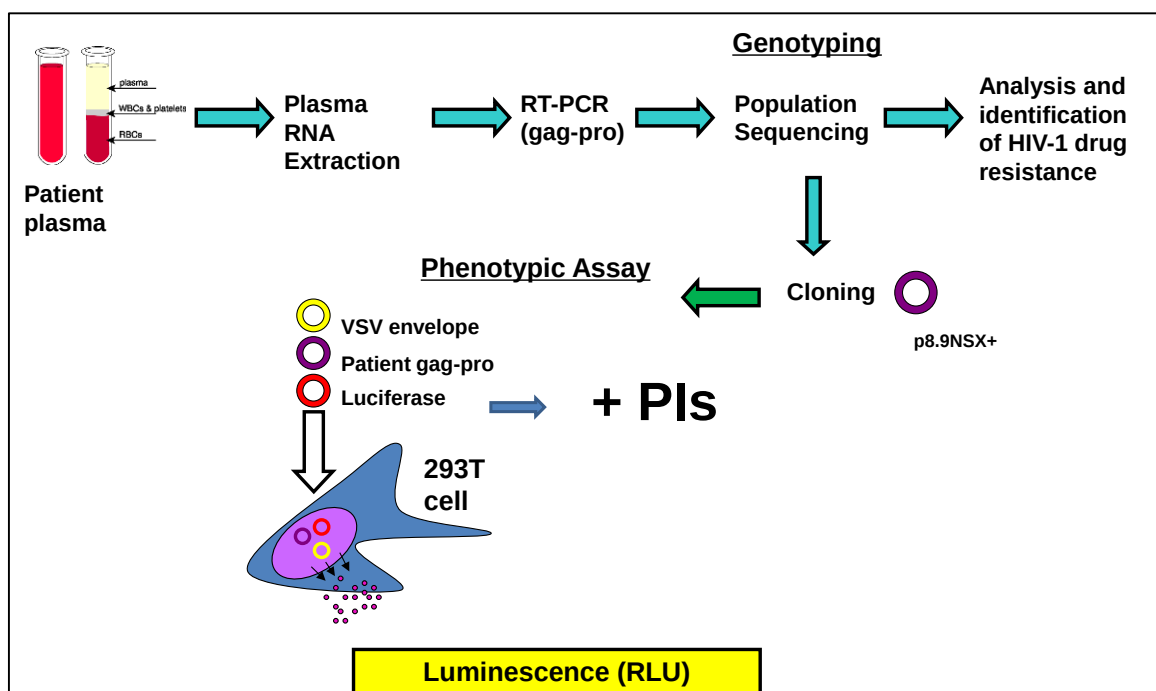


Figure 2.6. Illustration of the genotyping and phenotyping of patient-derived *gag-protease* genes into p8.9NSX+

Table 2.13. Final concentration of protease inhibitors

Drug	Final drug concentration
APV	1000nM
ATV	100nM
DRV	200nM
LPV	200nM
RTV	200nM
SQV	100nM

Table 2.14. Phenotypic assay fold change cut-offs for patient-derived *gag-protease*

	Phenotypic classification of patient-derived <i>Gag-Protease</i>		
	Susceptible	Reduced Susceptibility	Resistant
LPV	< 3.8	3.8 – 10.0	>10
RTV	< 3.2	3.2 – 10.0	>10
APV	< 4.0	4.0 – 10.0	>10
ATV	< 1.6	1.6 – 10.0	>10
DRV	< 4.9	4.9 – 10.0	>10
SQV	< 3.4	3.4 – 10.0	>10

Abbreviations: LPV, lopinavir; RTV, ritonavir; APV, amprenavir, ATV, atazanavir; DRV, darunavir; SQV, saquinavir

Table 2.15. Calculation of lower cut-offs for each drug for patient samples

	LPV	RTV	APV	ATV	DRV	SQV
Average	0.0009056	0.0055222	0.0046857	0.0007964	0.00008	0.0004457
Standev	0.0003556	0.0022691	0.0026842	0.0001043	0.00004	0.0002168
95% Confidence interv.	0.0001643	0.0010483	0.0019884	0.00005	0.00002	0.0001606
Number (n=)	18	18	7	12	11	7
Minimum	0.0004	0.0023	0.0023	0.00055	0.00003	0.00023
1st Quartile (Q1)	0.000625	0.003675	0.00275	0.0007375	0.00005	0.00029
Median	0.0008	0.00595	0.0033	0.00082	0.00008	0.00036
3rd Quartile (Q3)	0.001225	0.007375	0.00605	0.0008725	0.00012	0.00058
Maximum	0.0015	0.0088	0.0096	0.00092	0.00017	0.00079
1st Percentile	0.000417	0.0023	0.002324	0.0005676	0.00003	0.0002306
99th Percentile	0.0015	0.008732	0.009408	0.0009186	0.000166	0.0007834
Lower FC cut-off	3.797122	3.196522	4.048193	1.61834	4.868035	3.397225

2.7. Phenotypic Analysis of *gag* and *protease* Mutants

To investigate the individual contribution of mutations in *protease* and *gag*, site-directed mutants were created and tested against the protease inhibitors in a vector backbone. This allows for the impact of an individual mutation and drug susceptibility to be tested.

2.7.1. Site-directed Mutagenesis

An expression vector (p8.MJ4) containing the *gag-pol* region of a subtype C reference strain (MJ4, GenBank: AF321523.1) was used for the construction of site-directed mutants (Ndung'u et al., 2001). Primers used for the generation of mutants are listed in Appendix II. *Protease* mutants created and tested were based on those shown to cause high-level resistance to the different PIs from the Stanford drug resistance database (Figure 2.7.). *Gag* mutations were selected from patient samples based on their known or potential involvement in PI resistance. Additional *gag* non-CS mutants, which were shown to develop under positive selection pressure were also created and tested.

	M46I	I47V	G48V	I54V	V82A	I84V
LPV						
RTV						
APV						
ATV						
DRV						
SQV						

Figure 2.7. Protease mutations chosen based on the Stanford drug resistance database

Protease mutations were created by site directed mutagenesis as single mutants or combination of multiple mutants. Red refers to mutations which have been associated with high levels of resistance to the relevant PI. Orange refers to mutations which contribute to resistance in combination with other PI-resistance mutations to the relevant PIs. Green refers to mutations which do not have an effect on resistance to the relevant PI.

The primers were designed using the QuikChange Primer Design online tool:

(<http://www.genomics.agilent.com/primerDesignProgram.jsp>) and were ordered from

Integrated DNA Technologies® (Coralville, Iowa, USA). Mutations were selected from

patient samples based on their known or potential involvement in PI resistance. The

QuickChange® II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, California,

USA) was used according to the manufacturer's protocol (Figure 2.8) to create the single

site-directed mutants in a subtype C plasmid p8.MJ4. The site-directed mutagenesis

reaction uses a *PfuUltra* high-fidelity DNA polymerase for replication of both plasmid

strands. During the reaction, the primers containing the desired mutation are extended by

the DNA polymerase to generate a plasmid containing staggered nicks. The reagents of

the mastermix are shown in Table 2.15. The thermal cycler was programmed for 1 minute

at 95°C for initial denaturation, followed by 18 cycles of 50s at 95°C for denaturation, 50

s at 60°C for annealing, 12 minutes and 30s at 68°C for extension, and 7 minutes at 68°C

for the final extension. The product is then treated with *Dpn* I endonuclease for 1 hour at

37°C to digest the parental DNA template and select for the mutation containing synthesized DNA. The vector DNA was then transformed into XL10-Gold ultracompetent cells as mention in section 2.6.1.2.5. Mutants with multiple mutations were created by successive rounds of site-directed mutagenesis. Clones were sequenced (section 2.3.4.) to confirm the presence of the correct mutant.

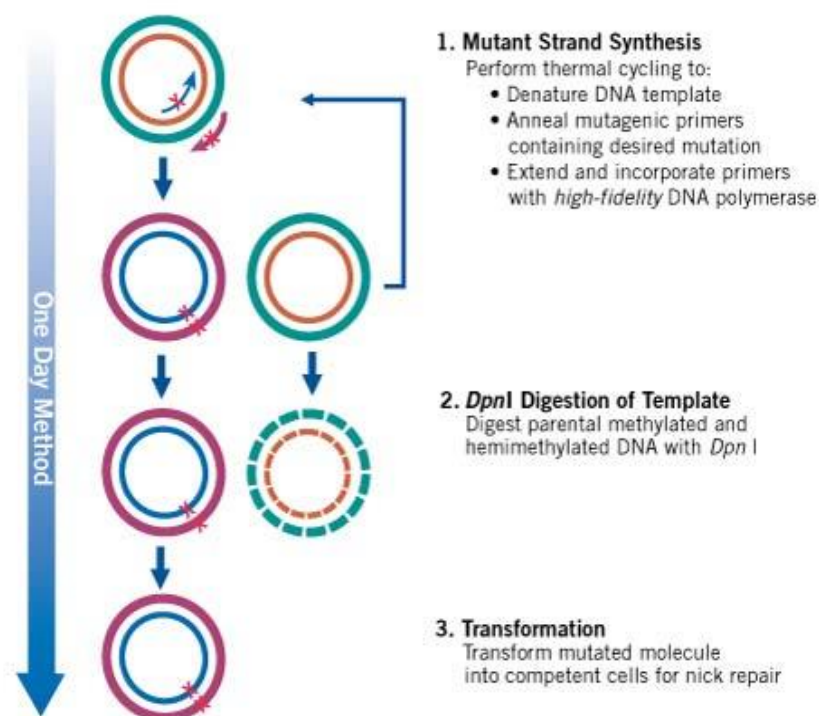


Figure 2.8. Overview of the QuikChange II XL site-directed mutagenesis method.
Taken from the QuikChange II XL site-directed mutagenesis kit insert.

Table 2.16. Site-directed mutagenesis mastermix

	Final Concentration	µl / reaction
10X Buffer	1x	5
dsDNA template	10 ng	2
Oligonucleotide Primer 1	125 ng	1.25
Oligonucleotide Primer 2	125 ng	1.25
dNTP		1
QuikSolution		3
Water	Make up to 50 µl	36.5
Total		50.00

2.7.2. Transfection of Site-directed Mutants

The correct clones were then tested against the PIs in the phenotypic assay described in section 2.6.2. The phenotypic susceptibility of mutants was expressed as a fold-change (FC) in IC_{50} , relative to that of p8.MJ4. Assay cut-offs are shown in Table 2.17. Calculation of the FC for each drug is shown in Table 2.18.

Table 2.17. Phenotypic assay fold change cut-offs for site-directed mutants

Site Directed Mutants			
	S	RS	R
LPV	< 3.8	3.8 – 10.0	> 10
RTV	< 3.2	3.2 – 10.0	> 10
APV	< 2.0	2.0 – 10.0	> 10
ATV	< 3.1	3.1 – 10.0	> 10
DRV	< 1.2	1.2 – 10.0	> 10
SQV	< 1.3	1.3 – 10.0	> 10

Abbreviations: S, susceptible; RS, reduced susceptibility; R, resistant; LPV, lopinavir; RTV, ritonavir; APV, amprenavir; ATV, atazanavir; DRV, darunavir; SQV, saquinavir

Table 2.18. Calculation of lower cut-offs for site-directed mutants

	LPV	RTV	APV	ATV	DRV	SQV
Average	0.00040	0.00285	0.00222	0.00058	0.00008	0.00032
Standev	0.00020	0.00102	0.00052	0.00023	0.00001	0.00003
95 % Confidence interv.	0.00008	0.000431	0.00026	0.00011	0.00001	0.00002
Number (n=)	22	22	15	15	9	12
Minimum	0.00018	0.00141	0.00141	0.00024	0.00005	0.00025
1st Quartile (Q1)	0.00024	0.00226	0.00183	0.00037	0.00008	0.00029
Median	0.00031	0.00265	0.00213	0.00063	0.00008	0.00031
3rd Quartile (Q3)	0.00053	0.00381	0.00269	0.00078	0.00008	0.00034
Maximum	0.00086	0.00482	0.00296	0.00100	0.00009	0.00038
1st Percentile	0.00019	0.00148	0.00142	0.00024	0.00007	0.00025
99th Percentile	0.00075	0.00481	0.00295	0.00079	0.00009	0.00035
Lower FC cut-off	3.88761	3.23450	2.07716	3.14507	1.19942	1.29363

2.8. Analysis of Phenotypic Results

Examples of phenotypic data (susceptible, reduced susceptibility and resistant) for patient samples and site-directed mutants are shown in Figure 2.9 and 2.10 respectively. For patient samples the IC_{50} of the sample at failure was compared to the IC_{50} of the matched

baseline sample to calculate the fold change (FC). p8.MJ4GP was included in all phenotypic assays containing patient samples as a wild-type reference. For the site-directed mutants, the IC_{50} of each mutant was compared to the IC_{50} of the p.MJ4 plasmid.

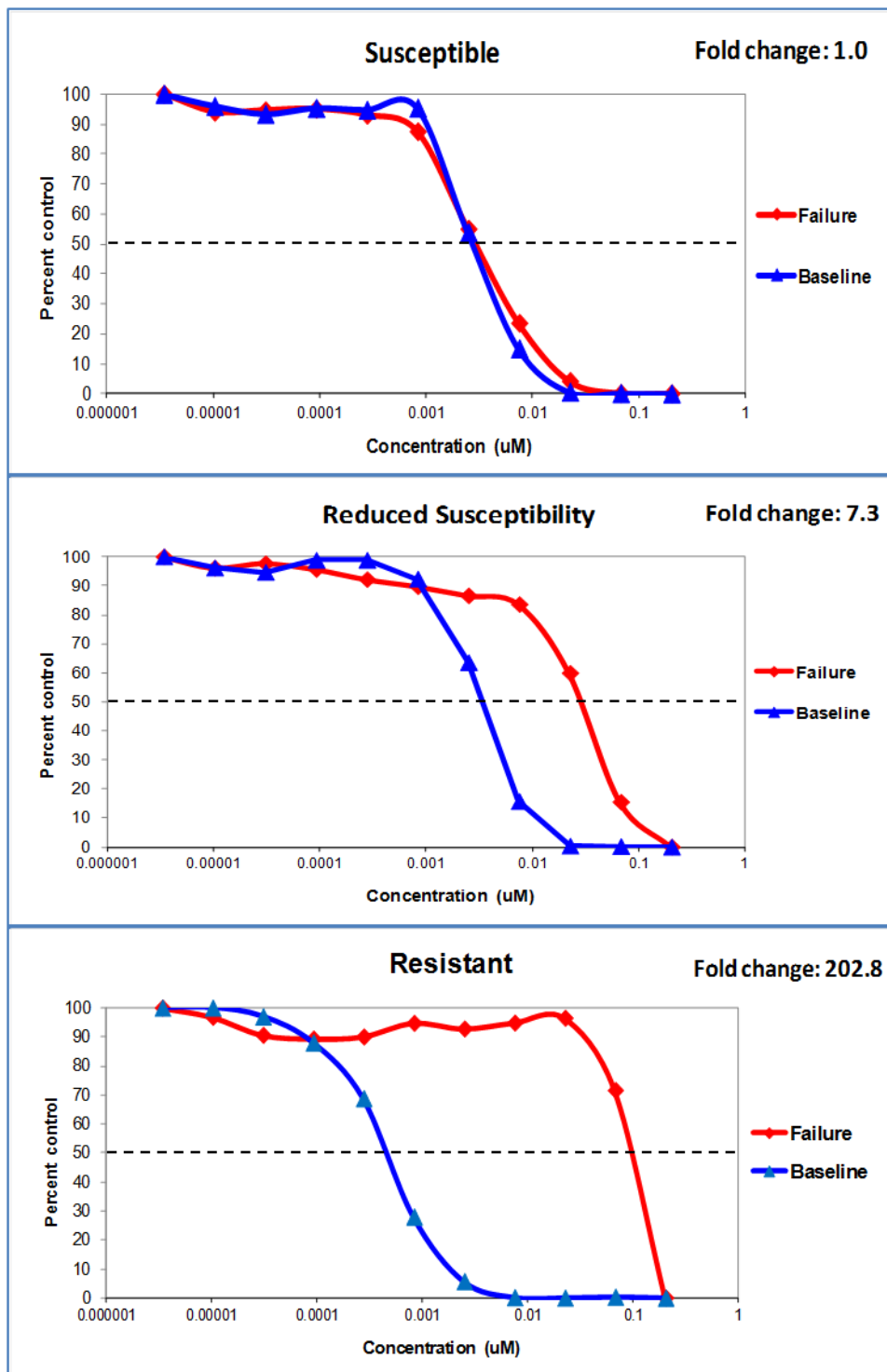


Figure 2.9. Phenotypic levels of susceptibility among patient samples to LPV
 The phenotypic susceptibility of each patient-derived virus at failure (red) was determined relative to the baseline (blue) sample. As the slope of the curve of the failing sample shifts further to the right, more drug is required to inhibit the virus, thereby resulting in increased resistance.

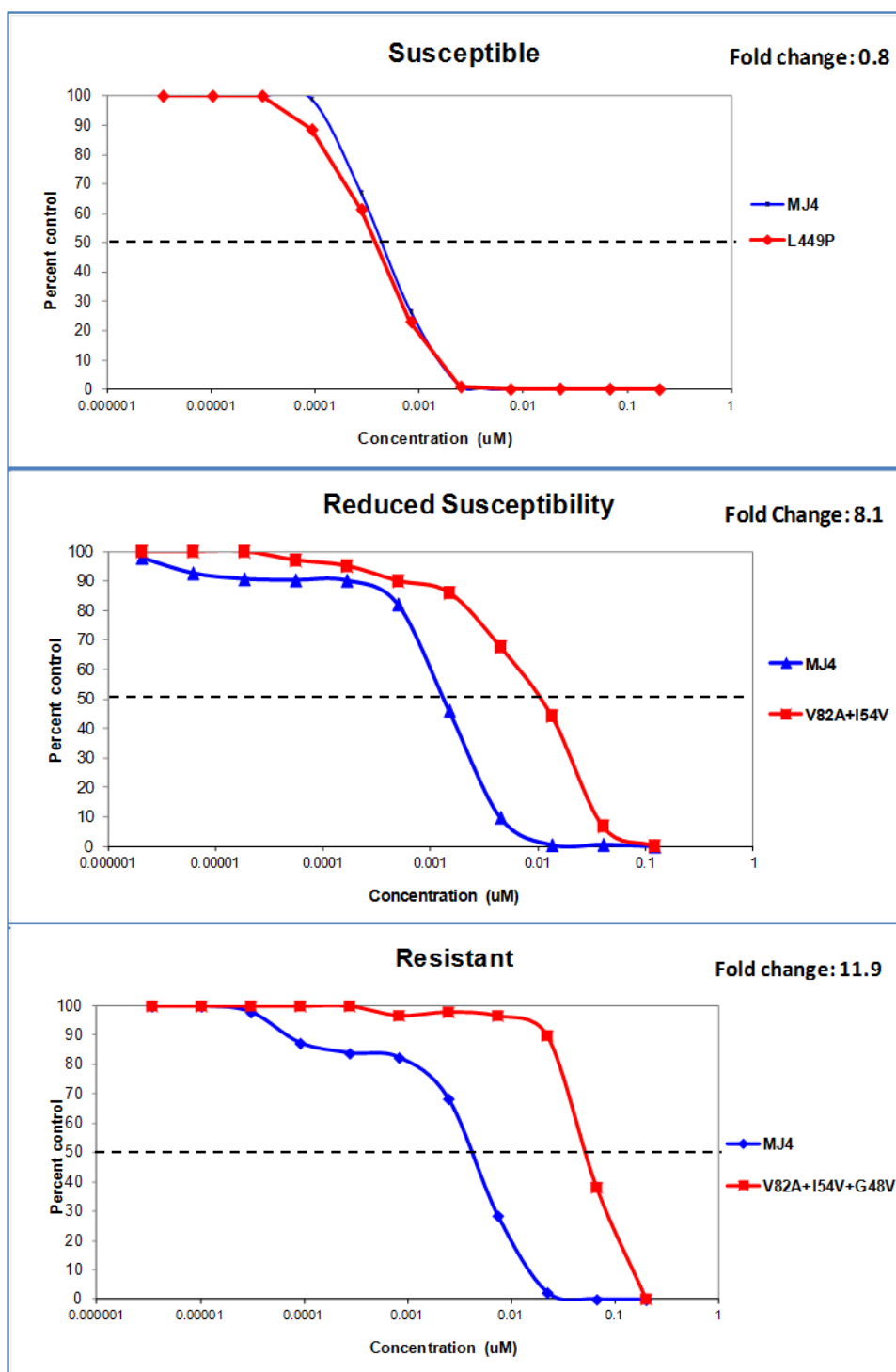


Figure 2.10. Phenotypic levels of susceptibility of site-directed mutants to LPV

The phenotypic susceptibility of each mutant (red) was determined relative to p.MJ4 (blue). As the slope of the curve of the failing sample shifts further to the right, more drug is required to inhibit the virus, thereby resulting in increased resistance.

Chapter Three

Results

Chapter 3.1.

Genetic Changes in HIV-1 *gag-protease* Associated with Protease Inhibitor-Based Therapy Failure in Paediatric Patients

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TOTAL WORD COUNT: 3346

3.1.1. Abstract

Introduction

Studies have shown a low frequency of *protease* mutations in patients failing protease-inhibitor (PI)-based therapy. Recent studies have identified mutations in *gag* as an alternate pathway for PI drug resistance in subtype B viruses.

Materials and Methods

We genotyped the *gag* and *protease* genes from 20 HIV-1 subtype C infected paediatric patients failing a PI-based regimen.

Results

Major *protease* resistance mutations (M46I, I54V and V82A) were identified in 8 (40%) patients, as well as *gag* cleavage site (CS) mutations (at codons 373, 374, 378, 428, 431, 449, 451 and 453) in 9 (45%) patients. Four of these *gag* non-CS mutations occurred in the absence of major *protease* mutations at PI failure. In addition, amino acid changes at *gag* non-CS were noted, some of which were under HLA/KIR immune-mediated pressure and/or drug selection pressure. Of these, 6 sites (15, 54, 69, 90, 339 and 386) were under positive selection pressure and could not be accounted for.

Conclusion

Changes in *gag* during PI failure suggest drug selection pressure and warrant further investigation of the *gag* gene and its role in PI failure in HIV-1 subtype C.

3.1.2. Introduction

South Africa has an estimated 410 000 children infected with HIV-1 of whom 58% were eligible and receiving antiretroviral treatment by the end of 2012 (UNAIDS, 2013). The national treatment guidelines follow World Health Organization recommendations to treat HIV-infected infants <3 years of age with a protease inhibitor (PI)-based regimen. This is due to the high rates of non-nucleoside reverse transcriptase inhibitor (NNRTI) resistance in infants treated for prevention-of-mother-to-child transmission (PMTCT) and the effectiveness of using a first-line PI-based regimen to reduce mortality and disease progression in children (Palumbo et al., 2010, Violari et al., 2012).

Lopinavir co-formulated with ritonavir (LPV/r) is used as part of a triple antiretroviral treatment regimen for infants >6 months of age. Prior to 2008, for infants <6 months of age or children receiving rifampicin for co-treatment of tuberculosis, ritonavir (RTV) alone was used. LPV/r was subsequently licensed for use in infants <6 months, and the boosting of LPV/r with additional RTV or double-dosing of LPV/r when using rifampicin introduced (Van Zyl et al., 2013).

PI drug resistance is mediated by an accumulation of major mutations in the *protease* gene resulting in reduced binding of the inhibitor to the protease enzyme (Lee et al., 2012). In addition, minor PI mutations, which have little or no effect on PI susceptibility, contribute to reduced susceptibility in combination with major mutations (Rhee et al., 2003). Studies have shown that mutations in *gag* cleavage sites (CS) also contribute to PI resistance development

(Fun et al., 2012, Maguire et al., 2002, Dam et al., 2009, van Maarseveen et al., 2012). HIV-1 Protease recognises and cleaves the Gag and Gag-Pol polyproteins to release the structural components: matrix (MA), capsid (CA), p6, nucleocapsid (NC), enzymes protease (PR), reverse transcriptase (RT), RNase H, integrase (IN), the small polypeptides p1 and p2, as well as transframe protein (TFP) (Pettit et al., 1994). Cleavage sites in *gag* comprise five amino acids flanking either side of the scissile bond, with position 1 (P1) located immediately upstream and position 1 prime (P1') located immediately downstream of the cleavage point (de Oliveira et al., 2003). *Gag* CS mutations such as A431V (CS NC/p1), L449P, and P453L (CS p1/p6^{Gag}) have been shown to co-evolve with the HIV-1 *protease* gene under drug selection pressure (Maguire et al., 2002, Dam et al., 2009, Fun et al., 2012). While these sites are the most commonly described, substitutions at all CS have been observed at PI failure (Fun et al., 2012, Maguire et al., 2002, Verheyen et al., 2006). In addition, studies have shown that certain non-CS mutations in *gag* can contribute to reduced susceptibility to PIs in the presence of major *protease* mutations *in vitro* (Adamson, 2012, Gatanaga et al., 2002). However the mechanism of resistance is not well understood since these mutations occur outside of the functionally related regions.

There is limited genetic data on paediatric patients infected with HIV-1 subtype C failing PI-based therapy, with no published data on *gag*. Given the large numbers of children initiating LPV/r -based regimens, identifying reasons for therapy failure is increasingly important. Recent studies in HIV-1 subtype C infected children and adults have shown a lack of *protease* mutations at failure, possibly suggesting alternate routes of PI resistance (Wallis et al., 2011, van Zyl et al., 2009). In this study, the *protease* and *gag* genes of paediatric patients were sequenced at baseline and at the time of virological failure to identify changes associated with PI failure taking into account both drug and immune selection pressures.

3.1.3. Materials And Methods

3.1.3.1. Study cohort

A retrospective cohort study was undertaken on 20 matched baseline and post-treatment plasma samples from children less than 24 months of age and failing PI-based therapy. These children were initiated on treatment and followed as part of the pre-randomization screening phase of a randomized clinical trial between 2005 and 2009. Because these children failed their initial LPV/r-based therapy they were not eligible for randomization as part of the trial which was designed to assess the feasibility of switching to an NNRTI-based regimen following one year of suppressive PI-based therapy (Coovadia et al., 2010, Taylor et al., 2011, Reitz et al., 2010). Children over 6 months of age were treated with lamivudine (3TC), stavudine (d4T) and ritonavir-boosted lopinavir (LPV/r). Those younger than 6 months of age and those receiving rifampicin-based regimens for treatment of tuberculosis or Bacille Calmette Guerin (BCG) disease, received ritonavir (RTV) until reaching 6 months of age and/or completing TB treatment, at which point they were initiated on or switched back to LPV/r. For children who suppressed and then rebounded, genotyping was done on the first sample where an elevated viral load was observed. For children who did not suppress, a sample within the first 3 to 6 months of treatment was selected. Whole blood specimens were collected at baseline (pre-treatment) and during regimen failure, defined as not having achieved a HIV-1 plasma RNA < 1000 copies/ml by 52 weeks on treatment. This study was approved by the institutional review boards of Columbia University and the University of the Witwatersrand.

3.1.3.2. Genotypic analysis of *gag-protease*

Viral RNA was extracted from plasma at baseline and failure using the QIAamp Viral RNA Mini Kit (QIAGEN, Belgium) and reverse transcribed using Thermoscript RT-PCR kit (Invitrogen, CA, USA). A 1.8 kb *gag-protease* fragment spanning HXB2 positions 792 to 2549 was amplified by nested PCR using Expand High Fidelity PCR kit (Roche Applied Science, Basel, Switzerland) as previously described. (Sutherland et al., 2013) Primers BKT03 (5' CGCAGGACTCGGCTTGC 3') and ProOutR (5' TTGGGCCATCCATTCCTGG 3') were used for first round PCR and primers GagNot+ (5'GCGGCGGCCGCAAGGAGAGAGATGGGTGCG3') and ProXhoR2 (5'CTGGTACAGTCTCGAGRGGACTRATKGG 3') for nested PCR. Population-based sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) on an ABI3130 Genetic Analyser (Applied Biosystems, Foster City, CA). Patient sequences were aligned using ClustalW in BioEdit version 7.0. (Hall, 1999) Minor and major *protease* mutations were defined according to the Stanford University Drug Resistance Database (<http://hivdb.stanford.edu/DR/PIResiNote.html>). Genetic changes in *gag* and *protease* were defined as those which developed at regimen failure compared to the baseline sequence, excluding those which reverted to the subtype C consensus sequence. Subtype C consensus sequence was downloaded from the Los Alamos Sequence Database (<http://www.hiv.lanl.gov/content/sequence/NEWALIGN/align.html>).

3.1.3.3. Prediction of sites selected by HLA- and KIR-mediated immune pressure

HLA-associated polymorphisms in *gag* were predicted from previously published mutation maps which presented the locations of common escape mutations in HIV-1 subtype B (Brumme et al., 2009). Briefly, the q-value threshold used for constructing the immune

escape maps was $q \leq 0.05$, meaning that only a 5% false-positive proportion was expected among associations displayed on the maps. Sites in *gag* selected by KIR-mediated immune pressure were identified as published (Alter et al., 2011).

3.1.3.4. Positive Selection Analysis

The fixed effects likelihood (FEL) test was performed using Datamonkey (Kosakovsky Pond and Frost, 2005) to test for selection pressure and estimate the ratio of synonymous to non-synonymous evolutionary changes (dN/dS). Calculations were performed for the entire dataset containing 20 *gag* baseline and failure nucleotide sequences. FEL estimates the probability of obtaining the dN and dS values under a neutral mode, with lower P-values indicating rejection of neutrality in favour of positive selection. Positions with a dN/dS ratio >1 were considered to be under positive selection pressure.

3.1.4. Results

3.1.4.1. Clinical characteristics of study population

Of the 20 children included in this study, 7 (35%) received both LPV/r and RTV while 7 (35%) received RTV as the only protease inhibitor and 6 (30%) were treated with LPV/r only. In addition to the PI, all children received two NRTIs (d4T and 3TC) (Table 3.1.1). The length of RTV-only treatment ranged from 27- 252 days. Of the 14 children that at some point received RTV as a single PI, 10 were co-treated for mycobacterial disease whilst 4 were <6 months of age. The mean viral load and CD4 count at time of measurement of drug resistance was 103, 880 copies/ml (range 1,240 - 750,000) and 28.4% (range 9.6 - 49.6) respectively. Of the 20 children, 2 (10%) had a CD4 count of $\leq 14\%$.

3.1.4.2. Genotypic analysis of *protease* and *gag* mutations at PI failure

Eight children (40%) had detectable major PI resistance mutations at failure, with V82A (n=8, 40%), I54V (n=4, 20%) and M46I (n=3, 15%) identified as the only major *protease* mutations (Figure 3.1.1 and Table 3.1.2). All patients who had at least one major PI mutation had received RTV-only therapy at some point, while patients who received LPV/r only did not have any major PI resistance mutations. Patients who developed ≥ 2 major protease mutations were more likely to have been on RTV therapy for a longer period of time. In addition, several minor PI mutations and polymorphisms (L10I, S12T, K20R, L33F, K43E, D60E, Q61H/E, L63P/T, K70R and N83S) were detected in both LPV/r- and RTV-treated patients.

When analysing the 20 patients at baseline and failing PI therapy, 10 showed no changes in *protease* after PI failure (highlighted in gray in Table 3.1.2), and six of these had received LPV/r exclusively. The majority of these patients had pre-existing baseline changes in the entire *protease* region, relative to consensus C sequence, that were also present following PI failure.

Nine of 20 patients had detectable *gag* CS mutations at failure, 5 of whom also had *protease* major mutations (Figure 3.1.2). HIV-1 drug resistance associated CS mutations A431V (NC/p1), L449P and P453L (both in p1/p6^{Gag}) were found in 4 of the 9 patients. The A431V mutation was selected in patients numbered 1 and 10 in the presence of major PI mutations I54V and V82A. Patients 11 and 20 had no major PI mutations, but both had mutations L449P and/or P453L. The most variable cleavage site was p2/NC where 4/9 patients had at least one mutation at this site at failure (positions N373A, T374A/S, M378A). Other CS mutations occurred at NC/p1 (position E428D) and p1/p6^{Gag} (position N451S). The most conserved cleavage sites were MA/CA, CA/p2, NC/TFP and TFP/P6^{pol} where no changes were observed.

Changes were also detected at *gag* non-CS at failure that differed from the consensus C. These changes were found in the majority of patients (17/20) and occurred mainly in the matrix and capsid regions in the N-terminus of *gag* (Supplementary Table 3.1.3).

3.1.4.3. Predicted HLA and KIR immune escape mutations in *gag*

Previously published immune escape maps were used to determine if some of the identified changes in *gag* that developed at failure were due to immune pressure. Seven of 20 patients had changes at the failure time point relative to baseline, at sites shown to be selected by known HLA A, B and C alleles reported to occur in South African Black and Caucasian populations (Table 3.1.4). In total, 8 sites were predicted to be associated with HLA or KIR-mediated immune pressure (Figure 3.1.3). One site at codon 374 was also a *gag* CS (p2/NC) mutation and another at codon 79 was at a *gag* non-CS that is associated with PI exposure (Parry et al., 2011). The E93D KIR-associated polymorphism in *gag* was identified in one patient (patient 8, Figure 3.1.3) and has been associated with HLA-mediated immune pressure (Alter et al., 2011, Brumme et al., 2009).

3.1.4.4. Positive selection in *gag*

A total of 53 amino acid residues were identified in *gag* that had changed between baseline and failure among these 20 patients (Figure 3.1.3). To determine which of these changes were under positive selection pressure following PI failure, a dN/dS analysis on the matched baseline and failing sample was performed (Table 3.1.5). A total of 7 sites in *gag* were found to be under significant selection pressure (Figure 3.1.3). Position 62, which has previously been reported to be associated with PI exposure, (Koh et al., 2009) had the highest dN/dS ratio of 6.033 ($p = 0.001$) and was found in 4 patients. The remaining 6 positively selected sites (positions 15, 54, 69, 90, 339 and 386) have not previously been associated with PI exposure nor predicted to be HLA/KIR. Two additional sites in *gag* have been reported to be associated with PI failure, (Parry et al., 2011, Gatanaga et al., 2002) including the HLA/KIR-selected position 79, but were not found to be positively selected in this analysis. In total, 23

of the 53 changes identified in *gag* following PI failure showed some evidence of selection pressure.

3.1.5. Discussion

This is the first study to describe the genotypic changes in both the *gag* and *protease* genes in HIV-1 subtype C paediatric patients failing PI-based therapy. The study showed that virologic failure during PI exposure may have led to the selection of *protease* resistance mutations as well as *gag* CS and non-CS mutations. Only patients on failing RTV regimens were observed to have developed all the major PI mutations and a majority of *gag* CS mutations. Mutations at *gag* CS were found in some cases to occur in the absence of major *protease* mutations implicating them as potentially resistance-conferring. Furthermore, sites in *gag* non-CS were found to be under various selection pressures following PI failure. Collectively these data warrant further investigation of the *gag* gene in contributing to PI drug resistance.

In this study, all PI resistance associated mutations were selected in patients who had received RTV compared to those treated with LPV/r exclusively, as previously reported (Taylor et al., 2011). Similar to previously published data, the major PI mutations detected at failure were M46I, I54V and V82A, all of which are well described as causing high-level resistance to multiple PIs (Johnson et al., 2011). Of interest was that nearly half of the patients in this study acquired changes at *gag* CS at failure. *Gag* and *protease* function as a single unit with enzyme and substrate co-evolving to optimise viral replication (Parry et al., 2009). Mutations at the *gag* CS A431V, L449P and P453L have been shown to contribute to protease inhibitor resistance (Dam et al., 2009, Maguire et al., 2002). A431V has been found to occur in combination with the *protease* mutation V82A and can be selected as compensatory or as a direct protease inhibitor mutation in the absence of mutations in

protease (Dam et al., 2009). The CS mutation L449P has been directly associated with exposure to ritonavir, indinavir, fosamprenavir, nelfinavir, saquinavir and atazanavir, while P453L has been found to enhance resistance in the presence of *protease* mutations I50V and I84V *in vitro* (Ali et al., 2010, Maguire et al., 2002, Fun et al., 2012). In this study these well described *gag*-CS mutations were found in 4 patients, 2 in the absence of *protease* mutations, and it is likely that they contributed to PI failure in these patients. In addition, 5 patients had *gag*-CS mutations that are less well defined, mainly at p2/NC, which is a more variable CS (de Oliveira et al., 2003). In some cases these also occurred in the absence of *protease* mutations. Whether these *gag* CS changes contributed to LPV/r and RTV failure in these patients is unknown and would require further phenotypic testing.

A large number of changes at *gag* non-CS were also detected at failure in this cohort. Eight sites were predicted to be under HLA/KIR-mediated immune pressure suggesting that they may be cytotoxic CD8⁺ T lymphocyte escape mutations (Brumme et al., 2009). One of these, codon 79, has previously been associated with PI exposure thus suggesting dual pressure from antiretroviral drugs and the immune system (Parry et al., 2011). HLA alleles associated with these predicted sites have all been identified in the South African population (Paximadis et al., 2012). However, it is important to note that the immune escape maps used to determine HLA-associated changes were based on HIV-1 subtype B *gag* sequences. Therefore this data may underestimate the sites associated with HLA immune pressure in subtype C patients. Only one KIR-associated polymorphism at position 93 in *gag* was identified in one patient suggesting this was not a major contributor to the *gag* changes. However, a more in-depth analysis involving high resolution HLA and KIR typing is required to determine whether these alleles were present in these patients and selected these mutations.

Nevertheless, this prediction analysis does suggest that some of the *gag* non-CS changes seen in this cohort are the result of immune selection pressures.

Positive selection analysis on the *gag* region identified seven sites to be under selection pressure, one of which was previously shown to be associated with PI exposure (position 62) (Koh et al., 2009). The remaining six sites under positive selection pressure occurred in 6 patients, 3 of whom developed *gag* CS changes at failure and one who had a pre-existing CS change from baseline. Therefore, these changes may be indicative of adaptive change possibly offering a fitness advantage and warrant further study. Of the 53 changes identified in this study, 23 could be accounted for by various selection pressures. The remaining thirty changes occurred mainly in the conserved matrix and capsid regions of *gag* and as such may have a function which is yet to be defined.

The availability of baseline and failure samples provides a robust analysis of drug resistance in patients failing treatment. However, the use of population sequencing may limit the detection of minority variants in *gag* already present at baseline. Furthermore, due to the small sample size, caution should be taken in generalising the findings to patients on RTV and/or LPV/r treatment. Almost half of the patients in this study failed RTV and LPV/r treatment without any changes in *protease* or *gag* CS. Poor adherence and sub-optimal drug levels in patients presenting with wild-type *protease* and *gag* cannot be excluded, but this data was not available. A recent study identified changes in gp41 of the *envelope* gene as an alternate route of PI resistance suggesting that sites outside of *protease* and *gag* may also be involved. (Rabi et al., 2013) Others have suggested that due to the short half life of PIs, there is a limited mutation selection window such that wild-type virus emerges faster than mutant virus resulting in restricted evolution of drug resistance. (Rosenbloom et al., 2012) Thus

further studies are needed to determine the reasons why patients fail PIs in the absence of known resistance mutations. Nevertheless, the availability of pre-and post- treatment samples allowed us to identify and correlate *gag* mutations selected with PI failure in the presence or absence of *protease* mutations in a sub-set of patients. This data suggests that it may be beneficial to include the *gag* gene when screening for protease inhibitor resistance. Further studies on the effects of *gag* mutations on protease inhibitor resistance, and development of drug resistance interpretation algorithms for both *gag* and *protease*, may prove beneficial.

3.1.6. Acknowledgements

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3.1.7. Sequence Data

Sequences used in this study correspond to Genbank accession numbers: KP056164-KP056203.

Table 3.1.1. Clinical characteristics and treatment information of HIV-1 infected infants and young children failing protease-inhibitor based antiretroviral therapy

Pt. No.	Time on Treatment (days)	Treatment ¹	Reason for RTV ²	Viral Load at Resistance Test	CD4%
1	365	RTV +LPV/r	< 6 months	7, 980	46.6
2	363	RTV +LPV/r	TB	48, 400	22.9
3	252	RTV +LPV/r	TB	17, 800	17.0
4	251	RTV +LPV/r	TB	28, 200	45.8
5	252	RTV +LPV/r	TB	15, 000	16.6
6	367	RTV +LPV/r	< 6 months,	1, 240	26.9
7	370	RTV +LPV/r	TB	100, 000	9.6
8	254	RTV	TB	15, 400	18.9
9	252	RTV	TB	4, 980	12.6
10	261	RTV	TB	66, 400	NA
11	84	RTV	TB	750, 000	NA
12	85	RTV	< 6 months,	750, 000	NA
13	27	RTV	< 6 months	6, 910	NA
14	161	RTV	TB	100, 000	20.4
15	254	LPV/r	-	100, 000	16.4
16	384	LPV/r	-	10, 100	25.4
17	369	LPV/r	-	2, 010	44.2
18	363	LPV/r	-	4, 220	41.2
19	253	LPV/r	-	8, 720	49.6
20	734	LPV/r	-	100, 000	31.0

¹ RTV + LPV/r: Patients received both RTV as a single PI as well as LPV/r;

RTV: Patients received RTV as a single PI; LPV/r: patients received LPV/r only

² < 6 months, age less than 6 months; TB, tuberculosis; NA, not available

Table 3.1.2. Genotypic characterisation of *protease* and *gag* from HIV-1 infected infants and young children failing protease-inhibitor based antiretroviral therapy

Pt. No.	Treatment ^a	<i>Protease</i> Genotype	<i>Gag</i> CS Genotype
1	RTV +LPV/r	<u>I54V, V82A</u> , K20R, L23I, E35D, L63P	<u>V378A, A431V</u>
2	RTV +LPV/r	<u>M46I, I54V, V82A, L10I</u> , K20R, E35D, <u>Q61H, L63P</u>	<u>E428D</u>
3	RTV +LPV/r	<u>V82A</u> , K20R, K45R	-
4	RTV +LPV/r	<u>M46I, I54V, V82A</u> , K20R, <u>L33F</u> , E35D, T74S	V128T, <u>N374S</u>
5	RTV +LPV/r	-	-
6	RTV +LPV/r	<u>K43E, N83S</u>	<u>S373A</u>
7	RTV +LPV/r	A71T	-
8	RTV	<u>V82A, S12T</u> , L10I, K20R, E35D, <u>D60E, Q61E</u>	<u>N451S</u>
9	RTV	<u>M46I, V82A, A63T</u>	V128I
10	RTV	<u>I54V, V82A</u> , I36M, N37S, L63P, V77I	<u>A431V</u>
11	RTV	N37S, L63S, V82I	V128I, <u>L449P, P453L</u>
12	RTV	<u>V82A</u> , K20R, E35D, R57K, D60E, L63P	-
13	RTV	E35D, L63V, T74S	E428K, L449P, P453L
14	RTV	L10M, I36M, N37K, K41I, L63T, <u>K70R</u>	-
15	LPV/r	K20R, E35D, L63V	-
16	LPV/r	I36M, K41R, T74S, V77I	I437L
17	LPV/r	L63V	-
18	LPV/r	V82I	Y132F
19	LPV/r	I36L, N37E, K41R, L63V	<u>T374A</u> , P453L
20	LPV/r	L63P, V77I	<u>L449P</u>

^aRTV + LPV/r: Patients received both RTV as a single PI as well as LPV/r at some point;

RTV: Patients received RTV as a single PI only; LPV/r: patients received LPV/r only

Protease mutations in bold represent major PI mutations

Mutations underlined represent changes which developed following PI-based therapy

For 10 patients there were no changes in *protease* at failure (highlighted in gray) although 9 had baseline *protease* polymorphisms

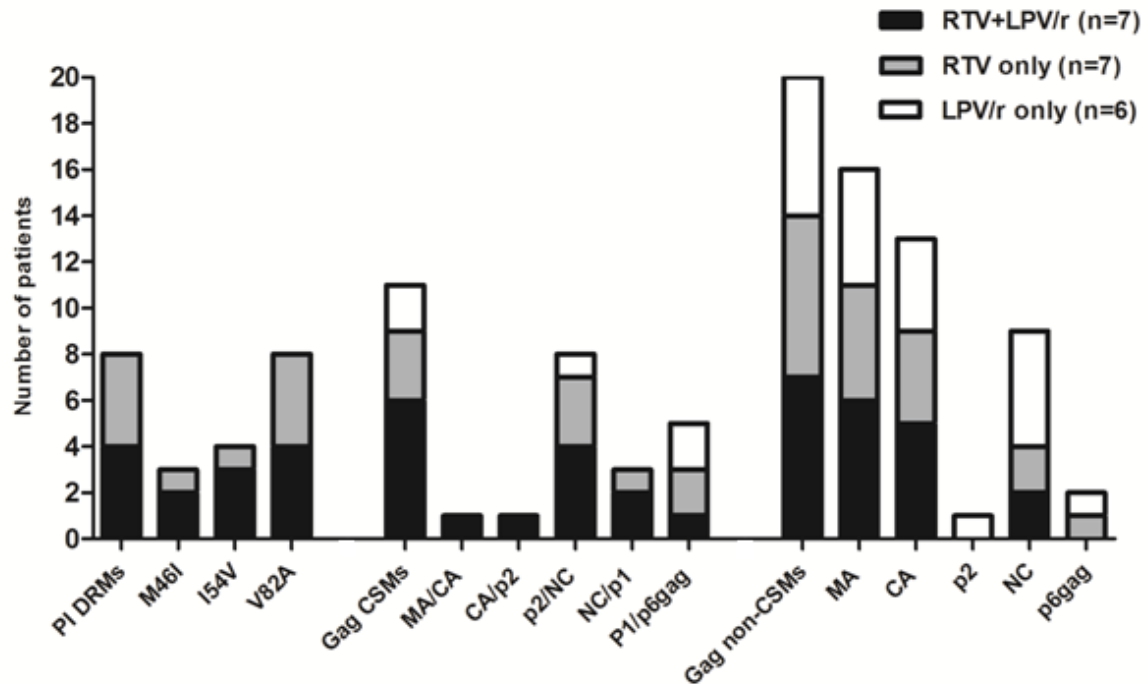


Figure 3.1.1. Frequency of *gag* and *protease* mutations in patient samples relative to their treatment. The number of patients with any PI drug resistance mutations (DRMs), any *gag* cleavage site mutations (CSMs) and any *gag* non-cleavage site mutations (non-CSMs) are shown. Individual PI mutations (M46I, I54V and V82A), and mutations occurring at *gag* CS and non-CS are shown. The proportion of patients on RTV+LPV/r is indicated by black bars, RTV only is indicated by grey bars and LPV/r only is indicated by white bars.

	Gag Cleavage Sites							Protease Major Mutations		
	MA/CA	CA/p2	p2/NC	NC/p1	p1/P6 ^{Gag}	NC/TFP	TFP/P6 ^{Pol}			
Consensus C	VSQNY/PIVQN	KARVL/AEAMS	NTNIM/MQRSN	ERQAN/FLGKI	RPGNF/LQNRP	ERQAN/FLREN	ENLAF/QQGEA	M	I	V
Amino acid	128..../....137	359..../....368	373..../....382	428..../....437	444..../....453	428..../....438	436..../....445	46	54	82
Pt. 1	/.....	/.....	/A....	...V./.....	/.....	/.....	/.....	.	V	A
Pt. 2	/.....	/.....	/.....	D..../.....	/.....	/.....	/.....	I	V	A
Pt. 4	/.....	/.....	.S.../.....	/.....	/.....	/.....	/.....	I	V	A
Pt. 6	/.....	/.....	A..../.....	/.....	/.....	/.....	/.....	.	.	.
Pt. 8	/.....	/.....	/.....	/.....	/..S..	/.....	/.....	.	.	A
Pt. 10	/.....	/.....	/.....	...V./.....	/.....	/.....	/.....	.	V	A
Pt. 11	/.....	/.....	/.....	/.....	/P...L	/.....	/.....	.	.	.
Pt. 19	/.....	/.....	.A.../.....	/.....	/.....	/.....	/.....	.	.	.
Pt. 20	/.....	/.....	/.....	/.....	/P....	/.....	/.....	.	.	.

Figure 3.1.2. Position of Gag CS amino acid changes in patients at PI failure

Nine patients with *gag* CS changes at PI failure are shown. Of these, 5 also had major PI mutations (positions 46, 54 and 82). *Gag* CS mutations are reported relative to the baseline sample. The sequences at consensus C cleavage sites are shown.

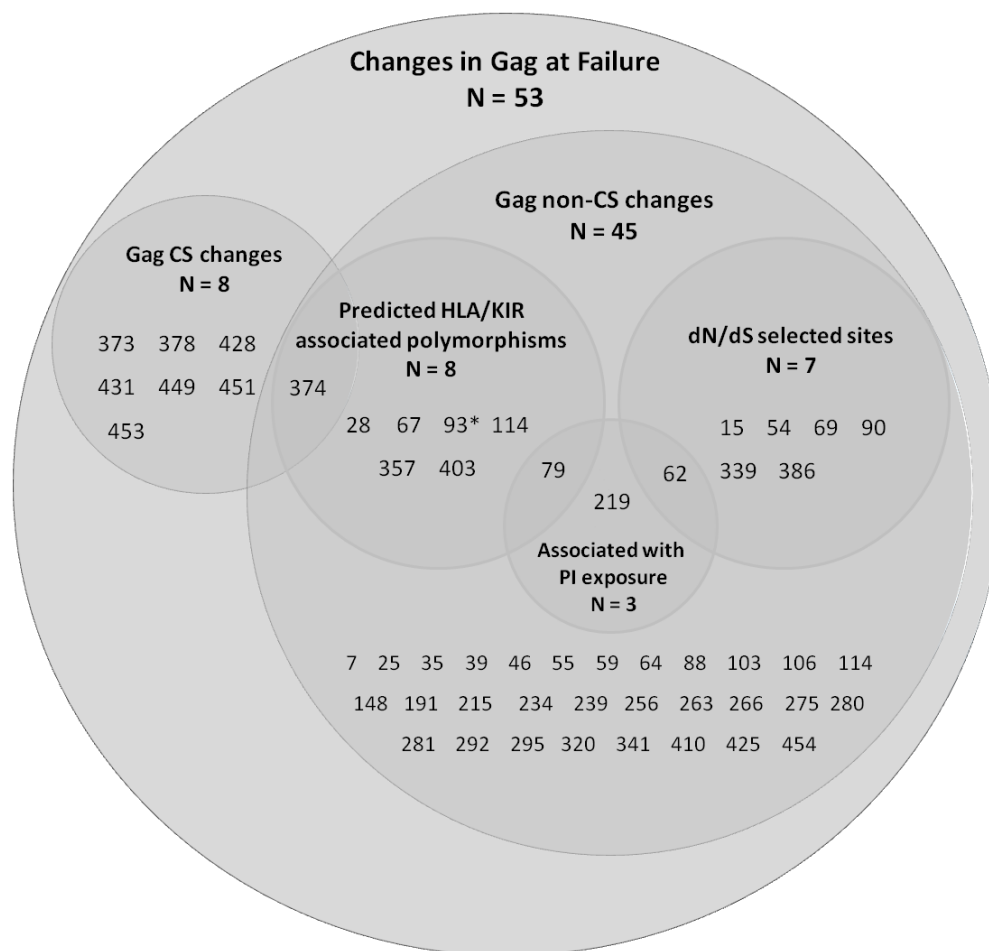


Figure 3.1.3. Changes in *gag* following PI-based therapy failure.

Amino acids positions in *gag* (n= 53) that change following PI-based therapy were classified according to those at CS (n= 8) and those at non CS (n= 45). Non-CS changes were further classified as those known to be associated with PI exposure from published literature (n= 3) those predicted to be HLA/KIR -associated polymorphisms (n= 8) and those under positive selection pressure (n= 7). *Position 93 was predicted to be an HLA and KIR associated polymorphism. The remaining 30 *gag* non-CS changes may represent natural polymorphisms.

Table 3.1.3. Genotypic characterisation of *gag* non-cleavage sites from HIV-1 infected infants and young children failing protease-inhibitor based antiretroviral therapy

Pt. No.	Additional Changes at Failure
1	-
2	-
3	G62S, H219Q
4	V88A
5	A55T, T62A, K69R, S148L
6	-
7	V35A, E106G, A357G
8	S54A, L64I, N90K, E93D, K103T, V191I, I256V, D295E
9	I7V, D15E, G25E, Y79H, S234G,
10	R28K
11	A67S, P339Q, K410R, K411G
12	E454G
13	R275K, S281N, A341S
14	Q69K
15	K263R, I266V, A280V, P292T, K411R
16	Q59R, A62Q, L215I, T239A, D425E
17	R39G, L46P, E454G
18	K62N, T320A
19	P386S, R403K
20	K114R, L215I

Table 3.1.4. Positions predicted to be under HLA-mediated immune pressure

Pt. No.	Amino Acid Change	HLA Allele	Prevalence in South African Population ^a	
			Blacks	Caucasians
7	A357G	B07	1 – 4%	1 – 14%
8	E93D	B40	1%	1 – 2%
9	Y79H	C14	1%	1%
10	R28K	A03, A30	6%; 1 - 10%	12%; 1 – 2%
11	A67S	A02	2 – 8%	1 – 26 %
19	R403K	A03, A30	6%; 1 - 10%	12%; 1 – 2%
	T374A	B45	1 – 6%	1%
20	K114R	C03	1 – 5%	1 – 5%

^aA range of frequencies are given since there are > 1 subgroup for the HLA alleles eg. B07:02, B07:06

Table 3.1.5. Sites in *gag* found to be positively selected using the FEL test

Gag Amino Acid	No. Of Patients	dN/dS	P value
62*	4	6.033	0.001
90*	1	3.470	0.077
15*	1	3.445	0.031
54*	1	2.711	0.006
69*	2	2.702	0.001
386*	1	1.256	0.068
339*	1	1.060	0.078
453	1	0.788	0.123
7	1	0.577	0.409
148	1	0.575	0.193
106	1	0.426	0.313
374	2	0.295	0.167
263	1	0.222	0.466
55	1	0.217	0.887
35	1	0.214	0.415
67	1	0.205	0.427
341	1	0.203	0.428
275	1	0.194	0.460
266	1	0.173	0.690
281	1	0.167	0.533
357	1	0.034	0.975
403	1	0.032	0.976
46	1	-0.042	0.950
103	1	-0.097	0.886
191	1	-0.103	0.900
431	2	-0.146	0.812
114	1	-0.294	0.713
215	2	-0.326	0.698
219	1	-0.351	0.143
454	2	-0.366	0.538
25	1	-0.369	0.464
39	1	-0.532	0.257
410	1	-0.533	0.408
292	1	-0.597	0.562
320	1	-0.755	0.208
234	1	-0.943	0.215
64	1	-1.038	0.219
59	1	-1.260	0.183

280	1	-1.263	0.068
451	1	-1.384	0.077
88	1	-1.745	0.041
93	1	-1.873	0.100
239	1	-1.977	0.045
79	1	-2.287	0.303
449	2	-2.322	0.065
425	1	-2.810	0.022
295	1	-3.218	0.009
428	1	-3.444	0.003
256	1	-4.061	0.030
28	1	-5.514	0.387
411	2	-6.270	0.008
373	1	-25.108	0.001
378	1	-26.494	0.030

Significance set at 0.1

Codons are ranked according to the dN/dS ratios.

Codons that were under positive selection pressure
are shown in bold with an asterisk.

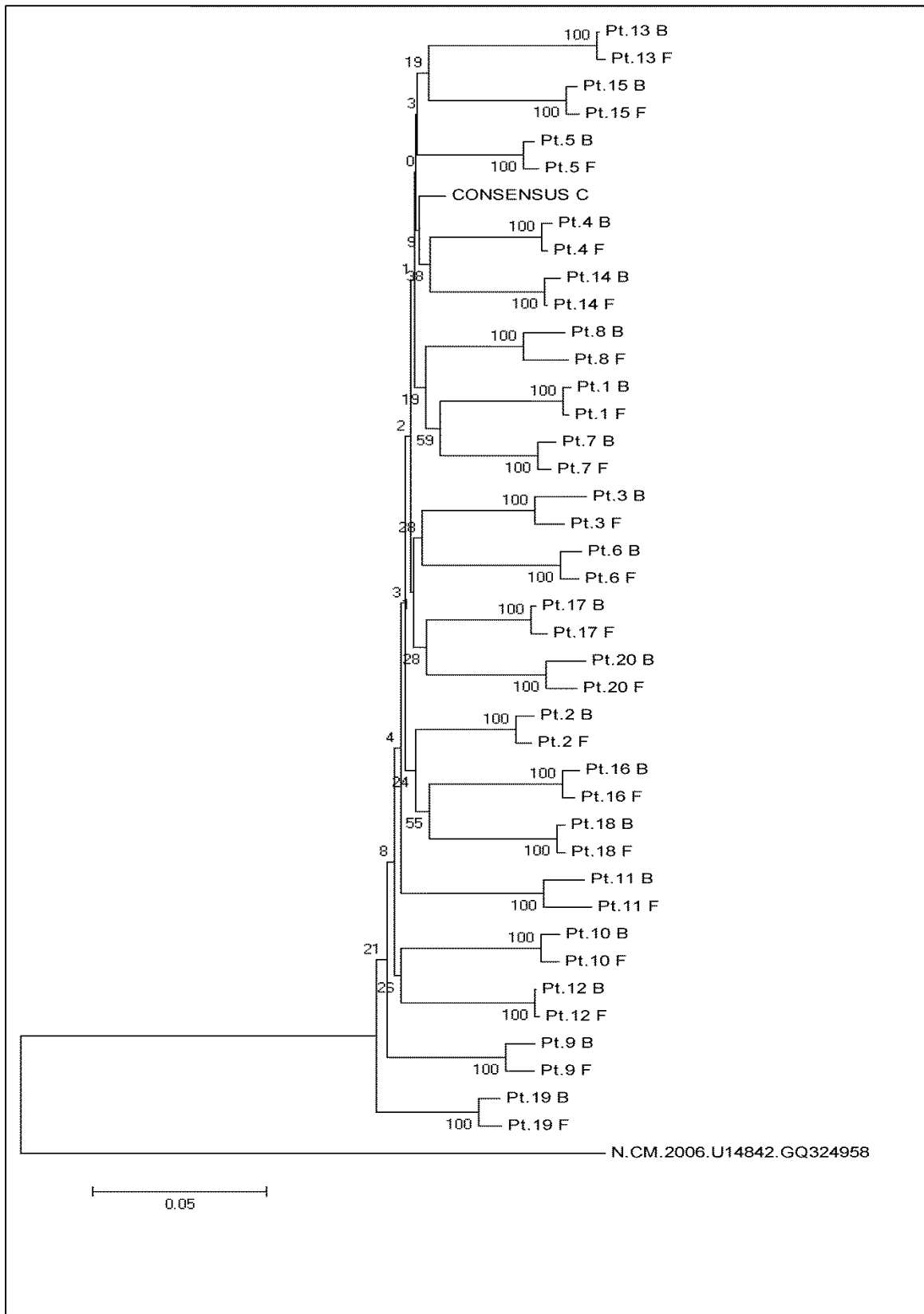


Figure 3.1.4. Phylogenetic tree showing clustering of the samples at baseline and failure
 Neighbour joining tree constructed using MEGA using 1000 bootstrap replications. All 1.8kb *gag-protease* sequences were aligned according to consensus C downloaded from the Los Alamos Sequence Database. Group N 1.8kb *gag-protease* sequence was used to root the phylogenetic tree.

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Chapter 3.2.

Phenotypic Analysis of HIV-1 *gag* and *protease* Genes in Paediatric Patients Failing Protease-Inhibitor Based Therapy

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TOTAL WORD COUNT: 4599

3.2.1. Abstract

Introduction

There is a low frequency of protease inhibitor (PI) resistance in patients failing PI-based therapy. We have previously reported on genotypic changes in *gag-protease* of paediatric patients failing PI-based therapy. Here we investigated the phenotypic effects of these changes on lopinavir and ritonavir susceptibility.

Materials and Methods

The *gag-protease* regions from pre- and post-treatment time points of 20 subtype C paediatric patients failing a lopinavir (LPV) and/or ritonavir (RTV)-based regimen were analysed in a non-replicative *in vitro* assay. *Gag* and *protease* mutations associated with PI resistance, were reconstructed by site-directed mutagenesis and tested.

Results

Phenotypic resistance to LPV was observed in 40% (n=8/20) of patients while 50% (n=10/20) were phenotypically resistant to RTV. Phenotypic resistance to LPV and/or RTV was associated with prior RTV treatment. Three patients showed a reduced PI susceptibility in the absence of PI mutations but had changes at *gag* cleavage sites (CS) (V128I, L449P and P453L) and/or non-CS. Site-directed mutagenesis showed that these changes in *gag* contributed to PI resistance but only in the presence of major PI mutations.

Conclusion

These results suggest that *gag* mutations in combination with major *protease* mutations can contribute to PI resistance. However, in some patients with wild-type *protease*, changes in *gag* may be responsible for reduced PI susceptibility. These findings warrant further investigation of the *gag* gene and its role in PI resistance.

3.2.2. Introduction

Protease inhibitors (PI) are potent antiretroviral drugs which inhibit HIV-1 Protease and prevent viral maturation (Santos and Soares, 2010). The South African national treatment guidelines recommend the use of ritonavir-boosted lopinavir (LPV/r) as a first-line regimen for paediatric patients <3 years of age and a second-line regimen for adults and older children (Department of Health, 2013). Prior to 2008, ritonavir (RTV) was used as a single PI to treat paediatric patients <6 months of age and those receiving rifampicin-based regimens for the treatment of tuberculosis (van Zyl et al., 2011). RTV monotherapy has been characterised by the selection of major *protease* resistance mutations during regimen failure and is therefore no longer recommended (Taylor et al., 2011, Orrell et al., 2013). Presently, RTV is used to boost LPV-based therapy and increase LPV bioavailability. Due to high viral loads and challenges with accurate dosing, paediatric patients are at a greater risk for developing HIV-1 drug resistance than adult patients on PI treatment (Gupta et al., 2009).

Resistance to PIs is characterised by the accumulation of various major and minor mutations in the *protease* gene (Johnson et al., 2011), including M46I, I54V and V82A. However, many patients failing protease inhibitor-based therapy do so in the absence of *protease* mutations (Levison et al., 2012, Wallis et al., 2011, Bartlett, 2013). While poor adherence is often associated with therapy failure in the absence of genotypic resistance mutations, it has been suggested that regions outside of the *protease* gene may contribute to PI resistance in some of these cases (Fun et al., 2012, Gupta et al., 2009, Orrell et al., 2013, Rabi et al., 2013, Siliciano and Siliciano, 2013). HIV-1 Protease recognises and cleaves the Gag and Gag-pol poly-proteins at 10 distinct cleavage sites (CS) to generate viral structural components such as matrix (MA), capsid (CA) and nucleocapsid (NC), and enzymes protease (PR), reverse

transcriptase (RT) and integrase (IN), necessary for viral replication (de Oliveira et al., 2003). Protease inhibitors act by binding to HIV-1 Protease and preventing cleavage of the Gag and Gag-Pol polyproteins (Ali et al., 2010). Changes at *gag* CS and non-CS have been identified that act as compensatory mutations to restore viral fitness in the presence of *protease* mutations, or directly by affecting PI susceptibility (Dam et al., 2009, Gupta et al., 2010, Fun et al., 2012, Verheyen et al., 2006, Kolli et al., 2009). *Gag* CS mutations such as those at positions 431, 436 and 437 have been associated with reduced PI susceptibility and virological failure during PI therapy in the absence of PI mutations (Nijhuis M, 2007, Fun et al., 2011). A recent study has shown the interdependency between a nelfinavir (NFV) resistant *protease* (D30N/N88D) and p1/p6^{Gag} cleavage site mutations (L449F and S451N) (Kolli et al., 2014). Furthermore, it has been suggested that this association may strengthen the inter-molecular interactions between the substrate and the active site of *protease* to allow for co-evolution of the substrate and mutant *protease*.

The genetic changes in 20 subtype C paediatric patients failing a PI-based regimen were previously reported (Giandhari et al., 2015). Changes in both *protease* and *gag* were identified. In this study, phenotypic testing of the 20 paediatric patients was performed to understand the role of *gag* mutations in paediatric PI resistance.

3.2.3. Materials and Methods

3.2.3.1. Samples from HIV-1 infected children

Twenty matched baseline and post-treatment plasma samples from children less than 2 years of age and failing PI-based therapy were studied (Chapter 3.1.3.1). Briefly, these children were initiated on treatment and followed as part of a randomized clinical trial between 2005 and 2009. Briefly, children <6 months of age and those receiving rifampicin for concomitant tuberculosis were treated with a ritonavir (RTV)-based regimen. Once the patients had passed 6 months of age or the tuberculosis had cleared, they were switched onto a ritonavir-boosted lopinavir (LPV) regimen. This study was approved by the institutional review boards of Columbia University and the University of the Witwatersrand.

3.2.3.2. Construction of patient derived test vectors

Phenotypic susceptibility to LPV and RTV was determined by using a retroviral vector system, capable of a single infectious event as previously described (Gupta et al., 2010, Parry et al., 2009). HIV-1 expression vector p8.9NSX+ (Parry et al., 2009, Sutherland et al., 2013) contained a *NotI* restriction site at the beginning of *gag* (HXB2 position 772) and a *XhoI* restriction site at the end of *protease* (HXB2 position 2563) to allow for the cloning of a 1.8kb *gag-protease* fragment from patient samples. Restriction enzymes (*Not I* and *Xho I*) were used to digest p8.9NSX+ and allow for cloning of patient-derived (baseline and failure) and p8.MJ4 derived *gag-protease* resulting in patient-derived expression vector and p8.9MJ4GP respectively. Viral RNA was extracted from plasma using the QIAamp Viral RNA Mini Kit (QIAGEN, Belgium) and reverse transcribed using Thermoscript RT-PCR kit (Invitrogen, CA, USA). A 1.8 kb *gag-protease* fragment spanning HXB2 positions 792 to 2549 was amplified by nested PCR using Expand High Fidelity PCR kit (Roche Applied

Science, Basel, Switzerland) (Chapter 3.1.3.2). Second round primers GagNot+ (5'GCGGCGGCCGCAAGGAGAGAGATGGGTGCG3') and ProXhoR2 (5'CTGGTACAGTCTCGAGRGGACTRATKGG 3') were used to introduce the *NotI* and *XhoI* restriction sites (underlined) into the 1.8 kb *gag-protease* amplicons during the second round of amplification. Amplicons were sequenced and analyzed for changes in the *gag* and *protease* genes. *Protease* sequences were submitted to the Stanford HIVdb Drug Resistance database (hivdb.stanford.edu/) to identify PI resistance mutations and predict phenotypic PI susceptibility. Polymorphisms in the *protease* and *gag* genes were defined as changes in amino acid sequence between the failing and baseline sequences. In addition, baseline and failing *protease* sequences were submitted to the Stanford Drug Resistance database to determine the predicted phenotype for LPV/r. For the Stanford predicted phenotype, each mutation present in the patient sample is assigned a mutation penalty score to determine the predicted level of phenotypic resistance for that particular drug. Predicted phenotype for RTV was not available since use of RTV on its own is no longer recommended.

The second-round products were cloned into the pCR-2.1 TOPO cloning vector (TOPO TA Cloning Kit, Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Clones containing the *gag-protease* inserts of the correct size were sub-cloned into the p8.9NSX+ expression vector by *NotI/XhoI* restriction digest (Roche, Basel, Switzerland) and ligation with the Rapid DNA Dephos and Ligation Kit (Roche, Basel, Switzerland). Ligation products were transformed into *Escherichia coli* (*E.coli*) XL10-Gold ultra-competent cells (Stratagene, La Jolla, California, USA) by a standard heat-shock method. All pCR-2.1 TOPO and p8.9NSX+ clones were sequenced, as described above, to confirm the presence of the relevant mutations and polymorphisms.

3.2.3.3. Site-directed mutagenesis

A 3,6 kb *gag-protease-reverse transcriptase* fragment (base pairs 775 to 4,403 of HXB2) in the p8.9NSX+ expression plasmid was replaced with the corresponding fragment restricted from the pMJ4 (GenBank: AF321523.1) subtype C reference strain to create p8.MJ4 (Ndung'u et al., 2001). This plasmid was utilized for the construction of site-directed mutants. *Protease* and *gag* mutations were selected from patient samples based on their known or potential involvement in PI resistance. Site-directed mutants were created in the p8.MJ4 expression plasmid by use of the QuickChange® II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, California, USA). The mutagenesis primers were designed using the QuikChange Primer Design online tool:

(<http://www.genomics.agilent.com/primerDesignProgram.jsp>) and were ordered from Integrated DNA Technologies® (Coralville, Iowa, USA). Site-directed mutants containing additional *gag* non-CS mutants, that have previously been shown to develop under positive selection pressure, (Chapter 3.1.)(Giandhari et al., 2015), were also created. Mutants with multiple mutations were created by successive rounds of site-directed mutagenesis, and the presence of mutations confirmed by population-based sequencing. Sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) on an ABI3130 Genetic Analyser (Applied Biosystems, Foster City, CA). p8.9MJ4 was used as a control in all patient PI susceptibility assays.

3.2.3.4. Phenotypic assessment of protease inhibitor susceptibility

A vector-based non-replicative phenotypic assay was used as previously described (Parry et al., 2009, Gupta et al., 2010), with minor modifications. Briefly, 1.25×10^6 HEK293T cells were transfected with 300 ng of plasmid pMDG (encoding the vesicular stomatitis virus G

protein), 500 ng of plasmid pCSFLW (encoding the firefly luciferase protein) and 300 ng HIV-1 expression vector containing patient-derived inserts or site-directed mutations) using 3.3 μ g polyethylenimine (PEI) (Polyscience, Inc., Warrington, Pennsylvania, USA). Transfected cells were harvested after 18 hours and seeded in the presence of serial dilutions of LPV and RTV. Supernatants were transferred after 24 hours to the corresponding wells of the 96-well culture plates that contained fresh 293T cells. The degree of infection was determined by measuring the expression of firefly luciferase in infected target cells, 48 hours after supernatant transfer. For each drug-virus combination, the 50% inhibitory concentration (IC_{50}) was calculated. For patient derived clones, the phenotypic susceptibility was expressed as a fold-change (FC) in IC_{50} , relative to that of the baseline virus. The fold change (FC) was calculated by comparing the IC_{50} of the sample at failure to that of the baseline sample. The standard phenotypic curves showed minimal change in IC_{50} for viruses showing PI susceptibility. Standard phenotypic curves of the resistant viruses typically show a greater shift to the right indicating that additional drug is required to inhibit the virus. An expression vector (p8.MJ4) containing the *gag-pol* region of a subtype C reference strain (MJ4, GenBank: AF321523.1) was used for the construction of site-directed mutants (Ndung'u et al., 2001). The assay-specific lower FC cut-off value for each drug was determined using the 99th percentile of the average IC_{50} for p8.MJ4GP and p8.MJ4 assessed in multiple repeat screens of each drug. An upper assay cut-off value of 10 FC was arbitrarily chosen. The phenotypes were classified as sensitive (S) (LPV: <4.4 FC, RTV: <3.2 FC), having a reduced susceptibility (RS) (LPV: 4.4 - 10 FC, RTV: 3.2 – 10 FC) or as resistant (R) (LPV: >10 FC, RTV: >10 FC). The classifications susceptible, reduced susceptibility and resistant serve to rank the response of the PIs used in our assay. The upper cut-off was arbitrarily chosen to distinguish those showing reduced susceptibility from those phenotypically resistant.

3.2.3.5. Statistical Analysis

Two-way analysis of variance (ANOVA) was used to identify significant differences in FC values between of site-directed mutants using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California, USA).

3.2.4. Results

3.2.4.1. Phenotypic susceptibility of patient-derived *protease* and *gag* genes

We previously reported the genotypic changes in *gag* and *protease* among 20 paediatric patients failing PI-based therapy. In order to examine the impact of these mutations on phenotypic resistance, the *gag-protease* fragment from each patient's baseline and failing sample were cloned into the HIV-1 expression vector. A single-cycle phenotypic assay was used to assess the impact of mutations in *gag* and *protease* on PI susceptibility. Treatment history, genotypic profiles, predicted phenotypes and *in vitro* phenotypic drug susceptibility of the 20 children are shown in Table 3.2.1. All changes in *protease* and *gag* CS are reported in the failing samples, relative to their corresponding baseline samples.

Seven of the eight patients with major PI mutations showed reduced susceptibility or high-level resistance to LPV and RTV. One patient (Pt. 12) with V82A only that was predicted to be resistant by the database, was shown to be susceptible in our assay. Five of these 8 patients also harboured *gag* CS mutations. A further 9 patients carrying *gag* CS and non-CS mutations only, showed susceptibility to LPV and RTV. Phenotypic resistance to LPV and/or RTV was only seen in patients that had received RTV only at some time-point. Interestingly, the remaining 3 patients (Pt. 5, 11 and 14) showed reduced susceptibility in the absence of PI mutations to RTV, one of which (Pt. 11) also showed reduced susceptibility to LPV. These three patients had *gag* CS and/or non-CS mutations. All *gag* non-CS changes were previously reported (Chapter 3.1) (Table 3.1.3.).

The phenotypic classification of resistance for the 20 patients was similar between LPV and RTV except for two patients. Patient 5 showed a reduced susceptibility to RTV (FC

6.0) but was susceptible to LPV (FC 4.2). This patient did not have any changes in *protease* or *gag* CS but did have four *gag* non-CS changes (A55T, T62A, K69R and S148L). Patient 14 showed a reduced susceptibility to RTV (FC 3.8) while remaining susceptible to LPV (FC 2.6). This patient had no major *protease* or *gag* CS mutations, but did contain a *gag* non-CS change (Q62K).

The phenotypic curves of the *gag-protease* regions of the 20 patients for LPV and RTV are shown in Figures 3.2.1 and 3.2.2 respectively. Differences were observed in the IC₅₀ values of the baseline samples (LPV: range 0.0003-0.0142, mean- 0.00345; RTV: range 0.0019-0.0687, mean 0.0174), indicating the possible effect of patient-specific polymorphisms in *protease* and *gag* on PI susceptibility.

When comparing the predicted phenotype (Stanford) to the *in vitro* phenotype, discordance was observed for six patient samples (Pt. 1, 3, 4, 8, 11 and 12). All six patients had received RTV as a single PI at some point. Patient 1 showed high-level phenotypic resistance to LPV (FC 109.0) although intermediate resistance was predicted by the database. This patient had two major PI mutations (I54V and V82A) in addition to *gag* CS mutations V378A and A431V. High-level resistance to LPV (FC 68.0) was seen in patient 3, although the database predicted low-level resistance. One major *protease* mutation (V82A) and non-CS changes (G62S, and H219Q) were present in this patient. Reduced susceptibility to LPV (FC 5.2) was shown for patient 4 although it was predicted to have high-level resistance due to the presence of 3 major PI mutations (M46I, I54V and V82A). In addition to the major PI mutations, one *gag* CS mutation (N374S) and one non-CS change (V88A) was also present. High-level resistance to LPV (FC 12.1) was shown for patient 8, although a low-level of resistance was predicted. This patient had one major PI mutation (V82A), one *gag* CS

mutation (N451S) and non-CS changes (S54A, L64I, N90K, E93D, K103T, V191I, I256V and D295E). Although the database had predicted a susceptible phenotype for patient 11, the *in vitro* phenotypic assay showed a reduced susceptibility (FC 5.9). Although no major PI mutation was observed in this patient sample, *gag* CS mutations (L449P and P453L) and non-CS mutations (A67S, P339Q, K410R and K411G) were present. Phenotypic susceptibility to LPV (FC <4.4) was observed in patient 12, whilst the database had predicted low-level resistance due to the presence of a single PI mutation (V82A). This patient had one *gag* non-CS mutation (E454G).

3.2.4.3. Phenotypic susceptibility of site-directed mutants with *protease* and/or *gag* mutations

In order to evaluate the contribution of specific *gag* and *protease* mutations to PI resistance, site-directed mutagenesis was used to recreate these mutations in p8.MJ4, individually or in combination. A total of 22 mutants were generated and tested for phenotypic susceptibility to LPV and RTV, as shown in Figures 3.2.4 and 3.2.6. The *protease* mutations (M46I, I54V and V82A) and *gag* CS mutations (N374S, A431V, L449P, S451N and P453L) were selected based on their presence in the patient samples. *Gag* non-CS mutations (Q62S and F79H), which were shown to be under positive selection pressure or associated with PI exposure (Chapter 3.1.) (Giandhari et al., 2015), were also included.

A comparison of the fold change in resistance to LPV and RTV between the patient samples and the mutants based on them are shown in Table 3.2.2 and 3.2.3 respectively. Mutants tested against LPV generally showed a lower fold change in resistance compared to the respective patient samples. Most mutants tested against RTV showed a lower fold change compared to the respective patients on which they were based. Patients 1 and 2 showed high-

level resistance to RTV but had a fold change ~3 times lower than that of the patient samples. However, mutant N374S+M46I+I54V+V82A had a 4 times higher fold change in resistance to RTV compared to patient 4.

High-level resistance to RTV (Figure 3.2.5 and 3.2.6) was seen in 4 mutants containing *gag-protease* mutations (E428D+M46I+I54V+V82A, A431V+K20R+I54V+V82A, N374S+M46I+I54V+V82A, A431V+I54V+V82A), and 1 mutant containing 3 *protease* mutations (M46I+I54V+V82A). Mutant I54V+V82A caused a reduced susceptibility (FC 4.06) to RTV. No resistance was displayed to LPV by any mutants (Figure 3.2.4). A reduced susceptibility to LPV was seen in 4 mutants (I54V+V82A, M46I+I54V+V82A, N374S+M46I+I54V+V82A and E428D+M46I+I54V+V82A) (Figure 3.2.3). No decrease in PI susceptibility was observed for any of the single *gag* or *protease* mutations. Double *protease* mutant I54V+V82A showed a reduced susceptibility to both LPV (FC 6.0) and RTV (FC 4.0) while M46I+I54V and M46I+V82A were susceptible to LPV and RTV. The triple *protease* mutant M46I+I54V+V82A showed a reduced susceptibility to LPV (FC 4.7) (Figure 3.2.3 and 3.2.4) and a high-level resistance to RTV (FC 13.7) (Figure 3.2.5 and 3.2.6).

Gag mutants (L449P+P453L and F79Y+L449P+P453L) and a combination of *gag-protease* mutants (Q62S+V82A, M46I+V82A+F79H and V82A+S451N) showed susceptibility to both LPV and RTV. Addition of N374S to M46I+I54V+V82A caused a further decrease in susceptibility to LPV ($p > 0.001$) and caused an increased resistance (FC 22.1) to RTV ($p < 0.001$). The addition of the A431V *gag* CS mutation to I54V+V82A increased susceptibility to LPV (FC 3.2) while significantly ($p < 0.001$) reducing RTV susceptibility (FC 11.3). Further addition of the minor PI mutation K20R to I54V+V82A+A431V did not alter susceptibility to either LPV or RTV ($p > 0.001$). Interestingly, addition of E428D to

M46I+I54V+V82A did not alter susceptibility to LPV but caused an increase in resistance (FC 16.4) to RTV ($p < 0.05$) (Figure 3.2.4).

The frequency of these mutations in the patient samples and the database are shown in Table 3.2.4. The *gag* CS mutations N374S and A431V, and non-CS mutation Q62S and F79H occurred only in the samples from failing patients in our cohort. Of these 4 mutations, N374S had a high frequency of 21% in the downloaded subtype C naive sequences suggesting that it may be a polymorphism. *Gag* mutations L449P and P453L were observed in both baseline and failure time-points. L449P occurred in 3 patients (15%) at failure and in one of these patients (5%) it was also present at baseline. P453L occurred in 3 patients (15%) at failure and was present in two of these patients at baseline. Both L449P and P453L have a low frequency ($< 5\%$) in subtype C naive patients. Major PI mutations were only observed in the samples failing PI therapy with V82A (40%), I54V (20%) and M46I (15%). The Stanford drug resistance database reported that major PI mutations (M46I, I54V and V82A) occurred at higher frequencies in PI-treated patients, 9.1%, 11% and 12%, respectively.

3.2.5. Discussion

Recent studies have shown a low frequency of *protease* drug resistance mutations in the majority of patients failing PI-based therapy (Siliciano and Siliciano, 2013, Wallis et al., 2011, Levison et al., 2012). It has been suggested that regions outside of *protease* may contribute to treatment failure (Siliciano and Siliciano, 2013). Previous genotyping of these paediatric patients showed the presence of changes at *gag* CS and non-CS at PI failure implicating the role of *gag* in PI resistance. Overall, the majority of patients in the current study that lacked PI major mutations showed complete phenotypic susceptibility to LPV and RTV. However, in some patients, changes in *gag* caused a reduced susceptibility to LPV and RTV in the absence of PI mutations, or a further decrease in PI susceptibility in the presence of major PI mutations. This study therefore suggests that changes in *gag* could have an impact on PI susceptibility in some cases but are unlikely to be the reason for PI failure in most patients.

Phenotypic analysis showed that most patients with PI resistance mutations had reduced susceptibility or high-level resistance to LPV and/or RTV. However, a reduced susceptibility to both drugs was also seen in three patients with *gag* CS and or non-CS changes in the absence of PI mutations. This suggests that *gag* has an effect on PI susceptibility in the absence of PI mutations. Dam et al. (2009) had similarly shown the direct effect of *gag* CS A431V mutation in highly PI-experienced patients on PI susceptibility. From our dataset, there were no associations found between *gag* CS and non-CS changes which caused reduced susceptibility to the PIs since each patient's *gag* genotype differed. However, few studies have investigated the effects of non-CS mutations on PI susceptibility. Further studies on full-length *gag* will be beneficial in providing more insight on the role of

non-CS mutations. The *gag* non-CS mutations may in combination with CS mutations influence PI susceptibility as seen with the three patients having no major PI mutations but reduced phenotypic susceptibility.

Individual *gag* CS and non-CS mutants created by site-directed mutagenesis did not show any effect on PI susceptibility. However, the combination of *protease* and *gag* mutations showed a decreased susceptibility to RTV similar to what was seen with the patient samples. In the presence of *protease* mutations, *gag* appeared to contribute to decreased PI susceptibility. The addition of *gag* CS mutations N374S and E428D to major *protease* mutations caused a slight increase in resistance to LPV and a 3- and 8-fold increase in phenotypic resistance to RTV respectively. The site-directed mutagenesis results did not correlate directly to the phenotypic data of the patient samples. This may be due to the background polymorphisms present in the patient samples which may in combination with other changes in *gag* and *protease* influence PI susceptibility. Nevertheless, the combination of *gag-protease* mutations did show the contributing effect of certain *gag* mutations to PI resistance. The results also demonstrated that an accumulation of mutations is required to develop resistance to PIs.

Although this study has shown that *gag* may in some cases contribute to PI resistance, in the presence or absence of *protease* mutations, the phenotypic susceptibility shown by some patient samples (n=9, 45%) in the absence of PI mutations does not support drug resistance as a cause for failure. Studies have suggested other possible reasons for PI-based therapy failure in the absence of *protease* mutations. The first suggests the involvement of PIs at viral entry where the *envelope* gene was identified as a possible site for PI resistance (Rabi et al., 2013). A second suggests that the sharp dose-response curve and short half-life

of PIs provides only a short window period in which resistance mutations can develop (Rosenbloom et al., 2012). This is likely to result in little resistance mutations observed at failure. Implicitly, this phenotypic data confirms that the lack of PI mutations corresponds with a lack of phenotypic resistance.

The phenotypic susceptibility to LPV and RTV *in vitro* varied amongst patients with different treatment histories. Most samples from patients treated with RTV or RTV+LPV/r showed a reduced susceptibility or resistance to LPV and/or RTV while all patients that received LPV/r remained susceptible to both drugs. Those exposed to RTV+LPV/r and RTV monotherapy contained mutations in both *protease* and *gag* while those exposed to LPV/r did not. Previous studies have also shown the selection of major PI mutations by RTV (Orrell et al., 2013, van Zyl et al., 2009), therefore RTV monotherapy is no longer recommended. The increased selection of *protease* and *gag* CS mutations by RTV monotherapy, as observed in the patient samples indicates that inaccurate dosage of the RTV component of LPV/r treatment may result in the selection of mutations.

Differences were observed between the phenotypes determined in this study and the predicted phenotypes reported by the Stanford HIV-1 Drug Resistance Database. This may be due to the inclusion of *gag* in our analysis compared to the database which only considers mutations in the *protease* gene. Additionally, the database determines the levels of resistance based on mutation penalty scores which have been based on previously published phenotypic data as compared to the assay which uses the patient's baseline sample as a reference to calculate the IC₅₀. The baseline resistance level to LPV and RTV vary widely between most patients. Therefore, changes which pre-exist in the baseline sample may influence the fold change. Currently, patient-specific changes in the *gag* gene are not considered in standard

genotypic or phenotypic resistance assays. Our study has shown that this may result in an inaccurate assessment of PI resistance. Furthermore, since certain *gag* CS are highly variable and differ between the various subtypes, this may create difficulty in establishing a list of *gag* resistance associated mutations to help easily identify these sites (de Oliveira et al., 2003). Therefore, further research on a larger number of patients at baseline and failing PI-based therapy is warranted to understand the role and mechanism by which *gag* CS and non-CS mutations affect PI susceptibility. Also further work involving the generation of *gag* chimeras to determine the individual contribution of *gag* to PI susceptibility would be valuable.

In conclusion, this study found that changes in *gag* contributed to PI resistance only in the presence of PI major mutations. A few patients showed the effect of *gag* mutations directly on PI resistance causing reduced susceptibility in the absence of PI major mutations. Therefore, further investigation of the *gag* gene in a larger cohort of patients failing PI-based therapy in the absence of major PI mutations is warranted to understand the role of *gag* in PI resistance. Nearly half of the patients showed a lack of PI mutations and had *in vitro* phenotypic susceptibility to LPV and RTV indicating that resistance was not the reason for PI-therapy failure in this subset of patients. This suggests sub-optimal adherence in these patients and switching to a new regimen may not prevent future therapy failure. Therefore, especially for paediatric patients who have limited treatment options, improving the tolerability and palatability of drugs will help increase adherence and retention on regimen.

3.2.6. Acknowledgements

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Table 3.2.1. Phenotypic resistance of paediatric patient samples to lopinavir and ritonavir

Pt. No.	Treatment ^a	Protease Genotype	Predicted Phenotype ^b	Gag-CS Genotype	LPV Phenotype		RTV Phenotype	
					FC (S<4.4)	Level ^c	FC (S<3.2)	Level ^c
1*	RTV +LPV/r	I54V, V82A , K20R, L23I, E35D, L63P	Intermediate	<u>V378A</u> , A431V	109.0	R	32.0	R
2	RTV +LPV/r	M46I, I54V, V82A , L10I, K20R, E35D, <u>Q61H</u> , L63P	High	<u>E428D</u>	191.0	R	44.4	R
3*	RTV +LPV/r	V82A , K20R, K45R	Low-level	-	68.0	R	26.0	R
4*	RTV +LPV/r	M46I, I54V, V82A , K20R, <u>L33F</u> , E35D, T74S	High	V128T, <u>N374S</u>	5.2	RS	5.0	RS
5	RTV +LPV/r	-	Susceptible	-	4.2	S	6.0	RS
6	RTV +LPV/r	<u>K43E</u> , N83S	Susceptible	<u>S373A</u>	1.3	S	0.8	S
7	RTV +LPV/r	A71T	Susceptible	-	0.8	S	0.8	S
8*	RTV	V82A , S12T, L10I, K20R, E35D, <u>D60E</u> , <u>Q61E</u>	Low-level	<u>N451S</u>	12.1	R	49.0	R
9	RTV	M46I, V82A , A63T	Intermediate	V128I	6.0	RS	5.7	RS
10	RTV	I54V, V82A , I36M, N37S, L63P, V77I	Intermediate	<u>A431V</u>	7.6	RS	3.4	RS
11*	RTV	N37S, L63S, V82I	Susceptible	V128I, <u>L449P</u> , P453L	5.9	RS	7.4	RS
12*	RTV	V82A , K20R, E35D, R57K, D60E, L63P	Low-level	-	1.2	S	2.3	S
13	RTV	E35D, L63V, T74S	Susceptible	E428K, L449P, P453L	0.1	S	1.0	S
14	RTV	L10M, I36M, N37K, K41I, L63T, <u>K70R</u>	Susceptible	-	2.6	S	3.8	RS
15	LPV/r	K20R, E35D, L63V	Susceptible	-	0.4	S	1.2	S
16	LPV/r	I36M, K41R, T74S, V77I	Susceptible	I437L	1.3	S	1.6	S
17	LPV/r	L63V	Susceptible	-	0.4	S	0.4	S
18	LPV/r	V82I	Susceptible	Y132F	2.1	S	1.9	S
19	LPV/r	I36L, N37E, K41R, L63V	Susceptible	<u>T374A</u> , P453L	1.0	S	1.4	S
20	LPV/r	L63P, V77I	Susceptible	<u>L449P</u>	0.1	S	0.3	S

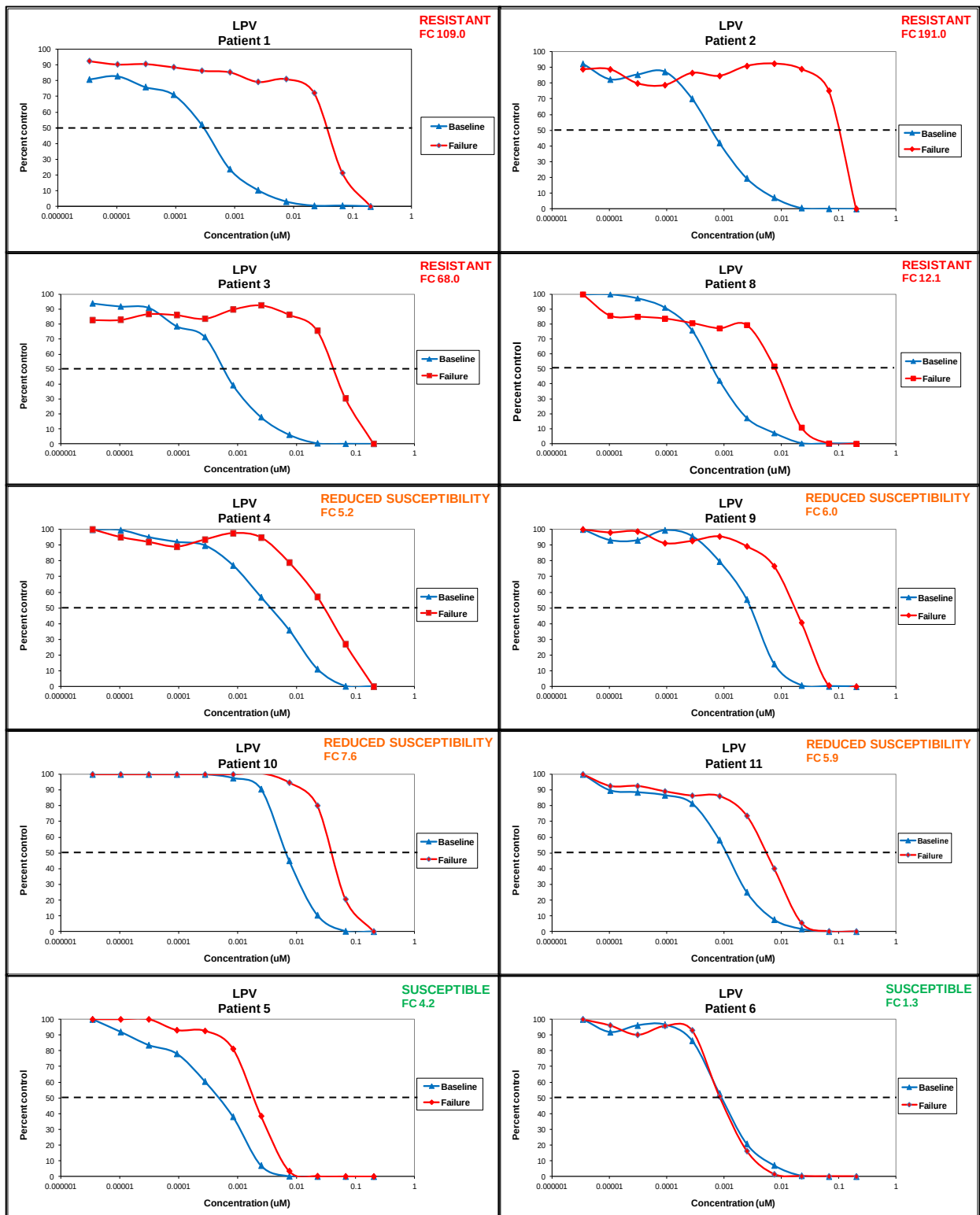
^a RTV + LPV/r: Patients received both RTV as a single PI as well as LPV/r at some point; RTV: Patients received RTV as a single PI only;

LPV/r: patients received LPV/r only

^b Predicted phenotype according to Stanford drug resistance database. Six patients (indicated by an asterisk) showed discordance between the predicted and actual phenotype

^c Levels of drug resistance: S= sensitive; RS= reduced susceptibility, H= High-level resistance (Highlighted by different levels of shading). The assay cut-offs were determined to be: sensitive (S) (LPV: <4.4 FC, RTV: <3.2 FC), reduced susceptibility (RS) (LPV: 4.4 - 10 FC, RTV: 3.2 – 10 FC) and resistant (R) (LPV: >10 FC, RTV: >10 FC).

Protease mutations in bold represent major PI mutations. Mutations underlined represent changes which developed following PI-based therapy



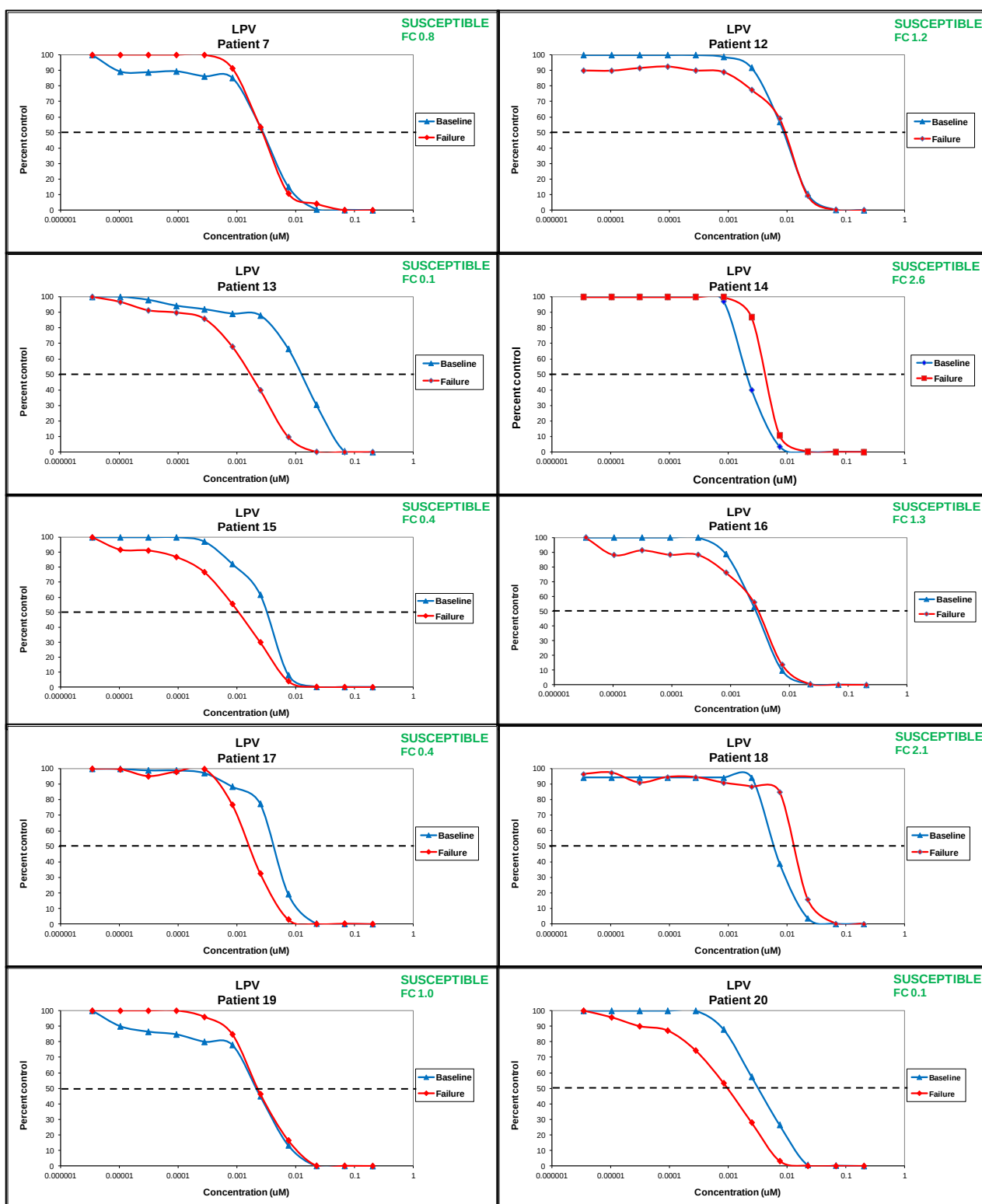
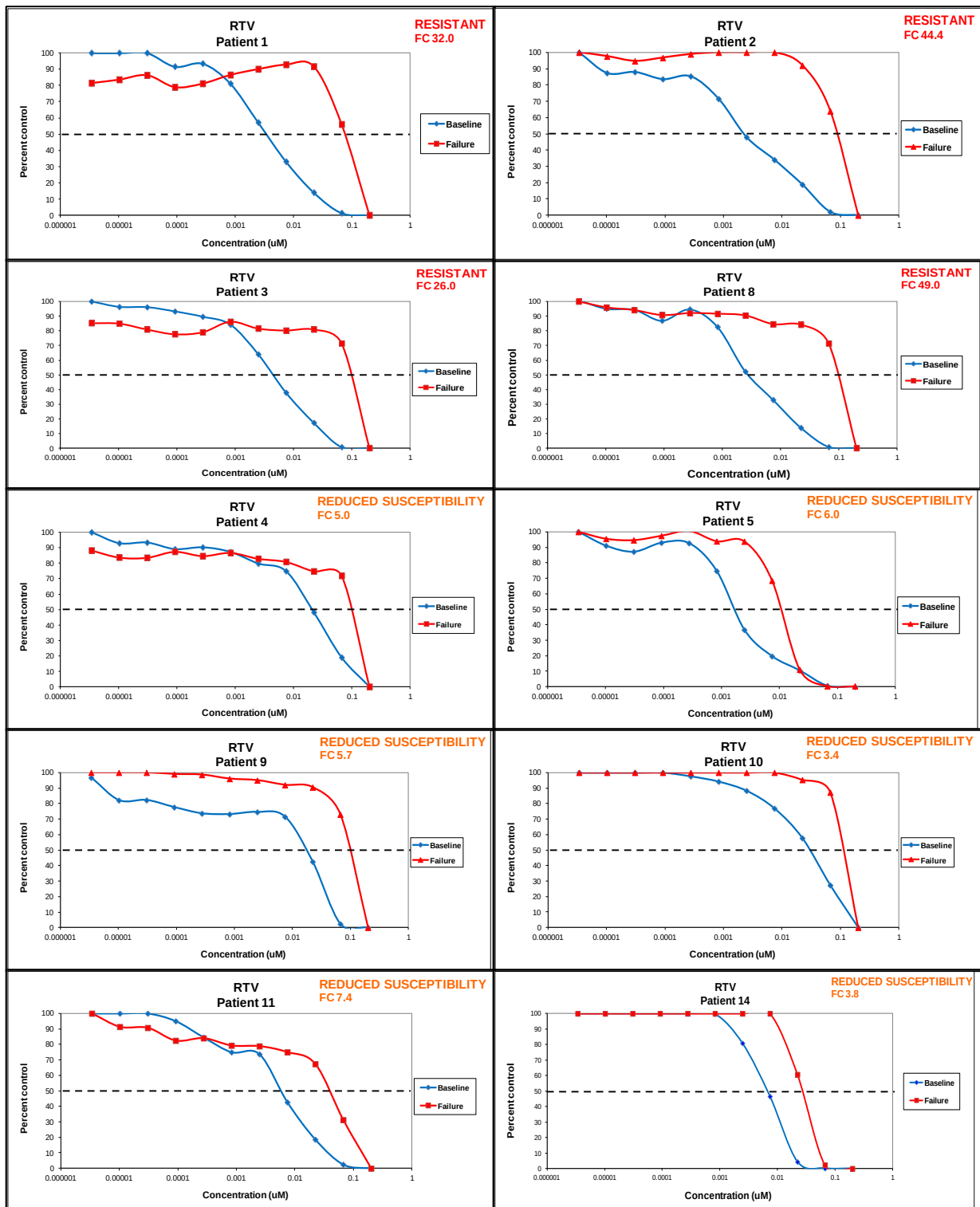


Figure 3.2.1. Phenotypic assay standard curves for patient samples to LPV

Fold changes are calculated based on the IC₅₀ value of the failing sample (red line) relative to the baseline sample (blue line). Each patient sample was tested in duplicate and the average of that is represented by the curve. The 4 resistant patients are shown in red at the top, the 4 with reduced susceptibility in orange and the 12 susceptible in green.



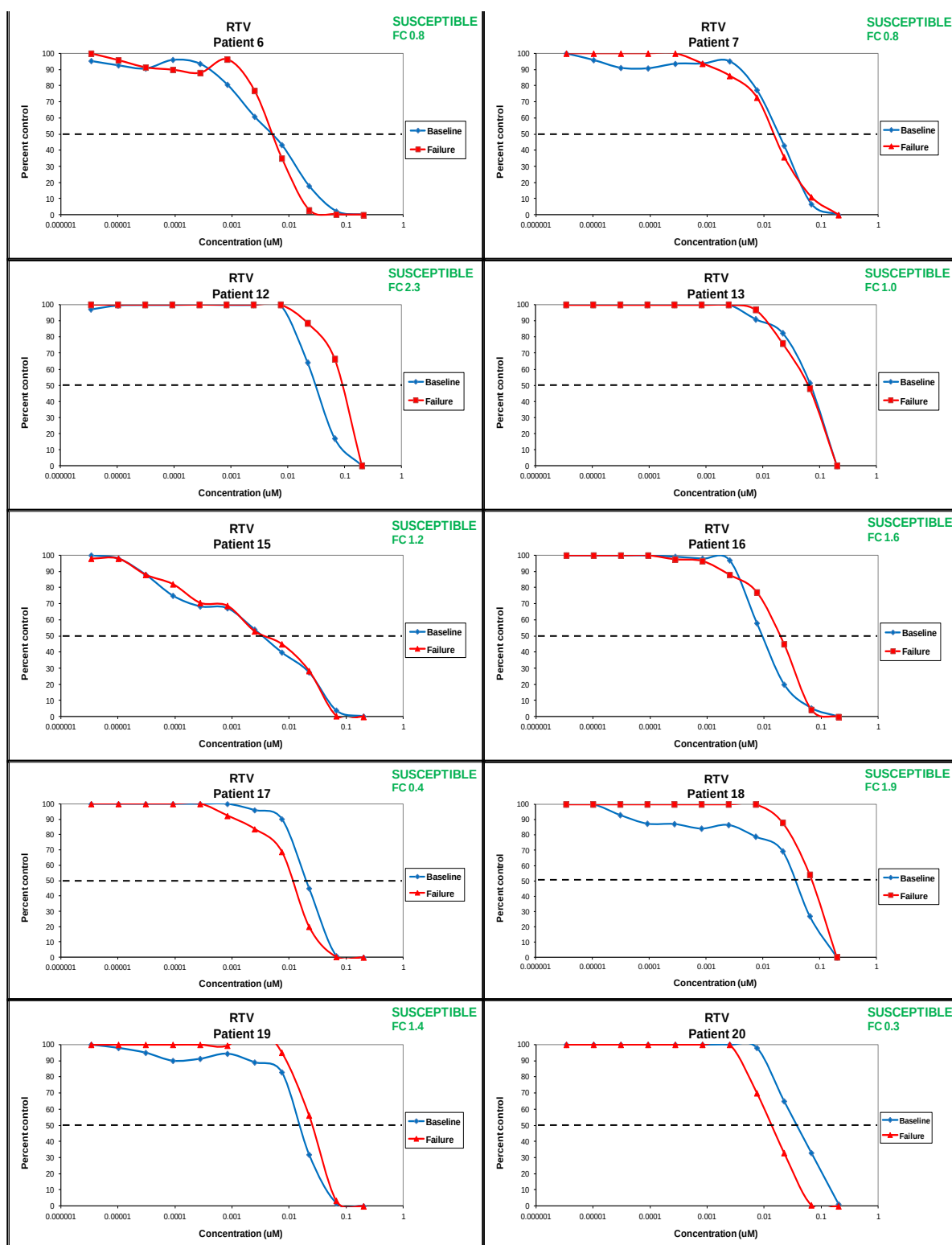


Figure 3.2.2. Phenotypic assay standard curves for patient samples to RTV

Fold changes are calculated based on the IC₅₀ value of the failing sample (red line) relative to the baseline sample (blue line). Each patient sample was tested in duplicate and the average of that is represented by the curve. The 4 resistant patients are shown in red at the top, the 6 with reduced susceptibility in orange and the 10 susceptible in green.

Table 3.2.2. Comparison of fold change between patient samples and *gag-protease* mutants based on patient samples for LPV

Patient	Fold change	SDM	Fold change
1	109.0	A431V+K20R+I54V+V82A	3.9
2	191.0	E428D+M46I+I54V+V82A	5.9
3	68.0	Q62S+V82A	0.7
4	5.2	N374S+M46I+I54V+V82A	5.9
8	12.1	N451S+V82A	1.4
9	6.0	F79H+M46I+V82A	1.7
10	7.6	I54V+V82A	6.0
11	5.9	L449P+P453L	0.8
12	1.2	V82A	1.3
20	0.1	L449P	0.7

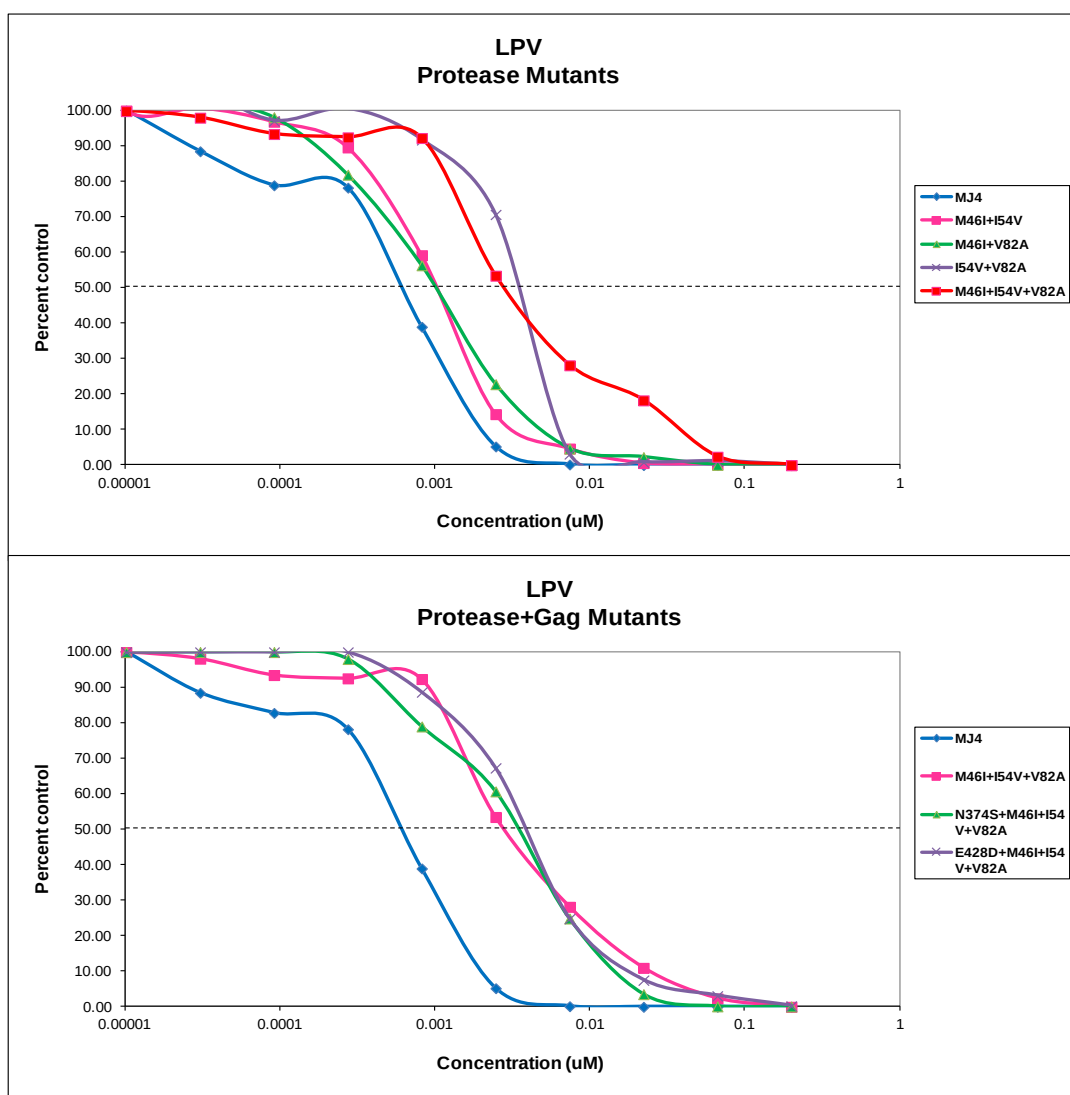


Figure 3.2.3. Phenotypic assay standard curves for *protease* and *gag* mutants to LPV

Multiple mutants were created by successive rounds of site-directed mutagenesis. The fold change of each mutant was calculated relative to p8.MJ4. The *protease* only mutants in the upper graph show that I54V+V82A and M46I+I54V+V82A mutants show reduced susceptibility. From the combination of *protease* and *gag* mutants, the addition of *gag* mutations was shown to have minimal change on LPV susceptibility.

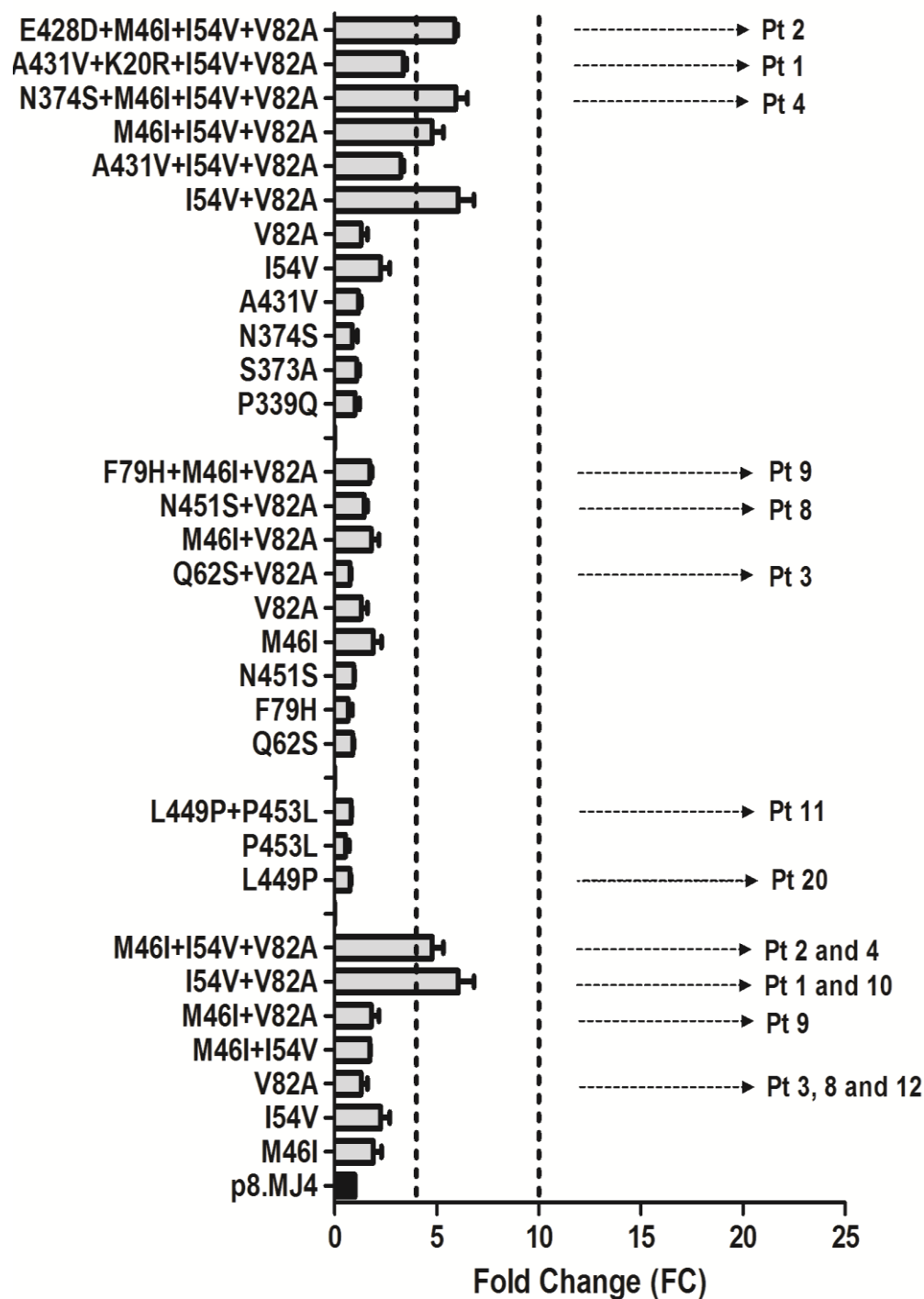


Figure 3.2.4. Phenotypic susceptibility of *protease* and *gag* mutations to LPV

Phenotypic response of single and multiple site-directed mutations created in p8.MJ4 subtype C HIV-1 expression plasmid to LPV. Phenotypic susceptibility was determined with a single-cycle non-replicative phenotypic assay. *Protease* and *gag* mutations were selected from patient samples based on their known or potential involvement in PI resistance. The patient sample to which each SDM is associated with is indicated on the graph. The fold change of each mutant was calculated relative to p8.MJ4. The lower and upper cut-offs are indicated by dotted lines. Error bars indicate the standard error of the mean (SEM). Each mutant was tested in at least two independent experiments, in duplicate.

Table 3.2.3. Comparison of fold change between patient samples and *gag-protease* mutants based on patient samples for RTV

Patient	Fold change	SDM	Fold change
1	32.0	A431V+K20R+I54V+V82A	10.8
2	44.4	E428D+M46I+I54V+V82A	16.4
3	26.0	Q62S+V82A	1.1
4	5.0	N374S+M46I+I54V+V82A	22.1
8	49.0	N451S+V82A	1.0
9	5.7	F79H+M46I+V82A	3.0
10	3.4	I54V+V82A	4.0
11	7.4	L449P+P453L	1.5
12	2.3	V82A	1.2
20	0.3	L449P	0.6

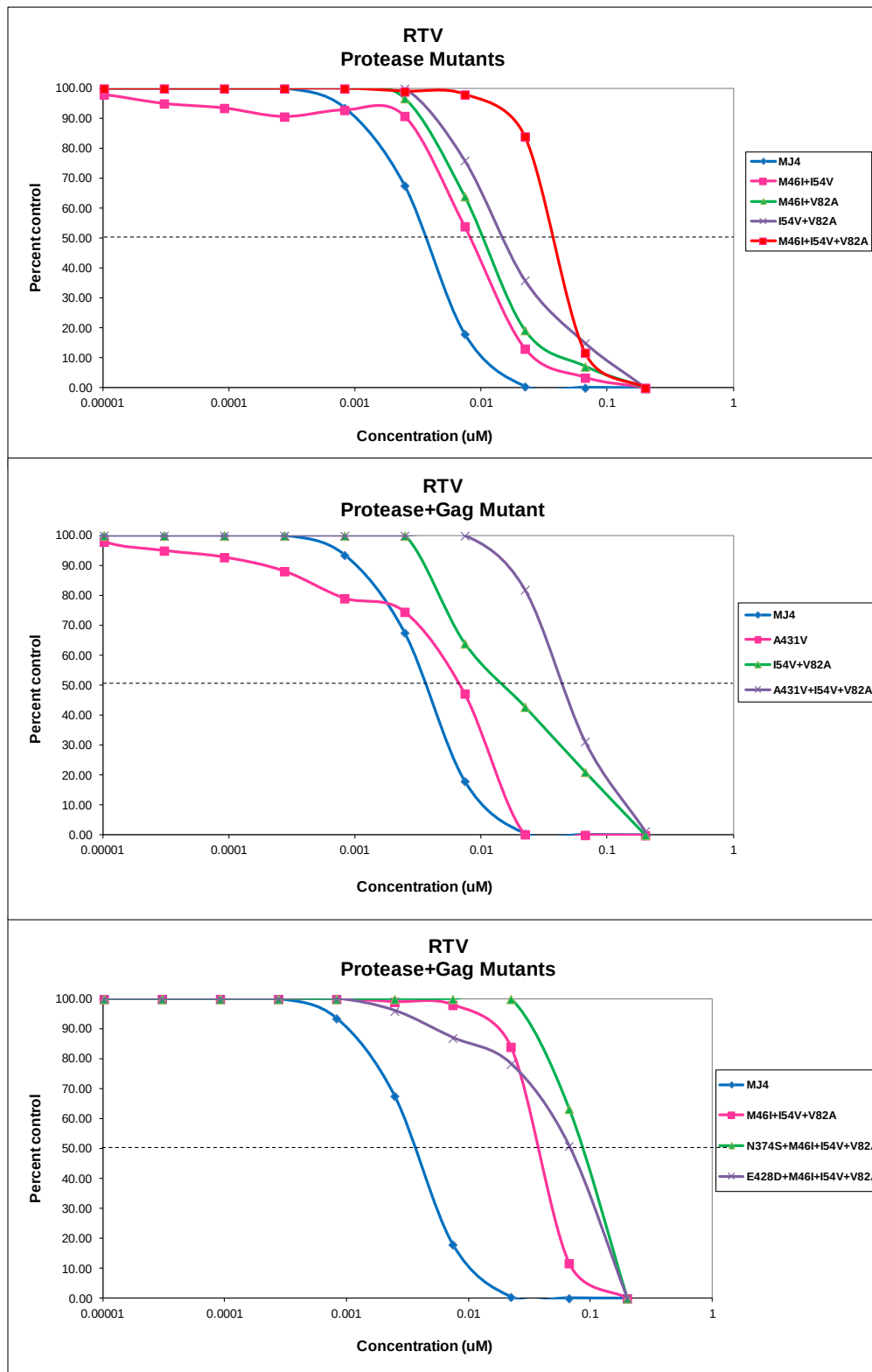


Figure 3.2.5. Phenotypic assay standard curves for *protease* and *gag* mutants to RTV

Multiple mutants were created by successive rounds of site-directed mutagenesis. The fold change of each mutant was calculated relative to p8.MJ4. The *protease* only mutants in the upper graph show the triple mutant M46I+I54V+V82A being the least susceptible to RTV. Addition of *gag* mutations N374S and E428D caused a distinct increase in resistance to RTV (second graph). Addition of *gag* mutation A431V to I54V+V82A caused a further increase in resistance to RTV.

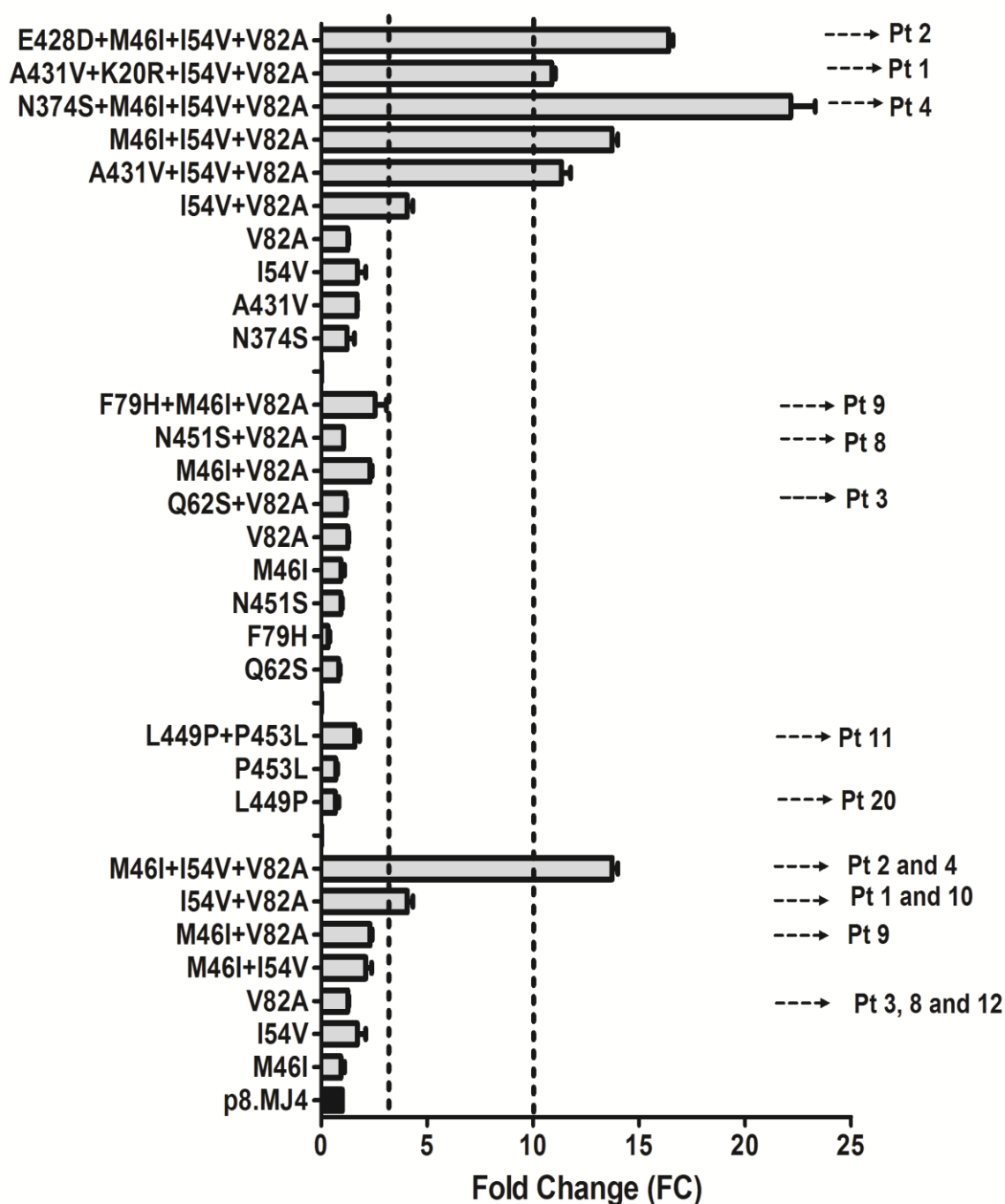


Figure 3.2.6. Phenotypic resistance of *protease* and *gag* mutations to RTV

Phenotypic response of single and multiple site-directed mutations created in p8.MJ4 subtype C HIV-1 expression plasmid to RTV. Phenotypic susceptibility was determined with a single-cycle non-replicative phenotypic assay. *Protease* and *gag* mutations were selected from patient samples based on their known or potential involvement in PI resistance. The patient sample to which each SDM is associated with is indicated on the graph. The fold change of each mutant was calculated relative to p8.MJ4. The lower and upper cut-offs are indicated by dotted lines. Error bars indicate the standard error of the mean (SEM). Each mutant was tested in at least two independent experiments, in duplicate.

Table 3.2.4. Frequency of site-directed mutations in patient samples and in the Stanford drug resistance database

Resistance database

Mutation	Frequency in Cohort (%)		Frequency in HIV-1 Subtype C in the Stanford database (%)	
Protease				
	Baseline (n=20)	Failure (n=20)	PI Naive (n=10466)	PI Treated (n=1208)
M46I	0.0	15.0	0.2	9.1
I54V	0.0	20.0	0.0	11.0
V82A	0.0	40.0	0.1	12.0
Gag				
	Baseline (n=20)	Failure (n=20)	PI Naive* (n=2152)	
Q62S	0.0	5.0	0.8	
F79H	0.0	5.0	2.2	
N374S	0.0	5.0	21.0	
A431V	0.0	10.0	0.1	
L449P	5.0	15.0	0.0	
P453L	10.0	15.0	3.2	

* The subtype C naive sequences used to determine the frequency of the *gag* mutations were obtained from the Los Alamos Sequence Database.

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Chapter 3.3.

HIV-1 subtype C viruses from children failing first line PI therapy: are they sensitive to other PIs?

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TOTAL WORD COUNT: 3851

3.3.1. Abstract

Introduction

The South African national guidelines recommend the use of ritonavir boosted-lopinavir (LPV/r) as a first line regimen for HIV-infected children <3 years of age. After failure of protease inhibitor (PI)-based therapy, options are limited for the treatment of paediatric patients. Here we investigated the phenotypic susceptibility of patients failing a RTV and/or LPV/r-based regimen to other PIs.

Materials and Methods

A phenotypic analysis of the *gag-protease* genes from 20 children failing a LPV/r and/or ritonavir (RTV)-based regimen was performed to determine their susceptibility to other PIs.

Results

Among the 20 patients tested, phenotypic susceptibility was shown for lopinavir (LPV) (n= 12), ritonavir (RTV) (n= 10), amprenavir (APV) (n= 16), atazanavir (ATV) (n= 11), darunavir (DRV) (n= 18), saquinavir (SQV) (n= 16). Four patients showed multi-drug resistance to LPV, RTV, APV, ATV and SQV. A reduced susceptibility in the absence of PI mutations was seen in 4 patients. These 4 patients contained *gag* cleavage site (CS) and/or non-CS changes. The majority of samples remained susceptible to DRV, with only 2 patients showing reduced susceptibility. One of these patients had a wild type *protease* with mutations in *gag*.

Conclusion

Mutations in *protease* acquired from RTV and LPV/r-based therapy, cause cross-resistance to other PIs. DRV, which remained phenotypically active against most mutants, is an effective alternative option for patients failing LPV/r-based therapy. Furthermore, since *gag* could influence the sensitivity of viruses to PIs the analysis of *gag* in addition

to *protease* could be useful in identifying effective drugs for use in future PI-based regimens.

3.3.2. Introduction

The use of protease inhibitors (PIs) for treatment of HIV-1-infected infants is recommended following possible exposure to NNRTI-based regimens for PMTCT, and has been adapted as standard-of-care in South Africa. In most resource-limited countries LPV/r is used in PI-based regimens. While LPV/r has a high genetic barrier to resistance, its effectiveness is limited due to drug-drug interactions and poor palatability resulting in poor adherence and treatment failure. Unfortunately, paediatric patients face limited subsequent PI options upon failure on a LPV/r-based regimen. Since HIV-1 infected children require life-long antiretroviral treatment, optimal adherence and identification of effective PIs will limit the need for unnecessary regimen change.

PIs target the HIV-1 protease enzyme and function by inhibiting the cleavage of HIV-1 Gag and Gag-Pol polyproteins resulting in the formation of immature viruses (Arts and Hazuda, 2012). There are currently 10 PIs approved for use by the US Food and Drug Administration (National Institutes of Health, 2014). Only 5 of these PIs (LPV/r, tipranavir (TPV/r), fosamprenavir (FTC), ritonavir (RTV) and nelfinavir (NFV)) have formulation options that are approved for use in children <3 years of age. In addition, mutations that are selected during LPV/r-based therapy, such as M46I, I54V and V82A, provide cross-resistance to the other PIs (Rhee et al., 2010). *Protease* mutation M46I has been associated with a decreased susceptibility to LPV, APV, IDV, and NFV. I54V decreases susceptibility to LPV, ATV, IDV, NFV AND SQV while V82A decreases susceptibility to LPV and IDV (Rhee et al., 2010).

Failure on PI-based regimens in the absence of mutations in *protease* have been reported in several studies (Zanoni et al., 2012, Levison et al., 2012, Orrell et al., 2013, Babiker et

al., 2011). Non-adherence is often the cause of the absence of resistance mutations, however, certain changes occurring at the *gag* CS have been shown to contribute to or directly cause resistance to PIs even in the absence of mutations in *protease* (Dam et al., 2009, Fun et al., 2012, Nijhuis M, 2007). Furthermore, an increased prevalence of *gag* CS mutations has been observed in PI treated patients compared to treatment naive patients (Verheyen et al., 2006, Nijhuis M, 2007). The *gag* CS mutations A431V and I437V/T have been associated with DRV resistance (Lambert-Niclot et al., 2008). A431V was associated with selection of *protease* mutation L76V during DRV therapy while I437V/T was associated with a lower rate of DRV resistance mutations. The exact role of *gag* non-CS mutations is not well understood, however it has been speculated that changes at non-CS may render the CS more available to protease via conformational changes in *gag* (Gatanaga et al., 2002). A study looking at the effect of non-CS mutations on PI susceptibility identified novel mutations in *gag* (codons 12, 75, 219, 390 and 409) which increased resistance to APV and delayed the emergence of resistance to RTV and NFV (Aoki et al., 2009).

Since HIV-1 Protease and its substrate Gag appear to co-evolve during resistance selection, both should be considered as a single functional unit when investigating PI resistance (Kolli et al., 2014, Gupta et al., 2010). To identify PIs that remain effective after LPV/r failure and investigate the role of *gag* in resistance to these PIs, patient-derived samples and *gag-protease* site-directed mutants were assessed for phenotypic susceptibility to APV, ATV, DRV and SQV.

3.3.3. Materials and Methods

3.3.3.1. Samples from HIV-1 infected children

Samples from 20 HIV-1 infected subtype C paediatric patients at baseline and failing a PI-based regimen were collected and analysed (Chapter 3.1.3.1.). All children >6 months of age were treated with ritonavir-boosted lopinavir (LPV/r) and two NRTIs. Children <6 months of age and those receiving treatment for tuberculosis, were treated with ritonavir (RTV) and two NRTIs. This study was approved by the institutional review boards of Columbia University and the University of the Witwatersrand.

3.3.3.2. Construction of patient-derived test vectors

A retroviral vector system, capable of a single infectious event was used to determine the phenotypic susceptibility to PIs (Gupta et al., 2010, Parry et al., 2009) (Chapter 3.2.3.2). Briefly, 1.8kb *gag-protease* amplicon from patient samples containing *NotI* and *XhoI* restriction sites was sequenced and cloned into the HIV-1 expression vector p8.9NSX+. Sequences were submitted to the Stanford HIVdb drug resistance algorithm (hivdb.stanford.edu) for identification of protease inhibitor resistance mutations and to predict phenotypic PI resistance. Polymorphisms in the *protease* and *gag* genes were defined as changes in amino acid sequence between the failing and baseline sequences.

3.3.3.3. Site-directed mutagenesis

An HIV-1 expression vector containing the *gag-protease-reverse transcriptase* fragments of the MJ4 subtype C reference strain (GenBank: AF321523.1) (Ndung'u et al., 2001) was used for construction of site-directed mutants (Chapter 3.2.3.3). The *protease* mutations were chosen based on the genotype of the patient samples and those in the Stanford Database which showed high-level resistance to each PI. The *gag* changes were selected from patient samples based on their known or potential involvement in PI resistance. Additional *gag* non-CS mutants, which were shown to develop under positive selection pressure (Chapter 3.1), were also created. Briefly, single site-directed mutants were created by use of the QuickChange® II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, California, USA). Mutants with multiple mutations were created by successive rounds of site-directed mutagenesis.

3.3.3.4. Phenotypic assessment of PI susceptibility

A single cycle phenotypic assay was performed as previously described (Parry et al., 2009, Gupta et al., 2010), with minor modifications (Chapter 3.2.3.4). Briefly, HEK293T cells were transfected with 300 ng of pMDG, 500 ng of pCSFLW and 300 ng of patient derived expression vector using 3.3 µg polyethylenimine (PEI) (Polyscience, Inc., Warrington, Pennsylvania, USA). Transfected cells were harvested and seeded in the presence of serial dilutions of PIs. Infectious supernatants were used to infect fresh HEK293T cells. The degree of infection was determined by measuring the expression of firefly luciferase in infected target cells at 48 hours post-infection. The phenotypic susceptibility of patient's samples at PI failure was expressed as a fold-change (FC) relative to the baseline sample. The phenotypic susceptibility of site-directed mutants was expressed as a FC relative to p8.MJ4. The lower cut-off values differed for the patient samples and site-directed mutants due to the different controls used in each assay. For the

patient samples, the p8.9NSX+ vector containing the *gag-protease* fragment from p8.MJ4 creating p8.MJ4GP was used as a control. For the site-directed mutants, p8.MJ4 was used as a control since all mutants were created in p8.MJ4. The phenotypes were classified as sensitive (S), reduced susceptibility (RS) and resistant (R). Sensitive referred to FC which was < the lower cut-off, reduced susceptibility referred to FC > lower cut-off but less than 10 FC, and resistant referred to FC >10. A 10 FC was arbitrarily chosen as an upper cut-off value. The assay cut-offs for the patient samples and site-directed mutants are shown in Table 3.3.7.

3.3.3.5. Statistical Analysis

Two-way analysis of variance (ANOVA) was used to identify significant differences in FC values between the site-directed mutants using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California, USA).

3.3.4. Results

3.3.4.1. Phenotypic susceptibility of patient-derived *protease* and *gag* genes

Genetic changes in the *gag* and *protease* genes of paediatric patients were previously reported (Chapter 3.1) (Giandhari et al., 2015). In addition these changes were phenotypically tested to examine the impact of these changes on LPV and RTV susceptibility (Chapter 3.2). To determine the impact of these changes on other PIs the baseline and failing samples were tested against APV, ATV, DRV and SQV. Protease inhibitor exposure, phenotypic drug susceptibility and genotypic profiles of the 20 children are shown in Table 3.3.1. All *protease* and *gag* changes were based on the failing sample relative to the baseline. The *gag* non-CS mutations were previously reported in section 3.1.3.

Of the 7 patients treated with RTV+LPV/r, one patient (Pt. 7) showed phenotypic susceptibility to 6 of the PIs tested. Patient 7 contained no major PI mutations or *gag* CS mutations. Three patients (Pt. 1, Pt. 2 and Pt. 3) treated with RTV+LPV/r showed cross-resistance to ≥ 5 PIs. All 3 of these patients had major PI mutations and *gag* changes. Of the 7 patients treated with RTV monotherapy, 2 patients (Pt. 12 and Pt. 13) showed susceptibility to all PIs tested while 4 patients (Pt. 8, Pt. 9, Pt.10 and Pt. 11) showed cross-resistance to ≥ 3 PIs. Of these 4 patients, 3 had PI mutations while one only had changes in *gag*. All 6 patients treated with LPV/r that lacked PI mutations were phenotypically susceptible to all the PIs tested.

Of the 20 patients tested, phenotypic susceptibility to DRV was seen in 18 (90%), to APV in 16 (80%), to and to SQV in 16 (80%), to LPV was seen in 12 (60%), to ATV in 11 (55%) and to RTV in 10 (50%). Reduced susceptibility was seen in 8 (40%) patients to

LPV, 10 (50%) patients to RTV, 4 (20%) patients to APV, 9 (45%) patients to ATV, 2 (10%) patients to DRV and 4 (20%) patients to SQV. DRV showed the least phenotypic resistance with 2 patients having reduced susceptibility of 5.5 FC and 5.3 FC. The phenotypic curves of all patients are shown in Figures 3.3.1, 3.3.2, 3.3.3 and 3.3.4 for APV, ATV, DRV and SQV. The phenotypic curves for LPV and RTV were previously shown in Chapter 3.2. The fold change for each failing patient was calculated relative to its matched baseline sample.

Four patients (Pt. 5, Pt. 6, Pt. 11 and Pt. 14) showed reduced susceptibility to PIs in the absence of PI mutations. Reduced susceptibility to RTV (6.0 FC) and ATV (3.8 FC) in the presence of *gag* non-CS mutations was seen in patient 5. Although marginally above the cut-off, reduced susceptibility to ATV (2.4 FC) was seen in patient 6 in the presence of *gag* CS and non-CS changes. Interestingly, patient 11 showed reduced susceptibility to 4 PIs: ATV (FC 11.5), LPV (FC 5.9), RTV (FC 7.4) and DRV (FC 5.3), whilst having only *gag* changes. Reduced susceptibility to RTV (FC 3.8) was seen in patient 14 in the presence of *gag* non-CS changes.

Patients with both major PI mutations and *gag* CS mutations showed greater reduced susceptibility to all the PIs compared to patients with only *protease* mutations, *gag* CS mutations and those with only *gag* non-CS mutations (Figure 3.3.5). This demonstrated the contribution of *gag* CS to PI resistance in the presence of PI mutations. Patients with only *gag* CS mutations showed increased susceptibility to LPV, APV, DRV and SQV.

3.3.4.2. The predicted phenotype versus the phenotypic assay

The predicted phenotype of each patient to 5 PIs (LPV, APV, ATV, DRV and SQV) was determined using the Stanford HIV-1 Drug Resistance database. Phenotypic predictions

for RTV are not available on the database. Discordance between the database and the assay were defined as: i) any sample showing susceptibility in the phenotypic assay and any level of resistance predicted by the database; ii) any sample having predicted susceptibility by the database and showing either reduced susceptibility or high-level resistance in the assay. Discordance between the database prediction and the phenotypic assay was seen for 8 patients (Pt 2, 4, 5, 6, 9, 10 and 11) as shown in Table 3.3.2 and Figure 3.3.6. For 4 patients (Pt 2, 5, 6 and 11) the database had predicted susceptibility however the phenotypic assay showed reduced susceptibility for these patients. The inclusion of *gag* in our assay may be responsible for this reduced susceptibility. The remaining 4 discordant patients (Pt 4, 9, 10 and 12) the database predicted low to high levels of resistance to the different PIs however the phenotypic assay predicted susceptibility.

3.3.4.3. Phenotypic susceptibility of control *protease* site-directed mutants

Single, double and triple *protease* mutants were created in p8.MJ4 and tested for phenotypic susceptibility to LPV, RTV, APV, ATV, DRV and SQV (Figures 3.3.7 and 3.3.8). These mutants included those which occurred in the patient samples (M46I, I54V and V82A) as well as additional combinations of mutations (G48V, I47V and I84V) which were predicted by the Stanford database to show resistance to each drug (Chapter 2.2, Figure 2.7). From the single *protease* mutants G48V showed reduced susceptibility to APV (FC 2.1) and SQV (7.6 FC) ($P < 0.001$). Mutant I54V showed reduced susceptibility (1.5 FC) to SQV modestly above the cut-off. M46I+I47V showed reduced susceptibility to APV (FC 5.8) which was statistically significant ($P < 0.001$) compared to ATV, DRV, LPV, RTV and SQV. I54V+V82A caused reduced susceptibility to LPV (FC 6.0) and RTV (FC 4.0). For LPV, the reduced susceptibility was significantly ($P < 0.001$) higher compared to RTV, APV, ATV, DRV and SQV. Triple *protease* mutant

M46I+I54V+V82A showed resistance to RTV (FC 13.7) and reduced susceptibility to ATV (FC 3.6) and LPV (FC 4.7). For RTV, the increase in resistance was significant ($P < 0.001$) compared to LPV, APV, ATV, DRV and SQV. For ATV, a significant ($P < 0.001$) change in susceptibility was seen compared to APV, DRV and SQV. The reduced susceptibility to LPV was significant ($P < 0.001$) compared to APV, DRV and SQV. M46I+I54V+I84V showed reduced susceptibility to RTV (FC 3.2) ($P < 0.05$) compared to ATV, DRV and LPV. G48V+I54V+V82A showed reduced susceptibility to RTV (FC 6.9) and high-level resistance to ATV (FC 16.8), LPV (FC 10.6) and SQV (FC 26.9). All *protease* mutants showed susceptibility to DRV.

3.3.4.4. Phenotypic susceptibility of *gag* site-directed mutants

Single and multiple *protease* and *gag* mutants were created in p8.MJ4 and tested for phenotypic susceptibility to APV, ATV, DRV and SQV (Figure 3.3.10, 3.3.12, 3.3.14 and 3.3.16). These mutations and combinations were based those found in the patient samples. A comparison of the fold change in resistance between the patient samples and the mutants based on them are shown in Tables 3.3.3, 3.3.4, 3.3.5 and 3.3.6. All mutants had a lower fold change compared to the respective patient samples.

Cross-resistance conferred by site-directed mutants were noted primarily with the PI major mutations (M46I, I54V and V82A) to ATV and SQV. Of the single *gag* mutants, cleavage site mutations N374S showed reduced susceptibility to SQV and P453L showed reduced susceptibility to DRV (Figure 3.3.13) which were marginally above the assay cut off. *Gag* mutants (L449P+P453L and F79Y+L449P+P453L) and *gag-protease* mutants (Q62S+V82A, F79H+M46I+V82A and S451N+V82A) showed susceptibility to all PIs tested. Reduced susceptibility and resistance was seen with mutants carrying ≥ 3 *gag-protease* mutations. Mutant N374S+M46I+I54V+V82A caused reduced susceptibility to

LPV (5.9 FC) and high-level resistance to RTV (22.1 FC). The resistance caused by N374S+M46I+I54V+V82A to RTV was significant ($P < 0.001$) compared to APV, ATV, DRV and SQV. Mutant A431V+I54V+V82A caused resistance (11.3 FC) to RTV which was significant ($P < 0.05$) compared to APV, ATV, DRV and SQV. A431V+I54V+V82A caused reduced susceptibility to SQV (Figure 3.3.15) slightly above the cut off (1.2 FC). Addition of minor *protease* mutation K20R to A431V+I54V+V82A did not affect resistance to RTV (10.8 FC) or any of the other PIs. Interestingly, for APV, there was a slight increase in FC seen with the addition of K20R to A431V+I54V+V82A (Figure 3.3.9.). Mutant E428D+M46I+I54V+V82A caused reduced susceptibility to LPV (5.8 FC) and SQV (1.4 FC) (Figure 3.3.15) and high-level resistance to RTV (16.4 FC). Resistance caused by E428D+M46I+I54V+V82A to RTV was significant ($P < 0.001$) compared to APV, ATV, DRV and SQV. For ATV, although not significant, the addition of *gag* mutations to *protease* mutations slightly increased the FC (Figure 3.3.11). Overall, all mutants were susceptible to APV and ATV. Mutants had a minimal effect on sensitivity to DRV and SQV with fold changes < 1.5 .

In comparison to the patients' phenotypic susceptibility, differences were observed for some mutations by site-directed mutagenesis. For APV and DRV the mutants created based on the patients samples did not correlate with the patient's phenotype as most mutants showed susceptibility to the PIs. For ATV, M46I+I54V+V82A based on patient 2 and 4, showed reduced susceptibility compared to patient 2 which showed high-level resistance. In patient 4, susceptibility to ATV was seen while the triple *protease* mutant showed reduced susceptibility. Interestingly, with a further addition of *gag* CS mutations E428D and N374S an increase in susceptibility was seen. For SQV, the triple *protease* mutant (M46I+I54V+V82A) based on patient 2, showed reduced susceptibility whereas

patient 2 had a phenotype showing high-level resistance. Further addition of *gag* CS mutation E428D reduced susceptibility to SQV marginally above the cut-off.

3.3.5. Discussion

Protease inhibitors are potent antiretroviral drugs used to treat HIV-1 infection, with LPV/r being the most widely used in resource limited settings. Recent studies have shown a low frequency of *protease* mutations in patients failing LPV-based therapy. Earlier studies have shown that changes in *gag* at failure contributed to resistance to LPV and RTV in the presence and absence of PI mutations in some patients. This data shows that major PI mutations from a prior RTV-based regimen caused cross-resistance to other PIs. Additionally, in some patients with no PI mutations, changes in *gag* were found to cause reduced susceptibility to some of the PIs. However, patients who remained susceptible to the PIs in the absence of PI mutations reflect what is currently seen in clinical settings where majority patients failing PI therapy have a wild-type *protease*.

As previously shown with LPV and RTV, phenotypic resistance was higher in patients who had received prior RTV monotherapy compared to those treated with LPV/r exclusively (Chapter 3.2). This was primarily due to the selection of PI mutations in the RTV treated patients. Some patients carrying PI mutations showed cross-resistance to five of the six PIs tested. In these patients, only DRV showed efficacy suggesting that this may be a good PI for future use in LPV/r failures. Interestingly one patient with wild type *protease* showed cross-resistance to 4 PIs i.e LPV, RTV, ATV and DRV. In this patient changes in *gag* were present both at CS and non-CS. Screening of relevant site-directed mutants did not disclose a particular pattern of PI mutations and/or *gag* changes that could be associated with PI resistance. However, as an entity the presence of PI and *gag* CS mutations were associated with greater phenotypic resistance compared to patients with PI mutations only. A comparison between the Stanford database and the phenotypic assay showed that half of the discordant samples in the database had under estimated the

phenotypic response which may be due to the inclusion of *gag* in the phenotypic assay. Since the database assigns a score to every mutation for a particular PI, the phenotypic prediction is based on the accumulative mutation scores. However, the presence of polymorphisms and their effects are not taken into account by the database and this may be a reason for differences seen with our phenotypic assay.

Since the patients did not have *protease* mutations associated with resistance to all PIs a subset of additional PI mutations were created and tested based on the Stanford drug resistance database. Each drug showed reduced susceptibility to high-level resistance with ≥ 2 mutants except for DRV which showed complete susceptibility further confirming the effectiveness of DRV in patients failing LPV/r with PI mutations. Individual *gag* site-directed mutants did not show resistance to the PIs, but in combination with *protease* mutations, high-level resistance was seen to RTV confirming the weakness of RTV as a single PI. The results from the site-directed mutagenesis show that *gag* contributes to PI resistance especially when tested against RTV as shown by mutants A431V+I54V+V82A, N374S+M46I+I54V+V82A and E428D+M46I+I54V+V82A. Thus suggesting that most *gag* mutations present in the patient samples may have been selected due to RTV monotherapy.

For some patients, treatment failure could not be explained by using patient-derived *gag-protease* in the phenotypic assay. In these patients reasons for failure may include poor adherence to regimen especially since these were paediatric patients. This would result in the absence of PI mutations and susceptibility of patients to PIs as was shown in our study. Incorrect dosing of the RTV component in LPV/r may have led to decreased plasma concentrations of LPV resulting in reduced drug selection pressure.

Protease mutations conferring resistance to one or more PIs have been well described and supported by databases (Rhee et al., 2010, Rhee et al., 2003, Shafer, 2006). However, sufficient data to identify specific *gag* determinants of PI susceptibility is still required. Most studies have focused on specific *gag* CS in the context of PI susceptibility, but it has been shown that full-length *gag* should be considered for PI resistance especially since changes outside of the CS also influence PI susceptibility (Gupta et al., 2010, Gatanaga et al., 2002).

This study has shown the importance of including *gag* in addition to *protease* when determining PI susceptibility. In patients failing RTV and/or LPV/r-based therapy, the pre-existence of PI and *gag* mutations may result in cross-resistance to other PIs thereby limiting effective PI options. These results demonstrate the importance of genotypic testing of PI-based therapy failures to guide future PI therapy options.

There is an increased need for more PIs to be made available for children who are failing PI therapy especially since they have limited options available (van Zyl et al., 2011, Gupta et al., 2009). This study demonstrated the *in vitro* effectiveness of other PIs on paediatric patients failing LPV/r therapy. Currently ATV and APV are not available for children < 6 years and SQV and DRV are not recommended in children <2 and <3 years respectively (National Institutes of Health, 2014). We are faced with the pharmacokinetic challenges and tolerability of these drugs in paediatric patients. Especially in children where adherence is a concern, improving the palatability of drugs is important. In addition, making more PIs more easily accessible in resource limited settings is essential.

3.3.6. Acknowledgements

This study was funded by grants from the Medical Research Council (MRC), Poliomyelitis Research Foundation (PRF), KwaZulu-Natal Research Institute for Tuberculosis and HIV (K-RITH) and the Faculty Research Council (FRC) of the University of Witwatersrand.

We would like to thank Didier Trono, EPFL, Lausanne, Switzerland, for the p8.9 and pMDG plasmids, and Nigel Temperton for the pCSFLW plasmid encoding luciferase. The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: APV, ATV, DRV, LPV, RTV and SQV.

Table 3.3.1. Phenotypic susceptibility and genotypic changes in patient samples

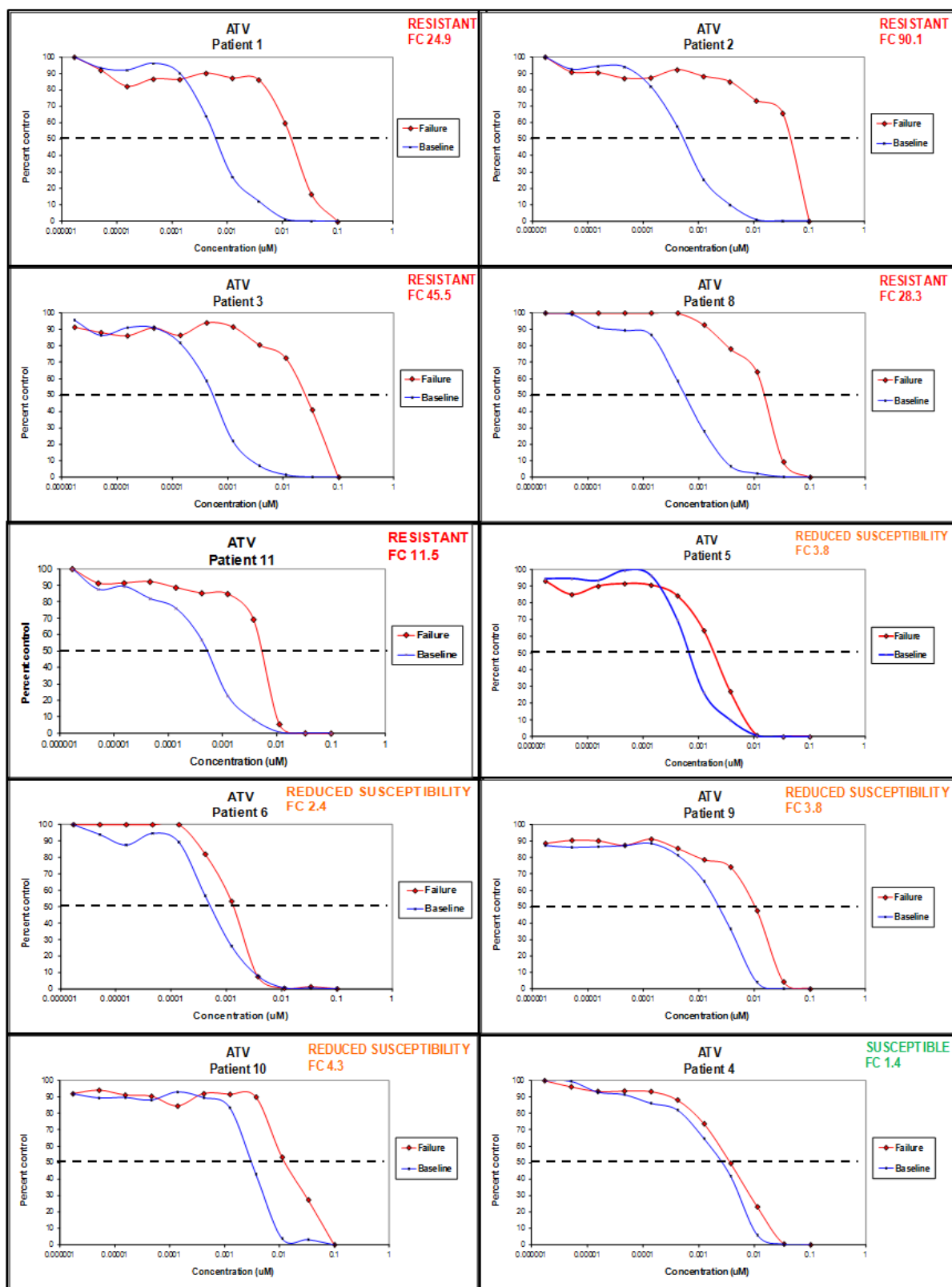
Pt. No.	Treatment	PI Susceptibility						Genotype	
		LPV (S< 4.4)	RTV (S< 3.2)	APV (S< 4.0)	ATV (S< 1.6)	DRV (S< 4.9)	SQV (S< 3.4)	Protease ^a	Gag-CS
1	RTV +LPV/r	109.0	32.0	8.7	24.9	3.0	7.7	<u>I54V, V82A</u> , K20R, L23I, E35D, L63P	<u>V378A, A431V</u>
2	RTV +LPV/r	191.0	44.4	51.8	90.1	5.5	14.9	<u>M46I, I54V, V82A, L10I</u> , K20R, E35D, <u>Q61H, L63P</u>	<u>E428D</u>
3	RTV +LPV/r	68.0	26.0	13.5	45.5	4.0	8.0	<u>V82A</u> , K20R, K45R	-
4	RTV +LPV/r	5.2	5.0	0.8	1.4	0.6	1.1	<u>M46I, I54V, V82A</u> , K20R, L33F, E35D, T74S	V128T, <u>N374S</u>
5	RTV +LPV/r	4.2	6.0	1.7	3.8	1.8	0.9	-	-
6	RTV +LPV/r	1.3	0.8	0.2	2.4	0.3	0.1	<u>K43E, N83S</u>	<u>S373A</u>
7	RTV +LPV/r	0.8	0.8	0.6	0.9	1.2	1.3	A71T	-
8	RTV	12.1	49.0	4.8	28.3	2.2	4.1	<u>V82A, S12T</u> , L10I, K20R, E35D, <u>D60E, Q61E</u>	<u>N451S</u>
9	RTV	6.0	5.7	1.4	3.8	1.3	0.5	<u>M46I, V82A, A63T</u>	V128I
10	RTV	7.6	3.4	0.8	4.3	1.4	0.9	<u>I54V, V82A</u> , I36M, N37S, L63P, V77I	<u>A431V</u>
11	RTV	5.9	7.4	1.2	11.5	5.3	0.9	N37S, L63S, V82I	V128I, <u>L449P, P453L</u>
12	RTV	1.2	2.3	1.5	1.0	1.6	0.6	<u>V82A</u> , K20R, E35D, R57K, D60E, L63P	-
13	RTV	0.1	1.0	0.5	1.5	1.4	0.8	E35D, L63V, T74S	E428K, L449P, P453L
14	RTV	2.6	3.8	1.0	0.9	0.9	0.2	L10M, I36M, N37K, K41I, L63T, <u>K70R</u>	-
15	LPV/r	0.4	1.2	0.6	0.3	0.4	0.6	K20R, E35D, L63V	-
16	LPV/r	1.3	1.6	0.7	1.1	0.6	2.2	I36M, K41R, T74S, V77I	I437L
17	LPV/r	0.4	0.4	0.3	0.5	0.5	0.5	L63V	-
18	LPV/r	2.1	1.9	0.5	1.4	0.8	0.5	V82I	Y132F
19	LPV/r	1.0	1.4	1.4	1.4	1.3	1.3	I36L, N37E, K41R, L63V	<u>T374A</u> , P453L
20	LPV/r	0.1	0.3	0.4	0.1	0.2	0.2	L63P, V77I	<u>L449P</u>

^aRTV + LPV/r: Patients received both RTV as a single PI as well as LPV/r; RTV: Patients received RTV as a single PI only; LPV/r: patients received LPV/r only

LPV, Lopinavir; RTV, Ritonavir; APV, Amprenavir; ATV, Atazanavir; DRV, Darunavir; SQV, Saquinavir
Protease mutations in bold represent major PI mutations. Mutations underlined represent changes which developed following PI-based therapy

^a Mutations highlighted in bold represent major PI mutations according to Stanford Drug Resistance Database

Levels of drug resistance: S= sensitive; RS= reduced susceptibility, H= High-level resistance (Highlighted by different levels of shading)



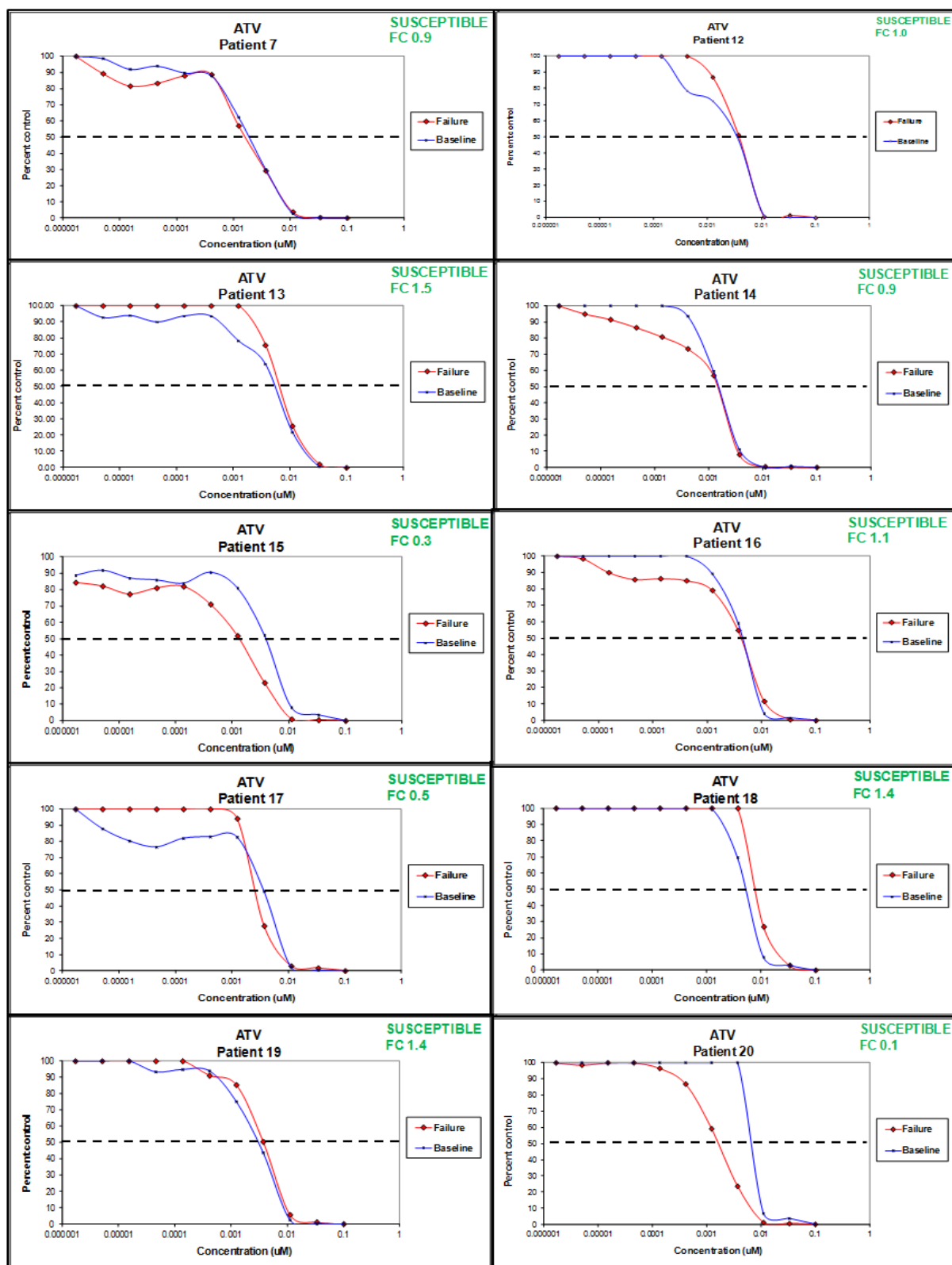
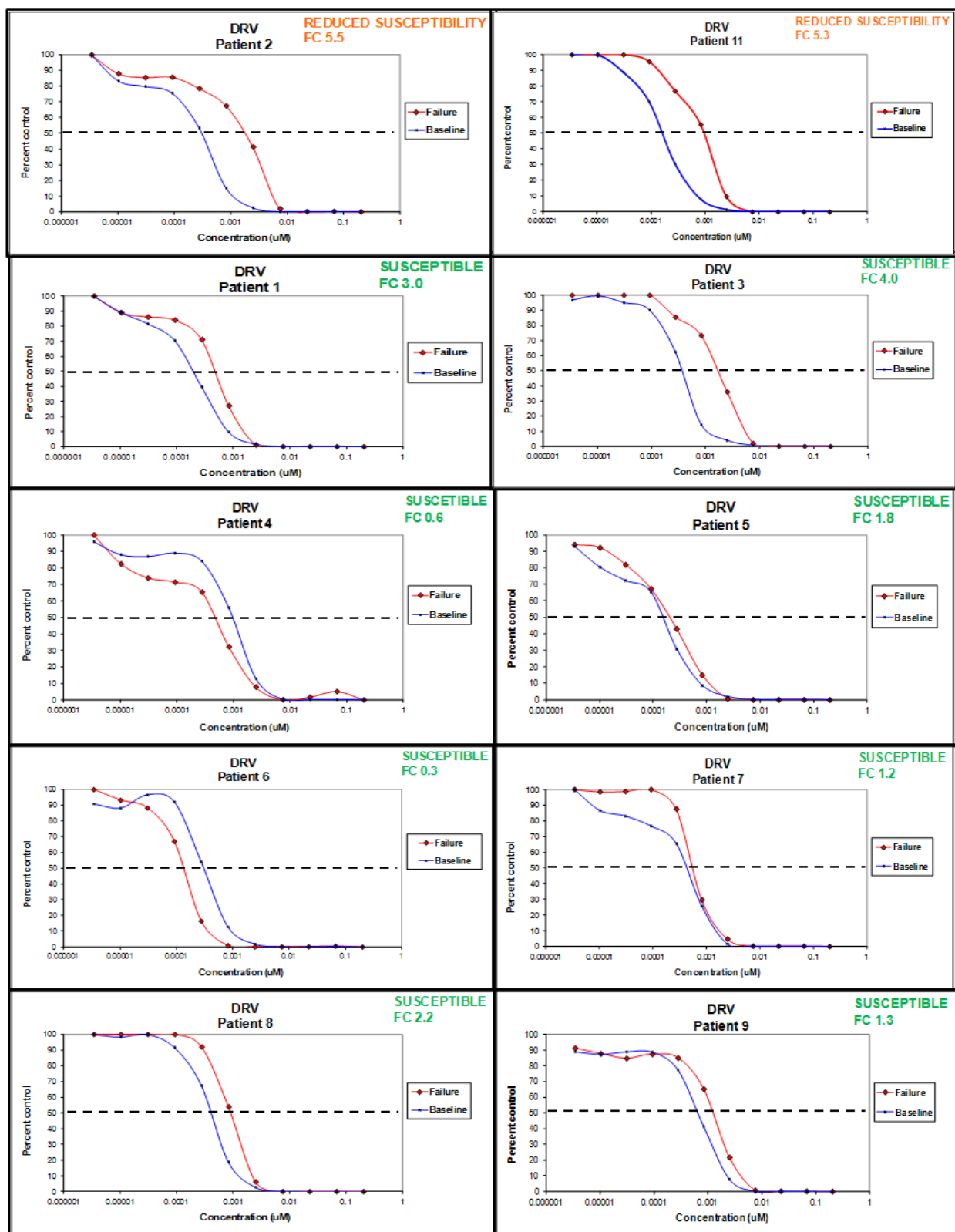


Figure 3.3.1. Phenotypic assay standard curves for patient samples to ATV

Fold changes are calculated based on the IC₅₀ value of the failing sample (red line) relative to the baseline sample (blue line). Each patient sample was tested in duplicate and the average of that is represented by the curve. The 5 resistant patients are shown in red at the top, the 4 with reduced susceptibility in orange and the 11 susceptible in green.



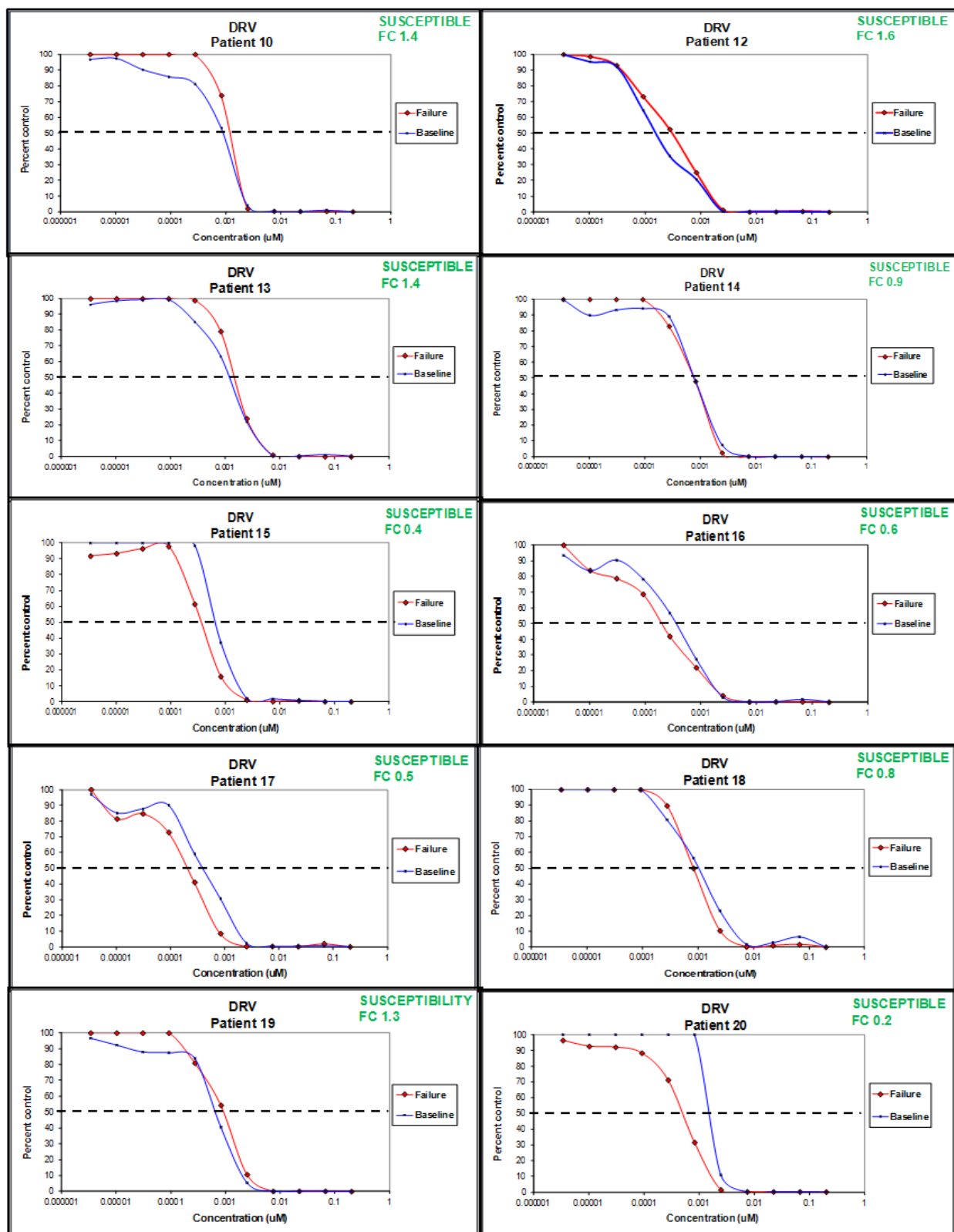
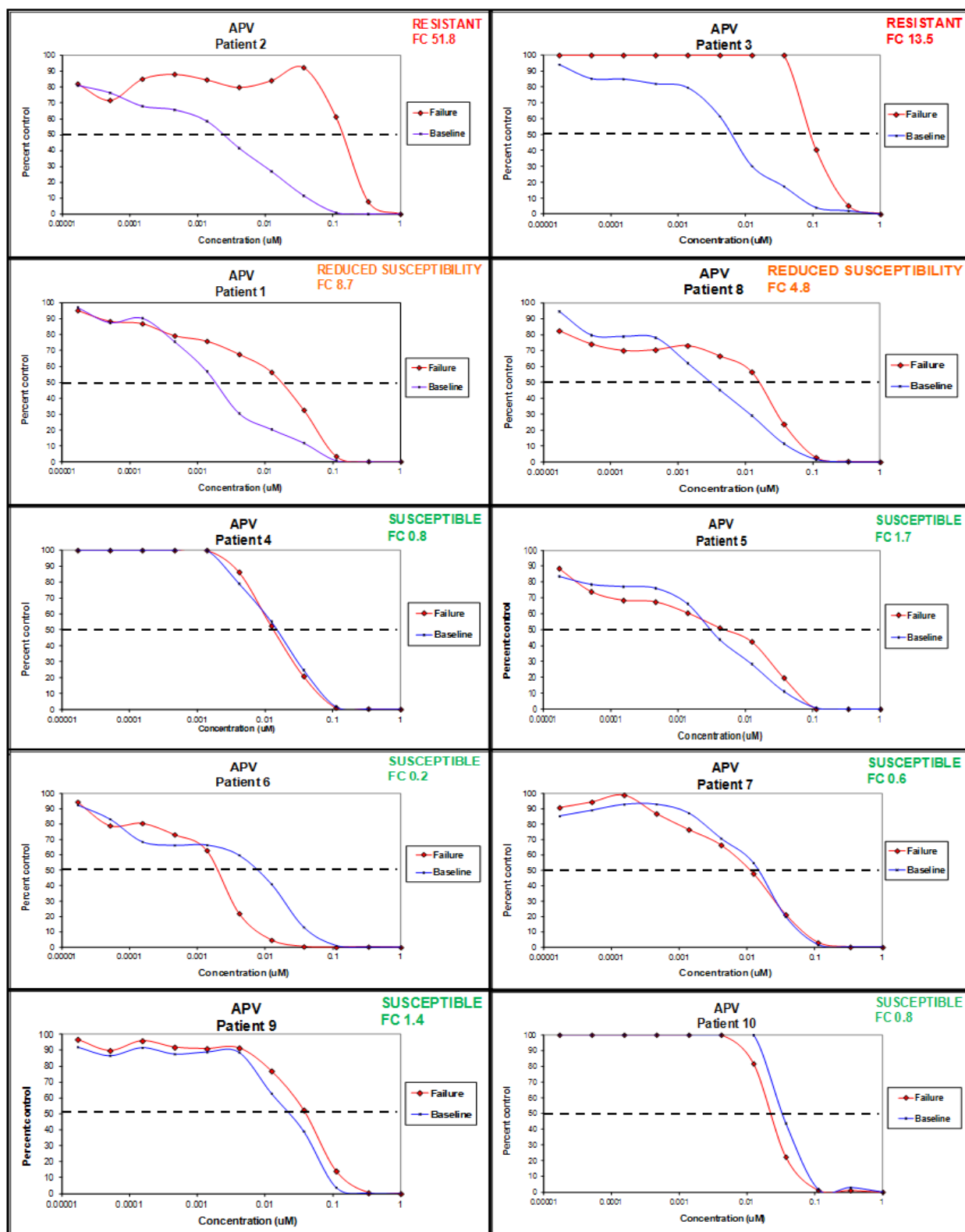


Figure 3.3.2. Phenotypic assay standard curves for patient samples to DRV

Fold changes are calculated based on the IC₅₀ value of the failing sample (red line) relative to the baseline sample (blue line). Each patient sample was tested in duplicate and the average of that is represented by the curve. The 2 patients with reduced susceptibility are shown in orange and the 18 susceptible in green.



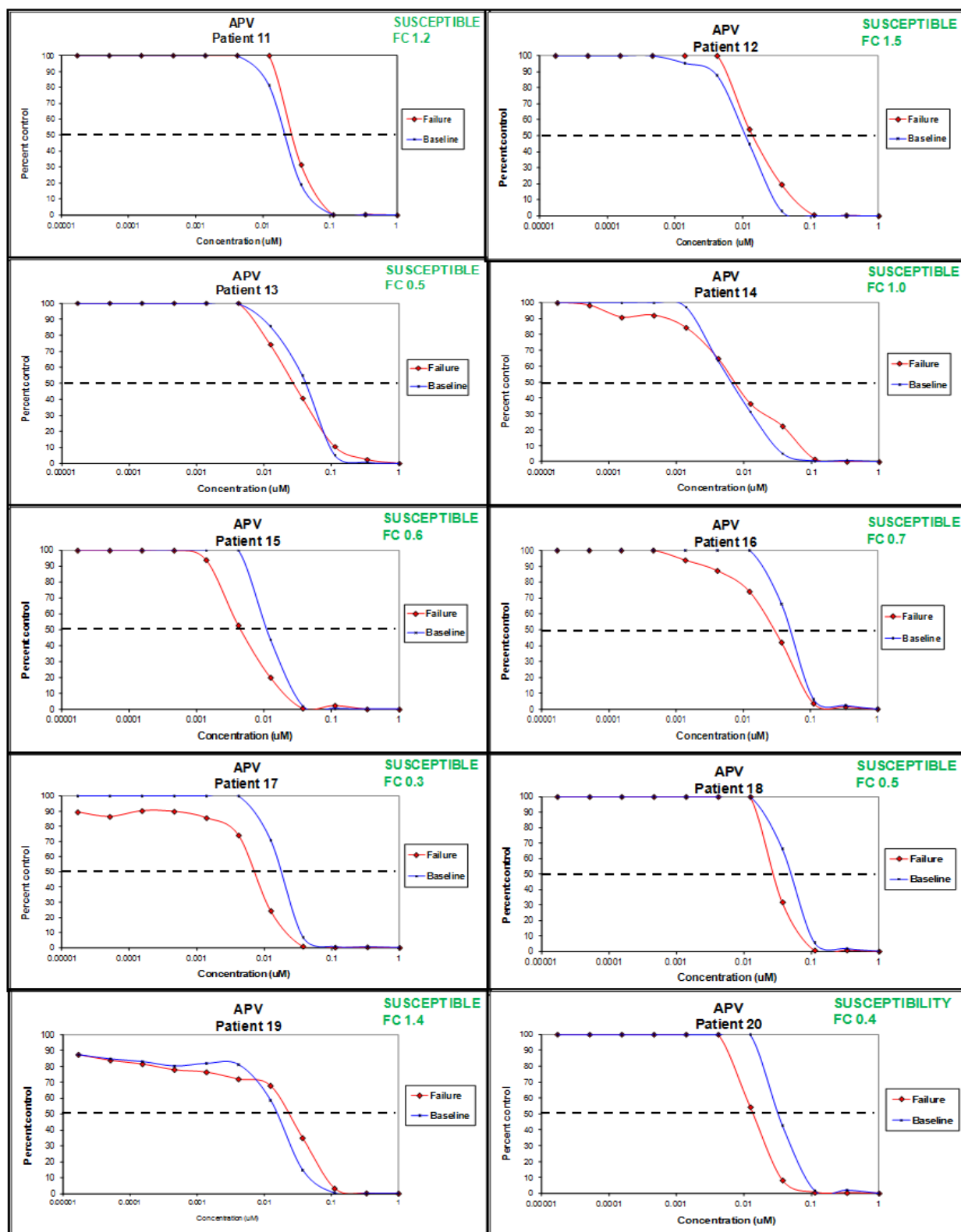
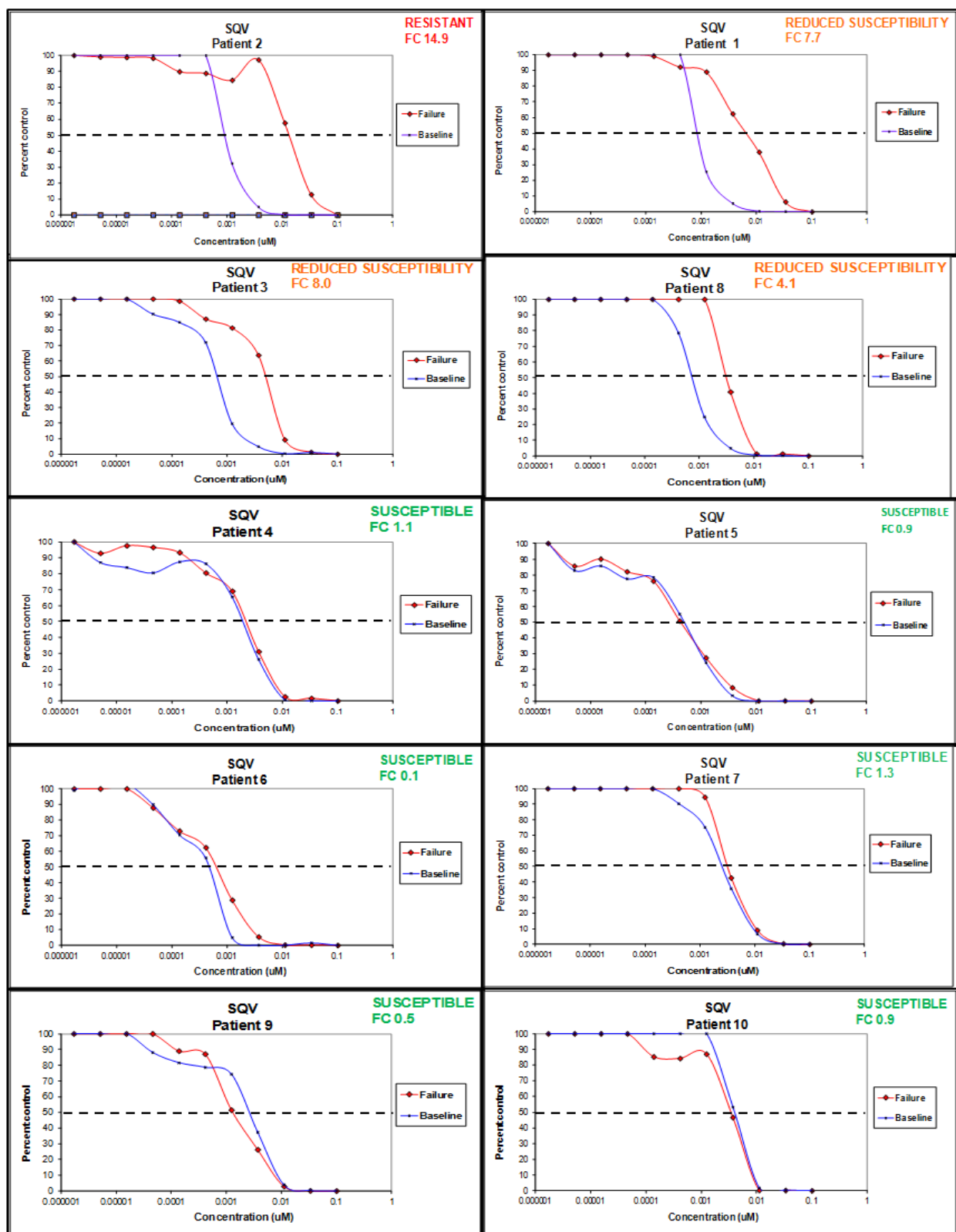


Figure 3.3.3. Phenotypic assay standard curves for patient samples to APV

Fold changes are calculated based on the IC₅₀ value of the failing sample (red line) relative to the baseline sample (blue line). Each patient sample was tested in duplicate and the average of that is represented by the curve. The 2 resistant patients are shown in red at the top, the 2 with reduced susceptibility in orange and the 16 susceptible in green.



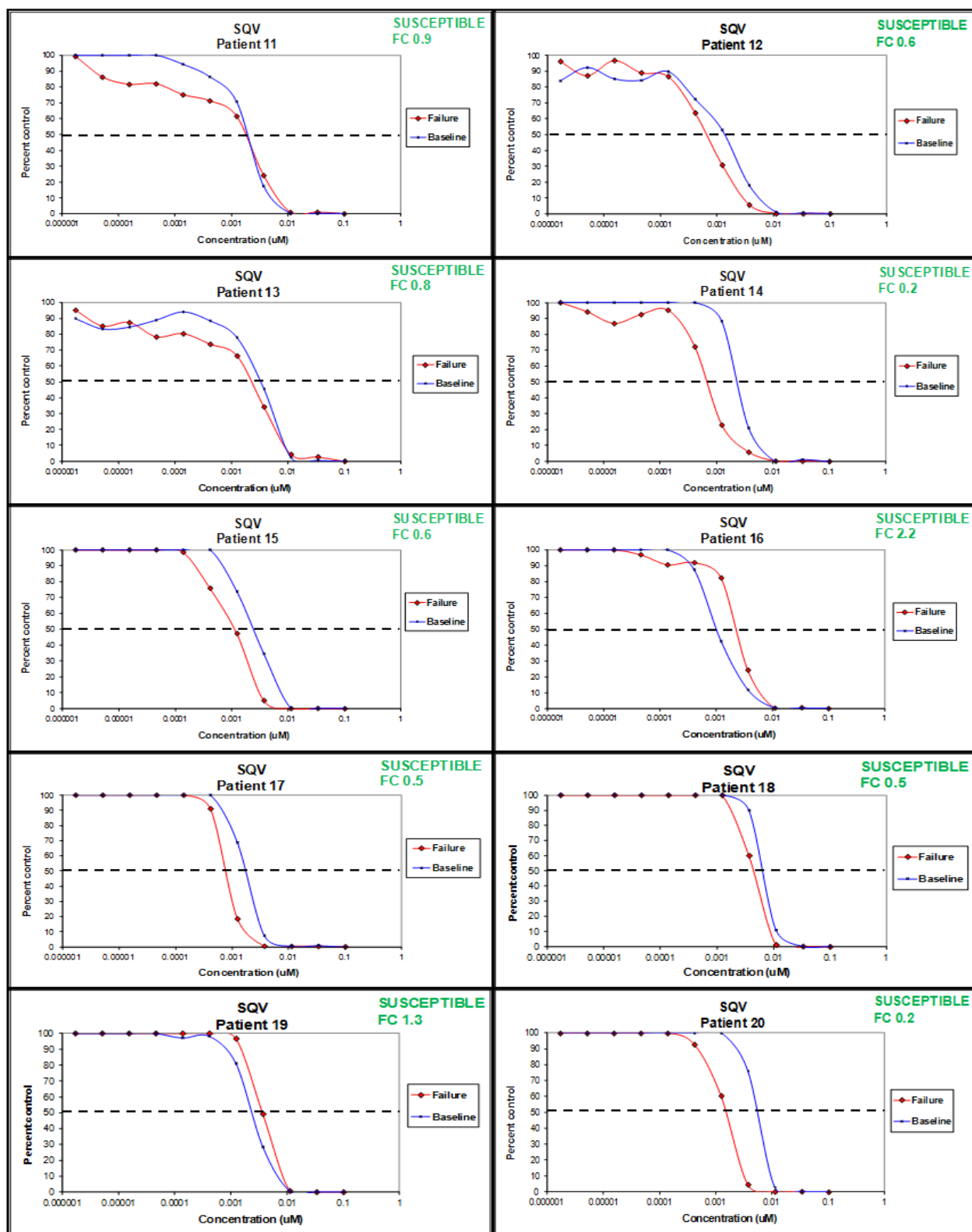


Figure 3.3.4. Phenotypic assay standard curves for patient samples to SQV

Fold changes are calculated based on the IC50 value of the failing sample (red line) relative to the baseline sample (blue line). Each patient sample was tested in duplicate and the average of that is represented by the curve. The 1 resistant patient is shown in red at the top, the 3 with reduced susceptibility in orange and the 16 susceptible in green.

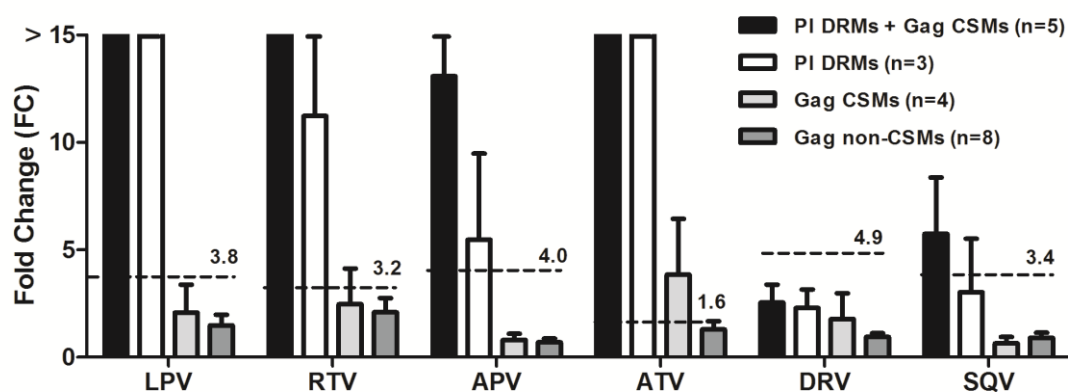


Figure 3.3.5. Association of genotype with phenotypic response to protease inhibitors.

Patients with both PI drug resistance mutations (DRMs) and *gag* CS mutations (CSMs) are shown by black bars, patients with PI DRMs only are shown by white bars, patients with only *gag* CSMs by light grey bars and patients with *gag* non-CSMs only are shown by dark grey bars. The lower cut-offs for each drug are indicated by a dashed line.

Table 3.3.2. Phenotypic susceptibility of patient-derived *gag-protease* relative to the Stanford Drug Resistance Database prediction for Protease

Pt. ID	LPV		APV		ATV		DRV		SQV	
	Predicted	Assay	Predicted	Assay	Predicted	Assay	Predicted	Assay	Predicted	Assay
1	IM	R	IM	RS	IM	R	S	S	IM	RS
2	H	R	IM	R	H	R	S	RS	IM	R
3	Low	R	Low	R	Low	R	S	S	Low	RS
4	H	RS	H	S	H	S	S	S	IM	S
5	S	S	S	S	S	RS	S	S	S	S
6	S	S	S	S	S	RS	S	S	S	S
7	S	S	S	S	S	S	S	S	S	S
8	Low	R	Low	RS	Low	R	S	S	Low	RS
9	IM	RS	IM	S	IM	RS	S	S	Low	S
10	IM	RS	IM	S	IM	RS	S	S	IM	S
11	S	RS	S	S	S	R	S	RS	S	S
12	Low	S	Low	S	Low	S	S	S	Low	S
13	S	S	S	S	S	S	S	S	S	S
14	S	S	S	S	S	S	S	S	S	S
15	S	S	S	S	S	S	S	S	S	S
16	S	S	S	S	S	S	S	S	S	S
17	S	S	S	S	S	S	S	S	S	S
18	S	S	S	S	S	S	S	S	S	S
19	S	S	S	S	S	S	S	S	S	S
20	S	S	S	S	S	S	S	S	S	S

Predicted: Predicted phenotype based on the Stanford drug resistance database; S, susceptible; Low, low level resistance;

IM, intermediate resistance; H, high-level resistance

Assay: Phenotypic assay; Level, level of resistance; S, susceptible; RS, reduced susceptibility; R, resistant

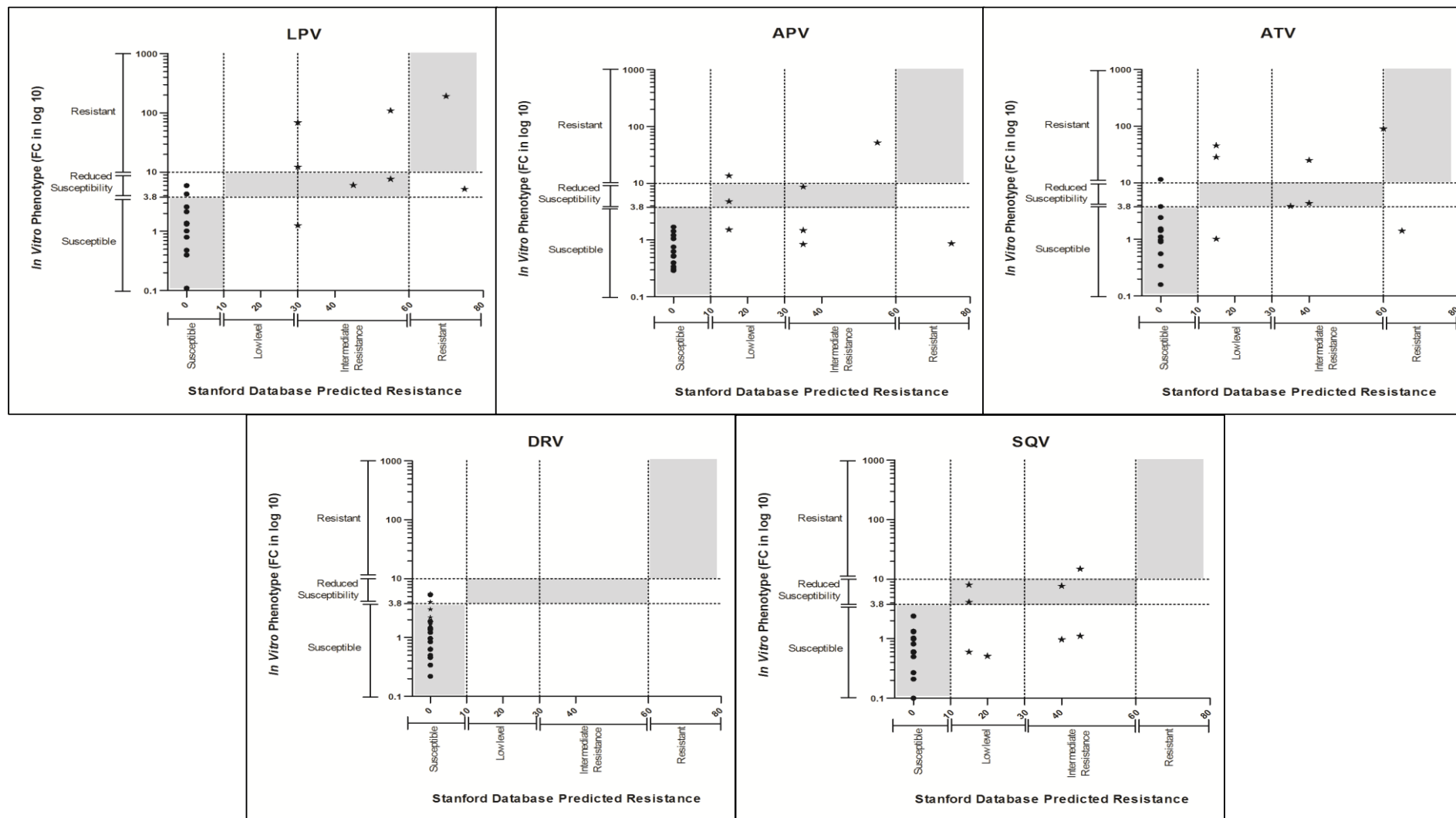


Figure 3.3.6. Phenotypic susceptibility of patient-derived *gag-protease* relative to the Stanford Drug Resistance Database prediction for Protease
 Grey shaded areas represent levels of phenotypically similar resistance. Points represented by a star indicate patients with major PI mutations.

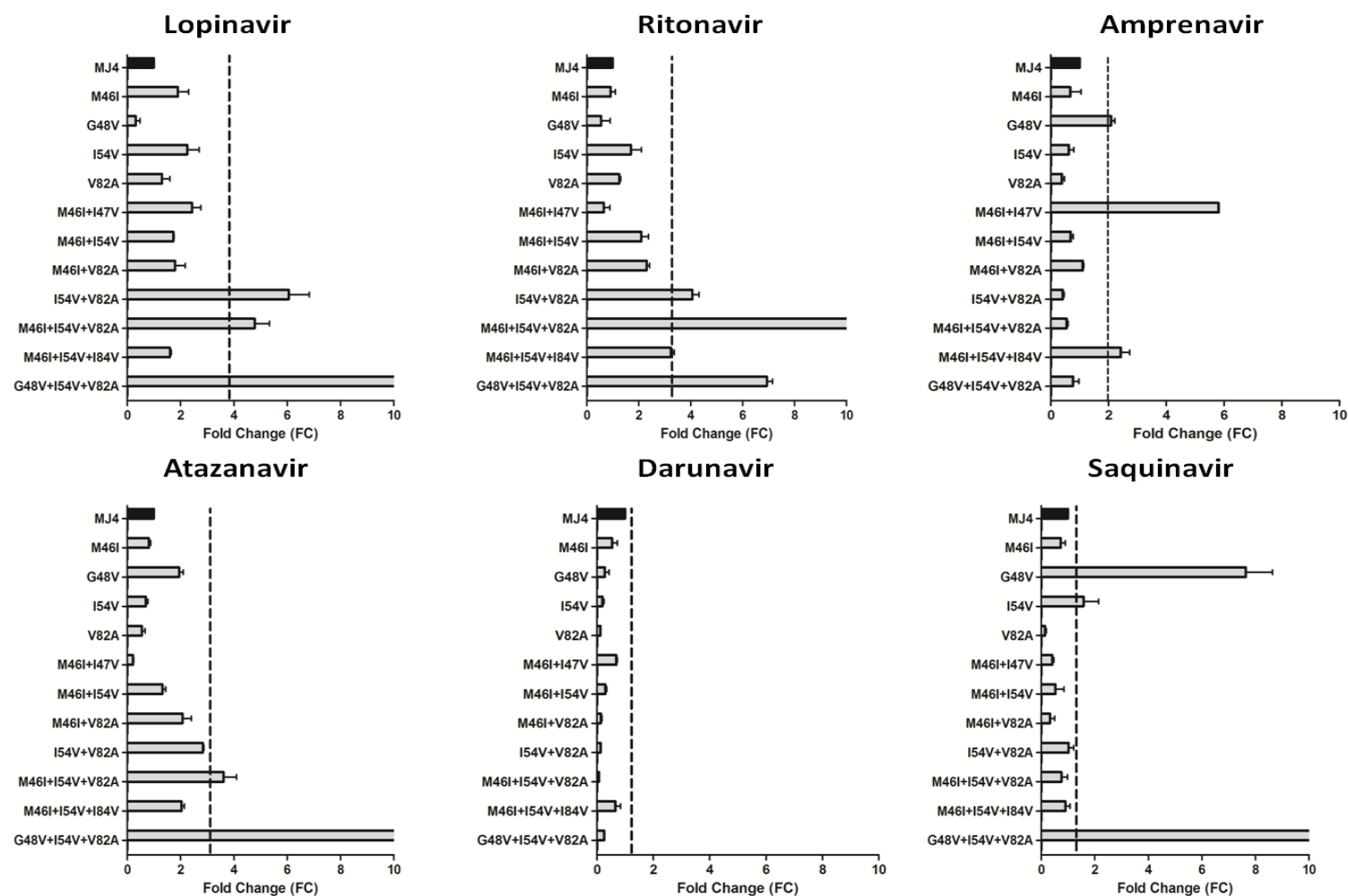


Figure 3.3.7. Protease inhibitor susceptibility of control *protease* mutants Susceptibility of *protease* mutants created in p8.MJ4 was determined using a single-replication cycle drug susceptibility assay. Data displayed are fold-change in IC_{50} values in comparison to the reference strain (p8.MJ4) for each of six PIs: LPV, RTV, APV, ATV, DRV and SQV. Each mutant was repeated at least twice.

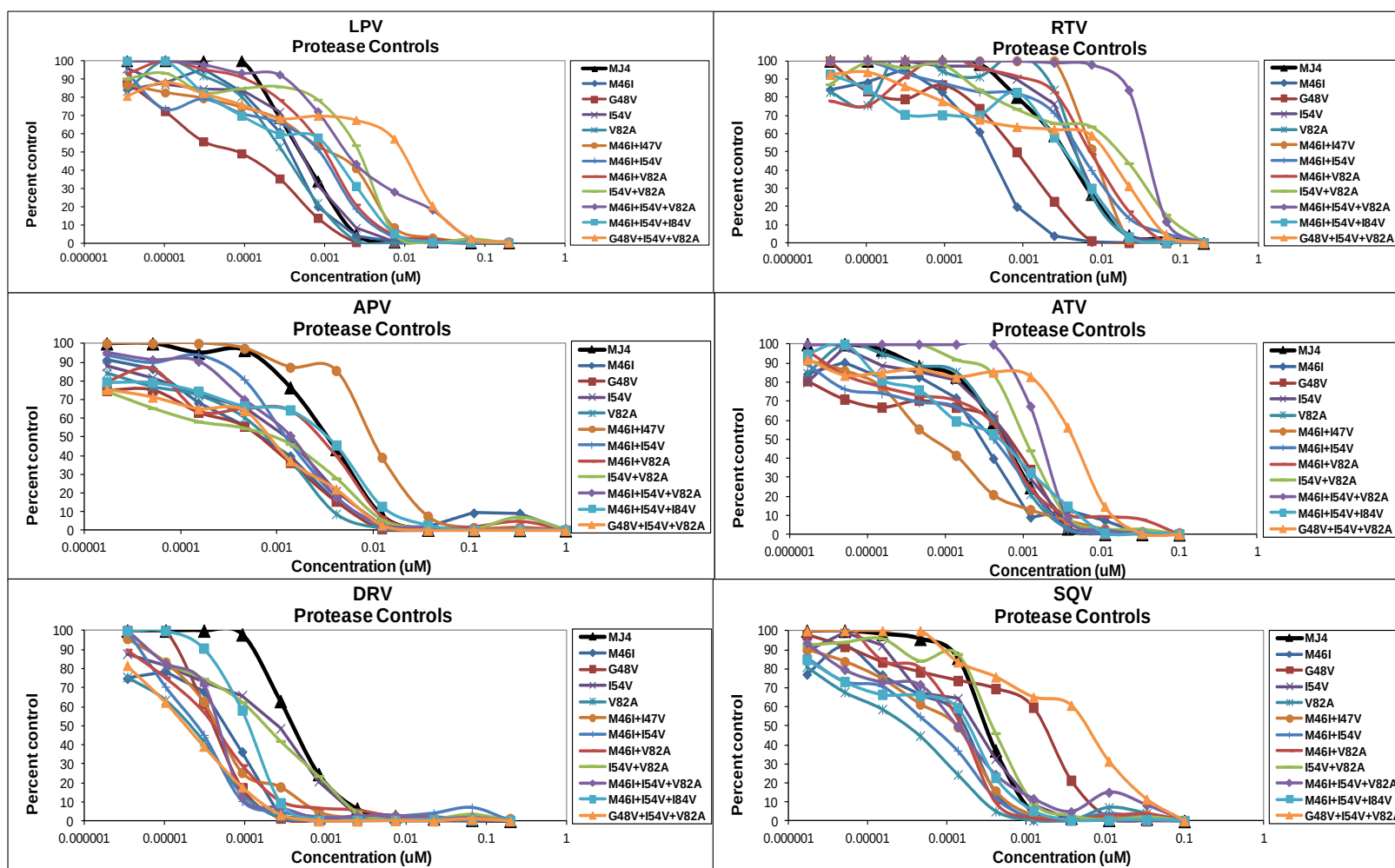


Figure 3.3.8. Phenotypic susceptibility standard curves of the control *protease* mutants

All curves of the *protease* mutants were plotted against the assay control p8.MJ4 (black line). The fold change was calculated by comparing the IC_{50} of each mutant to the IC_{50} of the control.

Table 3.3.3. Comparison of fold change between patient samples and *gag-protease* mutants based on patient samples for APV

Patient	Fold change	SDM	Fold change
1	8.7	A431V+K20R+I54V+V82A	1.5
2	51.8	E428D+M46I+I54V+V82A	0.5
3	13.5	Q62S+V82A	0.3
4	0.8	N374S+M46I+I54V+V82A	0.6
8	4.8	N451S+V82A	0.4
9	1.4	F79H+M46I+V82A	0.7
10	0.8	I54V+V82A	0.4
11	1.2	L449P+P453L	0.7
12	1.5	V82A	0.4
20	0.4	L449P	0.7

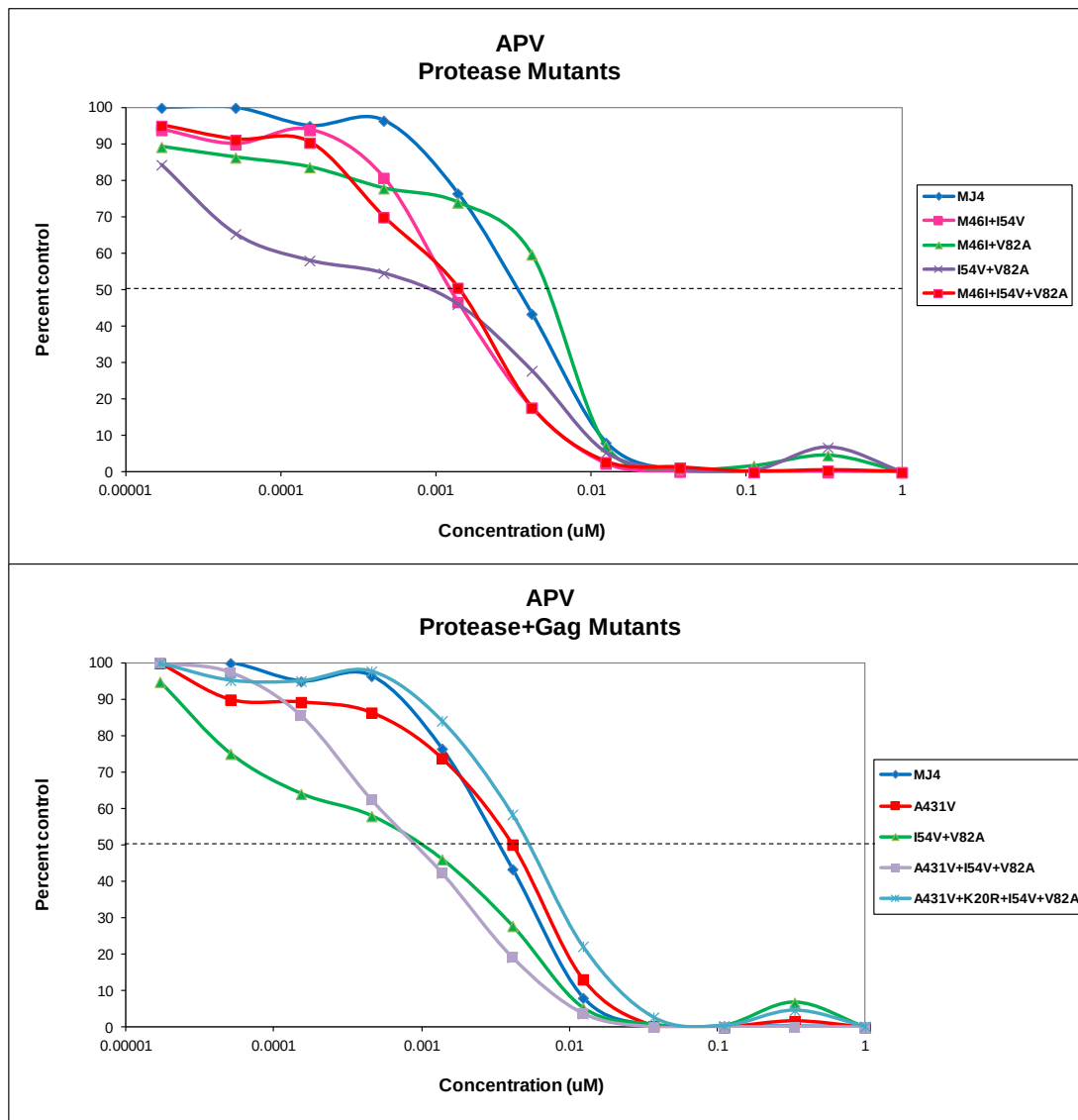


Figure 3.3.9. Phenotypic susceptibility standard curves of *gag* and *protease* site-directed mutants for APV

Multiple mutations were created by successive rounds of site directed mutagenesis. The top graph represents standard curves of *protease* mutants. The bottom graph demonstrates changes in susceptibility to APV when *gag* mutation A431V is added to *protease* mutations.

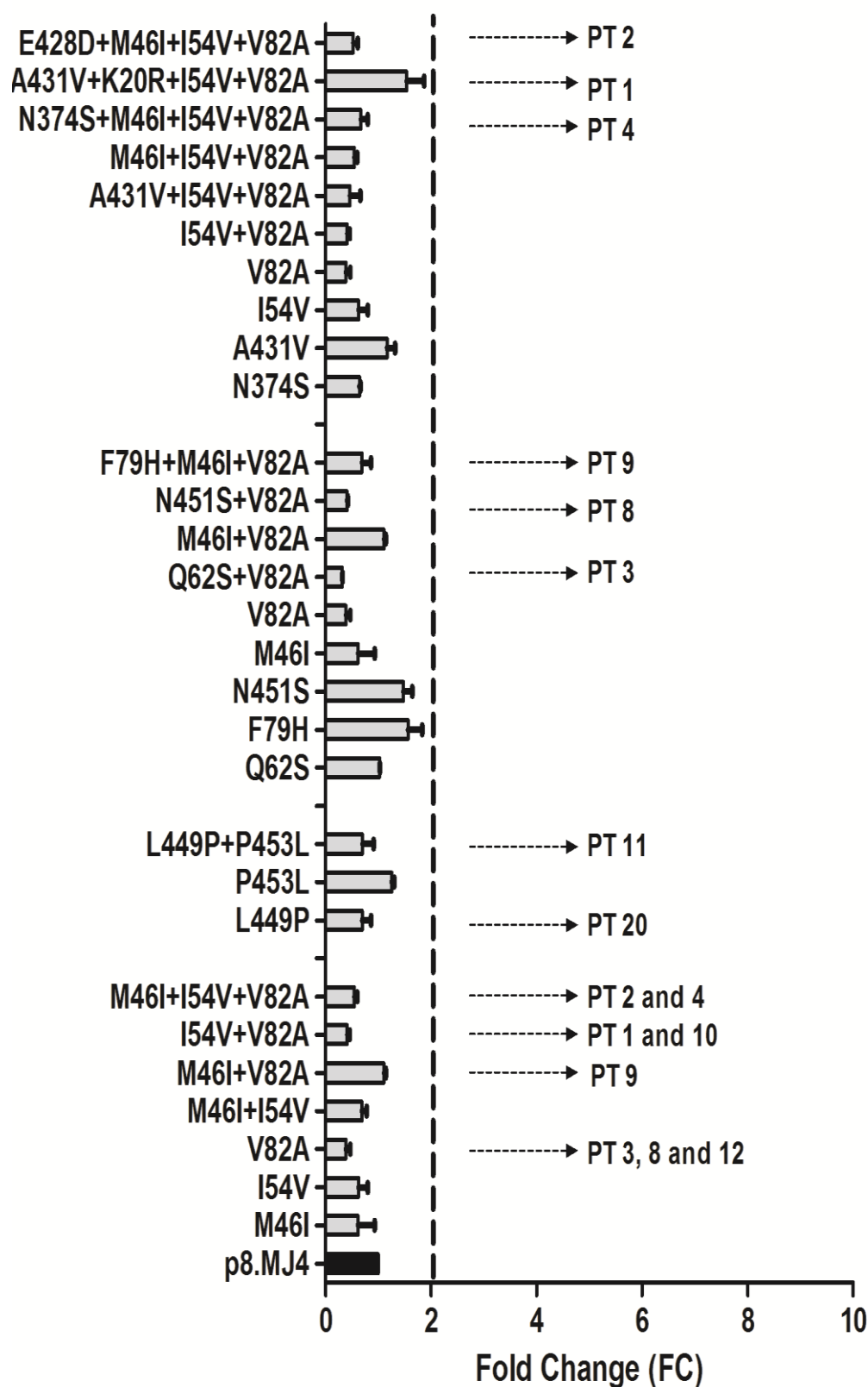


Figure 3.3.10. Phenotypic susceptibility of *protease* and *gag* site-directed mutants to APV

Susceptibility of *gag* mutants created in p8.MJ4 was determined using a single-replication cycle drug susceptibility assay. Data displayed are fold-change in IC_{50} values in comparison to the reference strain (p8.MJ4). The patients on which these mutations were based on are indicated.

Table 3.3.4. Comparison of fold change between patient samples and *gag-protease* mutants based on patient samples for ATV

Patient	Fold change	SDM	Fold change
1	24.9	A431V+K20R+I54V+V82A	1.4
2	90.1	E428D+M46I+I54V+V82A	1.5
3	45.5	Q62S+V82A	0.5
4	1.4	N374S+M46I+I54V+V82A	1.4
8	28.3	N451S+V82A	0.5
9	3.8	F79H+M46I+V82A	0.5
10	4.3	I54V+V82A	2.8
11	11.5	L449P+P453L	0.8
12	1.0	V82A	0.5
20	0.1	L449P	0.5

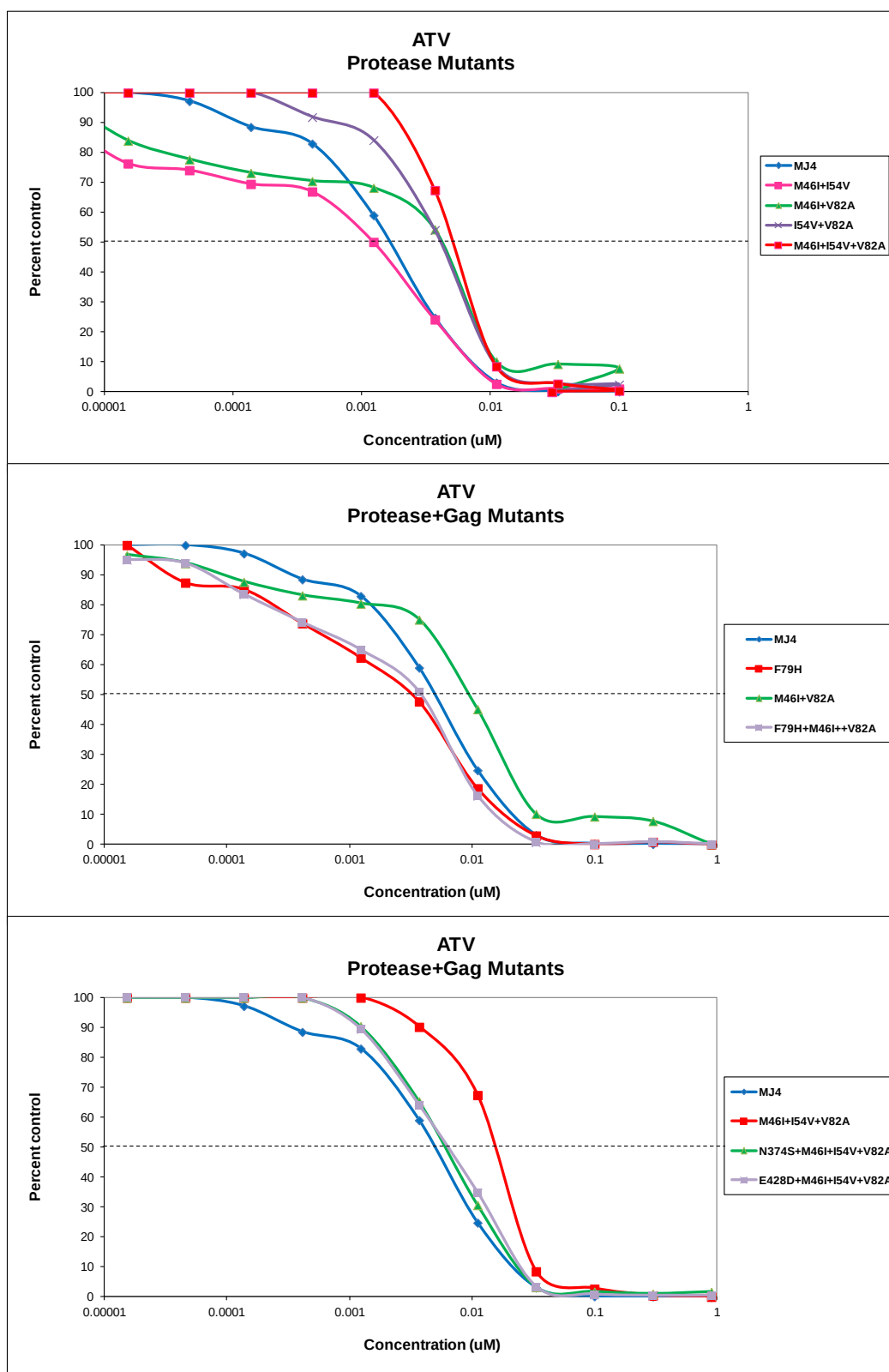


Figure 3.3.11. Phenotypic susceptibility standard curves of *gag* and *protease* site-directed mutants for ATV

Multiple mutations were created by successive rounds of site directed mutagenesis. The top graph represents standard curves of *protease* mutants showing reduced susceptibility to the triple *protease* mutant M46I+I54V+V82A. The middle and bottom graphs demonstrate changes in susceptibility to ATV when *gag* mutations are added to *protease* mutations.

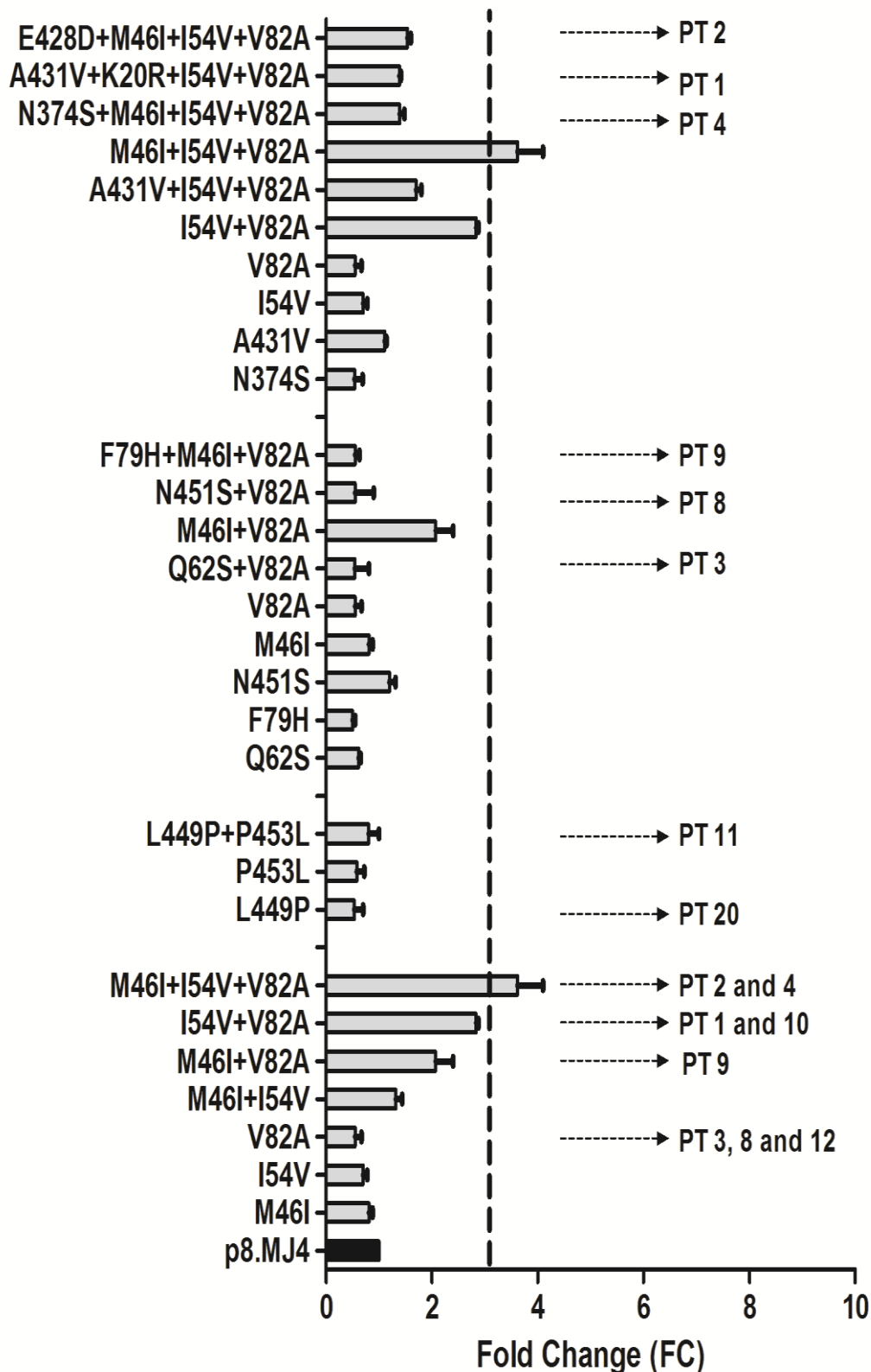


Figure 3.3.12. Phenotypic susceptibility of *protease* and *gag* site-directed mutants to ATV
 Susceptibility of *gag* mutants created in p8.MJ4 was determined using a single-replication cycle drug susceptibility assay. Data displayed are fold-change in IC_{50} values in comparison to the reference strain (p8.MJ4). The patients on which these mutations were based on are indicated.

Table 3.3.5. Comparison of fold change between patient samples and *gag-protease* mutants based on patient samples for DRV

Patient	Fold change	SDM	Fold change
1	3.0	A431V+K20R+I54V+V82A	0.1
2	5.5	E428D+M46I+I54V+V82A	0.2
3	4.0	Q62S+V82A	0.3
4	0.6	N374S+M46I+I54V+V82A	0.6
8	2.2	N451S+V82A	0.3
9	1.3	F79H+M46I+V82A	0.4
10	1.4	I54V+V82A	0.1
11	5.3	L449P+P453L	0.6
12	1.6	V82A	0.1
20	0.2	L449P	0.7

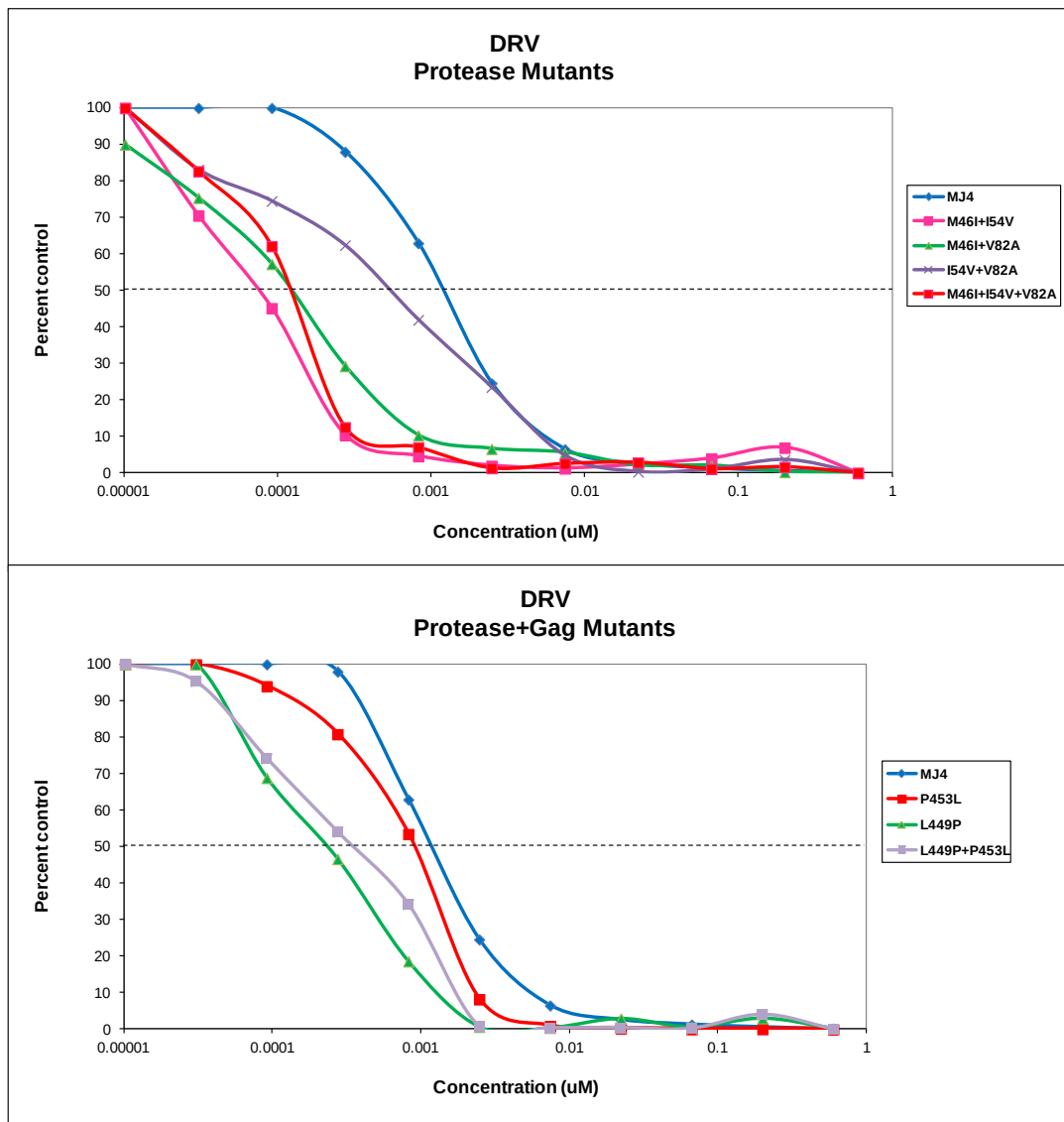


Figure 3.3.13. Phenotypic susceptibility standard curves of *gag* and *protease* site-directed mutants for DRV

Multiple mutations were created by successive rounds of site directed mutagenesis. The top graph represents standard curves of *protease* mutants. The bottom graph demonstrates no notable change in susceptibility by combining *gag* CS mutations L449P and P453L.

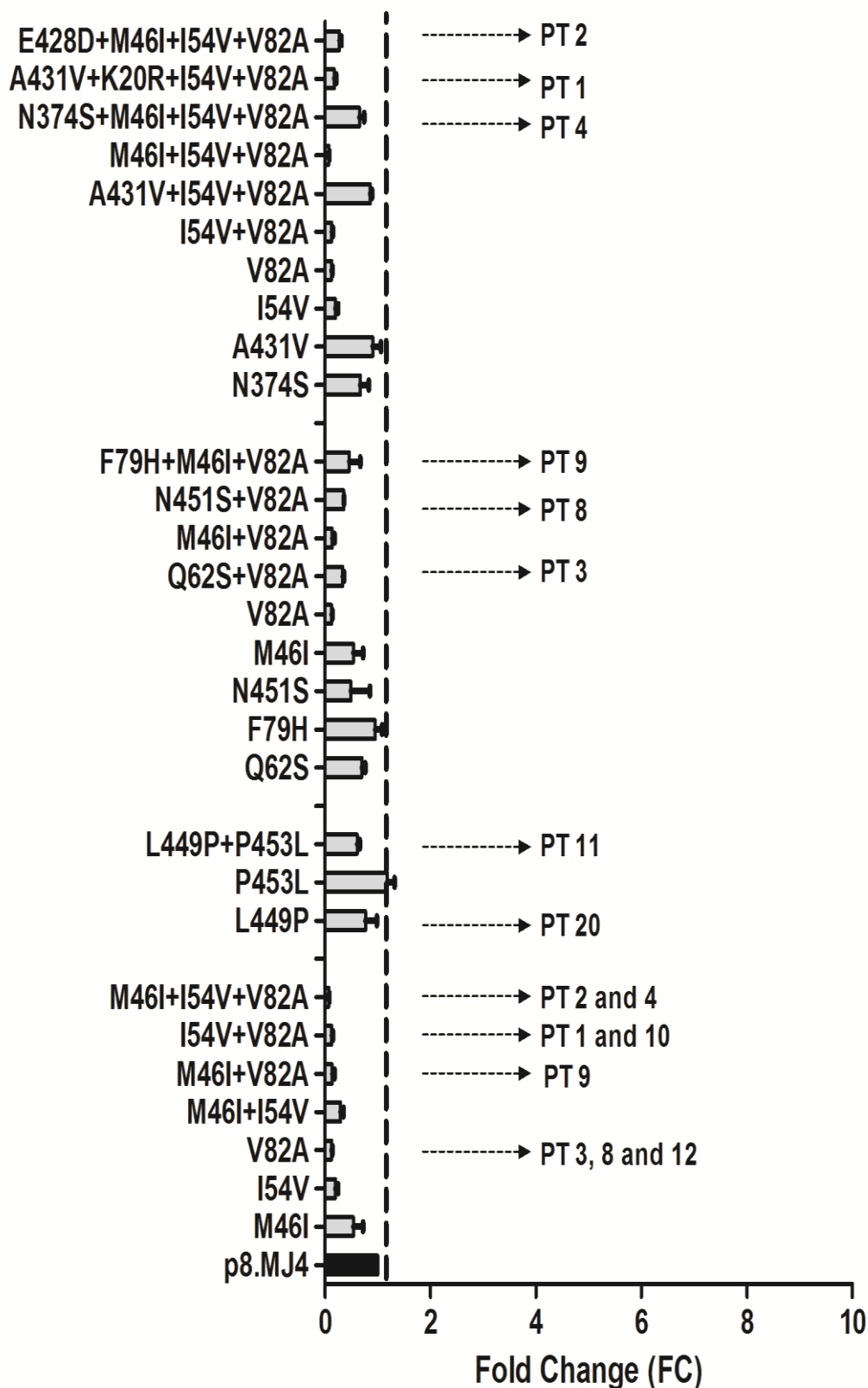


Figure 3.3.14. Phenotypic susceptibility of *protease* and *gag* site-directed mutants to DRV
 Susceptibility of *gag* mutants created in p8.MJ4 was determined using a single-replication cycle drug susceptibility assay. Data displayed are fold-change in IC_{50} values in comparison to the reference strain (p8.MJ4). The patients on which these mutations were based on are indicated.

Table 3.3.6. Comparison of fold change between patient samples and *gag-protease* mutants based on patient samples for SQV

Patient	Fold change	SDM	Fold change
1	7.7	A431V+K20R+I54V+V82A	0.1
2	14.9	E428D+M46I+I54V+V82A	1.4
3	8.0	Q62S+V82A	0.1
4	1.1	N374S+M46I+I54V+V82A	0.7
8	4.1	N451S+V82A	0.2
9	0.5	F79H+M46I+V82A	0.3
10	0.9	I54V+V82A	1.0
11	0.9	L449P+P453L	0.3
12	0.6	V82A	0.1
20	0.2	L449P	0.1

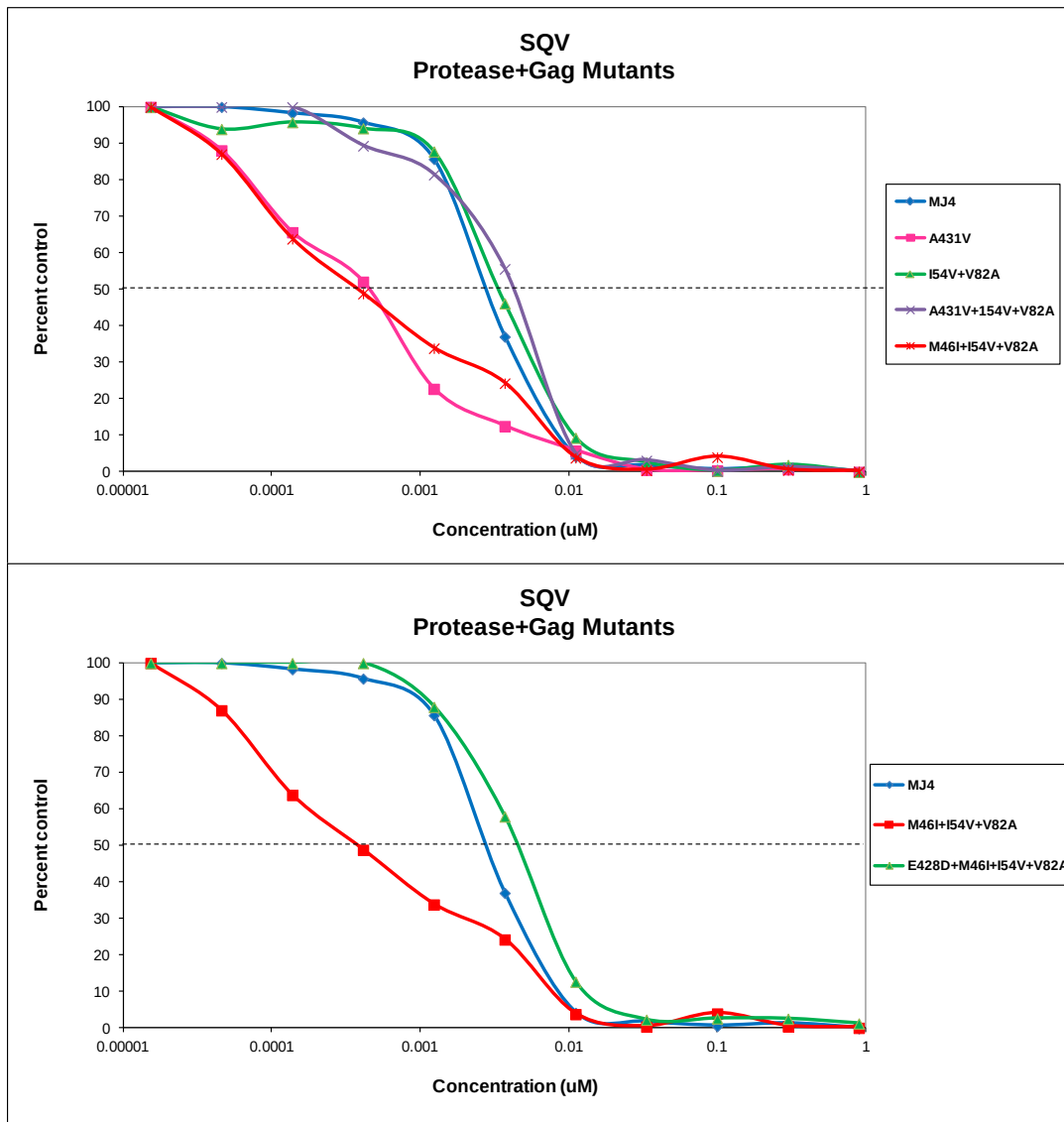


Figure 3.3.15. Phenotypic susceptibility standard curves of *gag* and *protease* site-directed mutants for SQV

Multiple mutations were created by successive rounds of site directed mutagenesis. The top graph represents standard curves of *protease* mutants. The bottom graph demonstrates reduced susceptibility to SQV when E428D is added to the triple *protease* mutant M46I+I54V+V82A.

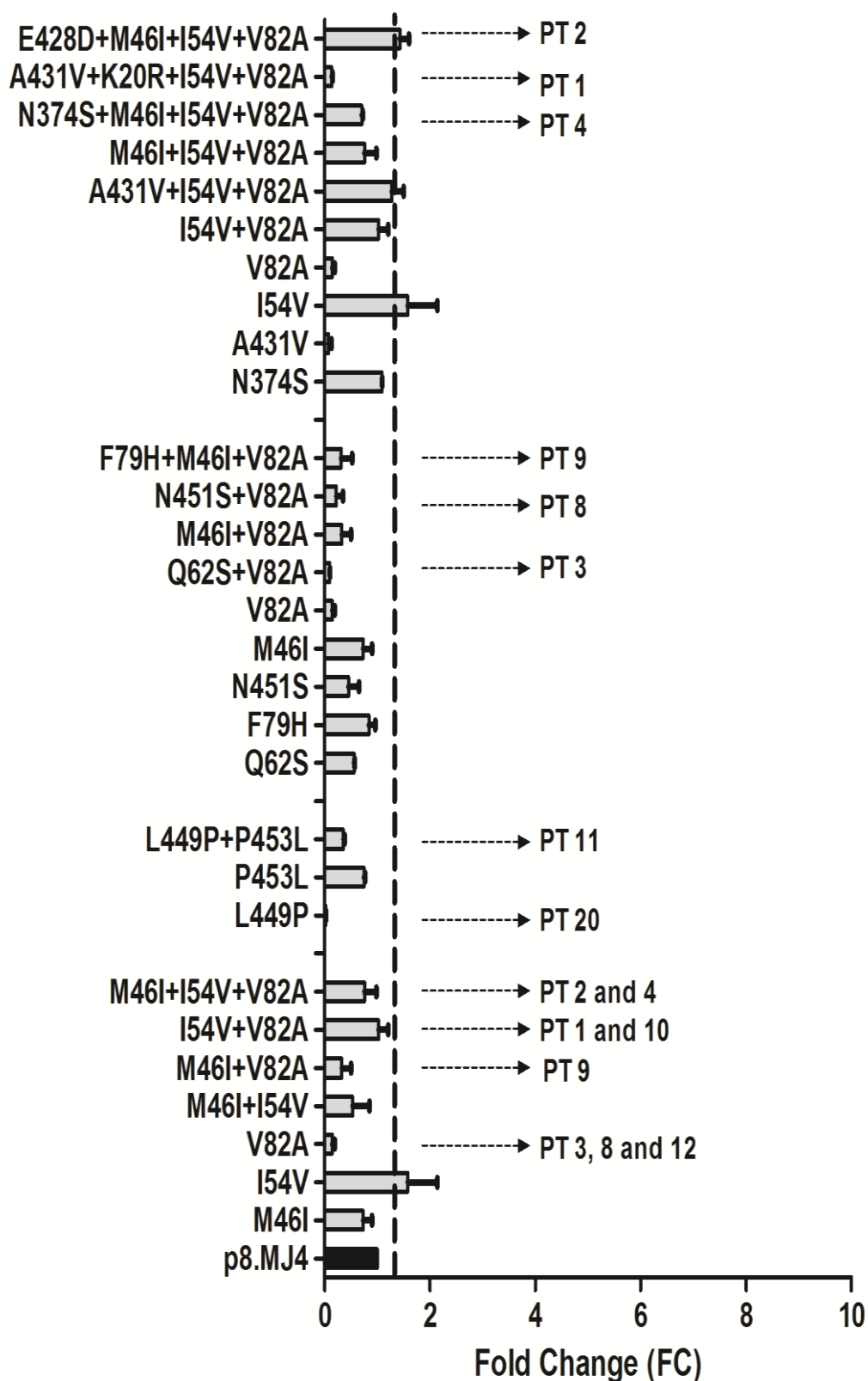


Figure 3.3.16. Phenotypic susceptibility of *protease* and *gag* site-directed mutants to SQV

Susceptibility of *gag* mutants created in p8.MJ4 was determined using a single-replication cycle drug susceptibility assay. Data displayed are fold-change in IC_{50} values in comparison to the reference strain (p8.MJ4). The patients on which these mutations were based on are indicated.

Table 3.3.7. Phenotypic assay cut-offs for patient-derived *gag-protease* and site-directed mutants

	Patient-derived <i>Gag-Protease</i>			Site-directed mutants		
	S	RS	R	S	RS	R
LPV	< 4.4	4.4 – 10.0	>10	< 3.8	3.8 – 10.0	>10
RTV	< 3.2	3.2 – 10.0	>10	< 3.2	3.2 – 10.0	>10
APV	< 4.0	4.0 – 10.0	>10	< 2.0	2.0 – 10.0	>10
ATV	< 1.6	1.6 – 10.0	>10	< 3.1	3.1 – 10.0	>10
DRV	< 4.9	4.9 – 10.0	>10	< 1.2	1.2 – 10.0	>10
SQV	< 3.4	3.4 – 10.0	>10	< 1.3	1.3 – 10.0	>10

Abbreviations: S, susceptible; RS, reduced susceptibility; R, resistant; LPV, lopinavir; RTV, ritonavir; APV, amprenavir ATV, atazanavir; DRV, darunavir; SQV, saquinavir

3.3.7. References

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Chapter Four

Discussion

Paediatric HIV-1 infection remains of major concern with an estimated 700 children newly infected each day, globally (UNAIDS, 2013). In accordance with the WHO guidelines, there has been a shift to earlier initiation of paediatric treatment to maximise long-term growth, neurodevelopment and immunologic health (Penazzato et al., 2014). As more children are initiated on ART, the challenges and effects of long-term treatment need to be considered. Therapy of paediatric patients are complicated by high viral loads and the challenges experienced with accurate dosing (Gupta et al., 2009). Given the relatively few options available for subsequent treatment in paediatric patients failing PI-based therapy, there is an urgent need to make more drugs paediatric-friendly and easily accessible (Mofenson and Cotton, 2013). Studies evaluating more palatable sprinkles for LPV/r found that LPV/r exposure from sprinkles was comparable with syrup and that sprinkles were more acceptable than syrups for younger children (Musiime et al., 2014). Since HIV-1 paediatric patients are facing a chronic illness requiring life-long therapy (Pillay et al., 2014), it is important to improve and maintain adherence to these drugs to avoid development of drug resistance and unnecessary regimen switching. In addition, as children develop, ARV dosages need to be adjusted adequately to ensure optimal drug concentrations. Furthermore, with entry into adolescence, children are faced with many psychosocial factors that may affect their adherence to ART (Gupta et al., 2009). Some adults have been on ART for 15-20 years and are still doing well so this bodes well for long-term usage combined with good adherence.

Diagnosis of HIV infection in infants is performed using HIV PCR at 6 weeks of age. A recent study in South Africa found that with a scale-up of PMTCT, there has been a remarkable decline in the vertical transmission rate from 23% in 2003 to 2.4% in 2012 (Sherman et al., 2014). Following revision of the WHO guidelines for PMTCT in 2010, it was recommended to use one of two options, either Option A or Option B. Option A and

Option B start as early as 14 weeks gestation and last up until one week after the cessation of breastfeeding (Thyssen et al., 2013). With Option A, HIV-1 positive pregnant women receive prophylaxis at antepartum (AZT), intrapartum (sdNVP+ first dose of AZT/3TC), postpartum (daily AZT/3TC for 7 days) and with Option B, HIV positive pregnant and breastfeeding women receive HAART (TDF+3TC (FTC) +EFV) intrapartum and through childbirth. In 2013, a further recommendation was proposed by the WHO to offer all pregnant women lifelong ART (Option B+) irrespective of CD4 cell count. This is considered to be more beneficial since it could result in less HIV mother-to-child transmission (Thyssen et al., 2013). The effectiveness of Option B+, if implemented successfully in South Africa gives promise for eliminating vertical transmissions as seen in developed countries.

Current guidelines for the use of ARV agents in paediatric patients recommend that resistance testing should be considered in newly diagnosed infants <12 months of age prior to initiation of therapy (Rojas Sánchez and Holguín, 2014). It is necessary to perform surveillance of paediatric drug resistance in order to inform future paediatric treatment guidelines and improve health outcomes. A few studies which have been reported on HIV-1 infected paediatric patients failing PI-based therapy in South Africa have shown a low frequency of *protease* resistance mutations (Wallis et al., 2011, van Zyl et al., 2009, Orrell et al., 2013, Levison et al., 2012, Pillay et al., 2014). Often, the absence of *protease* mutations in patients failing PI therapy is associated with poor adherence. However, some patients do present with mutations to other drug classes within their regimen suggesting drug exposure and hence the possibility of alternate routes of PI resistance. Regions outside of the *protease* gene have been shown to contribute to PI therapy failure and impact future PI options.

Interestingly, almost half of the children in our study did not have any major PI mutations despite failing a PI regimen. Those children who developed PI mutations had prior exposure to RTV. The *gag* region was investigated since it is the major substrate of the protease enzyme. Interestingly, changes were observed at both *gag* CS as well as non-CS. Although changes in *gag* CS have been well described in the literature, data on *gag* non-CS mutations are limited. It was found in three of the twenty patients that *gag* CS, in combination with *protease* mutations, contributed towards PI resistance. The selection of both *protease* and *gag* CS mutations in certain patients suggests co-evolution of the enzyme and substrate in the presence of PIs. A recent study described the co-evolution of the *gag* CS p1/p6^{Gag} with NFV resistance mutations D30N/N88D (Kolli et al., 2014). This interdependency between the substrate and enzyme demonstrated an improved fit of the substrate within the active site. The co-evolution of *gag* and *protease* may allow for sustained substrate recognition and cleavage in the presence of PI resistance mutations (Kolli et al., 2014). The compensatory role as well as contributing function of *gag*-CS mutations to PI resistance has also been described in other studies (Verheyen et al., 2006, Nijhuis M, 2007, Dam et al., 2009).

Although the PIs used for first-line treatment in paediatric patients varies, LPV/r remains the most widely used PI in resource-limited settings. Therefore it was important to determine the effects of other PIs in patients presenting with LPV/r selected mutations. Patient-derived viruses containing major LPV and RTV *protease* mutations showed cross-resistance mainly to ATV and APV. The least amount of resistance was seen with DRV implying that DRV may be a good option following failure on a PI-based therapy. Furthermore, one of these patients conferred cross-resistance to 4 PIs and contained a wild type *protease* with changes in both *gag* CS and non-CS. This suggests that *gag* may act as an alternate route to PI

resistance in this patient. It is important to note that the natural variation of the phenotypic assays is not well established and further tests are required to ascertain confidence intervals. Nevertheless, these findings highlight the shortfall of current genotypic resistance tests that exclude *gag* from predicted PI resistance scores. Under these circumstances, PI resistance would be under-estimated in some patients and could lead to the initiation on an ineffective PI.

In addition to the development of signature LPV/r mutations upon sub-optimal adherence, the use of LPV/r has also been associated with long-term metabolic toxicity (Prendergast, 2013). A recent study compared the long-term metabolic effects of children maintained on a LPV/r-based regimen or switched to a NNRTI-based regimen (Arpadi et al., 2012). It was found that although the children had undetectable viral loads and good immune reconstitution, those on LPV/r-based regimen had more lipid abnormalities compared to those on NNRTI-based regimen. The possibility of developing insulin resistance was also a cause for concern in this group of children due to their lifelong therapy. Although the benefits of ART outweigh these adverse effects, long-term toxicity needs to be addressed with the introduction of potent drugs, that have less severe adverse effects (Prendergast, 2013). However, the approval of new drugs is a lengthy process, especially in paediatric patients that require specialized formulations. In addition, delays are usually experienced in making new drugs available and accessible in resource-limited settings. The timeous switching of failing patients to drugs that are currently available should take preference over maintenance on an ineffective regimen. Although a PI-boosted first-line regimen is recommended for paediatric patients, studies have shown that the response to a NVP-based paediatric therapy is not affected when NNRTI mutations were present <25% and NVP therapy was preceded by a suppressive boosted PI-regimen (Moorthy et al., 2011, Hunt et al., 2011, Kuhn et al., 2012).

Therefore, the re-use of NVP in these patients will help preserve LPV/r for future treatment while minimising metabolic toxic effects (Kuhn et al., 2012). In this scenario, genotypic resistance testing of paediatric patients prior to treatment initiation would be essential as to identify NNRTI mutations. Furthermore, for the switch to remain effective, regular viral load testing would be necessary to monitor virologic outcomes (Kuhn et al., 2012).

Apart from PI resistance, many paediatric patients also present with resistance mutations to RT inhibitors. NRTIs form the backbone of triple drug regimens in which PIs and NNRTIs are used. Treatment with NNRTIs (NVP) during PMTCT or in first-line therapy can lead to the rapid selection of Y181C mutation in paediatrics (Martinson et al., 2007). Treatment with a thymidine analogue usually leads to the development of thymidine analogue mutations (TAMs) which confer cross-resistance to other NRTI thymidine analogues compromising their efficacy in future regimens (Iyidogan and Anderson, 2014). Examples of TAM mutations include M41L, D67N, K70R, T210W, T215FY and K219QE (Rhee et al., 2003). D4T was commonly used in first-line therapy which selected for T69Ins and Q151M which causes cross-resistance among ABC, DDI, TDF, D4T and AZT. Most patients on 3TC develop the M184V mutation and although it renders the drug ineffective, it cripples the virus and subsequently decreases viral load. A study reported on the use of 3TC monotherapy in children as a holding strategy until effective ART becomes available (Lazarus et al., 2013). This study found that 3TC monotherapy helped maintain clinical and immunological status in majority of children. It was suggested that 3TC monotherapy could be used to preserve second and third-line regimens while addressing adherence issues (Lazarus et al., 2013). Treatment with NVP in an NNRTI-based regimen selects for many mutations such as L100I, K101P, K103NS, V106M, V108I, Y181C, Y188L and G190A, which also cause cross-resistance to EFV. The use of EFV in children is only introduced in those ≥ 3 years of age due

to toxicity; therefore earlier exposure of NVP in first-line therapy may result in EFV being ineffective. New NNRTIs such as ETV and RVP show promise in patients with NVP/EFV resistance mutations however genotypic resistance testing would need to be done since K101P and Y181C also cause cross-resistance ETV and RVP (Rhee et al., 2003).

Although ineffective by themselves in a majority of patients, *gag* CS and non-CS changes were found to contribute to cross-resistance to one or more PI. Larger studies are needed to confirm the role of *gag* in PI resistance. The development of algorithms that help to predict PI resistance in conjunction with *gag* mutations will be an important outcome of such studies. Going forward, it is also important to develop ARV drugs which are tolerable and accessible to paediatrics since maintenance on an ineffective regimen will only compromise their future treatment options.

Chapter Five

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Appendix I

Preparation of Reagents

0.7% Agarose Gel

Dissolve 0.7 g agarose (Invitrogen, Spain) in 1X TBE (Tris/Acetic Acid/EDTA Buffer) buffer by heating in a microwave. Cool and add 2.5 µl Ethidium Bromide (10 mg/ml). Swirl to mix and pour into a gel casting tray.

Agar Plates

Dissolve 32.0 g LB Agar (Invitrogen, Carlsbad, CA) in 1L distilled water. Autoclave at 37°C for 20 minutes. Allow to cool before adding 1ml Ampicillin (100mg/ml). Swirl to mix, pour onto plates and allow to set.

LB Ampicillin

Dissolve 25.0 g Luria Broth (Affymetrix, Ohio) in 1L distilled water. Autoclave at 37°C for 20 minutes. Allow to cool before adding 1ml Ampicillin (100mg/ml). Swirl to mix.

Preparation of DMEM

500 ml DMEM (Gibco, NY, USA)

50 ml FBS (Gibco, NY, USA)

12.5ml HEPES (Gibco, NY, USA)

2.5 ml Gentamicin (Gibco, NY, USA)

To the DMEM, the FBS and antibiotics (HEPES and Gentamicin) are added. The DMEM was then swirled to ensure complete mixing of FBS and antibiotics in media. The DMEM was stored at 4 °C to preserve the antibiotics.

Appendix II

Sequences of SDM primers

SDM Primers (5' → 3')

Protease Mutations	
M46I	agtttgcaggaaaatggaaacaaaaataataggaggaattggag
I47V	tcaggaaaatggaaacaaaaatggtaggaggaattggagg
G48V	ggaaaatggaaacaaaaatgatagtaggaattggaggttttattaaag
I54V	tgataggaggaattggagggtttgttaaagtaagacagtatgatcaa
V82A	gtgttaataggacctactcctgccacataattggaagaaatatgt
I84V	aataggacctactcctgtcaacgtaattggaagaaatatgttgac
M46I+I54V	ggaaacaaaaataataggaggaattggaggttttatgaaagtaaga
M46I+I47V	gagtttgcaggaaaatggaaacaaaaatagtaggaggaattggagg
Gag Mutations	
Q62K	gaaggctgtaaacaataatgaaacagcttcaaccagctctt
Q62S	acatcagaaggctgtaaacaataatgtcacagcttcaaccagctct
F79H	ggaacagaggaacttagatcattacacaacacagtagcaactctctatt
F79Y	agacaggaacagaggaacttagatcattatataacacagtagcaactc
P339Q	ccattttaagagcattaggacaaggggctacattagaagaaat
S373A	ggcaatgagccaagcaaacgctacaagtatactgatgcag
N374S	aatgagccaagcaaacagttcaagtatactgatgcagag
E428D	aattagcctgcctatcagtacagctttcatttgggtgc
A431V	agactgtactgagaggcagggttaatttttagggaaaatctg
L449P	gccaggggaattccctcagagcaggccag
S451N	tgttggctctggcctgttctgaaggaaattccc
P453L	ccttcagagcaggctagagccaacagccc
L449P+P453L	gccaggggaattccctcagagcaggcttcagagcaggc

Appendix III

Ethics Clearance Certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Mrs Jennifer Giandhari

CLEARANCE CERTIFICATE

M110971

PROJECT

Genetic Changes in HIV-1 Gag and Pol Genes
Associated with Protease Inhibitor-Based
Therapy Failure in Paediatric and Adult Patients

INVESTIGATORS

Mrs Jennifer Giandhari.

DEPARTMENT

AIDS Virus Research Unit

DATE CONSIDERED

30/09/2011

M110971DECISION OF THE COMMITTEE*

Approved unconditionally

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

DATE 30/09/2011

CHAIRPERSON
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Dr Gillian Hunt

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.

I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

Appendix IV

Turnitin Report

Turnitin Originality Report

Thesis22February2015TurnitinSub.docx by Jennifer Giandhari

From Turnitin v1

(SVLeZ3JMOXo4tN4Zh3X6YuUM6tzC9067Qrie8ViOlf0GC125MSLZ1Cuo226FMZGkSM3bgypG6Ez6k1Qlk5O2vsaAAezQYTX18b0)

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Barbara Saatkamp Taylor. "Rapid development of antiretroviral drug resistance mutations in HIV-infected children initiating protease inhibitor-based therapy less than 2 years of age in South Africa", AIDS Research and Human Retroviruses, 02/24/2011

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8 < 1% match (publications)

[Iyidogan, Pinar and Anderson, Karen S.. "Current Perspectives on HIV-1 Antiretroviral Drug Resistance", Viruses \(1999-4915\), 2014.](#)

9 < 1% match (Internet from 29-Dec-2014)

<http://hivr4p.org/phocadownloadpap/abstracts/hiv-r4p-abstract-book.pdf>

10 < 1% match (publications)

[Teasdale, Chloe A, Elaine J Abrams, Ashraf Coovadia, Renate Strehlau, Leigh Martens, and Louise Kuhn. "Adherence and Viral Suppression among Infants and Young Children Initiating Protease Inhibitor-Based Antiretroviral Therapy :", The Pediatric Infectious Disease Journal, 2012.](#)

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<http://www.plosone.org/article/info:doi/10.1371/journal.pone.0006687>

12 < 1% match (Internet from 07-Oct-2009)

<http://jvi.asm.org/cgi/content/full/72/7/5680>

13 < 1% match (publications)

[Ravindra K Gupta. "Full-length HIV-1 Gag determines protease inhibitor susceptibility within in-vitro assays :", AIDS, 07/2010](#)

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