Impact of Viral and Host Genetic Factors on Antiretroviral Treatment Outcome in South African HIV-1 subtype C infected AIDS patients

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A thesis submitted to the Faculty of Health Sciences,
University of the Witwatersrand,
in fulfillment of the requirements for the degree
of
Doctor of Philosophy
Johannesburg, 2009

DECLARATION

I, Carole Lorraine Wallis declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

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……..day of……..2010

DEDICATION

This thesis is dedicated to my family, my parents, Norman and Shirley, my sisters, Elizabeth and Lindsay, without their love, support and understanding this thesis would have not been possible. From them I have learnt that success is not a destination; but a journey.

PUBLICATIONS & PRESENTATIONS ARISING FROM THIS STUDY

Publications:

Papers:

1. **Wallis C.L.**, Bell C., Rubel D., Papathanasopoulos M.A., Venter F., Sanne I., Stevens W. 2007. Emerging HIV-1 Drug Resistance Patterns from two Johannesburg clinics on the South African ARV rollout program. Southern African HIV Medical Journal, June, 41-2.
2. **Wallis C.L.,** Erasmus L., Varughese S., Ndiweni D., Stevens W. 2009. Surveillance of drug resistant mutations in HIV-1 subtype C children failing antiretroviral therapy. The Pediatric Infectious Disease Journal, 28, 1123-5.
3. **Wallis C.L.,** Mellors J.W., Venter F., Sanne I., Stevens W. 2009. Varied HIV Drug Resistance Patterns Among Patients on Failing Antiretroviral Therapy in the South African National Roll-out Programme. In press, Journal of AIDS.
4. **Wallis C.L.,** Papathanasopoulos M.A., Lakhi S., Karita E., Kamali A., Kaleebu P., Sanders E., Anzala O., Bekker L.G., Stevens G., Rinke de Wit T.F., Stevens W. 2009. Affordable in-house antiretroviral drug resistance assay with good performance in HIV-1 subtype C. In press, Journal of Virological Methods.

Conference presentations:

1. **Wallis C.L.**, Papathanasopoulos M.A., Sanne I., Stevens W. 2006. Monitoring HIV-1 Drug Resistance in South Africa. WITS Faculty of Health Science Research Day, Johannesburg, South Africa.

2. **Wallis C.L.**, Bell C., Boulme R., Papathanasopoulos M.A., Venter F., Sanne I., Stevens W. 2007. Emerging HIV-1 Drug Resistance Patterns from two clinics on the South African ARV rollout program, CROI, Los Angelos, USA.
3. **Wallis C.L.**, Papathanasopoulos M.A., Rinke de Wit T.F., Stevens W. 2007. Subtype C specific In-house HIV-1 Drug Resistance Assay, 5th European HIV Drug Resistance Workshop, Cascais, Portugal.
4. **Wallis C.L.,** Erasmus L., Varughese S., Ndiweni D., Stevens W. 2008. Surveillance of drug resistant mutations in HIV-1 subtype C children failing antiretroviral therapy. CROI, Boston, USA.
5. **Wallis C.L.**, Stevens W., Venter F., Sanne I. 2008. Antiretroviral-Drug Resistance Patterns from patients failing the national roll-out programme in South Africa. Antiviral therapy 13 suppl 3: A164.
6. **Wallis C.L.,** Sanne I., Venter F., Mellors J.W., Stevens W. 2009. Varied Drug Resistance Profiles following First-line Regimen Failure in the South Africa Antiretroviral Program. CROI, Montreal, Canada.
7. **Wallis C.L.**, J.W. Mellors, W.D.F. Venter, Sanne I., Stevens W. 2009. Varied patterns of HIV-1 drug resistance among patients on failing first-line antiretroviral therapy in the South African national roll-out programme Antiviral Therapy 14 Suppl 1:A181.
8. **Wallis C.L.**, Varughese S., Technau K., Stevens W. 2009. HIV drug resistant mutations in HIV-1 subtype C children failing antiretroviral therapy in South Africa Antiviral Therapy, 14 Suppl 1:A184.

9. **Wallis C.L.**, Mellors J.W., Venter W.D.F, Sanne I., Stevens W. 2009. Protease inhibitor resistance is uncommon in patients on failing second-line lopinavir/r containing regimen in South Africa Antiviral Therapy; 14 Suppl 1:A183.
10. **Wallis C.L.**, Papathanasopoulos M.A., Conradie F., Ive P., Orrell C., Zeinecker J., Sanne I., Wood R., McIntyre J., Stevens W. 2010. Early Switch based on virological failure reduces complexity of HIV-1 drug resistance. CROI, San Francisco, USA.

ABSTRACT

Background: The availability of highly active antiretroviral (ARV) treatment in the South African government sector has reduced the morbidity and mortality associated with HIV-1 infection. However, ARV drug resistance and toxicity are major obstacles to achieving and maintaining virus suppression, but there is no provision for ARV drug resistance testing in the public sector. To date, most studies of ARV drug resistance in HIV-1 *reverse transcriptase* (RT) and *protease* (PR), are based on sequence data from HIV-1 subtype B, whereas subtype C is the predominant circulating subtype in South Africa. Moreover, host genetic polymorphisms associated with ARV drug toxicity have not been investigated in South African populations. This study evaluated viral and host genetic factors associated with ARV treatment outcome in 812 ARV drug-naive South African AIDS participants enrolled on the CIPRA-SA study from Johannesburg and Cape Town.

Methodology: An affordable in-house genotyping protocol (subtype C specific) was established and validated to monitor the emergence of ARV drug resistance. This assay was used to genotype all CIPRA-SA participants failing the first- and second-line ARV drug regimens. Allellic discrimination assays to identify the G1344A, A6986G, G516T and C3435T SNPs in *CYP3A4, 3A5, 2B6* and *MDR-1,* respectively, associated with ARV metabolism and absorption were performed.

Results: The in-house ARV drug resistance assay successfully genotyped 95% of patient samples, including non-C subtypes from 8 African sites. Treatment failure was experienced in 371 participants, mainly due to toxicity (n=134) or virological failure

(n=83). Overall, CIPRA-SA participants with a lower CD4+ T-cell count at study onset were more likely to experience viral failure. Genotyping using the in-house assay revealed that 6 participants had ARV drug resistance mutations at study entry. Treatment failure of 58 participants was a result of ARV drug resistance mutations, whereas 19 had no known ARV drug resistance mutations. The most frequent mutations were M184V (67%) and K103N (50%). K65R was present (3%) and one participant harboured TAMs. Longitudinal genotypic analysis showed that NNRTI mutations accumulated at a rate of one per three months left on failing therapy. No PR mutations were detected amongst participants experiencing second-line failure. The four SNPs analysed occured in similar frequencies between a background and the CIPRA-SA cohort. Furthermore, no statistically significant association could be found between these four SNPs and viral failure and/or toxicity.

Conclusion: Overall, HIV-1 subtype C-infected individuals receiving ARV therapy develop many of the known subtype B drug resistance mutations. However, the ARV drug resistance patterns in the closely monitored CIPRA-SA cohort were less complex compared to published data from the region, confirming that more frequent viral load monitoring, genotyping, and a virological failure cut-off value of 1000 RNA copies/ml ensure a better prognosis, and preserve future ARV treatment options.

ACKNOWLEDGEMENTS

- I would like to thank my supervisors, Professor Wendy Stevens and Dr Maria Papathanasopoulos for their continual guidance, encouragement and support.
- National Institute of Health (NIH, USA) sponsored Comprehensive International Program of Research on AIDS (CIPRA) network, Grant U19 AI53217 ‘‘Safeguard the Household’’ study for funding and sample material.
- The National Health Laboratory Service (NHLS) and The Department of Molecular Medicine and Hematology, University of the Witwatersrand for funding.
- The staff of the genotyping laboratory, namely, Catherine Bell, Esrom Legadima, Lindiwe Skhosana and Christinah Mutuku for their support.
- Furthermore, I would like to thank the staff of the department of Molecular Medicine and Haematology for their encouragement and interest in my work, particularly Mrs Pamela Horsfield and Dr Lesley Scott.

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NOMENCLATURE

3TC-Lamivudine
ABC=Abacavir
ACTG-Adult AIDS Clinical Trial
AIDS-Acquired Immunodeficiency Syndrome
ALT-alanine aminotransferase
ARV-antiretroviral
AST-aspartate aminotransferase
AZT-zidovudine
CDC-Centre for Disease Control
cDNA-complimentary DNA
CPZ-chimpanzee
CRF-circulating recombinant forms
CYP450-cytochrome P450
d4T-stavudine
DAIDs-Division of AIDS
DART-Development of Antiretroviral Therapy in Africa
ddC-zalcitabine
ddI-didanosine
DDT-Dithiothreitol
DLV-delaviridine
FBC=Full Blood Count
EFV-efavirenz
EI-entry inhibitors
EQA-external quality assurance
ETR-Etravarine
FTC=Emtricitabine
gp-glycoproteins
gp120-glycoprotein 120
gp41-glycoprotein 41
HAART-highly active antiretroviral therapy
HIV-Infection with Human Immunodeficiency Virus
IAS-International AIDs Society
Kaletra-lopinavir boosted with ritonavir
KAVI-Kenya AIDs Vaccine Initiative
LTR-long terminal repeat
MDR-1-Multi-drug resistant type 1
MGPs-Magnetic Glass Particles

MuLV-Murine Moloney
NA-Not Available
ND-Not Done
NHLS-National Health Laboratory Service
NIH-National Institute of Health
NNRTI-non- Nucleoside Reverse Transcriptase Inhibitors
NRTI-Nucleoside Reverse Transcriptase Inhibitors
NVP-nevirapine
PBMCs-Peripheral Blood Mononuclear Cells
p17-protein 17
p24-protein 24
PHRU-perinatal HIV research unit
P-gp-P-glycoprotein
PIs-Protease Inhibotors
PR-protease
RT-reverse transcriptase
SGS-single genome sequencing
SIV- Simian immunodeficiency virus
SM-sooty mangabey
SNPs-single nucleotide polymorphisms
SOWETO-South West Township
TAMs-thymidine analogue mutations
TB-*M. tuberculosis*
TNF=tenofovir
UNAIDS-United Nations Program on HIV/AIDS
UGT-UDP-glucuronosylatransferases
V-valine
Vpr-viral proteins R
VQA-Virology Quality Assurance
WHO-World Health Organization
w-week

PREFACE

Several events initiated and steered the direction of this study. At the time the study was planned (January, 2005), the South African government had just initiated the antiretroviral (ARV) roll-out program (April, 2004) and 50000 HIV-1 infected individuals were receiving ARV therapy. It was therefore envisaged that to support these treatment programs, affordable and appropriate laboratory monitoring is essential. Currently, the South Africa government has enrolled 870000 HIV-1 infected patients onto ARVs and a greater understanding of host genetic and viral factors that impact on ARV treatment outcome in South Africa is required.

1. Chapter 1: Introduction

1.1. Background

Infection with Human Immunodeficiency Virus (HIV), the causative agent of Acquired Immunodeficiency Syndrome (AIDS) has resulted in a worldwide pandemic, with reports from the Joint World Health Organization (WHO) and the United Nations Program on HIV/AIDS (UNAIDs) estimating that a total of 33.4 million individuals worldwide were living with HIV infection by the end of 2008, with an additional 2.7 million newly infected during 2008 (UNAIDs, 2009). Furthermore, there were an estimated 2.0 million HIV/AIDS related deaths of which 1.7 million were adults during 2008 (UNAIDs, 2009). These staggering numbers ensure that the HIV-1 pandemic remains a serious public health challenge throughout the world.

Although in some areas of the world, the HIV-1 epidemic appears to be on the decline, this is not the case in most of sub-Saharan Africa where the prevalence of HIV-1 is still increasing. Furthermore, 67% of all adults and children infected with HIV-1 reside in sub-Saharan Africa (UNAIDs, 2009). South Africa remains the epicenter of the HIV pandemic, with an estimated prevalence of 10.6% and 5.21 million people infected, with approximately one-fifth of reproductive South African women being HIV-1 positive (Statistics South Africa, 2009).

1.2. Introduction to HIV

HIV exhibits remarkable genetic diversity, resulting in several different types and subtypes. HIV is divided into two distinct types namely, HIV-1 and HIV-2 (Centres for Disease Contol, 1982). HIV-1 accounts for the majority of infections globally, as a result of increased virulence and ease of transmission (Centres for Disease Contol, 1982); whereas HIV-2 is mainly concentrated in foci in West Africa and co-infection with HIV-1 are common in these areas (Gottlieb et al., 2003). HIV-1 infection resulted from chimpanzee (SIV_{cpz})-to-human transmission, and HIV-2 infection resulted from sooty mangabey (SIV_{sm})-to-human transmission. HIV-1 is classified into three groups: the "major" group M, the "outlier" group O and the "new, or non M non O" group N, each group reflecting a different cross-species transmission. Both group O and N are restricted to west and central Africa and are extremely rare; whereas group M makes up approximately 90% of the infections worldwide (Wainberg, 2004). Group M is composed of nine subtypes (A, B, C, D, F, G, H, J and K), two sub-subtypes (A1, A2 and F1, F2) and 43 circulating recombinant forms (CRFs; see http://www.hivweb.lanl. gov/CRFs /CRFs.html for updates). In Europe, North and South America, most HIV-1 infections are with subtype B (Figure 1.1). By contrast, in Africa most subtypes are represented, with subtype A and D occurring in the highest frequency in central and eastern Africa and subtype C in Southern Africa (Papathanasopoulos et al., 2003; Figure 1.1). HIV-1 subtype C is responsible for over 50% of all infections worldwide (Buonaguro et al., 2007).

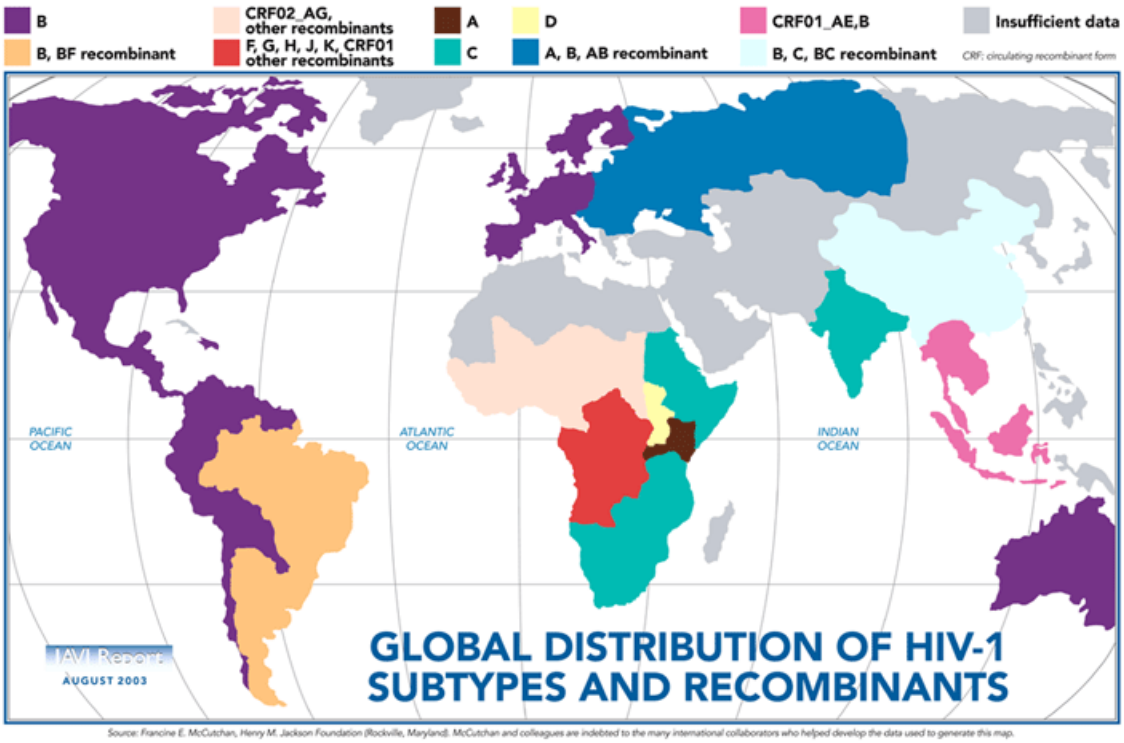


Figure 1.1: HIV-1 group M subtype distribution. Subtype B is found predominantly in North and South America, Europe and Australia; whereas subtype C is found predominantly in Southern and Eastern Africa and India (https://pathmicro.med.sc.edu/lecture/hiv6.htm).

1.2.1. Structure of HIV-1

HIV-1 is a retrovirus and belongs to the lentivirus genus. The virus is spherical and comprised of an envelope and the core. The envelope consists of a lipid bilayer, made up of approximately 72 viral envelope glycoprotein complexes (arranged as trimers) and different host derived proteins (Collier and Schwartz, 1999). Trimeric viral glycoproteins (gp) present in the lipid bilayer include the external glycoprotein 120 (gp120) and the transmembrane glycoprotein 41 (gp41). Below the lipid membrane is the matrix protein 17 (p17) containing the cone shaped viral capsid (p24; Gelderblom et al., 1998). The viral capsid surrounds two single-stranded HIV-1 RNA molecules, as well as viral

proteins necessary for replication, such as nucleoprotein p7-gag, p9-gag, the reverse transcriptase p51(RT), RNase H (p66), protease p15 (PR) and integrase p32. All the enzymes in the particle facilitate integration of viral genetic material into the host's genome (Figure 1.2). The HIV-1 genome is approximately 9.7 kb and made up of two identical long terminal repeat (LTR) regions which flank three major genes *gag, pol* and *env.* There are a further 2 regulatory genes *tat* and *rev,* and 4 accessory genes *vif, vpu, vpr* and *nef,* which are not an absolute requirement for viral replication *in vitro*.

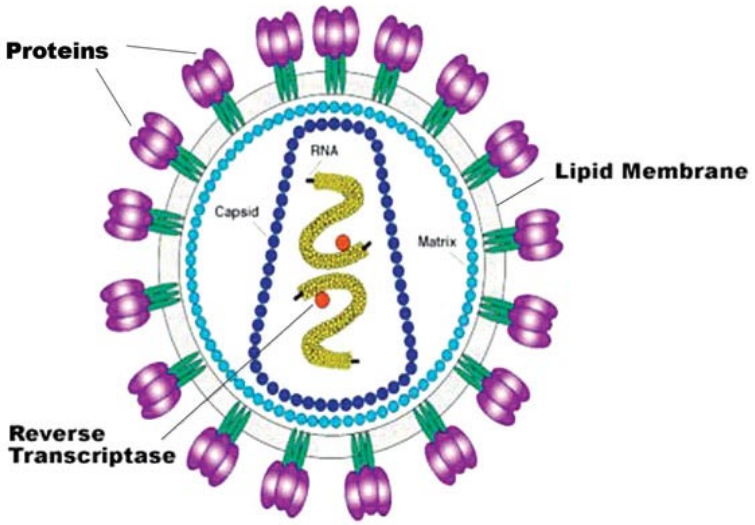


Figure 1.2: Cross sectional representation of the structure of HIV-1. The lipid bilayer containing the gp41 and gp120 trimeric proteins, and the capsid which encompasses the two single stranded RNA molecules and viral enzymes (adapted from https://www.aidsfactsheet.com).

1.2.2. Lifecycle of HIV-1

HIV-1 has an extremely successful replication cycle, which takes approximately 2.5 days. Over ten billion HIV-1 particles can be produced and cleared each day in an average HIV-infected person. A schematic diagram of the viral life cycle is depicted in Figure 1.3 (Turner and Summers, 1999).

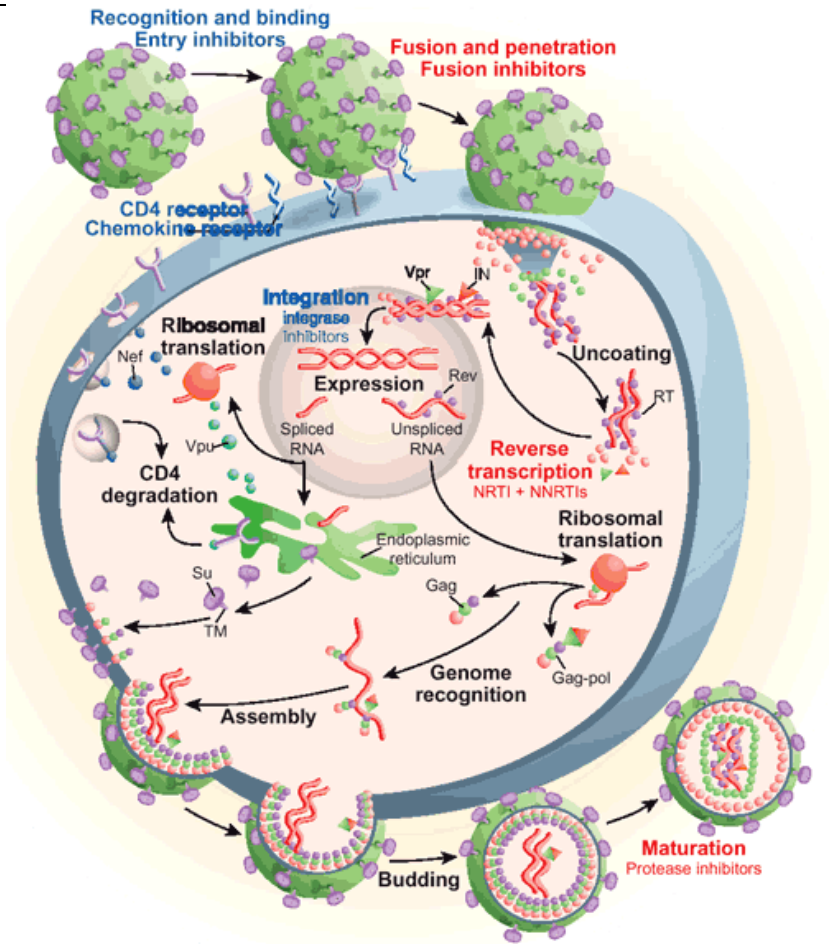


Figure 1.3: The lifecycle of HIV-1. The HIV-1 lifecycle is depicted from 1) attachment, fusion and entry into the cellular cytoplasm to reverse transcription, integration into host DNA and assembly, budding and final maturation to make infectious virions. Modified from (Turner and Summers, 1999).

1.2.2.1. Viral Attachment and Entry

Once the HIV-1 particle enters the body it infects T-lymphocytes and/or macrophages expressing the CD4 receptor (Dalgleish et al., 1984, Klatzmann et al., 1984) by binding of the viral envelope gp120 to the CD4 receptor (King, 1994). The gp120-CD4 interaction results in a conformational change in gp120 which exposes the gp120 coreceptor binding sites that are able to bind to either CCR5 or CXCR4, or both (Alkhatib et al., 1996, Dragic et al., 1996; Figure 1.3). The binding of the viral particle to the co-receptors results in further conformational changes in the envelope gp41. The gp41 forms a six helix bundle resulting in fusion of the viral and host membranes.

1.2.2.2. Uncoating and Reverse Transcription

After fusion of the cellular and viral membranes, the HIV-1 core containing viral proteins enters the host cytoplasm (Turner and Summers, 1999; Figure 1.3). The viral capsid disintegrates releasing the single-stranded viral RNA and the viral enzymes: RT, integrase and PR. The RT transcribes the single-stranded RNA into complementary DNA (cDNA), and the viral RNA is degraded through the RNase H activity. The RT is extremely error-prone, introducing mistakes with each replication cycle (estimated misincorporation rate of $3x10^{-5}$ per base per replication cycle), which give rise to numerous quasispecies circulating within the same patient.

1.2.2.3. Viral Integration

In the cytoplasm, integrase binds to specific sequences located at the ends of the viral DNA. This binding 'recruits' viral proteins such as Vpr (viral proteins R) and host cellular factors which form the preintegration complex. The integrase enzyme then excises two nucleotides from the 3' ends of the viral DNA (3' end processing) in preparation for strand transfer. The preintegration complex then moves into the nucleus by the aid of cellular co-factors, where it integrates into the host DNA. Integrase nicks the host DNA, strand transfer takes place and the host DNA repair mechanisms join the viral and host DNA together (Fish et al., 2010).

1.2.2.4. Transcription and Translation

The integrated viral DNA either becomes latent or is transcribed into at least 30 mRNA species (Peterlin and Trono, 2003). The transcribed mRNA is spliced several different ways and moved into the cytoplasm were it encodes for the early regulatory proteins Tat and Rev. This facilitates the production of the remainder of the HIV-1 proteins. The alternative splicing patterns ensure all the structural and accessory components that are required for infectious virions are produced. The viral proteins (at least 16) are all translated within the cytoplasm, and some undergo post-translational modifications such as glycosylation and myristolation. The viral protease partially cleaves the Gag and Gag-Pol polyprotein, while host cellular proteases cleave the gp160 into the gp120 and gp41 components (Peterlin and Trono, 2003).

1.2.2.5. Assembly, Budding and Maturation

All the viral proteins then travel to the plasma membrane where immature virions assemble in cholesterol rich lipid rafts and start budding. Gag is pivotal in assembly of the virions by recruiting the required host and viral factors. As each new virus buds from the host cell, it takes away with it the host cell's outer lipid membrane, within which the viral gp120 and gp41 is embedded. Once the virion is released, complete processing of the Gag protein by the viral protease is required to make a mature infectious HIV particle capable of infecting another CD4+ T-cell (Turner and Summers, 1999).

1.2.2.6. Consequences of the HIV-1 replication strategy

The short replication cycle, high numbers of virions produced daily and the error prone RT lead to the rapid evolution of HIV-1 (high genetic diversity). At least one error is introduced per virion during each replication cycle. Thus genomes or quasispecies with each possible mutation are generated daily. This ultimately allows HIV-1 to escape host immune surveillance, establish antiretroviral (ARV) drug resistant variants, affect accurate diagnoses and impact on the development of effective ARV drugs, microbicides and HIV-1 vaccines.

1.3. HIV-1 Disease Progression to AIDS

Adults generally acquire HIV-1 by sexual transmission, blood transfusions and sharing of intravenous needles for drug usage. In infants, transmission can occur vertically from mother to child *in utero*, during birth, or through breastfeeding.

The 3 major stages of HIV-1 disease progression to AIDS are shown in Figure 1.4a.

1.3.1. Acute Infection

The acute phase of infection can last up to six months post transmission. During this stage the virus infects CD4+ T-cells and results in a rapid depletion of the memory CD4+ T-cells, in the gut-associated lymphoid tissue of the gastrointestinal tract (Brenchley et al., 2004, Guadalupe et al., 2003, Veazey et al., 2001). This depletion results in a loss of most of the mucosal CD4+ memory T-cells, which is thought to contribute to the disease progression to AIDS (Douek et al., 2006). Furthermore, there is a high level of viral replication (measured as viral load; RNA copies/ml) as there is no or limited host immune control. There is an initial depletion of peripheral CD4+ T-cells, but as the host immune system becomes activated (particularly the anti HIV-1 CD8+ cytotoxic T lymphocytes) it is able to control the viral replication, resulting in a rapid decline of viral load and restoration of the CD4+ T-cell counts. Furthermore, proviral DNA integrated into host DNA persists as viral reservoirs in resting CD4+ T-cells (Chun et al., 1995). At least 50% of new infections (transmissions) occur during the acute phase.

1.3.2. Asymptomatic Phase

The viral load reaches a set point, which is indicative of the rate of disease progression to AIDS (Fauci et al., 1996). This phase can last for up to ten years and during this time the patient generally has no symptoms, but the virus continues to replicate at low levels and infects CD4+ T-cells. Over time, the immune system loses the battle against HIV-1, and CD4 + T-cells gradually decline.

1.3.3. Symptomatic Phase and progression to AIDS

CD4+ T-cell depletion is characterized by the emergence of opportunistic infections. When the CD4+ T-cell counts reach a critical level the individual is diagnosed with AIDS. Without the initiation of ARV therapy, people die within 2 years of an AIDS diagnosis. AIDS is classified by the Centre for Disease Control (CDC) as a positive HIV-1 test and fewer than 200 CD4+ T-cells per microlitre, or the development of any 26 opportunistic infections (common opportunistic infections such as: *Pneumocystis jiroveci* pneumonia; *Mycobacterium avium* complex; cytomegalovirus, *M. tuberculosis* (TB), toxoplasmosis; cryptosporidiosis and human papillomavirus or cancers that affect HIV-1 positive individuals (AIDS defining diseases; Figure 1.4a; http://www.cdc.gov/hiv/resources/brochures/livingwithhiv.htm).

1.3.4. Initiation of highly active antiretroviral therapy

When an AIDS diagnosis is made, highly active antiretroviral therapy (HAART) consisting of a combination of three antiretrovirals is introduced to slow or reverse the progression to AIDS and death. If HAART is successful it can effectively suppress viral replication and reduce the viral load to below detectable limits of commercially available assays (Figure 1.4b) within an average of 12 weeks (Fauci et al., 1996). This viral suppression can be maintained with optimal treatment and compliance for several years, however, in some cases viral rebound can occur during HAART.

1.3.5. Factors influencing disease progression to AIDS:

Disease progression to AIDS can vary between individuals and can be as a result of host and viral genetic differences, age, co-infection with other microbes and the virulence of the virus which has infected an individual. There have been reported cases of patients that progress to AIDS within a short period of time after infection and these are known as rapid progressors, moreover, there are individuals which appear to remain healthy for an extended period of time after infection and are known as long-term non-progressors (5%), and there are a group with spontaneous control that maintain a viral load of less than 50 RNA copies/ml and have been termed elite controllers (1 in 300; http://www.elitecontrollers.org).

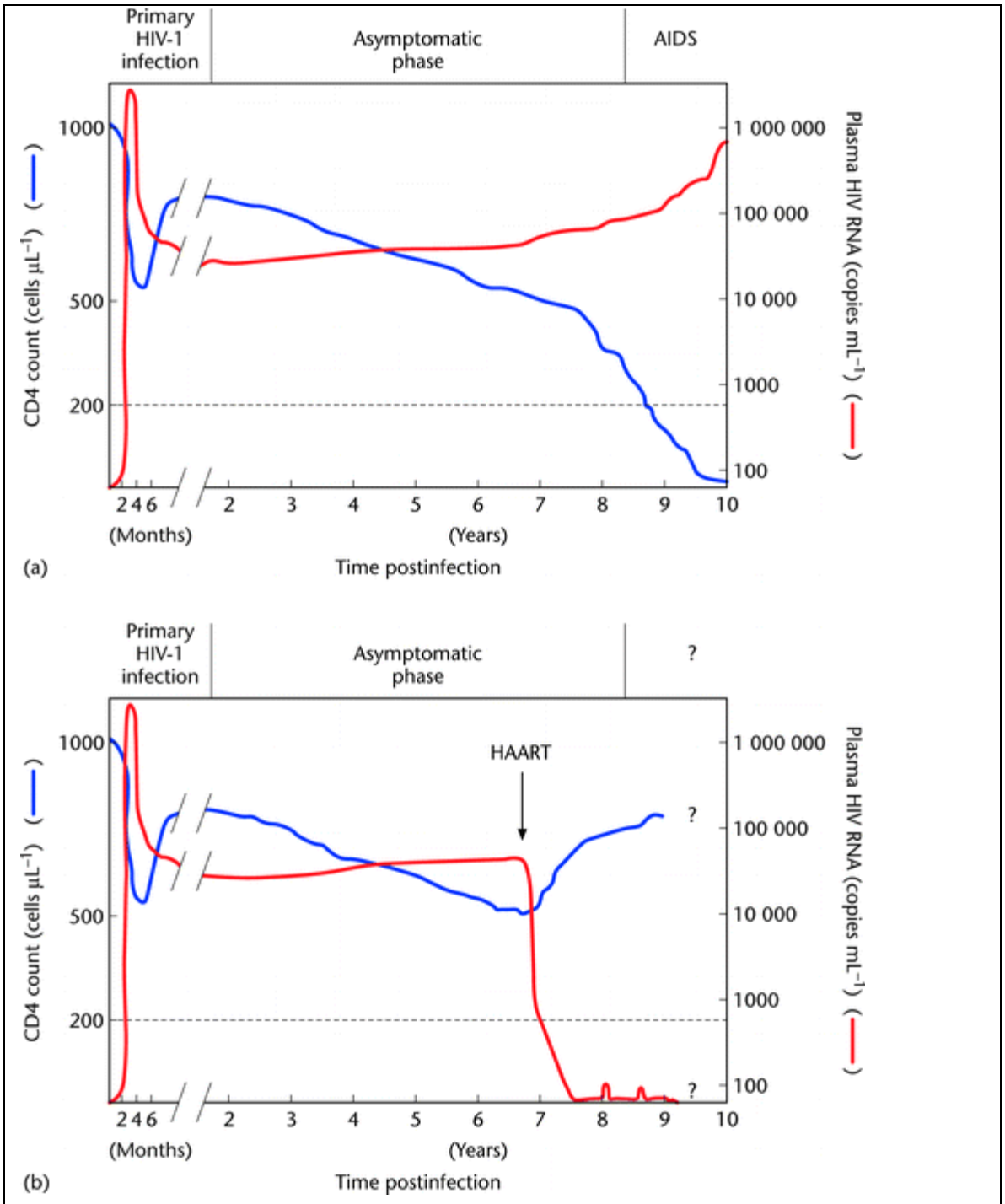


Figure 1.4 HIV-1 disease progression from primary infection to AIDS. a) in the absence of HAART; b) with the intervention of HAART, however, viral rebound can occur as a result of several different factors (Adapted from Fauci et al., 1996).

1.4. Antiretroviral Agents

Extensive analysis of the HIV-1 lifecycle has resulted in the identification of several drug targets, namely, 1) viral entry (entry inhibitors [EI] such as Fusion Inhibitors and CCR5 coreceptor antagonists); 2) viral replication (Nucleoside Reverse Transcriptase Inhibitors [NRTIs] and non-NRTIs [NNRTIs]); 3) integration (Integrase inhibitors) and 4) viral processing (Protease Inhibitors [PIs]; Table 1.1).

1.4.1. Targeting viral entry

Viral entry is a multistep process encompassing attachment, coreceptor binding and fusion. US Food and Drug Administrator (FDA) approved entry inhibitors include Enfuvirtide (T-20; fusion inhibitor), a synthetic peptide which competitively binds to the six helix bundle in gp41, thereby preventing the conformation changes required for fusion of the viral and cellular membranes, and Maraviroc (CCR5 coreceptor antagonist), a small molecule which binds to CCR5, thereby preventing binding of gp120 to the coreceptor. ARV drug resistance to entry inhibitors has been reported (Olson and Maddon, 2003).

1.4.2. Targeting the Reverse Transcriptase (RT) Enzyme

The RT enzyme is used by HIV-1 to transcribe single stranded viral RNA into viral cDNA in the cytoplasm (Figure 1.3). A functional RT is a heterodimer comprised of two subunits; p66 and p51. The p66 is made up of the N and C-terminal polymerase and

RNase H domains, respectively. The p51 is processed by proteolytic cleavage of p66 and corresponds to the N-terminal domain of p66. The RT crystal structure has been compared to a right hand, which contains three subdomains, namely, fingers, palm and thumb (Figure 1.5). The p66 subunit has three catalytic residues which are exposed in nucleic acid binding. The DNA/RNA-dependent DNA polymerase activity is catalysed by the p66. The thumb (made up of two alpha helices) and the fingers hold the nucleic acid in place over the palm, which contains the polymerase active site (Jacobo-Molina et al., 1993, Kohlstaedt et al., 1992). As mentioned previously, the RT enzyme is extremely error prone resulting in a high level of incorrectly incorporated bases. These errors constitute either polymorphisms or mutations, which may change the amino acid sequence of the virus, and result in a conformational or charge change in the proteins produced. This in turn can result in the development of resistance to ARV drugs targeting the RT enzyme.

The majority of currently used antiretroviral therapies (zidovudine [AZT], didanosine [ddI], ddC [zalcitabine], stavuidine [d4T] and Lamivudine [3TC]; Table 1.1) are nucleoside analogues that competitively inhibit RT activity. By contrast, the non-nucleoside, non-competitive RT inhibitors including efavirenz (EFV) and nevirapine (NVP) act by irreversibly binding to the enzyme.

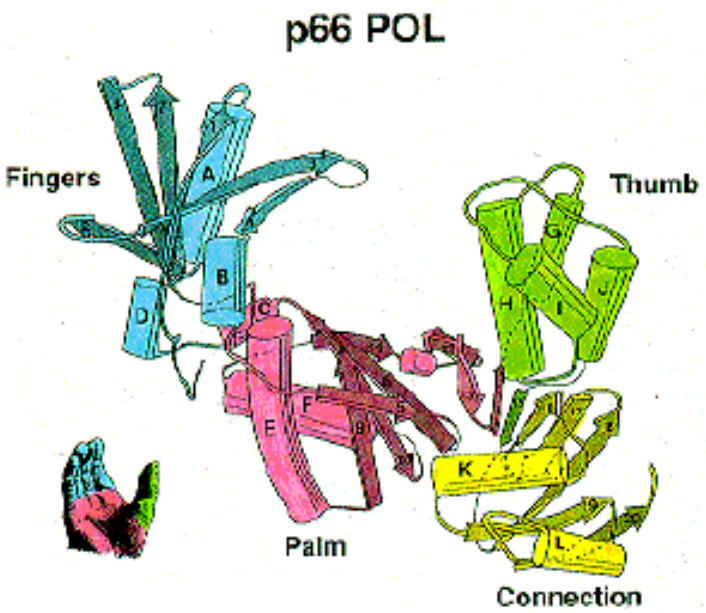


Figure 1.5: Reverse Transcriptase enzyme. The RT enzyme is in the shape of a hand with the 'palm' containing the catalytic site, and the 'thumb' and fingers hold the DNA in place during replication (http://www.biochem.ucl.ac.uk/bsm/xtal/teach/repl/rt.html).

These two classes of drugs which target the RT enzyme, nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) make up the majority of regimens used in HIV-1 treatment (see Table 1.1 for US FDA approved NRTIs and NNRTIs). NRTIs are essentially modified nucleotides/sides that when incorporated into the replicating strand result in chain termination. During ARV drug pressure the HIV-1 RT is able to develop resistance to these drugs by generating mutations.

The genetic pathways to develop resistance to NRTIs can occur in two ways. Firstly, RT-residues that encode amino acids on the tips of the fingers (Figure 1.5) that come into direct contact with the dNTPs or NRTIs can mutate. These mutations affect the rate of binding and incorporation of nucleotides. Primary mutations associated with this type of

resistance are K65R, L74V, Y115F, M184V/I and Q151M and its associated mutations. Primary mutations are amino acid substitutions in critical positions of the enzyme that cause an immediate decrease in susceptibility to the drug, ultimately leading to virological failure. Further work performed on the M184V mechanism of resistance has shown that the mutation results in a steric hindrance to the correct binding of both 3TC and FTC to the active site of the RT (Sarafianos et al., 1999, Schinazi et al., 1993). The second mechanism of resistance towards NRTIs is an increased rate of excision of the NRTIs (Arion et al., 1998). This process is driven by adenosine triphosphate (ATP) and is caused by thymidine analogue mutations (TAMs) that occur close to the triphosphate binding site. As the number of TAMs such as M41L, D67N, K70E, L210W, T215Y/F, K219Q/E/N/K increase in the RT, the level of resistance increases.

The NNRTIs (Table 1.1) are molecules which have a high affinity for the hydrophobic pocket of the RT enzyme (as described above; Figure 1.6). This results in the NNRTIs binding to the pocket, thereby inhibiting replication. Resistance develops when mutations occur in the hydrophobic pocket, which changes the overall charge of the protein and results in a decreased binding of the NNRTIs (Figure 1.6). The mutations that develop in the hydrophobic pocket result in cross-resistance to all first-generation NNRTIs (Table 1.1). Recently a second generation NNRTIs, Etravarine (ETR) has been released which unlike the first generation NNRTIs, is a highly flexible molecule resulting in a high genetic barrier to resistance. ETR is susceptible to viruses with the K103N mutation, which results in cross resistance to both EFV and NVP. The level of

susceptibility is determined using a weighted scoring system for each mutation (Lazzarin et al., 2007, Madruga et al., 2007).

Interestingly, the hydrophibic pocket of the RT enzyme has been found to be poorly conserved across different HIV-1 groups, resulting in HIV-1 group O being naturally resistant to the first-generation NNRTIs (Descamps et al., 1997). The impact of the variation in the RT hydrophobic pocket of different groups on second-generation NNRTIs is unknown.

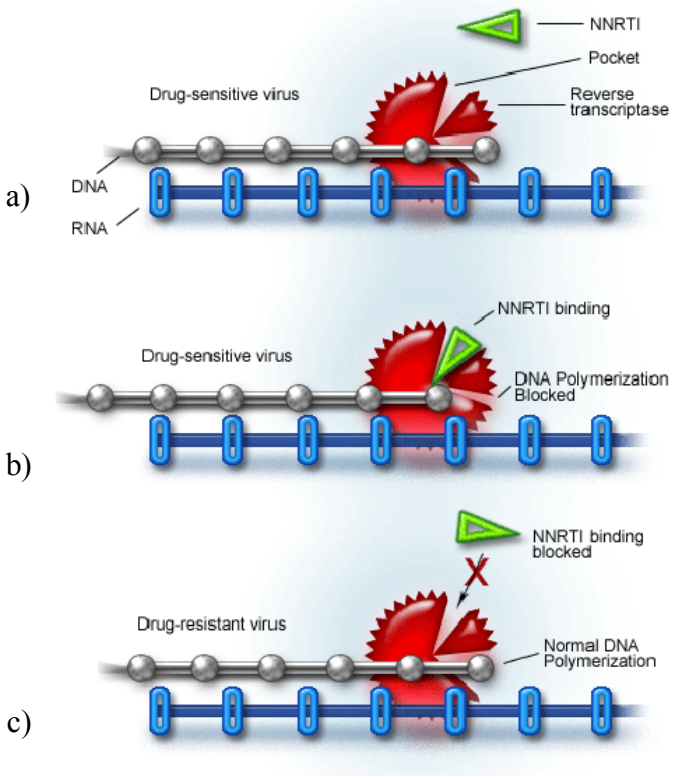


Figure 1.6: Diagrammatic representation of the viral reverse transcriptase (RT) transcribing its RNA to cDNA prior to integration (a). When a non-nucleotide reverse transcriptase (NNRTIs) is introduced it binds to the hydrophobic pocket of the RT resulting in inhibition of the polymerization of the cDNA (b). However, the RT can develop mutations which result in a change of charge or configuration resulting in the NNRTIs being unable to bind (c; http://www.thebodypro.com/thebody/images /nnrti_ fig1.gif).

1.4.3. Targeting the Integrase Enzyme

Once HIV-1 has infected the host cell and replicated the viral RNA into cDNA, the viral DNA is integrated into the host DNA (Figure 1.3). These steps are catalysed by the integrase enzyme. The integrase enzyme consists of three domains, namely, a catalytic core and the C- and N-terminal domains. All three domains are required for DNA integration, with the catalytic core being the initiator in the integration process (Figure 1.7). The C-terminus binds the host and viral DNA while the function of the N-terminus is still unknown (Fish et al., 2009).

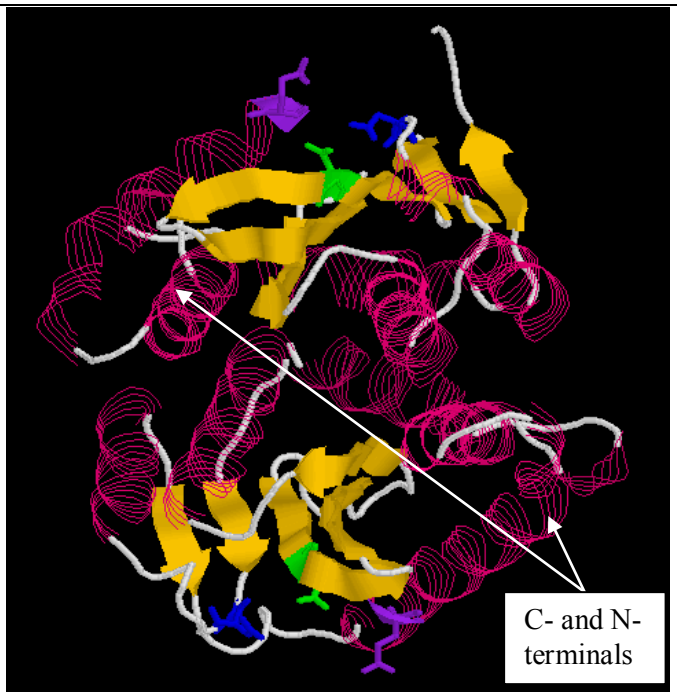


Figure 1.7: HIV-1 integrase enzyme. The catalytic site is represented by green, with the flanking C and N terminals in pink. (Image from http://www.web.chemistry.gatech.edu/~williams/ bCourse_Information/6521/protein/images/haller.gif).

There is only one US FDA approved ARV in this class, Raltegravir, a strand transfer inhibitor; which prevents viral cDNA integration into the host genome. Resistance

develops as a result of amino acid changes in the catalytic site. Second-generation integrase inhibitors are currently being investigated, which are active against some raltegravir mutants (Cooper et al., 2008, Steigbigel et al., 2008). An additional class of integrase inhibitors (quinolones) is being investigated which blocks both 3' processing and strand transfer, however, these have not yet reached clinical trials (Thibant et al., 2009).

1.4.4. Targeting the Protease Enzyme

The PR enzyme in HIV-1 is responsible for cleaving the newly transcribed viral Gag and Gag-Pol polyproteins to functional proteins essential for viral assembly (Figure 1.3). The PR enzyme is a homodimer consisting of two identical chains, referred to as A and B. These two chains are mirror images and are stabilized by the aliphatic residues in the hydrophobic core. This hydrophobic core surrounds the electronegative active site of the enzyme (Figure 1.8). There is a beta hairpin loop which covers the active site, which is flexible and allows substrate access to the active site by folding the glycine rich tips into the hydrophobic pocket. PR undergoes a conformational change as the cleft of the active site tightens around the neutral or positively charged substrate (Scott and Schiffer, 2000). This conformational change ensures proper cleavage of the substrate.

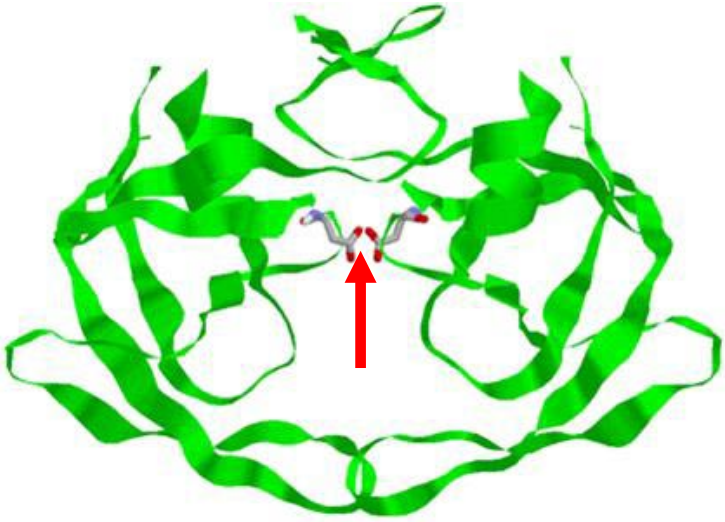


Figure 1.8: HIV-1 protease enzyme. The green represents the identical chains A and B which surround the hydrophobic active site (red arrow) (Modified from http://www.udel.edu/ chem/bahnson/chem645/websites/Mays/activesite.jpg)

PIs (Table 1.1) are a powerful class of drugs which bind more tightly to the active site of the PR enzyme than the natural substrates and act as preferred substrates. They are therefore competitive enzyme inhibitors, resulting in the PR enzyme being unable to cleave polyproteins, thus reducing the amount of mature virions that are produced. Interestingly, HAART regimens that include PIs are often boosted by low-levels of ritonavir. Resistance to this class of inhibitors is similar to that of NNRTIs with mutations developing in the active site and glycine tips prohibiting the binding of the PIs. PIs have a high genetic barrier for resistance, and require an accumulation of major mutations to lose complete susceptibility to the PIs.

Table 1.1: All FDA approved ARVS. Information adopted and incorporated from Clinical Care Options (http://www.clinicaloptions.com/HIV/)

Drug Class	Brand Name	Generic Name	Abbre-viation	Manufact-urer	Mode of Inhibition	Base	Activation Required	Metabololised /Catabolism by	Adverse Events	Genetic Barrier	Mode of resistance	Mutations (PI-major)	Mutations (PI-minor)
Reverse Transcriptase Inhibiotrs													
Nucleoside Reverse Transcriptase Inhibitors (NRTIs)													
	Retrovir	Zidovudine	AZT	GlaxoSmithKline	Chain Termination	Pyrimidine	Yes	Undergo cellular phosphorylation and catalysis	Nausea, headache, changes in body fat, anemia and bone marrow suppression	High	excision	M41L, D67N, K70R, L210W, T215Y/F, T219Q/E	NA
	Videx	Didanosine	ddI	Bristol-Myers Squibb	Chain Termination	Purine	Yes	Undergo cellular phosphorylation and catalysis	Diarrhea, headaches, vomiting, rash, peripheral neuropathy, pancreatitis and lactic acidosis	High	distinguish dNTP versus modified bases	K65R, L74V	NA
	Hivid	zalcitabine	ddC	Roche	Chain Termination	Pyrimidine	Yes	discontinued	discontinued	High	discontinued	discontinued	NA
	Zerit	Stavudine	d4T	Bristol-Myers Squibb	Chain Termination	Pyrimidine	Yes	Undergo cellular phosphorylation and catalysis	Peripheral neuropathy, lactic acidosis	High	excision	M41L, D67N, K70R, L210W, T215Y/F, T219Q/E	NA

Table 1.1: All FDA approved ARVS. Information adopted and incorporated from Clinical Care Options (http://www.clinicaloptions.com/HIV/)

	Epivir	Lamivudine	3TC	GlaxoSmithKline	Chain Termination	Pyrimidine	Yes	Undergo cellular phosphorylation and catalysis	Headache, Nausea, vomiting, malaise, fatique, sleeplessness, anorexia, dizziness, rash, depression, anemia, neutropenia, increase amylase	High	distinguish dNTP versus modified bases	K65R, M184V/I	NA
	Ziagen	Abacavir	ABC	GlaxoSmithKline	Chain Termination	Purine	Yes	Undergo cellular phosphorylation and catalysis	hypersensitivity	High	distinguish dNTP versus modified bases	K65R, L74V, Y115F, M184V	NA
	Viread	Tenofovir	TNF	Gilead Sciences	Chain Termination	Purine	NO	Undergo cellular phosphorylation and catalysis	Renal and Mild gastrointestinal adverse events	High	distinguish dNTP versus modified bases	K65R, K70E	NA
	Emtriva	Emtricitabine	FTC	Gilead Sciences	Chain Termination	Pyrimidine	Yes	Undergo cellular phosphorylation and catalysis		High	distinguish dNTP versus modified bases	K65R, M184V/I	NA

Table 1.1: All FDA approved ARVS. Information adopted and incorporated from Clinical Care Options (http://www.clinicaloptions.com/HIV/)

Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)													
First Generation NNRTI													
	Viramune	Nevirapine	NVP	Boehringer Ingelheim	Binds to active Pocket	NA	NO	Hepatic-CYP3A4	Rash, fever, potential for hypersensitivity reaction	Low	Change in charge of the hydrophobic pocket	L100I, K103N, V106A/M, V108I, Y181C/I, Y188C/L/H, G190A	NA
	Rescriptor	Delavirdine	DLV	Pfizer	Binds to active Pocket	NA	NO	Hepatic-CYP3A4, CYP2D6	Rash	Low	Change in charge of the hydrophobic pocket	K103N, V106M, Y181C, Y188L, P236L	NA
	Sustiva	Efavirenz	EFV	Bristol-Myers Squibb	Binds to active Pocket	NA	NO	Hepatic-CYP2B6	Central Nervous System, Rash, Tetrageneic	Low	Change in charge of the hydrophobic pocket	L100I, K103N, V106M, V108I, Y181C/I, Y188L, G190S/A, P225H	NA
Second Generation NNRTI													
	Intelence	Etravirine	ETR	Tibotec	Binds to active Pocket	NA	NO	Hepatic-CYP3A4, CYP2C9, CYP2C19	Rash, potential for hypersensitivity reaction	High	Change in charge of the hydrophobic pocket	Weighted Scoring System	NA

Table 1.1: All FDA approved ARVS. Information adopted and incorporated from Clinical Care Options (http://www.clinicaloptions.com/HIV/)

Protease Inhibitors (PIs)													
	Fortovase/Invirase	Saquinavir	SQV	Roche	Binds to active Pocket	NA	NO	Hepatic-CYP3A4	Diarrahea, nausea, dyspepsia, abdominal pain, dyslipidemia	High	Change in charge of the hydrophobic pocket	G48V, L90M	10, 24, 54, 62, 71, 73, 77, 82, 84
	Norvir	Ritonavir-used in low conc. to boost PI metabolism	RTV	Abbott	Binds to active Pocket	NA	NO	Hepatic-CYP3A4	Dyslipidemia, GI adverse events	High	Change in charge of the hydrophobic pocket	NA	NA
	Crixivan Sulfate	Indinavir	IDV	MERCK	Binds to active Pocket	NA	NO	Hepatic-CYP3A4	Kidney stones, hyperbilirubinemia, GI adverse events, insulin resistance	High	Change in charge of the hydrophobic pocket	M46I/L, V82A/F/T, I84V	10, 20, 24, 32, 36, 54, 71, 73, 76, 77, 90
	Viracept	Nelfinavir	NFV	Agouron Pharmaceuticals	Binds to active Pocket	NA	NO	Hepatic-CYP3A4	GI adverse events	High	Change in charge of the hydrophobic pocket	D30N, L90M	10, 36, 46, 71, 77, 82, 84, 88
	Kaletra	Lopinavir/ritonavir	LPV/r	Abbott	Binds to active Pocket	NA	NO	Hepatic-CYP3A4	GI adverse events, hypertriglyceridemia	High	Change in charge of the hydrophobic pocket	V32I, I47V/A, V82A/F/T/S	10, 20, 24, 33, 46, 50, 53, 54, 63, 71, 73, 76, 84, 90

Table 1.1: All FDA approved ARVS. Information adopted and incorporated from Clinical Care Options (http://www.clinicaloptions.com/HIV/)

	Reyataz	Atazanavir	ATV	Bristol-Myers Squibb	Binds to active Pocket	NA	NO	Hepatic-CYP3A4	Hyperbilirubinemia	High	Change in charge of the hydrophobic pocket	I50L, I84V, N88S	10, 16, 20, 24, 32, 33, 34, 36, 46, 48, 53, 54, 60, 62, 64, 71, 73, 82, 85, 90, 93
	Lexiva	Fosamprenavir	FPV	GlaxoSmithKline	Binds to active Pocket	NA	NO	Hepatic-CYP3A4	GI adverse events, hyperlipidemia, rash	High	Change in charge of the hydrophobic pocket	I50V, I84V	10, 32, 46, 47, 54, 73, 76, 82, 90
	Aptivus	Tipranavir	TPV		Binds to active Pocket	NA	NO	Hepatic-CYP3A4	GI adverse events, intracranial hemorrhage, dyslipidemia	High	Change in charge of the hydrophobic pocket	L33F, Q58E, T74P	10, 13, 20, 35, 36, 43, 46, 54, 69, 83, 90
	Prezista	Darunavir	DRV	Tibotec	Binds to active Pocket	NA	NO	Hepatic-CYP3A4	Rash	High	Change in charge of the hydrophobic pocket	Accumulate over 3 major mutations	NA
Integrase Inhibitor													
	Isentress	Raltegravir	RAL	MERCK	Binds to active Pocket	NA	NO	Hepatic-UGT1A1	rhabdomyolysis or myopathy	Low	Change in charge of the hydrophobic pocket	Y143R/H/C, Q148H/K/R, N155H	NA

Table 1.1: All FDA approved ARVS. Information adopted and incorporated from Clinical Care Options (http://www.clinicaloptions.com/HIV/)

Fusion Inhibitor													
	Fuzeon (T-20)	Enfuviritide	ENF	Roche		NA	NO	Hepatic-exact mechanism unknown	Peripheral Neuropathy, Insomnia, depression, dyspnoea, hypersensitivity, hypotension		Disrupts fusion		NA
Entry Inhibitor													
	Selzentry	Maraviroc	MVC		Blocks CCR5 co-receptor	NA	NO	Unknown			Disrupts binding of CCR5 and HIV	CXCR4 usage	NA

1.5. Factors Influencing Treatment Outcome

There are several factors which can contribute to ARV treatment outcome. The overall outcome of ARV treatment is dependent on the interaction of the following factors: drug-drug interactions, non-compliance, environmental factors, ARV drug toxicity and an array of either viral or host genetic factors (Boulle et al., 2007, Carr and Cooper, 2000, Johnson et al., 2008, Haas et al., 2004, Haas et al., 2003, Rotger et al., 2005, Rotger et al., 2007). Drug-drug interactions can lead to insufficient drug bioavailability to adequately suppress viral replication. For example, drug interactions with concomitant therapy, in particular anti-tubercular drugs (Kashuba, 2005, Spradling et al., 2002) can alter the circulating concentration of ARV drugs. Non-compliance, as a result of complicated dosage requirements, adverse side effects or non-adherence leads to virological failure. Non-compliance can be monitored by clinical algorithms such as pill-count or treatment diaries, or in the laboratory by directly measuring the concentrations of PIs or NNRTIs in the patient's peripheral circulation and indirectly via continued virological suppression. Environmental factors such as alcohol consumption and recreational drug usage via non-adherence may impact on treatment outcome. Other factors that may contribute to altered drug concentrations in the local South African environment include the use of traditional remedies such as St. John's Wort and milk thistle (Henderson et al., 2002). Low drug concentrations result in continued viral replication, whereas high drug concentrations can lead to ARV toxicity in humans, both of these scenarios resulting in ARV treatment failure.

1.5.1. ARV drug Toxicity

ARV drug toxicities are the most common cause of treatment failure. ARV drug toxicity can be acute or chronic and can result in several different complications (See Table 1.1). Examples of acute complications are lactic acidosis, hepatic toxicity (commonly associated with the use of d4T and ddI; (Boubaker et al., 2001, Carr and Cooper, 2000, Carr et al., 2000) and pancreatitis. Chronic toxicity related complications such as metabolic complications, diabetes, altered fat distribution, and myopathies (Carpentier et al., 2005, Grinspoon and Carr, 2005) are also observed. These toxicities can contribute to non-compliance and often result in a change of ARV treatment regimens. Interestingly, drug toxicities have been shown to occur in 16.5% of South Africa patients accessing similar regimens to those prescribed in the national roll-out programme (Boulle et al., 2007, Rosen et al., 2008, Wood et al., 2009).

ARV drug toxicity is diagnosed by a combination of clinical and laboratory markers. Laboratory markers are routinely used to investigate pancreatic function (triglycerides and lipase); liver function (total bilirubin, aspartate aminotransferase [AST] and alanine aminotransferase [ALT]); renal function (creatinine) and lactate levels when lactic acidosis is suspected. Toxicity can be graded using systems such as the Adult AIDS Clinical Trial (ACTG) adverse events table (Appendix A). ARV drug toxicity is generally considered relevant when the ALT, AST or creatinine levels are greater than grade 2. All grades of lipase and lactate levels are classified as adverse events. All other

laboratory markers must be greater than grade 3 to be considered clinically relevant, necessitating treatment switches.

1.5.2. Viral Factors

1.5.2.1. HIV-1 Drug Resistance

Viral factors contributing to ARV treatment outcome are a direct result of the poor proof-reading capability of the HIV-1 RT enzyme during replication (see section on Lifecycle of HIV-1). As mentioned previously this results in either polymorphisms or mutations which reduce the efficacy of the ARV drugs. To date, most data looking at viral factors linked to treatment failure during ARV therapy has been generated from studies of patients infected with HIV-1 subtype B, or *in vitro* drug susceptibility assays using HIV-1 subtype B viral isolates. The emergence of HIV-1 genetic variants with mutations conferring resistance to the above mentioned ARV compounds (EIs, RTIs, Integrase Inhibitors and PIs) have been mapped to several genes in the viral genome. These substitutions are classified into primary mutations, leading to several-fold decreases in sensitivity to one or more ARV drugs, and secondary mutations, which may not result in significant decreases to ARV drug sensitivity but are associated with restoration of the original viral fitness in the presence of the inhibitors (Quinones-Mateu et al., 2008). Several HIV-1 databases constantly maintain and update detailed lists of known mutations conferring ARV drug resistance (genotypic), the degree of drug resistance associated with specific mutations (phenotypic) as well as providing algorithms for

predicting drug resistance (http://www.hiv.lanl.gov; www.hivdb.stanford.edu). For example, in HIV-1 subtype B the presence of T215Y confers resistance to d4T, as well as cross-resistance to AZT (Table 1.1) and tenofovir. By contrast, the presence of M184V causes resistance to Lamivudine (Table 1.1), but enhances susceptibility to AZT, d4T and tenofovir (Johnson et al., 2008). Furthermore, the presence of either the K103N or Y188L mutation can substantially reduce the clinical use of all currently available NNRTIs. Unlike the V108I and P225H which both confer resistance to EFV in combination with other NNRTI-associated mutations (Clavel et al., 2004; Table 1.1).

Emerging data suggests that ARV drug resistance profiles for non-B subtypes vary, and in many instances the known subtype B profiles should not be directly extrapolated to other HIV groups/subtypes. *In vitro* data have indicated that HIV-1 group O viruses, as well as HIV-2, are naturally resistant to NNRTIs (Descamps et al., 1997, Witvrouw et al., 1999). There are also indications of variation in drug susceptibility within HIV-1 group M. For example, some HIV-1 subtype G strains are less susceptible to PIs (Descamps et al., 1997). In HIV-1 subtype C, which is responsible for over 50% of new infections worldwide, the presence of naturally occurring polymorphisms which have been classified as minor mutations in HIV-1 subtpye B PR, for example, M36I and L63P, could impact on the efficiency of PI against subtype C (Kantor, 2006). The clinical relevance of this for HIV-1 subtype C infected patients remains unclear, and further *in vivo* and *in vitro* studies are necessary to delineate how these naturally occurring polymorphisms can affect treatment outcome. These polymorphisms may result in a predisposed resistance to the ARV or result in novel mutations (Grossman et al., 2004).

In the case of novel mutations, the amino acid valine (V) occurs at codon 106 in both subtype B and C RT, however, as a result of a natural polymorphism at a nucleotide level, a subtype C patient treated with EFV has a 50% chance of developing the V106M mutation. This mutation has never been reported in subtype B infected patients on EFV. By contrast, they are likely to develop the V106A mutation under NVP pressure (Brenner et al., 2003, Morris et al., 2003).

The K65R has recently been shown to occur in higher frequencies in the RT of HIV-1 subtype C patients administered d4T compared to HIV-1 subtype B (Hosseinipour et al., 2009, Orrell et al., 2009, Wallis et al., 2010). Recently, a study showed that this is a result of a homopolymeric region surrounding codon 65 resulting in the enzyme pausing and an increased frequency of the K65R mutation (Coutsinos et al., 2009).

Furthermore, studies have shown that even within one subtype there can be different primary mutations, both leading to resistance. For example, a genotypic study evaluating and comparing the frequency and patterns of PR mutations from HIV-1 subtype C infected patients in Botswana and Ethiopia showed that subtype C viruses from Botswana developed Nelfinavir resistance (D30N) via a different mutation pathway than those from Ethiopia (L90M; Grossman et al., 2004). A comparison of two clinical trials using single dose NVP to prevent mother to child transmission, namely the HIVNET 012 and NVAZ, indicated that the prevalence of the K103N drug resistance mutation in the RT of HIV-1 subtype C (69.2%) was significantly higher than that of subtype A (19.4%) or subtype D (36.1%; Eshleman et al., 2005b). Moreover, NVP resistance was more frequent in

subtype C infected infants from Malawi (87%) versus subtype A or D infected infants from Uganda (46%; Eshleman et al., 2005a).

1.5.2.2. Diagnostic Assays to monitor for the development of HIV-1 drug resistance

The emergence of HIV-1 drug resistance during HAART can be monitored in the laboratory through phenotypic and genotypic assays. Phenotypic assays are based on growing the HIV-1 primary virus isolates from the patient or pseudoviruses in the presence of different levels of ARVs. The amount of virus present is quantified and the level of susceptibility of the virus to each ARV is determined, as compared to a baseline sample or reference virus. Although phenotype testing gives a direct measure of resistance, it does not take into account the discrepancies that could arise from differences in metabolism of the ARVs between individuals (Shao et al., 2003). The tests are extremely expensive, time consuming, require highly skilled staff and as a result of the high risk to laboratory staff, need to be performed in bio-safety level 3 laboratories. This has resulted in population based sequencing assays (genotyping) becoming the gold standard. These assays are based on the generation of the PR, RT, Integrase or gp160 sequences followed by comparison to a consensus sequence to determine if there are any differences. Differences at codons linked to drug resistance are called mutations, and linked to the level of resistance for each ARV. There are currently two commercially available and FDA approved assays for monitoring ARV drug resistance to the RTIs and

PIs: ViroSeq™ HIV-1 Genotyping Assay version 2.0 (Celera Diagnostics, Alameda, CA, USA) and TruGene (Siemens Health Diagnostics, Deerfield, IL, USA). Both methodologies use population based sequencing and detect the presence of more than one nucleotide in the same sequence chromatogram position at proportions greater than 20%, reflecting the presence of quasispecies. These methods are fast, safe and reproducible. However, the commercially available assays are still expensive ($400 per test) and perform poorly with non-subtype B samples (Engelbrecht et al., 2007). Alternatively, there are more sensitive point mutation assays (not FDA approved) which can only detect a single known mutation at a time. These probes/primers are highly dependent on sequence similarity to ensure accurate detection (Beck et al., 2002, Palmer et al., 2003, Wallis et al., 2005). However, the sequence variability of HIV-1 poses numerous challenges for the implementation of these types of assays in different geographic regions.

Novel sequencing technologies that detect minority variants such as, single genome sequencing (SGS; Kearney et al., 2008, Palmer et al., 2005) and more recently pyrosequencing have been developed (Varghese et al., 2009). Both these methodologies are prohibitively expensive ($750); require a huge initial capital outlay and highly skilled staff. Furthermore, studies are only beginning to emerge which indicate the clinical significance of detecting minority variants (Le et al., 2009, Varghese et al., 2009).

1.5.3. Host Factors:

There are numerous host factors that have been linked to HIV-1 disease progression and treatment outcome (Carrington and O'Brien, 2003, Fellay et al., 2007, Gao et al., 2001, Gatanaga et al., 2007, Thomas et al., 2009, Torno et al., 2008). However, for the purposes of this review, only the host genetic factors linked to metabolism and absorption of ARVs used in the South African roll-out program are discussed below. NRTIs are not metabolized but undergo a complex homostatic relationship of activation through several phosphorylating and dephosphorylating steps once in the cell (Balzarini, 1994, Stein and Moore, 2001). All other classes of ARVs are metabolized by enzymes known as cytochrome P450 (CYP450) and UDP-glucuronosylatransferases (UGT) in the gastro-intestinal tract (Haas, 2005, Haas et al., 2005, Mutlib et al., 1999, Ward et al., 2003). Genetic polymorphisms in CYP450 and UGT in different population groups have been linked to variations in metabolism of PIs and NNRTIs.

The CYP450 superfamily is involved in transforming these compounds into reactive, electrophilic and water-soluble products (Lin and Lu, 1998). CYP450 enzymes are located in high concentrations in enterocytes and hepatocytes. The CYP450 enzymes 3A4/5, 2B6, 2C9, 2C19, 1A2, and 2D6 are induced and/or inhibited by the following ARVs: the NNRTIs-EFV, NVP or the PIs ritonavir, nelfinavir, indinovir amprenavir and saquinavir. Furthermore, the same enzymes are responsible for metabolizing these drugs. Enterocytes primarily contain CYP3A4/5 and hepatocytes contain all CYP450 enzymes (Lin and Lu, 1998).

CYP enzyme activity is exquisitely sensitive to environmental, chemical, and genetic influence. Both EFV and NVP act as inducers of CYP3A4, 5, 7 and CYP2B6 (Erickson et al., 1999, Fichtenbaum and Gerber, 2002, Hesse et al., 2001); whereas delaviridine (DLV) inhibits CYP3A4. Other PIs have mixed effects on CYP enzyme activity.

CYP3A5*3 is the most frequent polymorphism of CYP3A5, the presence of which results in no protein expression and thus no enzymatic activity (Table 1.2). Moreover, when expressed, CYP3A5 represents at least 50% of the total hepatic CYP3A content, making it the most important genetic contributor to inter-individual differences in CYP3A-dependent drug clearance (Kuehl et al., 2001). CYP3A5 is more frequently expressed in livers of African Americans (60%) than in Caucasians (33%; Kuehl et al., 2001).

Genetic variations have also been found to contribute to the expression and activity of CYP enzymes (see Table 1.2; Hustert et al., 2001, Kuehl et al., 2001, Givens et al., 2003). These polymorphisms can cause large inter-individual variability in ARV drug exposure and might account for the differences in drug response between different ethnic groups. For example, the CYP2B6 516TT genotype (conferring lower CYP2B6 activity) was associated with greater EFV exposure in a Swiss HIV Cohort Study (Rotger et al., 2005). This genotype is also found in greater frequency in African American and Ghanaian populations (Klein et al., 2005).

The CYP2B6 function can be impaired by single nucleotide polymorphisms (SNPs) present in the gene. Studies have shown that SNPs in CYP2A6 result in a decrease in EFV metabolism (di Iulio et al., 2009, Kwara et al., 2009b). Furthermore, EFV toxicity has been linked to the presence of SNPs in CYP2A6 and CYP2B6 (Wood et al., 2009). EFV dose adjustments in individuals with the genotype 516TT have been found to be beneficial (Gatanaga et al., 2007, Torno et al., 2008). However, recently it has been demonstrated that the UGT 2B7*1 and *2 alleles impact on the concentration of EFV in individuals (Kwara et al., 2009a), as a direct result of the glucuronidation process of UGT2B7 (Belanger et al., 2009).

Absorption of ARVs into cells can occur either via passive diffusion (in the case of NRTIs) or active cell mediated transport (Hediger et al., 2004, Haas et al., 2003). Currently, over 350 transporters have been identified including those from the ATP binding cassette family (for example: MDR-1, MRP-4, MRP-5, MRP-8; Borst et al., 2007, Guo et al., 2003). SNPs in the transmembrane protein P-glycoprotein (P-gp) encoded by the multi-drug-resistance transporter (*MDR-1*) gene located on chromosome 7 have been linked to altered absorption of ARVs, particularly PIs (Pan et al., 2007, Shaik et al., 2007). Specific SNPs linked to absorption of ARVs are *MDR-1* 1236, 2677 and 3435. Most importantly, numerous studies have associated the *MDR-1* C3435T polymorphism with P-gp expression (Zhu et al., 2004). A study by Saitoh et al., 2005 that evaluated P-gp polymorphisms in infants receiving HAART consisting of nelfinavir, EFV and an NRTIs, revealed that infants with the *MDR-1*-3435 CT genotype had a more rapid virologic response compared to those patients with the CC genotype.

By contrast, Haas et al., 2004 showed the *MDR-1*-3435 TT genotype was present in 20% of African Americans compared to 3% of Caucasians (Haas et al., 2004). This genotype has been linked to increased plasma and intracellular exposure of EFV, and increased plasma concentrations of NVP (Hasse et al., 2005, Rodriguez-Novoa et al., 2005, Rotger et al., 2005).

Table 1.2: Examples of known polymorphisms, frequencies within ethnic groups, and enzymatic activity of cytochrome P450s.

Polymorphism	Mutation	Frequency (%)					Enzymatic Activity
		Caucasian	African	Japanese	Chinese	Other	
CYP3A4*1	Wild-type	21	ND	ND	ND	ND	Decreased
CYP3A4*2	Unknown	2.7	0	ND	ND	ND	Unknown
CYP3A4*3	Unknown	2	0	ND	ND	ND	Unknown
CYP3A4*4	Unknown	0	ND	ND	ND	ND	Decreased
CYP3A4*5	Unknown	0	ND	ND	ND	ND	Decreased
CYP3A4*6	Frameshift	0	ND	ND	ND	ND	Decreased
CYP3A4*1A	Unknown	8	59	ND	ND	78 Senegalese 81 Ghana	Decreased
CYP3A4*1B[1]	A290G	4-5	54-82	ND	ND	ND	Increased
CYP3A5*1[2,3,4,]	A698	4.9-5.6	ND	2.8	25	40Asian	Increased
CYP3A5*2[3]	C27289A	1-1.9	ND	0	0	0 Asian	Unknown
CYP3A5*3[1,2,3,4]	698G	70-76	77.6	74-93	76	60 Asian	No Protein
CYP3A5*4	ND	0	ND	0	0.5	ND	Unknown
CYP3A5*5	ND	0	ND	0.4	0.5	ND	Unknown
CYP3A5*6 [1,2]	G14690A	0.1	10-22	0	0	0 Asian	No Protein
CYP3A5*7		ND	10-22	ND	ND	ND	Unknown
CYP3A5*3B	C3705T; 3709-10insG	ND	ND	ND	ND	ND	Unknown
3A7*1	Wildtype	92	38 Tanzania	ND	72	83 Saudi	Mainly expressed in infants
3A7*2 [5]	7*1/5*3	8	62 Tanzania	ND	28	17 Saudi	Increased (Mainly expressed in infants)
2B6*1	Wildtype	16	ND	68	ND	ND	ND
2B6*2 [6]	C64T	5.3	ND	4.7	ND	ND	Unknown
2B6*3 [6]	C777A	0.5	ND	0	ND	ND	Decreased
2B6*4 [6]	A785G	4-32	ND	9.3	ND	ND	Decreased
2B6*5[6,7]	C1459T	9-14	ND	1.1	ND	0.5 Mongolian	Decreased (In Females)
2B6*6[6,7,8]	G516T; A785G	16.4-25	ND	16.4	ND	20 Mongolian	Decreased (associated with increase of EFV concentration)
2B6*7	G516T; A785G; C1459T	3	ND	0	ND	ND	Decreased

[1](Atanasova et al., 2005), [2](Kuehl et al., 2001),[3](Hustert et al., 2001),[4](Givens et al., 2003),[5](Rodriguez-Antona et al., 2005),[6](Lang et al., 2001),[7](Davaalkham et al., 2009),[8](Rotger et al., 2005),^ND=Not Done

1.6. Guidelines for Monitoring HIV-1 Treatment Management

1.6.1. Developed Countries

In developed countries, patients are initiated onto HAART when their CD4+ T-cell count is 350 cells/mm^3; however, this is dependent on the patient and recommendations are for patients to initiate HAART when they become diagnosed. Prior to initiation of ARVs, it is recommended that HIV-1 drug resistance testing is performed to aid in optimal ARV choice (Hirsch et al., 2008). The International AIDs Society (IAS)-USA and European AIDS Clinical Society guidelines recommend PI-based regimens initially and three to four monthly viral loads and CD4+ T-cell/counts (Hammer et al., 2008, Reiss et al., 2009). When viral suppression is achieved, CD4+ T-cell monitoring can be done less frequently (6 monthly). Viral failure is determined as an increase in viral load above 500 RNA copies/ml on two consecutive visits and the possibility of non-adherence or the presence of HIV-1 drug resistance is investigated (Hirsch et al., 2008, Reiss et al., 2009).

1.6.2. Developing Countries

The recently published WHO guidelines for HIV-1 management and care have amended the immunological start point from 200cells/mm^3 to 350cells/mm^3, irrespective of clinical symptoms (WHO, 2009). The drug regimens recommended for first-line have also been changed to remove d4T, as a result of the disfiguring and potentially life threatening toxicity of this drug. It has been recommended that developing countries move towards

replacing d4T with AZT or TNF, depending on the cost implication in each country. Modifications to the time to switch from first- to second-line regimens (if available in country) have been made, recommending a switch in ARV drug regimen if the patient has a viral load of 5000 RNA copies/ml (when viral load monitoring is available). Second-line regimens should contain a PI and an NRTI-backbone as several studies have shown that PI monotherapy is linked to reduced viral suppression and an increase in viral rebound (Arribas et al., 2005, Cameron et al., 2008, Delfraissy et al., 2008, Escobar et al., 2006). Finally it has been recommended that developing countries begin to consider the development of third-line regimens.

Currently, however, in developing countries ARV treatment monitoring is generally determined by immunological failure (CD4+ T-cell count) and most do not have second- or third-line regimens available. In contrast, the South African government initiated the comprehensive HIV/AIDS treatment plan which started providing access to AIDS patients with a CD4+ T-cell counts <200 cells/ mm^3 to ARV drugs in April 2004. The regimen includes first-line: d4T (can be substituted for AZT if toxicity occurs), 3TC, NVP or EFV and ddI, AZT and Kaletra. These guidelines were revised to form the HIV and AIDS and STI strategic Plan for 2007-2011 (http://www.doh.gov.za/ docs/misc/stratplan-f.html). The South African President announced on World AIDS day (1st December 2009) that from April 2010 AIDS patients would be initiated on ARVs at 350 cells/mm^3.

Despite these advances, numerous challenges still face the Strategic Plan. For example, the first-line regimen still contains d4T, but recommendations have been made to change this to AZT or TNF. The appropriate cut-off to determine virological failure has not yet been established, and currently in South Africa this level is 5000 RNA copies/ml, but recent consensus is to change this to 1000 RNA copies/ml. No provisions to monitor for the emergence of ARV drug resistance have been made. Other limitations and difficulties have been encountered with this programme, for example, the initial cost of both procurement of the ARVs drugs and implementation of the diagnostic assays required to monitor disease progression. Increasing access to ARVs and diagnostic assay for monitoring HIV disease progression in remote areas of South Africa has also been very difficult and resource draining. Despite these obstacles, mid-year 2009 national statistics revealed that although approximately 870 000 HIV-1 infected adults and children were receiving HAART, but an additional 1 630 000 South Africans are still in need (Statistics South Africa, 2009).

Currently, the national ARV roll-out program has provision to perform routine chemistry and haematology tests such as liver and kidney function and lactose levels to monitor for adverse events associated with the ARVs prescribed (Table 1.1). The ARV roll-out programme also monitors the patients and viral response to the ARV drugs by performing CD4+ T-cell counts and viral load monitoring. To date, monitoring of the above mentioned parameters have identified approximately 10% of patients enrolled from mid 2004 to present have failed the first-line regimen as a result of either non-compliance,

possible emergence of viral resistance or ARV toxicity (Professor F. Venter personal communication).

However, there are still no guidelines in South Africa (or any developing country) that recommend the use of HIV-1 ARV drug resistance testing at initiation (screen for transmitted drug resistance or resistance from mother-to-child-transmission [MTCT] programmes) or at virological failure (see Table 1.1 for mutations associated with ARVs prescribed in the South African 1st and 2nd line regimens). This consideration in mainly based on the high cost of the assay, high cost of initial outlay to implement these tests and the level of skilled staff required.

As a result there is currently limited HIV-1 drug resistance data available in South Africa. A small study investigating ARV drug resistance mutations that develop from preventing MTCT programmes show that in 53 infants whose mothers received single dose NVP at birth, 45.3% developed HIV-1 ARV drug resistance mutations which confer resistance to NVP and EFV, namely, Y181C (75%), K103N (25%), and Y188C (12%; (Martinson et al., 2007). Further studies have shown that the drug resistance mutations that appear in women after single dose NVP, K103N and Y181C, can persist in low levels for over a year after exposure (Loubser et al., 2006, Palmer et al., 2006). Three studies have reported on the ARV drug resistance mutations occurring on the national ARV roll-out programmes and have shown that the mutation profiles are similar to those observed in HIV-1 subtype B (Marconi et al., 2008, Orrell et al., 2009, Wallis et al., 2010). The major mutations found in these cohorts were M184V, K103N and V106M.

Interestingly, these studies observed a higher than expected frequency of the K65R mutation in these predominantly HIV-1 subtype C infected cohorts, compared to what has been observed in HIV-1 subtype B, a result of subtype C specific nucleotide polymorphisms around this codon (Coutsinos et al., 2009).

Furthermore, a study by Wallis et al., 2010, looking at 226 patients on a failing first-line regimen in South Africa found that 11% of patients had more than 3 TAMs, this is in contrast to the 56% of patients with 3 or more TAMs reported from Malawi first-line failure data emerging from 94 patients on a failing first-line regimen (Hosseinipour et al., 2009). The increased frequency of TAMs in the Malawi study is attributed to an extended duration on a failing regimen prior to HIV-1 drug resistance testing or other factors. In contrast to data from HIV-1 subtype B patients with a longer duration on a failing regimen (Kuritzkes et al., 2004, Wallis et al., 2009b) showed that TAMs occurred in a higher frequency in the AZT- than the d4T-containing regimens.

Although the South African national ARV roll-out programme is well designed to treat HIV-1 infected patients, the overall data collection between the clinic and laboratory is disjointed, making it difficult to link the clinical and laboratory data. This has resulted in limitations of overall data analysis on treatment outcome and this is why the Comprehensive International Programme of Research in AIDs in South Africa (CIPRA-SA) program with its careful longitudinal monitoring of patients on ARV treatment represents a good opportunity to study these factors. The CIPRA-SA 'Safeguard the household' study is a controlled randomised study of ARV therapy in a resource poor

setting, with the primary objective of evaluating the care given by primary health care workers and doctors. The first-line and second-line regimens are the same as the national ARV roll-out program allowing for a unique opportunity to examine the effects of the South African ARV roll-out programme in a well monitored cohort. This study has two study sites, the Chris Hani Baragwanath site situated in Soweto, Johannesburg and the Masiphumelele site in a poor stable community on the outskirts of Cape Town. Preliminary data from the government ARV roll-out program indicates that HIV-1 infected South African patients have a rapid virological response to HAART (Lisgaris et al., 2005), while approximately 10% fail therapy each year. It is an intriguing possibility that the frequency and presence of polymorphisms in both CYPs and *MDR-1* influences response to HAART in this population. Thus the CIPRA-SA study offers the perfect cohort to investigate the impact of both viral and host genetics on ARV treatment outcome.

The overall objective of this study was to determine the impact of viral and host genetics factors on antiretroviral treatment outcome in South African HIV-1 Subtype C infected patients receiving HAART in a well-defined cohort, with the aim of guiding future government programs.

This was achieved by setting the following Secondary Objectives:

1. To develop a validated, affordable HIV-1 ARV drug resistance assay to monitor the development of ARV drug resistance on the CIPRA-SA cohort

2. To identify patients with virological failure on the CIPRA-SA study and establish the presence/absence of known ARV drug resistance mutations.
3. To establish the pharmacogenetic background with respect to cytochromes P450 and MDR-1 polymorphisms in the study cohort, as compared to a control cohort.
4. To correlate host genetic profiles (identified polymorphisms) to ARV treatment outcome (toxicity and viral failure).

2. Chapter 2: Materials and Methods

2.1. Participant samples used in this study

A total of 2335 participant samples were used for various analyses throughout the course of this study (Figure 2.1). Two hundred and ninety HIV-1 positive samples and 10 samples from two external quality assurance (EQA) panels were used to develop and validate the in-house HIV-1 drug resistance assay. One thousand two hundred and twenty three HIV-1 negative samples were used to set up the assays for host genetics, and determine the background frequency of these single nucleotide polymorphisms (SNPs; Figure 2.1a). Subsequently, the newly validated and established assays were used with 812 HIV-1 positive participants from the CIPRA-SA 'Safeguard the household' cohort to determine viral and host parameters associated with ARV metabolism and absorption (Figure 2.1b). Ethical clearance for the use of patient sample material was obtained through the human ethics committee of the University of the Witwatersrand, Johannesburg, South Africa using the ethics clearance number: M-061025 (Appendix B).

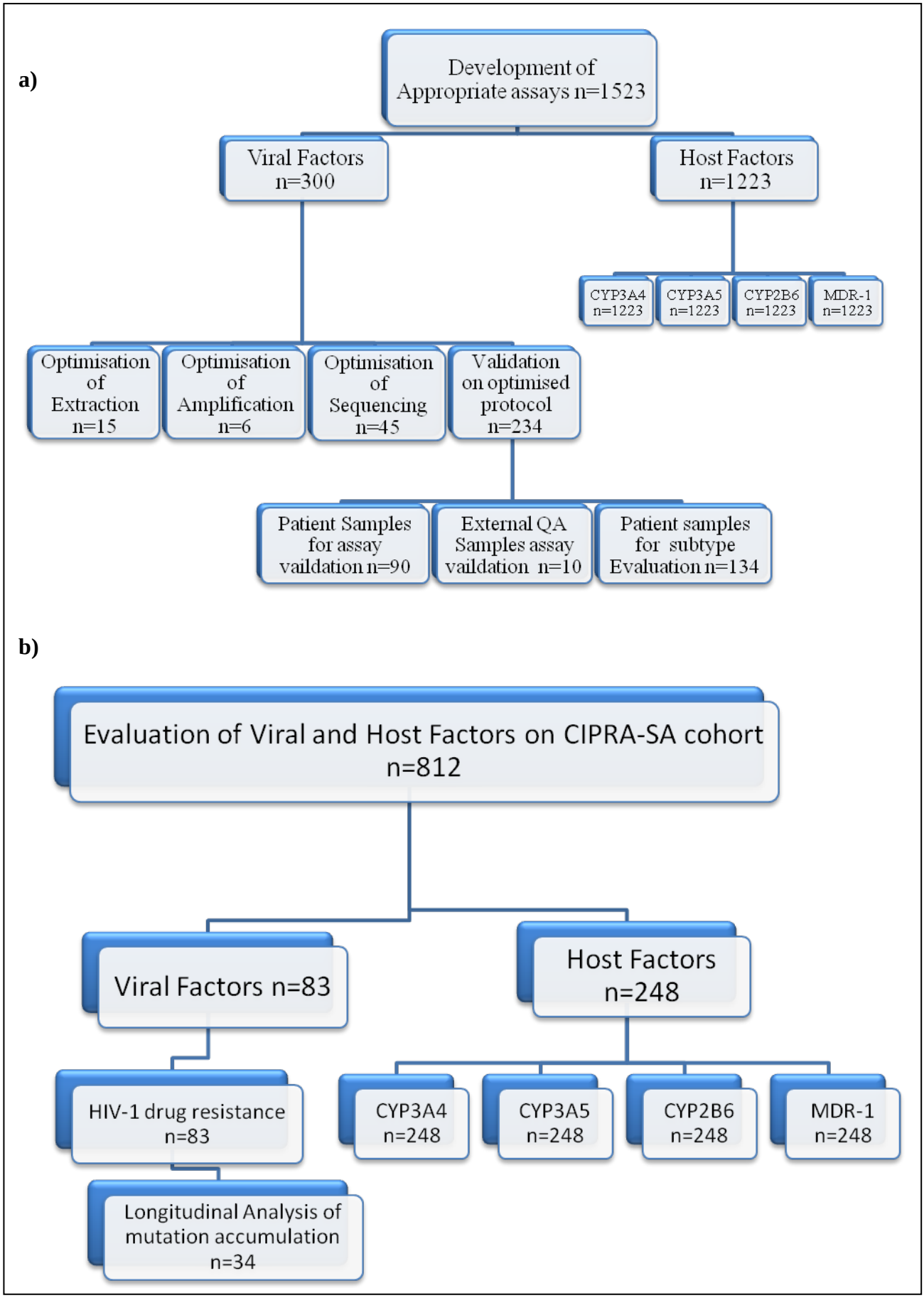


Figure 2.1: **Flow diagram of samples** a) used in the development of the appropriate assays; b) used in the evaluation of the host and genetic factors on the CIPRA-SA cohort.

2.1.1. Patient Samples used in the Development of appropriate assays

2.1.1.1. Viral Factors

2.1.1.1.1. Routine Patient Samples for in-house HIV-1 drug resistance assay development

Two hundred and ninety patient samples sent for routine HIV-1 testing were used in the development and validation of the in-house HIV-1 ARV drug resistance assay (section 2.2). One hundred and fifty six samples were obtained from patients accessing the national antiretroviral roll-out program that were sent for routine viral load (n=21) or HIV-1 drug resistance testing (n=135) as they appeared to be failing either the first (3TC, d4T, NVP/EFV) or second (AZT, ddI, Kaletra) line ARV drug regimens. All samples selected had a viral load greater than 1000 RNA copies/ml. A further, 134 samples from patients recently infected with HIV-1 that were sent for routine HIV-1 drug resistance testing and subtyping from countries throughout Africa were used to evaluate the effectiveness of the in-house assay on non-C subtypes circulating in the region.

2.1.1.1.2. External Quality Assurance Panels for in-house HIV-1 drug resistance assay validation

Ten well-characterised plasma samples with a range of viral loads (3557-57005 RNA copies/ml) and a variety of HIV-1 subtypes were obtained from the National Institutes of Health (NIH) Division of AIDS (DAIDS) Virology Quality Assessment Program (VQA) Laboratory, Rush Institute, USA (http://www.hanc.info/labs/Pages/VQA.aspx; (Huang et al., 2005) and used to validate the in-house drug resistance assay (section 2.2).

2.1.1.2. Host Factors

2.1.1.2.1. Control Group for host genetic single nucleotide polymorphisms (SNPs) study

One thousand, two hundred and twenty three anonymous, unlinked HIV-1 negative samples, sent for routine HIV-1 DNA testing in the PCR laboratory, National Health Laboratory Service (NHLS), Johannesburg, were randomly selected and processed in a blinded fashion as a control population because little data is available on the frequencies of certain of the cytochrome P450 (CYP450) and Multi-drug resistant type 1 (*MDR-1)* polymorphisms in the South African population. Thus, at the time of study design, the sample numbers required to determine statistically significant frequencies were unknown. For the purposes of this study, we assumed the prevalence of 15% occurrence in the population (http://www.ncbi.nlm .nih.gov/SNP/), thus an estimated minimum sample size of 1 223 individuals is sufficient to measure the true occurrence with 0.02 margin of error with 95% confidence intervals. The use of a control group is required to ensure there is no biased selection of a certain SNP examined in the HIV-1 positive patient group.

2.1.2. CIPRA-SA cohort used for the Evaluation of viral and host genetic factors

Eight hundred and twelve participants from the CIPRA-SA 'Safeguard the household' study were used to investigate specific viral and host factors that may impact on ARV treatment outcome. The CIPRA-SA project is an NIH funded study (CIPRA grant: U19 AI53217-01, Principal Investigator Prof J McIntyre) which allowed for the establishment of this cohort to investigate whether HIV-1 related care provided by primary health care nurses was non-

inferior to doctors in a resource constrained environment. Subjects were eligible to participate in the CIPRA-SA study if they were HIV-1 positive, had a CD4+ T-cell count below 350 cells/mm^3 and were ARV treatment naive (excluding previous single dose EFV that may have been administered to prevent MTCT). There were two clinic sites involved in this study, namely, the perinatal HIV research unit (PHRU), Chris Hani Baragwanath in South West Township (SOWETO) and Masiphumelele in the Cape region. The Chris Hani Baragwanath site was chosen because of the high HIV-1 prevalence in SOWETO. SOWETO was established in the 1930's and borders the mining belt of Johannesburg. The population has been overwhelmingly black and consists of approximately 1.3 million people. Masiphumelele was established in 1997 and is situated on the outskirts of Cape Town. It is a poor, stable community with about 10500 residents. The HIV clinic in Masiphumelele was established in 2000 and designed as a primary health care clinic, with a focus on medical care for women and children.

2.1.2.1. Schedule of events for participants enrolled in the CIPRA-SA study:

Participants presenting at each clinic were screened by the primary health care sisters or doctors, and only enrolled in the study if they were HIV-1 positive, had either a severe CDC category B AIDS defining illness or history of a CDC category B/C illness or a CD4+ T-cell count of less than 350cells/mm^3, were ARV drug-naïve, willing to give written informed consent and met all other study inclusion criteria. All 812 participants enrolled in the study were then subjected to physical examinations and blood draws (for laboratory tests and sample storage) as outlined in Table 2.1. The participants that met these criteria were started on ARV therapy as described in section 2.1.2.2.

Table 2.1: CIPRA-SA Schedule of events

Phase 1 Visits	Screening PreEntry	Baseline-W0*	W 2	W4	W 8	W 12	Every 12 weeks until study completed	Phase I Termination Visit
HIV ELISA	X							
Pregnancy Test (for all females of child bearing potential)	X	X		X	X	X	X	As Needed
Assess for Symptoms of Tuberculosis	X	X		X	X	X	X	X
Hepatitis B Surface Antigen	X							
Biochemistry/FBC ^	X	X	[X]	X	X	X	X	
Reflex Test (if indicated)			if on NVP[&]					
HIV-1 Viral Load	X	X		X	X	X	X	X
CD4+ T-cell Count	X	X		X	X	X	X	X
Dried Blood Spots		X		X	X			
Pill Count/syrup measurement				X	X	X	X	X
Compliance/ Adherence Discussion		X	[X] if on NVP	X	X	X	X	X
Resource Utilization	X	X	X	X	X	X	X	X
Well Being Questionnaire		X		X		X	X; 24 weekly after week 12	X
Dispense Study Drugs		X		X	X	X	X	
Virological Resistance Testing		X	WHEN CLINICALLY OR VIROLOGICALLY INDICATED					X
Pharmacokinetics Sample (trough)			X	X	X	X	X	X
Plasma/ PBMC[%] Storage	X		[X] if on NVP	X		X	X	X

*w=week; ^=Full Blood Count; [&]NVP=Nevirapine; [%]PBMC=Peripheral Blood Mononuclear Cell

2.1.2.2. ARV drug Treatment Regimens

The first-line regimen of the CIPRA-SA study contained d4T, 3TC and EFV for adults over the age of 16 years. However, if the woman was of child-bearing age and unwilling to use two forms of contraception, EFV was replaced by NVP. Lopinavir boosted with ritonavir

(Kaletra) replaced both EFV and NVP if the woman was pregnant when therapy was initiated. Furthermore, a one drug switch was permitted if drug toxicity was observed (section 2.1.4.4 below). The second-line regimen for participants not receiving TB-treatment consisted of a combination of AZT, ddI and Kaletra. Participants that required TB-treatment in either first- or second-line regimen were given an EFV-based regimen or were withdrawn from the protocol.

2.1.2.3. CIPRA-SA participants:

The 812 participants were enrolled into the CIPRA-SA study from the two sites over a period of 2 years (Feb 2005-Jan 2007). Participants were monitored longitudinally for a minimum period of 96 weeks (actual follow up was dependent on patient failure), up to a maximum of 3.5 years from baseline. The demographic data of the CIPRA-SA participants is shown in Table 2.2. The participants were randomized into two arms (primary health care sister vs. doctor monitoring) and monitored three monthly to determine whether HIV management from primary health care workers was non-inferior to doctors.

Table 2.2: Demographics of 812 participants at the two CIPRA-SA sites.

Site	Number of participants randomized	Male (%)	Female (%)
Johannesburg	449	126 (28.1 %)	323 (71.9 %)
Masiphumelele	363	113 (31.1 %)	250 (68.9 %)
Total	812	239 (29.4 %)	573 (70.6 %)

2.1.2.4. Toxicity, adherence, virological and immunological failure:

Toxicity (laboratory adverse events) noted at any of the visits were graded according to the DAIDs table for Grading the Severity of Adult and Pediatric Adverse Events (http://rcc.tech-res-intl.com, Appendix A). All laboratory indicators recorded above Grade 3 were reported as adverse events to the CIPRA-SA safety team, which took appropriate action.

Adherence monitoring was performed at every scheduled visit. This was done by pill counting and adherence was determined as a percentage of number of pills taken/total pills prescribed. A percentage greater than 90% was considered satisfactory adherence. Participants with adherence less than this underwent re-counselling.

Virologic failure was defined by either of the following criteria:

1) A less than 1.5 log drop in viral load measurements from baseline to week 12 or
2) Two consecutive viral load measurements of greater than 1000 RNA copies/mL after week 24

If viral failure was experienced, participants were switched to the PIs-based second-line regimen.

Immunological Failure was defined as a CD4+ T-cell drop of 15% from study entry with a viral load between 400 and 1000 RNA copies/ml. If immunological failure was experienced participants were switched to the PIs-based second-line regimen.

Whole blood was collected in EDTA and Heparin tubes and centrifuged, according to standard protocols (www.aactg.org/) to isolate plasma and peripheral blood mononuclear cells (PBMCs). The processed plasma and PBMCs were stored at -70^{o}C and -150^{o}C until used.

2.2. Development of an in-house assay for HIV-1 drug resistance testing

An RT-PCR and sequencing method to amplify the subtype C *pol* region implicated in ARV drug resistance was designed and optimized, and compared to the gold standard ViroSeq assay. Ninety of the 290 HIV-1 positive samples selected from samples sent for routine drug resistance were assessed using the new method as well as the gold standard ViroSeq.

2.2.1. Extraction of viral RNA

2.2.1.1. Manual

Briefly, 500ul of plasma was centrifuged for 1 hour at 23000xg at 4ºC, to pellet the HIV-1 viral particles. The viral pellet was resuspended in 600ul lysis buffer to lyse the viral particles and release the HIV-1 RNA. The RNA was purified by centrifugation using two 600ul washes using isopropanol and cold 70% ethanol, respectively and the supernatant discarded. The RNA pellet was eluted in 50ul elution buffer containing RNase inhibitors to ensure intact RNA was obtained. The eluted RNA was stored at -70ºC, until used.

2.2.1.2. Automated

Viral RNA was extracted from plasma separated from whole blood collected in EDTA tubes using the Roche MagNa Pure LC instrument (Roche, Mannheim, Germany) and the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche, Mannheim, Germany), according to manufacturer's instructions. Briefly, 200ul of plasma was aliquoted into the sample cartridge and placed onto the MagNa Pure analyser where the samples were lysed, proteins digested using proteinase K and the nucleic acids were bound to the silica surface of the Magnetic Glass Particles (MGPs) allowing for them to be magnetically separated. Unbound debris was removed from the beads with several washing steps and the isolated nucleic acids eluted in 100ul of elution buffer. The extracted RNA was stored at -70^{o}C until used in all other experiments (unless otherwise indicated). Fifteen samples were used to compare the manual and automated extraction samples. The automated extraction system allows for 32 samples (30 test samples and 2 controls) to be isolated in 90 minutes. The use of an automated system allows for an increase in throughput, improved purity of the isolated sample and decreases the risk of cross-contamination.

2.2.2. Amplification of extracted viral RNA to cDNA

An RT-PCR protocol was designed and optimized to amplify the 1.55 kB required *pol* region from extracted viral RNA. Amplification and sequencing primers were designed against sequences from the Los Alamos Database *(*www.hiv.lanl.gov/*)* and aligned against HIV-1 subtype C sequences to ensure there was a high homology between the primers and the HIV-1 subtype C sequences. Two PCR primers spanning nucleotides 2047 to 3594 according to

the HXB2 sequence, and five sequencing primers which result in the generation of fully bidirectional sequences were designed (Figure 2.2). These five sequencing primers ensure sequencing primer coverage according to the HXB2 sequence from nucleotide 1912 to 3576 (PR1-99 and RT1-240; with primer CWCS5 a back-up for primer CWCS4).

The extracted viral RNA was used to generate HIV-1 cDNA using the in-house RT-PCR protocol described below. Extracted viral RNA was synthesized into cDNA using the reverse primer CWR1 (Table 2.3) and the Expand RT kit (Roche, Mannheim, Germany). Briefly, 2.5mM of the reverse primer CWR1 (Table 2.3, Figure 2.2) and 8ul of viral RNA were added together and incubated for 10 minutes at 65^{o}C in the GeneAmp® PCR System 2700, (Applied Biosystems, Singapore). The samples were removed from the thermocycler and placed on ice. A mastermix containing 1mM dNTP (AB gene, Surrey, United Kingdom), 20U Protector RNase Inhibitor (Roche Mannheim, Germany), 10mM Dithiothreitol (DTT), 1x Expand RT buffer and 50U Expand RT (Roche Mannheim, Germany) were added to make a total volume of 20ul. The total volume of 20ul was incubated at 42^{o}C for 60 minutes in the GeneAmp® PCR System 2700, (Applied Biosystems, Singapore).

PCR amplification was performed using primers CWR1 and CWF1 (Table 2.3, Figure 2.2), 20ul of synthesized cDNA and the Expand High Fidelity^{PLUS}kit (Roche, Mannheim, Germany). This reaction was carried out in a total volume of 50ul containing 20ul cDNA, 0.4mM dNTPs (AB gene, Surrey, United Kingdom), 0.8umol of primers CWR1 and CWF1, 1xExpand High Fidelity^{PLUS}reaction buffer with 1.5mM $MgCl_2$ and 2.5U Expand High Fidelity^{PLUS}enzyme blend (Roche, Mannheim, Germany). The cycling conditions consisted of an initial denaturation step of 94^{o}C for 2 minutes, followed by 10 cycles of 94^{o}C for 30

seconds, 54.5°C for 30 seconds and 72°C for 2 minutes, followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 2 minutes increased by 10 seconds each cycle, and a final elongation step of 72°C for 10 minutes in the GeneAmp® PCR System 2700, (Applied Biosystems, Singapore). The PCR step was validated on 6 samples.

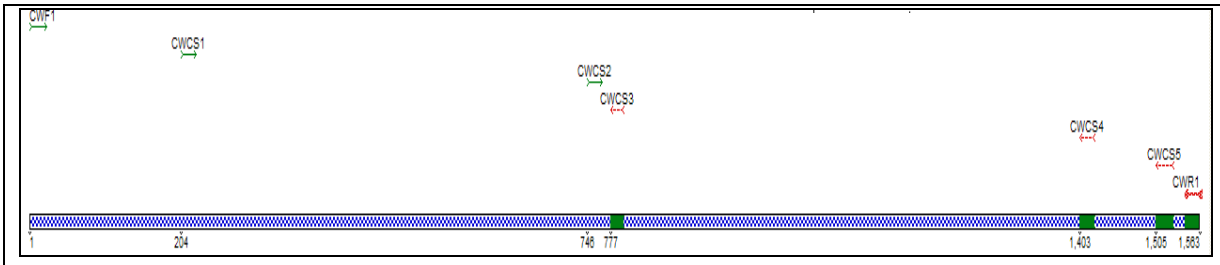


Figure 2.2: Graphic representation of the PCR and sequencing primers used in the in-house HIV-1 ARV drug resistance assay. An amplicon of approximately 1.55kb is generated in the amplification step. The sequencing primers CWCS1-5 ensure a 1.25kb sequence with bidirectional coverage. The forward primers (CWF1, CWCS1 and CWCS2) are represented by the green forward arrow. The reverse primers (CWR1, CWCS3-5) are presented by the red arrows. Position 1-204 and 204 to 1563 on the blue line are the *gag* and *pol* genes, respectively.

Table 2.3: Sequences of primers used for amplification and sequencing of nucleic acids

Name	Direction	Sequence	Location against the *pol* gene	Position on HXB2	Use
CWR1	Reverse	5'-GCA TAC TTY CCT GTT TTC AG-3'	RT349-355	3610-3594	RT and PCR
CWF1	Forward	5'-GAA GGA CAC CAA ATG AAA GAY TG-3'	68codons before PR1	2047-2066	PCR
CWCS1	Forward	5'-CCTCAAATCACTCTTTGGC-3'	PR1-8	2253-2271	Sequencing
CWCS2	Reverse	5'-AGAACTCAAGACTTTTGGG-3'	RT83-89	2796-2814	Sequencing
CWCS3	Forward	5'-TGCTGGGTGTGGTATTC-3'	RT93-99	2846-2830	Sequencing
CWCS4	Reverse	5'-TCCCTGTTCTCTGCCAATTC-3'	RT300-308	3472-3453	Sequencing
CWCS5	Reverse	5'-TGGTAAATTTGATATGTCCATTG-3'	RT336-343	3577-3555	Sequencing

Excess primers and buffers were removed from the 50ul of generated amplicon using microconcentrators, according to manufactures instructions (Celera Diagnostics, CA, USA). The PCR product was analyzed and quantified on a 2% agarose gel containing 30pmol ethidium bromide (Sigma, USA) in a 1xTAE buffer (Fermentas Life Sciences, Lithuania) and viewed under a UV transilluminator. The size and concentration of the DNA fragment was

identified by comparing it to a DNA Mass Ladder (MassRuler™ DNA ladder, Mix, ready-to-use Fermentas Life Sciences, Lithuania).

2.2.3. Dideoxy Sequencing

The samples were diluted according to their concentration and dideoxy (chain termination) sequencing performed. Cycle sequencing was performed using a final volume of 0.16uMol of one of the five primers CWCS1-5 (Table 2.3, Figure 2.2), 13ul of PCR product (section 2.2.2), 0.5x BigDye® Terminator v1.1/3.1 Sequencing Buffer and 0.5x Ready Reaction Premix from the ABI PRISM® BigDye® Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). This reaction was carried out in a total volume of 20ul. The cycling conditions consisted of an initial denaturation step of 96°C for 1 minute, followed by 25 cycles of 96°C for 10 seconds, 55°C for 5 seconds and 60°C for 4 minutes on a GeneAmp® PCR System 2700, (Applied Biosystems, Singapore).

After cycle sequencing, unincorporated ddNTPs were removed by adding 80ul of 80% isopropanol (MERCK, Wadeville, Gauteng, South Africa) to each sample. Incubation for 15 minutes at room temperature was followed by a centrifugation step to pellet the sequence amplicon (45 minutes at 4000xg). The plate was immediately inverted to remove the supernatant by centrifugation for 1 minute at 700xg. To the dried pellet 20ul of Hi-Di™ Formamide (Applied Biosystems, Warrington, UK) was added. The sequencing step was optimized using 50 samples.

2.2.4. Analysis of Generated Sequences

The DNA fragments now labeled with four different fluorescent dyes (one for each base; generated in sections 2.2.3) were loaded onto the ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, HITACHI, Foster City, USA). The sequence information was processed by the ABI PRISM ® 3100 Data Collection Software version 1.1 (Applied Biosystems, Foster City, USA). The sequence data was obtained from the ABI3100 genetic analyser and data was edited using the Sequencing analysis 3.3 program (Applied Biosystems, Foster City, USA), and the complete sequences encompassing the *pol* region of interest were assembled and manually edited using Sequencher version 4.7 (Genecodes, Ann Arbor, MI). Sequence data was submitted to the Stanford Database (http://hivdb.stanford.edu/index.html) to generate an HIV-1 ARV drug resistance report.

2.2.5. ViroSeq Assay

The patient plasma obtained from section 2.1 and 2.2 were sequenced using the ViroSeq™ HIV-1 Genotyping System Version 2.0, Pack 1 (Celera Diagnostics, Alameda, CA, USA), as per manufacturers' protocol. Viral RNA was extracted for each of the samples as described in section 2.2.1.1.

Twenty microliters of HIV-1 cDNA were generated from the isolated viral RNA using 10ul of reaction mix (Murine Moloney [MuLV] reverse transcriptase, RNase Inhibitor and RT mix containing a specific reverse primer of unknown sequence) and 10ul of RNA. The 20ul of

cDNA was subsequently used as a PCR template, and added to 30ul of amplification mixture (AmpliTaq Gold, dUTP/UNG and a PCR mix containing HIV-1 specific primers, of unknown sequence). The PCR reaction was used to generate an approximately 1.8kb fragment including the coding sequence for HIV-1 protease (the entire protein, amino acids 1-99) and part of the HIV-1 reverse transcriptase (amino acids 1-324). The amplicon was purified using columns provided in the ViroSeq kit, as per manufacturer's instructions.

Twelve microlitres of primer mix was added to 8ul of appropriately diluted amplicon. Seven sequencing reactions were set up for each sample, and included 4 sense (A, B, C, D) and 3 anti-sense (F, G, H) primers. The cycling conditions were: 96^{o}C for 10 minutes; 50^{o}C for 5 seconds, 60^{o}C for 4 minutes for 25 cycles (GeneAmp® PCR System 2700, Applied Biosystems, Singapore). After cycle sequencing unincorporated ddNTPs were removed as per the method described in section 2.2.3.

The sequences obtained were assembled and analyzed on the ViroSeq^{TM} HIV-1 Genotyping System Software Version 2.7 (Applied Biosystems, Foster City, USA). Manual reviewing and editing was performed, while the generated sequence was compared to a reference sequence. Once manual editing was completed the following files were saved: 1) FASTA file, containing the actual sequence; 2) GT file, containing a mutation list and 3) a report showing the mutations and ARV drugs no longer effective for the patient. The FASTA file was submitted to the REGA subtyping tool version 2 (http://dbpartners.stanford.edu /RegaSubtyping/).

2.2.6. Comparison of sequences obtained in the in-house and ViroSeq sequencing assays

The data generated from the ViroSeq and in-house assays were analyzed and compared to determine 1) assay sensitivity by comparing the participant viral load ranges amplified by each method; 2) nucleotide sequence homology between the two assays and 3) assay accuracy by comparison of the HIV-1 ARV drug resistance mutation profiles generated by the two methods to ensure the same clinical information was obtained.

2.2.7. Nucleotide sequence homology between the two assays

Nucleotide sequences generated from each of the samples by the two methodologies were aligned by the Clustal W method (http://www.ebi.ac.uk/clustalw/) to determine nucleotide mismatches between the two overlapping sequences. The mismatches were divided into two categories: partial and complete mismatch.

A partial mismatch was classified as a mixture of two of more bases observed in one sequence, whereas only one of the bases was observed in the other sequence (Figure 2.3). A complete mismatch was classified as two different nucleotides at the same position on the two different sequences (Figure 2.3). Sequence similarity between the sequences obtained from the 'in-house' and ViroSeq assays were compared using a Hamming distance, this was done by: [Total number of differences/Total overlapping sequence length]x100 and represented as a percentage. The closer the Hamming distance is to 100 the stronger the sequence similarity (Hamming, 1950).

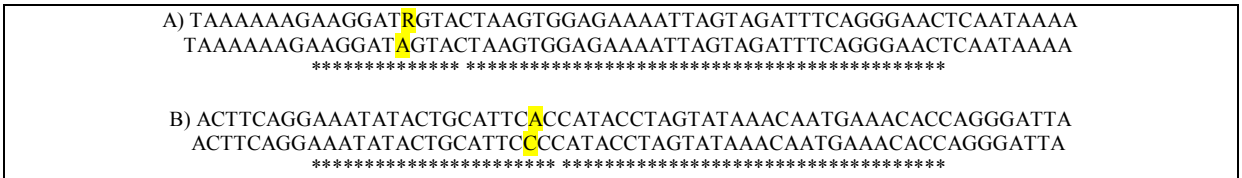


Figure 2.3: Alignment of two sequences, the asterisks represent sequences that are completely homologous, the yellow highlighted bases represent a partial mismatch in A and a complete mismatch in B.

2.2.8. ARV drug resistance mutation profiles

Mutation profiles of the sequences obtained from the in-house and ViroSeq methods were compared using HIV-1 drug resistance results obtained from the Stanford Database. The resistance reports generated were compared and samples that were not concordant in their mutation patterns had their chromatograms re-analysed.

2.2.9. Sequence Primer Mismatch analysis

This analysis was only performed for the in-house assay, as the primer sequences for the ViroSeq assay are proprietary knowledge and thus unknown. Sequences generated using the in-house assay not completely bidirectional were aligned to the primer that failed in an attempt to identify potential primer sequence mismatches The sample and oligonucleotide primer sequences (CWCS1-5; Table 2.3) were aligned using Clustal W (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

2.2.10. Subtype and Phylogenetic Analysis

Sequences generated were subtyped using the published REGA subtyping tool version 2 (http://dbpartners.stanford.edu/RegaSubtyping/). Sequences were aligned with reference sequences from HVI-1 subtype A to K (www.hiv.lanl.gov/), using Clustal X (version 1.83; http://www.ebi.ac.uk/Tools/clustalX). Neighbor-joining phylogenetic tree analysis was performed in MEGA version 4 (Tamura et al., 2007), using the Kimura two parameter model, to verify the REGA subtyping results. The stability of the nodes was assessed by bootstrap analysis (100 replicates), and bootstrap values greater than 70% were considered significant.

2.3. Viral Factors affecting ARV treatment outcome in the CIPRA-SA cohort.

2.3.1. Determination of HIV-1 ARV drug resistance in the CIPRA-SA cohort

Viruses from patients failing ARV therapy (section 2.1.2) were sequenced using the newly developed in-house assay described in section 2.2 to determine if virological failure was attributable to the presence of ARV drug resistance mutations. Mutations were classified according to the IAS-USA mutation list (Johnson et al., 2008). All participants demonstrating failure of a first-line regimen had their baseline samples sequenced to determine if HIV-1 drug resistance was present prior to the initiation of therapy. The failure sample was then sequenced to determine if the development of resistance contributed to viral failure.

2.3.2. Data Analysis

Subtype and phylogenetic analysis was performed as per section 2.2.8 and section 2.2.10. Statisitcal analysis was performed using Statistica version 8 (StatSoft Inc, Tulsa, OK, USA). The frequencies of the major and minor mutations were determined by dividing the total number of mutations over the total size of the cohort analysed. A non-linearized multivariate analysis (Mardia, 1967) was performed to determine if gender, age, viral load, CD4+ T-cell at baseline were linked to ARV treatment outcome with a 95% confidence level. The difference in frequency of the mutations associated with EFV and NVP exposure were compared using a non-parametric chi-Squared or Fishers Exact test (depending on the cohort size) with a 95% confidence level. A *p*-value of <0.05 was considered significant.

2.3.3. Emergence of HIV-1 ARV drug resistance mutations overtime

Samples classified with virological failure as a result of HIV-1 drug resistance mutations (section 2.3.1) had any additional time-points with viral loads greater than 1000 copies/ml prior to failure sequenced using the in-house assay (section 2.2). All serial sequences obtained from each patient were aligned and compared, to determine how mutation patterns evolved over time.

2.4. **Assays to detect 4 host single nucleotide (SNPs) that impact on ARV metabolism and absorption**

2.4.1. Genomic DNA extraction procedure

Genomic DNA was extracted from stored week 4 baseline PBMCs from 248 CIPRA-SA participants that consented for host genetic studies, and the 1223 control populations. Stored week 4 PBMCs were used because it did not compromise other potential studies. The PBMCs were defrosted and 100ul aliquoted (approximately 1 $x10^6$ cells) in a 1.5ml STARTAC tube. The cells were concentrated by centrifugation for 10 minutes at 2000g. The supernatant was removed and the cell pellet re-suspended in a total volume of 300ul of lysis buffer from the MagNaPure LC DNA Isolation Kit 1 (Roche Applied Science, Mannheim, Germany) and left at room temperature for 10minutes. The DNA was extracted using 200ul of the re-suspended cell pellets as per manufacturer's instructions.

The DNA from the 1223 HIV-1 negative controls were extracted from 100ul of whole blood using the MagNaPure LC DNA Isolation Kit 1 (Roche Applied Science, Mannheim, Germany), as per manufacturers instruction, and once HIV-1 negative status had been confirmed using the Roche Amplicor DNA version 1.5 (Roche Applied Science, Mannheim, Germany).

2.4.2. DNA Quantification

The quantity and quality of extracted DNA was determined using the Thermo Scientific NanoDrop^{TM} Spectrophotometer (Thermo Fisher Scientific, MA, USA). Once the quantity of DNA was determined it was diluted to 25ng/ul using distilled water and 20ul aliquoted into one well per sample in a 96 well plate. The DNA samples were left at room temperature for 24 hours, to lyophilize.

2.4.3. Identification of human genetic variants CYP3A4, 3A5, 2B6 and MDR-1

Genotyping of each participant for the presence of SNPs in their *CYP3A4, 3A5, 2B6 and MDR-1* genes was performed using fluorescently labeled minor groove binding (MGB) allele-specific probes purchased from the Assay-on-Demand TaqMan® SNP genotyping Assays (Applied Biosystems, https://products.appliedbiosystems.com). Participants were genotyped for CYP3A4 (1344G>T, 001_1344), CYP3A5 (6986 G>A, rs776746), CYP2B6 (516 G>T, rs3745274) and MDR-1 (3435 C>T, rs1045642).

Amplification was performed using allele specific probes that were 5'fluorescently tagged with 6-FAM and VIC (Assay-on-Demand TaqMan® SNP genotyping Assays, Applied Biosystems, https://products.appliedbiosystems.com), 20ul of lyophilized DNA and 1xTaqMan®Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems, Foster City, USA) to a total volume of 20ul. The cycling conditions consisted of an initial denaturation step of 95^{o}C for 10 minutes, followed by 50 cycles of 92^{o}C for 15 seconds and a

combined annealing and extension step for 1 minute at 60°C. All amplification reactions were performed on the ABI 9700 real-time machine (Applied Biosystems, Foster City, USA). Post analysis was performed using the allelic discrimination analysis option on the SDS version 2.3 sequence detection software (Figure 2.4; Applied Biosystems, Foster City, USA).

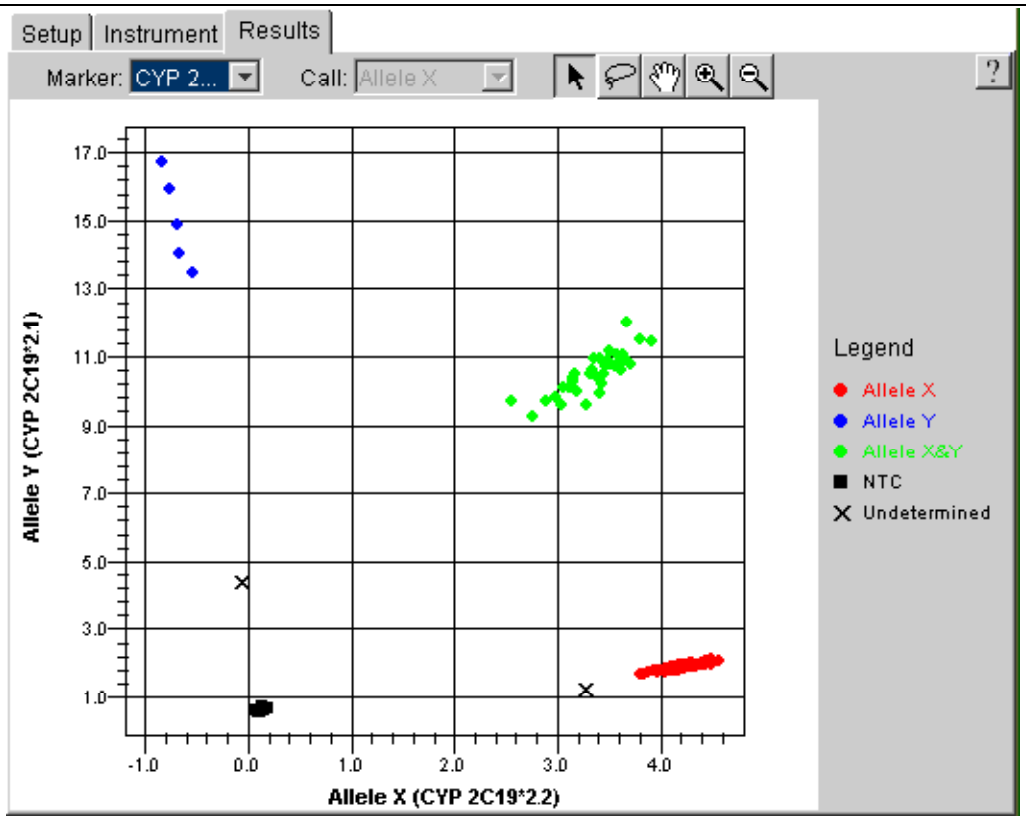


Figure 2.4: An example of a graphic representation of the allelic discrimination analysis software. This allows for easy identification of the 2 homozygous alleles (red and blue) and the 1 heterozygous allele (green). This clustering is determined by the fluorescence recorded in each well (sourced from examples in the SDS version 2.3 software, sequence detection systems, Applied Biosystems, Foster City, USA).

2.4.4. Data Analysis

Statistical analysis was performed using Statistica version 8 (StatSoft Inc, Tulsa, OK, USA). The frequencies of the genotypes of the four SNPs were determined by dividing the total number of genetic variations over the total size of the cohort analysed. The difference in

frequency of the genotypes for each SNP was compared using a non-parametric chi-Squared or Fishers Exact test (depending on the cohort size) with a 95% confidence level. A non-linearised univariate analysis was performed to determine if SNP genotype was linked to ARV drug toxicity or viral failure.

3. Chapter 3: Results

3.1. Development and Evaluation of an in-house HIV drug resistance assay

An optimised protocol to monitor the emergence of HIV-1 ARV drug resistance in a predominantly HIV-1 subtype C infected population was established. Overall, the protocol entailed an automated extraction of viral RNA and an in-house RT initiated PCR sequencing using the newly designed HIV-1 subtype C specific primers.

3.1.1. Viral RNA extraction

A comparison of the HIV-1 ARV drug resistance profiles obtained from the manual and automated extraction methods on 15 randomly selected patient samples are shown in Table 3.1. The mutation profiles on the resistance reports generated using the Stanford database for all 15 samples were shown to be 100% homologous for 11/15 (73%) samples. Samples 4, 7, 9 and 10 had different mutation patterns when using the automated extraction versus the manual (number of disagreeing mutations ranging from 1-3). In patient 4 (96% sequence homology), 2 of the 3 differences in the HIV-1 ARV drug resistance mutations were as a result of partial mismatches and 1 was due to a complete mismatch. In all 4 cases, more mutations were detected by automated extraction. The difference in ARV drug resistance profiles may be attributed to improved sample extraction when using the automated MagNaPure extraction method. Thus, the automated extraction method was selected for use

in all subsequent experiments since in a routine laboratory setting it is advantageous to have an automated extraction step that increases the sample throughput and reduces hand on time.

Table 3.1: Comparison of samples extracted using the manual ViroSeq extraction method versus the automated MagNaPure extraction method and amplified using ViroSeq. For each sample, the column for the HIV-1 ARV drug resistance profiles has the manual extraction method results (ViroSeq) above the automated extraction (In-house). Differences in mutation patterns are highlighted in bold.

Sample number	Viral Load (RNA copies/ml)	HIV-1 ARV* drug resistance profiles		Number of disagreeing mutations	Sequence Similarity
1	2300	ViroSeq	K65R, T69I, V75I, F77L, F116Y, V118I, Q151M, Y181C, G190A, K20R, M36I	0	98%
		In-house	K65R, T69I, V75I, F77L, F116Y, V118I, Q151M, Y181C, G190A, K20R, M36I		
2	2400	ViroSeq	M184V, K103N, M36I	0	99%
		In-house	M184V, K103N, M36I		
3	5500	ViroSeq	A62V, M184V, K103N, V106M, M36I, L63P	0	99%
		In-house	A62V, M184V, K103N, V106M, M36I, L63P		
4	9200	ViroSeq	M184V, K103N, Y181C, M36I, L63P	3	96%
		In-house	**T69N, V75I/A/T,** M184V, K103N, **V106M,** Y181C, M36I, L63P		
5	11000	ViroSeq	M184V, K101E, V106M, G190A, M36I	0	99%
		In-house	M184V, K101E, V106M, G190A, M36I		
6	37000	ViroSeq	M184V, V108I, Y181C, K20M, M36I	0	98%
		In-house	M184V, V108I, Y181C, K20M, M36I		
7	38000	ViroSeq	K65R, K219R, K103N, Y181C, G190A, K20R, M36I, L63P	1	99%
		In-house	K65R, **L74V**, K219R, K103N, Y181C, G190A, K20R, M36I, L63P		
8	53000	ViroSeq	M184V, K103N, V108I, P225H, K20R, M36I, L63P	0	99%
		In-house	M184V, K103N, V108I, P225H, K20R, M36I, L63P		
9	92000	ViroSeq	M36I, L63P	1	99%
		In-house	**D67G**, M36I, L63P		
10	130000	ViroSeq	V75I/A/T, M184V, V106M, Y188C, K20R, M36I	1	96%
		In-house	V75I/A/T, M184V, **K103N**, V106M, Y188C, K20R, M36I		
11	150000	ViroSeq	M36I	0	99%
		In-house	M36I		
12	600000	ViroSeq	Q151M, V106M, G190A, M36I	0	99%
		In-house	Q151M, V106M, G190A, M36I		
13	670000	ViroSeq	M36I, L63P	0	99%
		In-house	M36I, L63P		
14	800000	ViroSeq	L10I, M36I	0	99%
		In-house	L10I, M36I		
15	Unknown	ViroSeq	M36I	0	99%
		In-house	M36I		

*ARV=antiretroviral

3.1.2. RT-PCR amplification of viral RNA

An RT-PCR protocol for HIV-1 subtype C specific amplification of an approximately 1.55kb fragment encompassing the entire PR region and most of the RT was successfully established (Figure 3.1). As a result of decreased amplification of the HIV-1 subtype C samples on the ViroSeq Assay (Engelbrecht et al., 2007), the ViroSeq amplification step was substituted with the in-house amplification step, while using the ViroSeq extraction and sequencing methods. Results from 6 patient samples of this initial validation are shown in Table 3.2. The mutation profiles on the resistance reports from all six samples were shown to be exactly the same when using the two amplification methods (overall sequence similarity 97-99%). In addition, the RT-PCR protocol was shown to successfully amplify 100% of all HIV-1 subtype C samples (section 3.1.4).

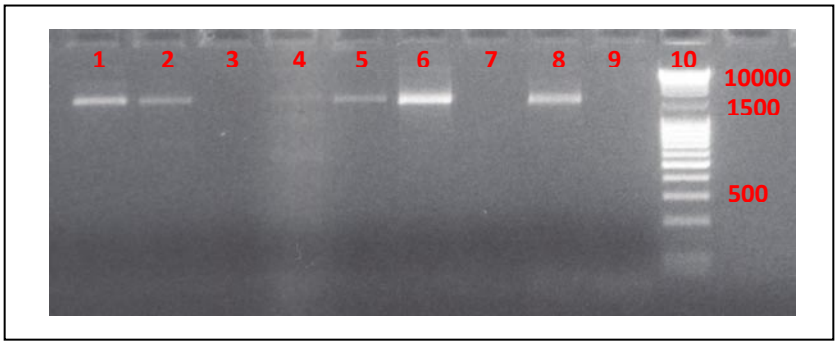


Figure 3.1: The 1.55kb fragment obtained with the in-house ARV drug resistance assay. Lane 1-7 are HIV-1 subtype C samples with viral loads ranging from 1990-42800 RNA copies/ml, lane 8 is the positive control and lane 9 is the negative control. The molecular mass ladder is shown in lane 10. Samples 3 and 7 did not amplify and had viral loads 170000 and 185000 RNA copies/ml, respectively.

Table 3.2: Sequence similarity between sequences obtained using the ViroSeq Amplification module and amplification with primers designed in-house.

Sample	Sequence Similarity (%)	Complete Mismatch of bases	Number of disagreeing Mutations
HIVDR1672	99%	0	0
HIVDR1674	99%	0	0
HIVDR1793	99%	0	0
HIVDR2269	99%	0	0
HIVDR3591	97%	0	0
HIVDR3696	99%	0	0
Average Similarity	**98.7%**	**0**	**0**

3.1.3. Cycle Sequencing and analysis

The performance of the newly designed in-house sequencing primers on 45 previously genotyped samples (26 manual and 19 automated extractions) were compared to the ViroSeq primers (Table 3.3). The percentage homologies ranged from 92% to 99.77% compared to 98.92% to 100% for the manual and automated extractions, respectively. Differences were mainly a result of partial mismatches.

Table 3.3: Sequence homology for 45 patient samples obtained from comparison of ViroSeq and in-house sequencing primers.

Sample Name (Manual)	Sequence Similarity
HIVDRMAN01	96.00%
HIVDRMAN02	97.00%
HIVDRMAN03	92.00%
HIVDRMAN04	96.00%
HIVDRMAN05	99.00%
HIVDRMAN06	98.80%
HIVDRMAN07	98.92%
HIVDRMAN08	99.36%
HIVDRMAN10	99.43%
HIVDRMAN11	99.77%
HIVDRMAN12	99.62%
HIVDRMAN13	99.13%
HIVDRMAN14	99.38%
HIVDRMAN15	99.77%
HIVDRMAN16	99.70%
HIVDRMAN17	99.04%
HIVDRMAN19	99.39%
HIVDRMAN20	99.53%
HIVDRMAN21	99.38%
HIVDRMAN22	95.00%
HIVDRMAN23	99.00%
HIVDRMAN24	92.00%
HIVDRMAN25	98.00%
HIVDRMAN26	98.00%

Sample Name (Automated)	Sequence Similarity
HIVDRAUTO27	99.38%
HIVDRAUTO28	99.62%
HIVDRAUTO29	99.78%
HIVDRAUTO30	99.69%
HIVDRAUTO31	99.46%
HIVDRAUTO32	99.23%
HIVDRAUTO33	99.16%
HIVDRAUTO34	99.39%
HIVDRAUTO35	99.69%
HIVDRAUTO36	99.00%
HIVDRAUTO37	99.54%
HIVDRAUTO38	99.77%
HIVDRAUTO39	100.00%
HIVDRAUTO40	99.31%
HIVDRAUTO41	99.16%
HIVDRAUTO42	99.31%
HIVDRAUTO43	99.46%
HIVDRAUTO44	99.39%
HIVDRAUTO45	98.92%

3.1.4. Full Validation of the in-house HIV-1 drug resistance assay on patient samples

Ninety previously genotyped patient samples, with viral loads ranging from 1300 to 1.6million RNA copies/ml, were used to successfully validate the in-house HIV-1 ARV drug-resistance assay. All 90 samples were successfully amplified using the in-house HIV-1 ARV drug resistance amplification step (Appendix D), whereas 9 of the 90 samples (10%) were

unable to be amplified using the ViroSeq methodology. The subtype distribution was as expected with 96.7% (n=87) of the samples being classified as HIV-1 subtype C (as determined by REGA). An example of REGA subtyping is shown in Figure 3.2. Phylogenetic tree analysis of all 90 sequences was performed using reference sequences from HIV-1 subtype A to K (www.hiv.lanl.gov; Figure 3.3). Table 3.4 shows the HIV-1 ARV drug resistance mutation patterns of the 9 samples obtained using the in-house HIV-1 ARV drug resistance assay that could not be amplified using ViroSeq. Five of the 9 had significant HIV-1 ARV drug resistance mutations. These 9 samples had viral loads ranging from 2300 to 627000 RNA copies/ml, indicating that primer mismatch in the amplification step rather than a low viral load were likely responsible for the lack of amplification, but this could not be unbundled further due to proprietary information. All 9 of these samples were HIV-1 subtype C.

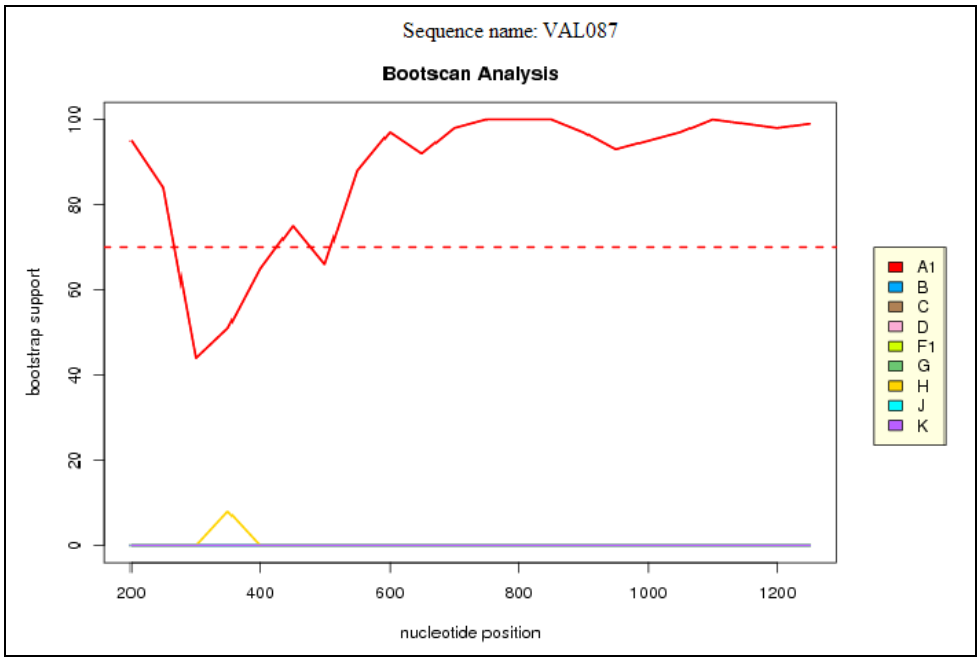


Figure 3.2: Subtype similarity plot for sample VAL087. The sample was classified as subtype A using the REGA HIV Subtyping tool version 2. Subtype was determined using a significance threshold of 0.8 (http://dbpartners.stanford.edu/RegaSubtyping/).

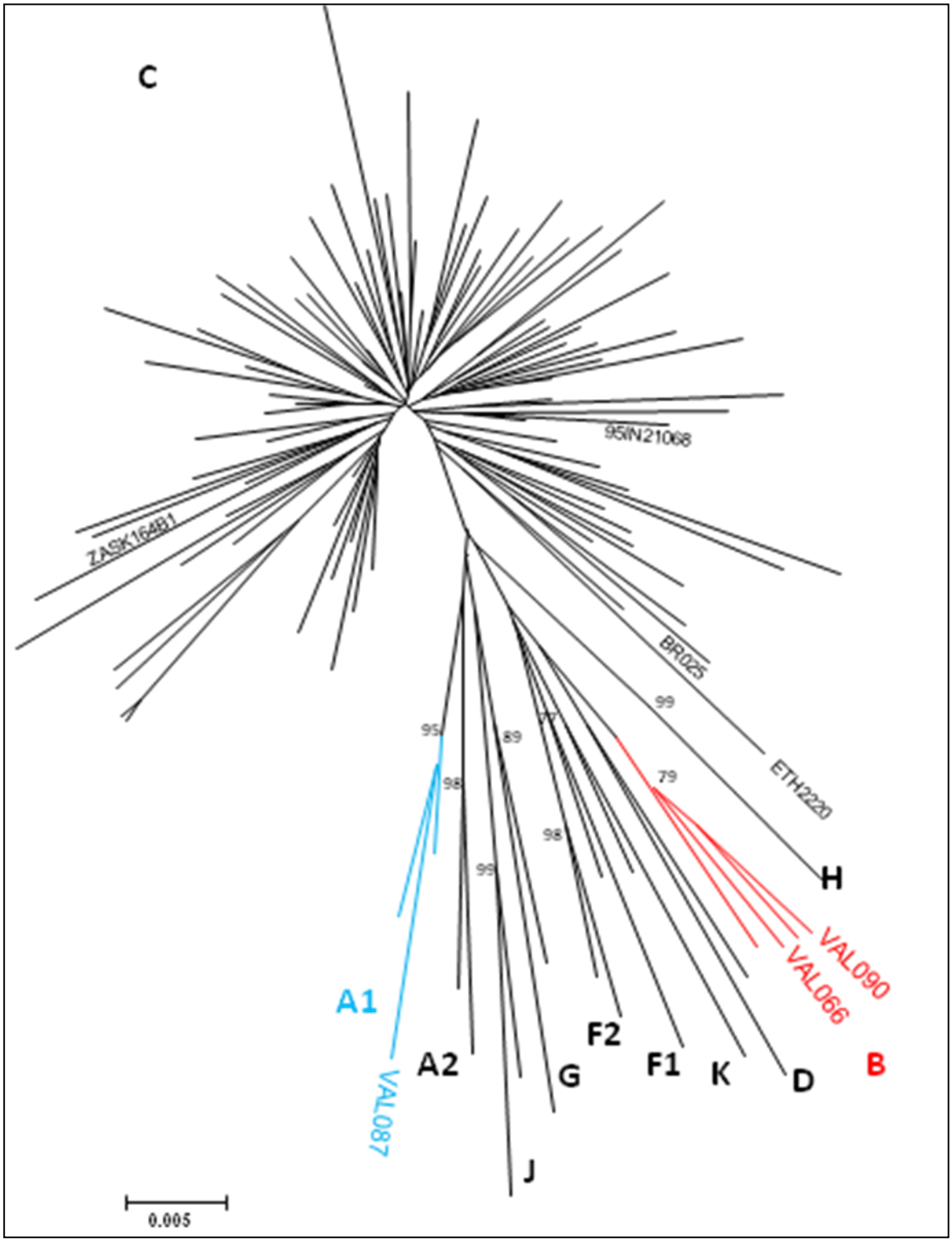


Figure 3.3: A radial phylogenetic tree was constructed from the 90 nucleotide alignments obtained from the in-house ARV HIV-1 ARV drug resistance assay, using neighbour-joining and the Kimura two-parameter distance matrix. Only bootstrap values of 70% or higher are shown. Sample VAL087 clusters with the representative subtypeA1 sequences (blue); and samples VAL066 and VA090 cluster with representative subtype B sequences (red); the remaining 87 sequences cluster with the subtype C representative sequences (numbers not shown due to size constraints). The scale represented the number of nucleotide changes between the sequences compared for every 1000 bases.

Table 3.4: ARV drug resistance mutation profiles and clinical significance of samples that successfully amplified using the in-house assay, but failed to amplify using the ViroSeq assay.

Patient Name	Viral load (RNA copies/ml)	NRTIs Mutations	Clinical Significance	NNRTIs Mutations	Clinical Significance	Subtype
VAL076	2300	D67N, M184V	High-level resistance to 3TC* and FTC[&] and low level resistance to ABC[#] and ddI^	K101E, K103N, V108I, G190A	High-level resistance to EFV[2] and NVP[3]	C
VAL077	2400	D67N, K70R, M184V	High-level resistance to 3TC and FTC and low level resistance to ABC, d4T[$], AZT[@] and ddI	Y188L, G190A, K238T	High-level resistance to EFV and NVP	C
VAL078	7400	M184V	High-level resistance to 3TC and FTC	K103R, V106M, V179D, M230L	High-level resistance to EFV and NVP	C
VAL079	14000	K65R, M184V	High-level resistance to 3TC, ABC and FTC and intermediate resistance ddI, TNF[1] and d4T	None	None	C
VAL080	32000	V118I, M184V	High-level resistance to 3TC, and FTC	None	None	C
VAL081	42000	None	None	None	None	C
VAL082	150000	K65R, D67N, T69I, K70R, M184V	High-level resistance to 3TC, ABC and FTC, intermediate resistance to d4t, ddI and TNF and low level resistance to AZT	K101P, K103N, Y318F	High-level resistance to EFV and NVP	C
VAL083	627000	V75I, M184V	High-level resistance to 3TC, and FTC, low level resistance to ddI and ABC	V106M, F227L	High-level resistance to EFV and NVP	C
VAL084	unknown	None	None	None	None	C

*3TC=Lamivudine; [&]FTC=Emtricitabine; [#]ABC=Abacavir; ^ddI=didanosine; [$]d4T=stavudine; [@]AZT=Zidovudine; [1]TNF=tenofovir; [2]EFV=Efavirenz; [3] NVP=Nevirapine

The 81 samples that were successfully amplified using both HIV-1 ARV drug resistance assays were found to have an average sequence homology of 99.20%. Detailed phylogenetic analysis revealed that sequences clustered with their partners from the two different sequencing methods and no contamination was observed (results not shown). Seventy-six of the 81 samples were found to have identical HIV-1 ARV drug resistance mutation profiles generated by the two assays. Of the 5 that were different 4 had additional mutations that were present in mixtures (Table 3.5; Appendix D). An example is shown in Figure 3.4.

Table 3.5: ARV drug resistance mutation profiles and clinical significance of samples that had differences in HIV-1 ARV drug resistance profiles.

Patient Name	Viral Load (RNA copies/ml)	HIV-1 ARV Drug Resistance Profiles		Number of Disagreeing mutations	Sequence Similarity	Clinical Significance	Subtype
VAL002	10000	ViroSeq	D67G,K70R, M184V, K219Q, V106M, V179D,**T215I/T**	1	98.92	None	C
		In-house	D67G,K70R, M184V, K219Q, V106M, V179D				
VAL003	36000	ViroSeq	D67N, K70R, M184V, K219E, A98G, K103N, V106M, Y188C, **V108I/V**	1	99.45	None	C
		In-house	D67N, K70R, M184V, K219E, A98G, K103N, V106M, Y188C				
VAL004	77000	ViroSeq	T74S, M184V, K103N, P225H, **V108I/V**	1	98.5	None	C
		In-house	T74S, M184V, K103N, P225H				
VAL005	300000	ViroSeq	L10I, K103N, V106M, **L100L/I**	1	99.11	None	C
		In-house	L10I, K103N, V106M				
VAL085	84000	ViroSeq	D67N, M184V, T215F, L100I, K103N, H221I, **K70R**	3	99.05	None	C
		In-house	D67N, M184V, T215F, L100I, K103N, H221I, **T215F, P225H**				

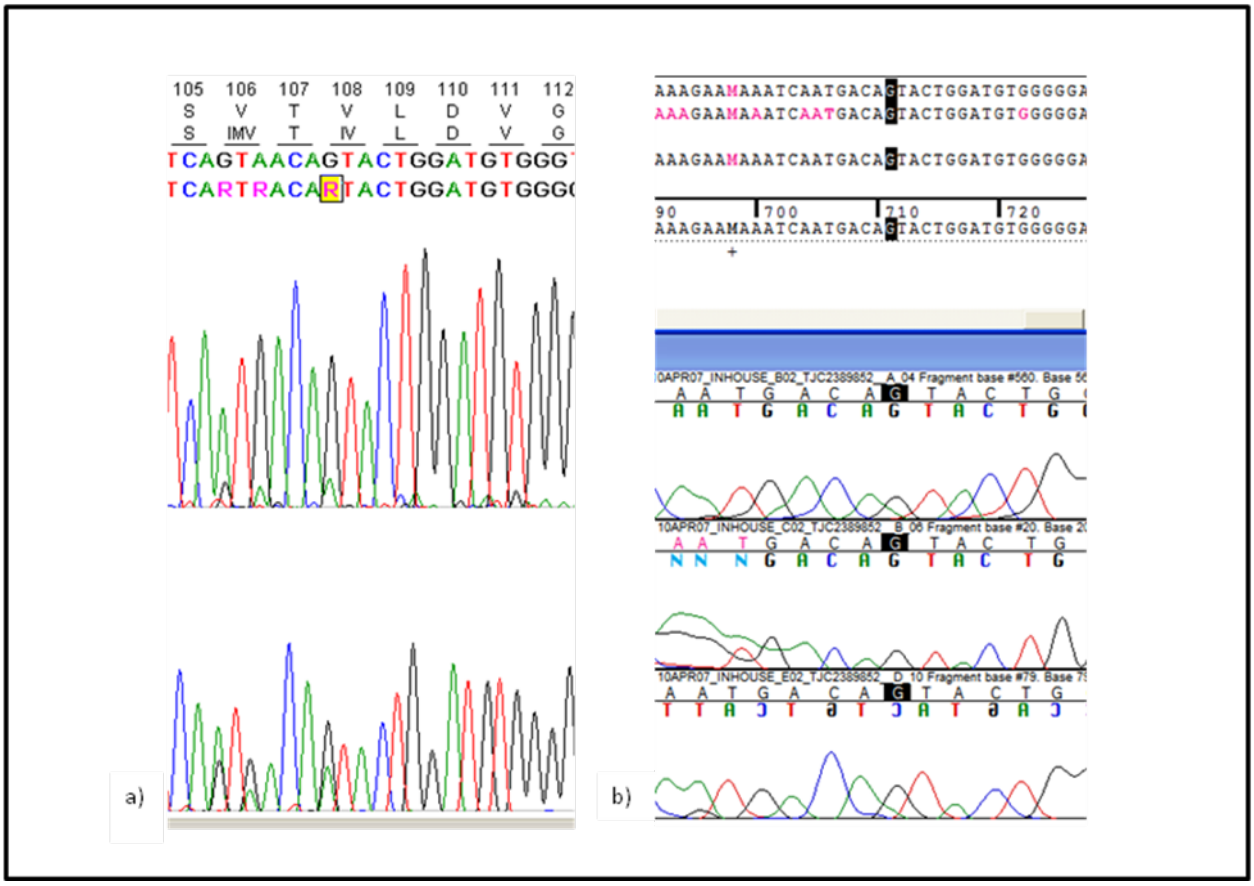


Figure 3.4: The ViroSeq chromatogram from patient VAL004 (a) indicates a mixed population at codon 108 (boxed) of the RT, resulting in a partial mutation V108V/I. This is not observed in the in-house sequence (highlighted; b).

Of the 5 in-house sequencing primers, CWCS1 was found to successfully sequence 90 out of 90 samples, CWCS2 was unable to sequence 12 of the 90 samples (13.33%), CWCS3 was unable to sequence 7 of the 90 samples (7.77%), CWCS4 was unable to sequence 3 of the 90 sequences (3.33%) and CWCS5 was unable to sequence 2 of the 90 samples (2.22%). Sequence primer mismatch analysis was only performed for the in-house assay as the primer sequences for ViroSeq are unknown. Although a primer may have not successfully sequenced a sample, sequences obtained from neighbouring primers from each sample were available. This allowed for the sequence primer mismatch analysis against the sequences obtained from the samples (Figure 3.5). However, CWCS5 could not be aligned as it binds at the 3'end of the PCR amplicon, and CWCS2 sequences did not extend that far.

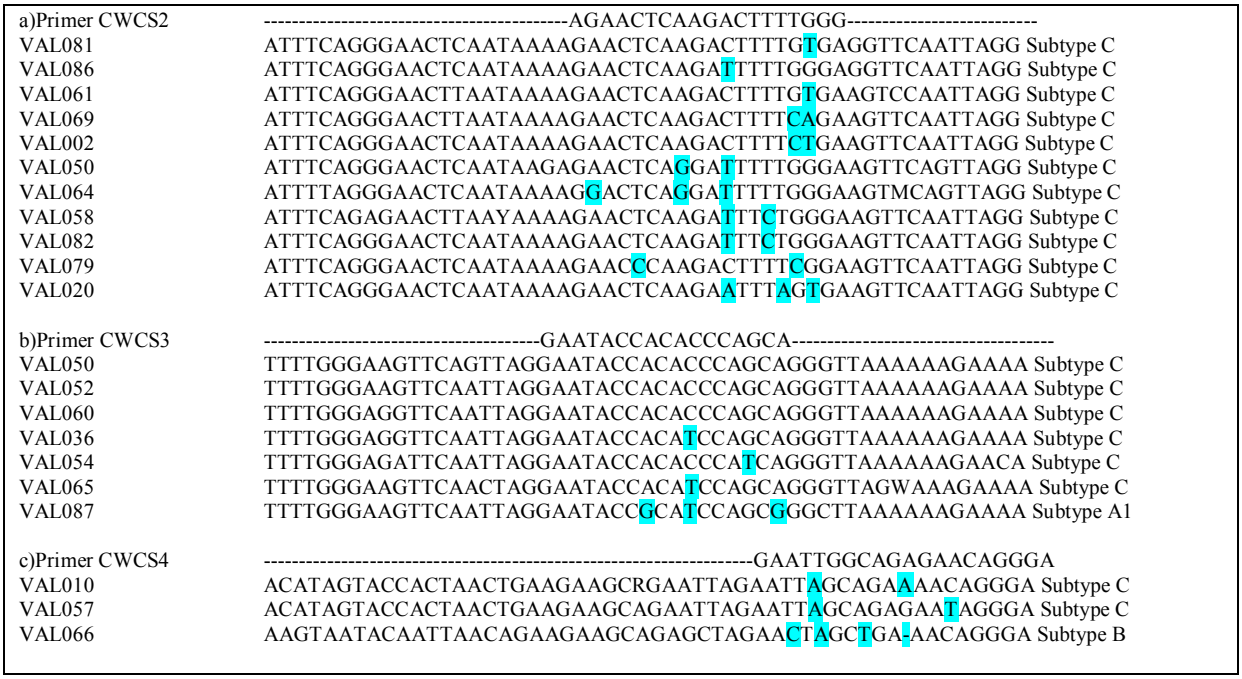


Figure 3.5 Alignment of sequencing primers and patient sample sequence. The majority of the samples had mismatches with the primers which could influence primer binding. Two of the samples which had mismatches were subtype B (VAL066) and A1 (VAL087).

3.1.5. In-house Validation with the EQA Panel Results

A comparison of performance of the in-house ARV HIV-1 drug resistance assay to ViroSeq with two VQA panels provided by the NIH resistance genotyping EQA Scheme is shown in Table 3.6. The mutation patterns for the 3 subtype C samples were identical using the IAS-USA mutation list (Johnson et al., 2008). In addition, the in-house HIV-1 ARV drug resistance assay gave identical patterns for 6 of 7 non-C subtypes when compared to the ViroSeq results. The VQA certified the in-house HIV-1 ARV drug resistance assay, and this assay was subsequently used to genotype patients on the CIPRA-SA cohort (section 3.2).

Table 3.6: Samples from the EQA panel that were sequenced with the ViroSeq and In-house assays, indicating subtype, mutation pattern and homology

Sample Number	Viral load (RNA copies/ml)	Subtype	Number of Disagreeing Mutations
VQA012g01	17775	C	0
VQA012g02	35450	B	0
VQA012g03	18525	B	2
VQA012g04	40500	B	0
VQA012g05	12580	C	0
VQA013g01	27810	A1	0
VQA013g02	148282	C	0
VQA013g03	24238	F	0
VQA013g04	3557	B	0
VQA013g05	57005	A2	0

3.1.6. In-house Validation with non-subtype C samples

The in-house assay was also evaluated on 134 patient samples obtained from eight African sites (Kigali, Lusaka, Copperbelt, MRC Uganda, Kemri, Cape Town, Kenya AIDs Vaccine Initiative [KAVI] and Entebbe). HIV-1 viral RNA loads of these patients ranged between 1 230 and 720 000 RNA copies/ml using the COBAS amplicor version 1.5 (Roche Diagnostics, Mannheim, Germany). One hundred and eighteen samples were successfully amplified using the in-house HIV-1 ARV drug resistance assay. Of the 118 samples sequenced with the in-house assay, 14 did not have bidirectional sequencing data; as a result of the primers used in cycle sequencing failing to amplify (Table 3.7). Subtype analysis using REGA revealed that across all eight sites the subtype distribution was 56.49% subtype C, 29.77% subtype A1, 4.58% subtype D and 11.84% were unassigned. The subtype distribution per site was as expected with the majority of subtype C samples occurring in sites situated in Southern Africa and the Eastern African sites containing predominantly subtype A, D and several unassigned subtypes (Table 3.8).

Table 3.7: The number and (percentage) of primers which failed to amplify per subtype.

Subtype	CWCS1	CWCS2	CWCS3	CWCS4	CWCS5
A1	0	6 (15%)	6 (15%)	0	2 (5%)
C	1 (1%)	2 (3%)	2(3%)	1 (1%)	4 (5%)
D	0	0	0	1 (17%)	1 (17%)
NA	0	0	0	1 (8%)	0

Sixteen samples that could not be amplified using the in-house assay; were successfully amplified using the ViroSeq assay. The 16 samples were found to have the following subtypes: subtype A1 n=8 (Kigali, Kemri, Entebbe); subtype C n=4 (Copperbelt and Lusaka); subtype D n=1 (Entebbe); and 2 subtypes were unassigned.

Table 3.8: Distribution of subtype per African site

Site (N)	Subtype C	Subtype A1	Subtype D	Unassigned
Kigali (24)	8.33%	83.33%	0.00%	8.33%
Lusaka (76)	97.26%	0.00%	0.00%	2.74%
Masaka (12)	0.00%	33.33%	33.33%	33.33%
Kemri (13)	0.00%	84.62%	0.00%	15.38%
Cape Town (1)	100.00%	0.00%	0.00%	0.00%
KAVI (2)	0.00%	50.00%	0.00%	50.00%
Entebbe (6)	0.00%	50.00%	33.33%	16.67%

Of the 134 samples 1 had the K103N mutation and two had the polymorphic I85V non-polymorphic PR mutation.

3.2. Emergence of HIV-1 drug resistance on the CIPRA-SA 'Safeguard the Household' project

3.2.1. Description of the CIPRA-SA Cohort

A total of 812 ARV naive participants were enrolled on the CIPRA-SA cohort, from 2 different geographical regions during 2005-2007. All 812 participants were older than 18 years of age and had no active opportunistic infection at time of enrolment. Seventy percent of the cohort was female (n=569) and 99% of the patients were of African descent. Five hundred and seventeen participants (64%) had CD4+ T-cell counts less than 200 cells/$mm^{3,}$ with the remaining having CD4+ T-cell counts below 350 cells/mm^3. The median baseline CD4+ T-cell and mean log viral load was 167 cells/mm^3 and 5.65 copies/ml, respectively. Thirty five percent of participants enrolled in the study had a CDC stage C classification, defined by CD4+ T-cell count, symptomatic conditions attributed to HIV-1 infection or a defect in cell-mediated immunity.

Of the 812 participants a total of 371 (45.7%) experienced treatment failure, defined by toxicity (n=134; 16.5%), virological failure (n=83; 10.2%), withdrawal of consent (n=39; 4.8%), defaulted (n=70; 8.62%), lost to follow-up (n=24; 2.96%) or death (n=21; 2.59%). Of the 83 patients that failed as a result of viral rebound, 54 of the 449 enrolled (n=12%) were from the Johannesburg site and 29 of the 363 enrolled (8%) were from the Cape Town site. The increased number of failures at the Johannesburg site may reflect a difference in adherence levels between the two sites. The majority of the patients on a failing regimen were receiving d4T, 3TC, EFV (49 of 83) followed by 22 on d4T, 3TC, NVP. Twelve patients (pregnant at time of study entry) were on a PI-based regimen at time of failure (n=3;

d4T, 3TC, NLF and n=9; d4T, 3TC, lopnavir/ritonavir). All 83 patients were of African descent.

To ascertain possible associations with virological failure, baseline CD4+ T-cell counts and viral loads were compared between the viral failure group and the remainder of the CIPRA-SA cohort (Appendix E; Table 3.9**)**. This comparison found that baseline viral load did not impact on subsequent virological failure (p=0.7781), whereas a lower CD4+ T-cell count at the time of therapy initiation were more likely to experience viral failure (p=0.0321). Of the 83 patients experiencing virological failure, 75% (n=62) had a CD4+ T-cell count less than 200 cells/mm^3. Age (p=0.3033) and gender (p=0.5395) did not appear to impact on viral outcome.

Table 3.9: Baseline characteristics of the CIPRA-SA Participants *

Variable	**All Patients (n=812)**	**Viral Failure (n=83)**	***p*-values**
Gender-number (%)			
Female	573 (70.6%)	62 (74.6)	0.5395
Male	239 (29.4%)	21 (25.3)	
Mean Age –year	33.2 (10-61)	32.3 (0-53)	0.3033
Median CD4+ T-cell count (cells/mm^3)	167 (2-734)	147 (16-354)	**0.0321**
Mean baseline HIV-1 RNA log $_{10}$copies/ml	5.65 (49->750000copies/ml)	5.6 (399->750000 copies/ml)	0.7781

*Percentages may not total 100 because of rounding.

3.2.2. Baseline sequences of the virologically failing samples

Of the 83 participants experiencing viral failure, 12 were not available for analysis (no amplification n=5; no storage n=7). The remaining 71 baseline samples were successfully sequenced and analysed for HIV-1 ARV drug resistance mutations (Appendix E). Six of the

71 participants were found to harbour resistance at time of study entry (Table 3.10), five of which were female. Of the six samples, 2 had mutations that resulted in reduced susceptibility to NNRTIs (K103N: n=1; K103N and V106M: n=1), 1 with mutations that impact on NNRTIs and PIs susceptibility (G190A, K101E, M46V and Q58E) and 3 that had mutations that reduce PIs susceptibility (M46I: n=1; M46L: n=1; Q58E: n=1; Table 3.10). All 3 of these participants with NNRTIs mutations were female, but only one (135671) had reported previous NVP exposure to prevent two separate cases of mother-to-child-transmission at the end of 2002 (36 months prior to study entry) and 2005 (1 month prior to study entry). Participant 266571 with the M46L mutation had also had previous NVP exposure in mid 2003 (36 months prior to study entry).

Seventeen participants had HIV-1 subtype C polymorphisms that in combination with other mutations result in resistance. The T74S HIV-1 subtype C polymorphism was observed in 10 of the 71 baseline samples (14%); one of these had the K103N mutation (Table 3.10). Two patients had the A71T polymorphism (3%) and one patient had the L10I mutation. The E138A and V179D polymorphism was observed in three and two participants, respectively (Table 3.10). The I85V polymorphism was observed in one participant.

Table 3.10: Baseline polymorphisms and mutations linked to HIV-1 ARV drug resistance of 22 CIPRA-SA participants who later experienced viral failure on the CIPRA-SA study.

Patient Name	Gender	Protease						Reverse Transcriptase					
		Codon 10	Codon 46	Codon 58	Codon 71	Codon 74	Codon 85	Codon 101	Codon 103	Codon 106	Codon 138	Codon 179	Codon 190
135671	Female					T74S			K103N				
137011	Female			Q58E									
165121	Female		M46V	Q58E				K101E	K103R				G190A
165621	Female								K103N	V106M/V	E138A		
166931	Male		M46I										
266571	Female		M46L										
135311	Male					T74S							
135431	Female						I85VI						
135641	Female					T74S							
135891	Female										E138A		
135941	Female					T74S							
136841	Female				A71T								
165351	Female					T74S							
165961	Male				A71T								
166141	Male	L10I											
166811	Female					T74S							
166911	Female										E138A	V179D	
236411	Male					T74S						V179D	
265651	Female					T74S							
265951	Male					T74S							
266262	Female								K103R				
266661	Female					T74S							

3.2.3. HIV-1 ARV drug resistance at viral failure time point

Of the 83 patients experiencing viral failure, 12 were considered failing as a result of not having a 1.5 log drop in viral load from study entry to week 12 and the remaining 71 as a result of two consecutive viral loads greater than 1000 copies/ml (Figure 3.6). Six samples for participants experiencing viral failure were not available for analysis (no amplification n=4; no stored samples n=2). Of the 77 samples available for analysis, 56 had ARV drug resistance mutations, while 21 had no known mutations (Appendix F). Results were analysed according to whether patients failed due to a less than 1.5 log drop by week 12 or two consecutive viral loads greater than 1000 RNA copies/ml (Figure 3.6). Of the 10 patient samples available for analysis failing at week 12, 50% (n=5) were female, 2 of which had received prior MTCT. Eight of the 10 (80%) patients were on a failing d4T, 3TC, EFV regimen; whereas the remaining 2 were on a PIs-based regimen (d4T, 3TC, LPV/r n=2). At baseline none of these patients had mutations that would have resulted in reduced susceptibility to the regimen they were accessing. There were no NRTIs mutations present at failure in any of the 10 patients analysed. Of the 8 failing on the d4T, 3TC, EFV regimen, 2 had NNRTIs mutations (135891-K103N and E138A-and had previous NVP exposure and 165461-K103N and V106M-with no known previous NVP-exposure). Of the 2 patients accessing a failing PIs-based regimen no PIs-associated mutations were observed and one female patient (136101) reported as having no previous MTCT exposure had 3 NNRTIs mutations (K103N, V106A and Y188C).

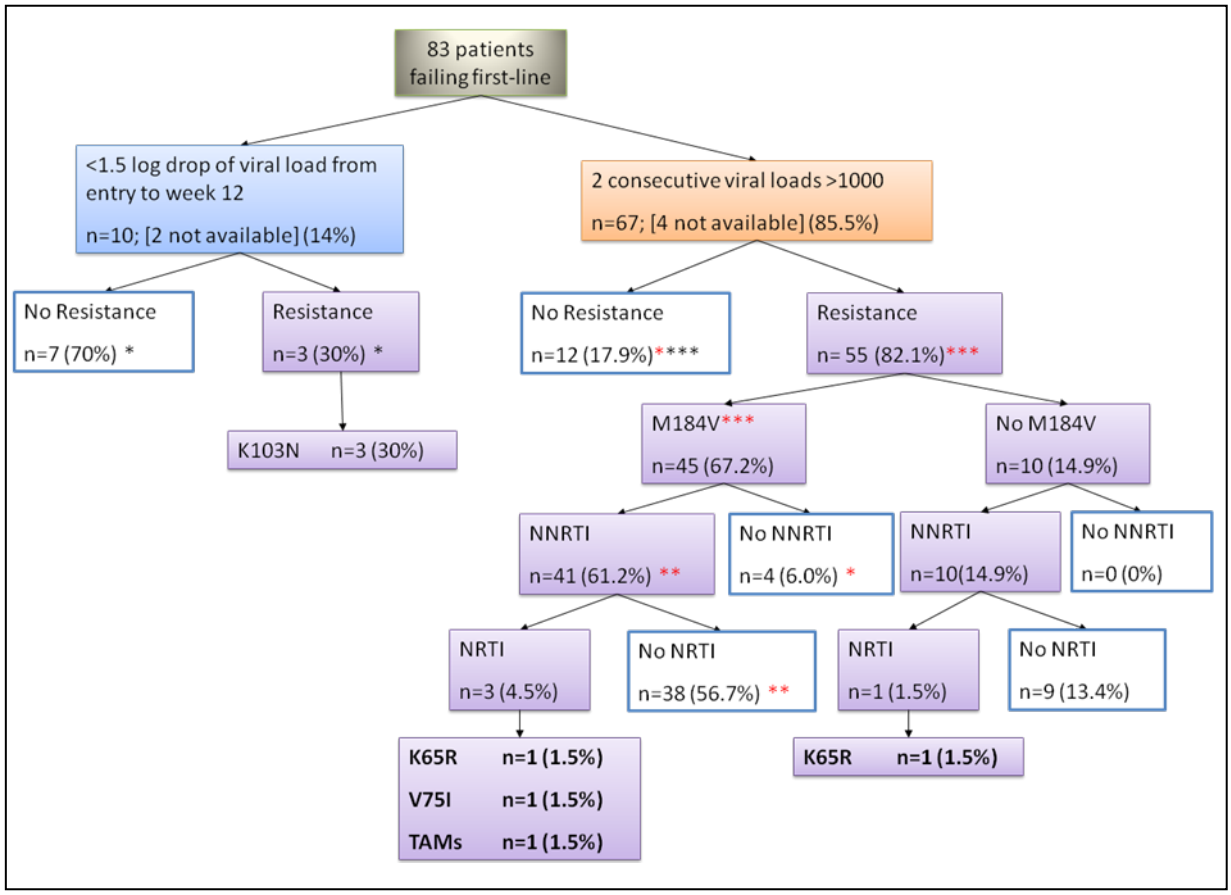


Figure 3.6: Diagrammatic representation of the CIPRA-SA patients with viral failure. Participants on a PIs-based regimen are represented by a black asterisks, if they were also exposed to NVP to prevent mother-to-child-transmission the asterisks is red. Of the 67 participants on a failing ARV therapy as a result of 2 consecutive viral loads greater than 1000 RNA copies/ml, all were accessing the same NRTIs-backbone consisting of d4T and 3TC with either EFV (n=40, 60%), NVP (n=20, 30%), LPV/r (n=5, 7%) and NLF (n=2, 3%).

Of the 67 participants on a failing ARV therapy as a result of two consecutive viral loads greater than 1000 RNA copies/ml, all were accessing the same NRTIs-backbone consisting of d4T and 3TC with either EFV (n=41; 57.7%), NVP (n=22; 31.0%), LPV/r (n=6, 8.5%) and NLF (n=2, 2.8%). The M184V mutation was the most prevalent NRTIs mutation occurring in 67% (n=45) of the participants (Figure 3.7) followed by the K65R mutation (3%). Eighteen percent (n=12) of the participants had no known or previously described mutations.

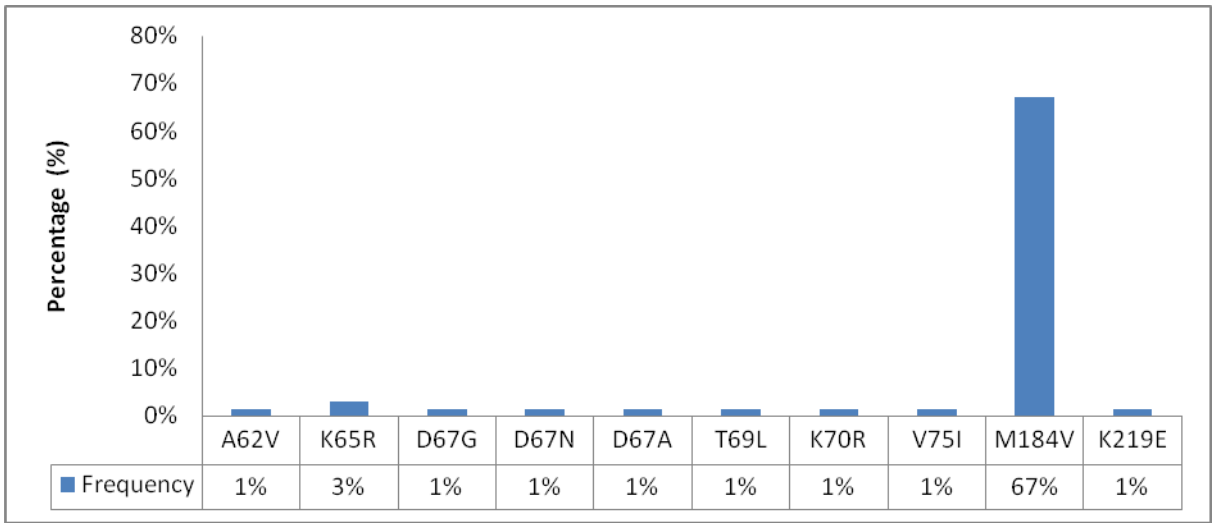
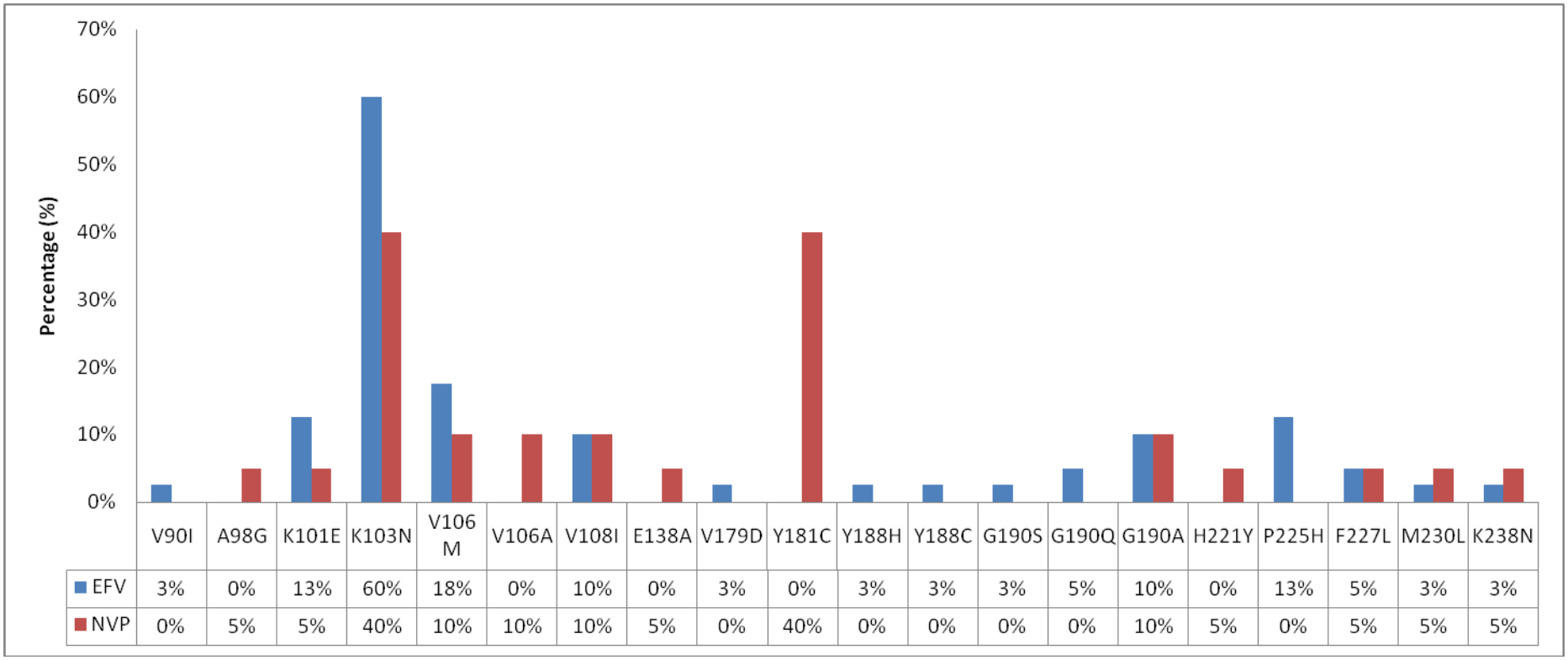


Figure 3.7: Frequency of the HIV-1 ARV drug resistance mutations associated with NRTIs resistance in the 67 CIPRA-SA participants failing ARV therapy as a result of 2 consecutive viral loads greater than 1000 RNA copies/ml.

Of the 63 participant accessing an NNRTIs based regimen, 60 were successfully amplified and differences in mutation profiles were observed between the EFV and the NVP-containing regimens (Figure 3.8). The Y181C mutation only occurred in the patients accessing a failing NVP-containing regimen (40%). As expected, the K103N mutation was more frequent (p=0.1432) with EFV (n=60%) than NVP (40%). The V106M mutation occurred at a higher frequency in subjects on EFV (18%) than NVP (10%), but this difference was not significant (p=0.4431).The V106A mutation only occurred in the NVP-containing regimen (10%). EFV appeared to select for a wider range of mutations in the RT region compared to NVP (Figure 3.8). Of the 20 NNRTIs resistance mutations detected; 8 were associated with both EFV and NVP therapy (Figure 3.8).



	V90I	A98G	K101E	K103N	V106M	V106A	V108I	E138A	V179D	Y181C	Y188H	Y188C	G190S	G190Q	G190A	H221Y	P225H	F227L	M230L	K238N
EFV	3%	0%	13%	60%	18%	0%	10%	0%	3%	0%	3%	3%	3%	5%	10%	0%	13%	5%	3%	3%
NVP	0%	5%	5%	40%	10%	10%	10%	5%	0%	40%	0%	0%	0%	0%	10%	5%	0%	5%	5%	5%

Figure 3.8: Distribution of NNRTI mutations from patients accessing a failing EFV- or NVP-containing regimens.

Of the 8 subjects failing a PIs-containing regimen, none had known resistance mutations to any PIs. Within the 6 participants failing lopinavir/r, 4 had no other mutations associated with resistance, one had the M184V mutation and one sample failed to amplify. Of the 2 failing NLF and NRTIs-based regimens both were female, with previous NVP-exposure and harboured NNRTIs mutations (participant 135641-K103N, V106M and participant 135241-K103N).

Well known naturally occurring HIV-1 subtype C PR polymorphisms were observed in patients experiencing virological failure. The T74S polymorphism was observed in 13% of patients. Mutations resulting in PIs resistance were observed in 3 patients, none of which were exposed to a protease inhibitor, but these mutations were present at baseline (section 3.2.2).

3.2.4. Accumulation of mutations

Additional resistance testing was performed on 34 of the patients experiencing virological failure that had sequential visits with detectable viral loads. Two of the patients were accessing a PIs-based regimen and were not analysed further. Nine of the 32 patients had 3 sequential visits and the remainder had 2 sequential visits. One patient had the K103N mutation at study entry (baseline). The NRTIs mutations were found to increase in number from the first detectable viral load to the second visit 1-12 weeks later (Figure 3.9). This increase was due to the development of the M184V mutation. NNRTIs mutations were found

to increase as the patient was left on a failing regimen and on average for every 3 months left on a failing regimen an additional NNRTIs mutation was observed (Figure 3.9).

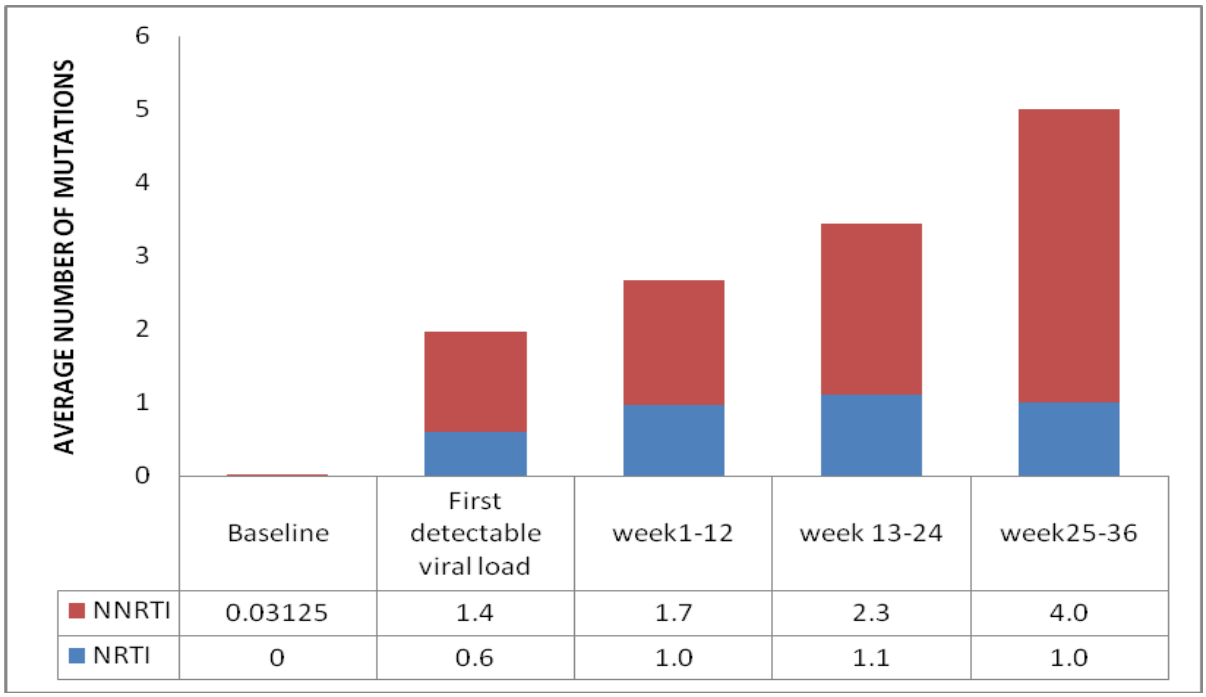


Figure 3.9: Development of NRTIs and NNRTIs mutations over time.

The NRTIs backbone was common for all 32 patients, but the 32 patients analysed either had EFV- or NVP exposure. Patients exposed to NVP- were found to have a greater increase of mutations overtime, compared to EFV-exposed patients (Figure 3.10). However, the variety of NNRTIs mutations in EFV-exposed (n=11) was greater than the NVP-exposed (n=8).

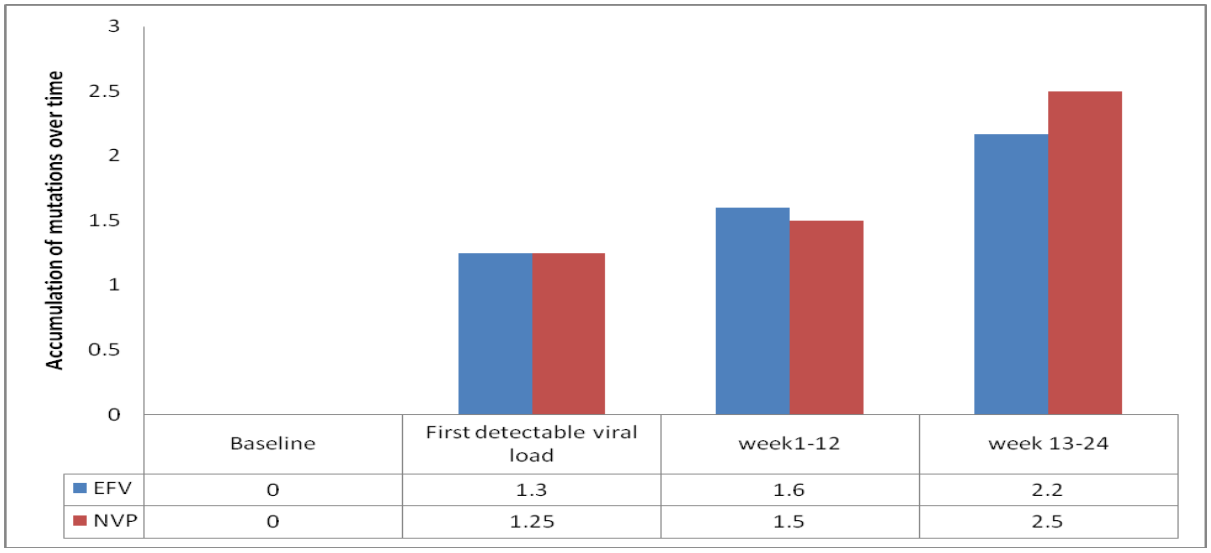


Figure 3.10: Accumulation of NNRTIs mutations in NVP- versus EVF- exposed patients.

A small sub-set of patients had previous MTCT exposure (n=12) prior to the onset of the CIPRA-SA study. The number of mutations that occurred increased in the EFV- versus the NVP-exposed patients, with a greater variety of NNRTIs mutations occurring in patients exposed to EFV (n=8) compared to NVP (n=4; Figure 3.11). No association with the pattern of NNRTIs mutations developing was observed.

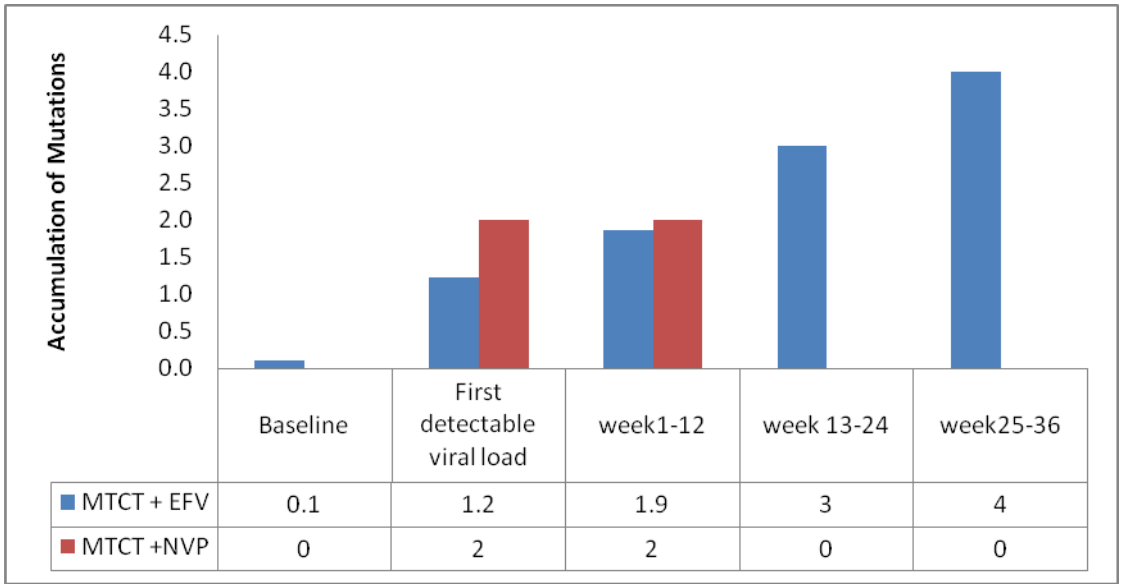


Figure 3.11: Accumulation of NNRTIs mutations in NVP- versus EFV-exposed patients, with previous MTCT.

Amino acid sequences from participants with longitudinal visits were aligned at the change in amino acids compared. Figure 3.12 is representative of the analysis performed. This patient had no mutations present at baseline and successfully suppressed the virus for 22 months. At the first detectable viral load since suppression only the K103N (yellow) mutation was present. Six months later the M184V (blue) and V108I (green) mutations had developed followed three months later by P225H (purple). No additional amino acid changes that are not associated with HIV-1 ARV drug resistance occurred during these nine months of viral failure.

```
                 *        90         *       110         *
CP50739(week 0) : KLVDFRELNKRTQDFWEVQLGIPHPAGLKKKKSVTVLDVGDAYFSVPLDE : 122
CP61906(week96) : ............................N.................... : 122
CP63892(week120): ............................N....I.............. : 122
CP64620(week132): ............................N....I.............. : 122

               130         *       150         *       170
CP50739(week 0) : NFRKYTAFTIPSTNNETPGIRYQYNVLPQGWKGSPAIFQCSMTKILEPFR : 172
CP61906(week96) : ..................................................... : 172
CP63892(week120): ..................................................... : 172
CP64620(week132): ..................................................... : 172

                 *       190         *       210         *
CP50739(week 0) : AQNPEIVIYQYMDDLYVGSDLEIGQHRAKIEELRKHLLKWGLTTPDKKHQ : 222
CP61906(week96) : ..................................................... : 222
CP63892(week120): ..........V.......................................... : 222
CP64620(week132): ..........V.......................................... : 222

                360         *       380         *       400
CP50739(week 0) : KEPPFLWMGYELHPDKWTVQPIQLPEKDSWTVNDIQKLVGKLNWASQIYP : 272
CP61906(week96) : ..................................................... : 272
CP63892(week120): ..................................................... : 272
CP64620(week132): ..H.................................................. : 272
```

Figure 3.12: **Amino acid sequence of the 4 longitudinal sequences obtained from participant 236081.** The highlighted amino acids represent HIV-1 ARV drug resistance mutation (Yellow-K103N; Green-V108I; Blue-M184V and Purple-P225H).

3.3. Second-line failures on the CIPRA-SA cohort

3.3.1. Baseline Mutations of the second-line CIPRA-SA patients

Sixty one of the 812 participants that experienced treatment failure (toxicity: n=20; viral failure: n=41) on the first-line of CIPRA-SA were changed to the second-line drug regimen (Table 3.11). Of the 21 participants that did not achieve viral control on second-line the baseline median age was 36 years, 76% (n=16) were female, with a mean second-line baseline viral load of 373000 RNA copies/ml. There was no statistically significant difference between the age, gender and second-line entry viral loads of the participants who subsequently virologically suppressed versus those that did not on the second-line regimen (Table 3.12). The second-line baseline resistance was examined and showed that the most prevalent mutation was M184V (60%), followed by K103N (30%; Figure 3.13). Of the 61 samples accessing the second-line regimen (past 4 weeks) treatment outcome on the second-line regimen was classified into 3 different groups: a) viral suppression; b) viral rebound or c) no viral suppression (Table 3.11).

Table 3.11: Second-line outcome of the 61 patients accessing second-line regimen

Second-line viral outcome **Reason for switch to second-line**	**Suppression**	**Rebound**	**No Suppression**	**Total**
Toxicity	16	3	1	**20**
Two consecutive VL > 1000 copies/ml after week 24	22	6	9	**37**
Less than1.5log drop in VL by week 12	2	0	2	**4**
Total	**40**	**9**	**12**	**61**

*VL-viral load

Table 3.12: Second-line Baseline Demographics of the 61 patients that were switched to a second-line regimen.

	Participants that suppressed on second-line	**Participants that did not suppressed or rebound on second-line**	**p-value**
Age year	35 (24-55)	36 (21-51)	0.0688
Gender-Female %	80%	76%	0.4854
Viral Load (RNA copies/ml)	61800 (399->750000)	37300 (399-335000)	0.2066

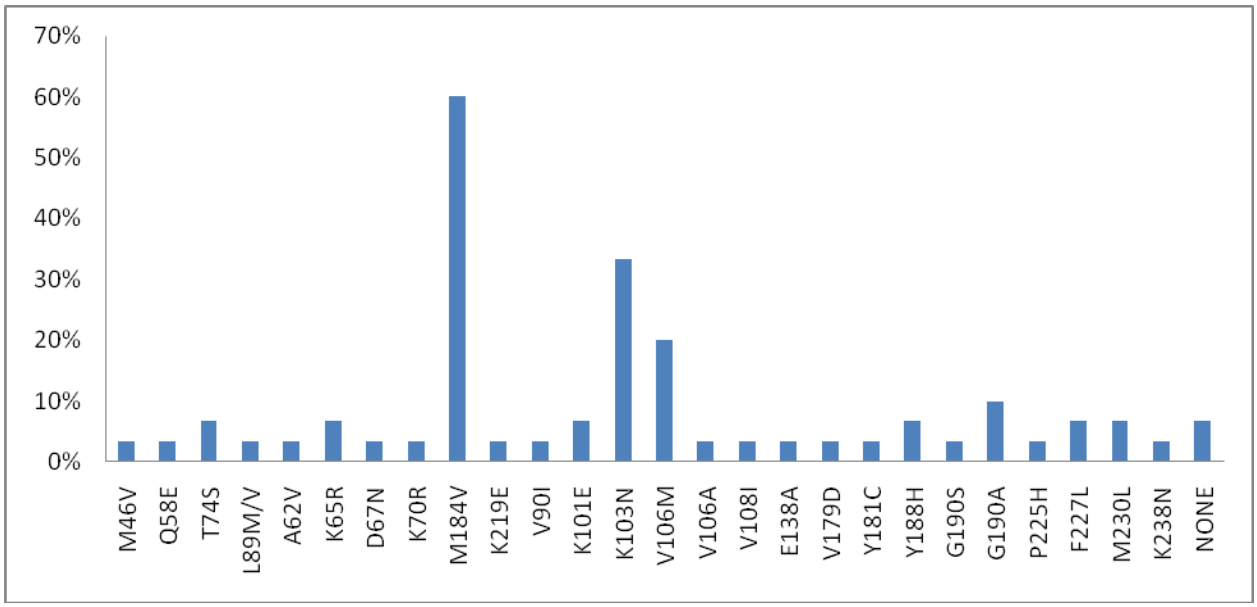


Figure 3.13: Distribution of second-line baseline mutations prior to starting the second-line regimen. The M184V and K103N mutations are the most prevalent mutations.

3.3.2. Failure Mutations of the second-line CIPRA-SA patients

Twenty one participants failed the second-line regimen, 12 of whom never achieved viral suppression at any stage of second-line therapy. No PIs or NRTIs mutations were observed in all 21 patients. Four participants had NNRTIs mutations (Table 3.13), with the most frequent being K103N (n=4; 19%). Of the 9 participants that experienced viral rebound, 3

had experienced toxicity to the first-line regimen, and had no mutations present at second-line failure.

Table 3.13: Baseline and failure characteristics of the 21 participants that experienced failure on the second-line regimen.

Participant Name	Reason for failure	Baseline Resistance	Time since regimen change	Outcome on Second-line	A98G	K103N	P225H	K103R
265842	Toxicity	NA*	5	Never Suppressed	1	1	1	
165461	<1.5LOG DROP	K103N	4	Never Suppressed		1		
135381	<1.5LOG DROP	NONE	7	Never Suppressed				
266262	VL>1000	M184V, K103E, V108I, Y181C	7	Never Suppressed				1
235681	VL>1000	K103N	8	Never Suppressed		1		
136611	VL>1000	V106M, Y188C	10	Never Suppressed				
135941	VL>1000	K103N	11	Never Suppressed				
265641	VL>1000	M184V	6	Never Suppressed				
266571	VL>1000	NA	7	Never Suppressed				
136251	VL>1000	NA	9	Never Suppressed				
136801	VL>1000	NONE	8	Never Suppressed				
135101	VL>1000	NONE	8	Never Suppressed				
165671	TOXICITY	NA	17	Rebound				
235051	TOXICITY	NA	44	Rebound				
166741	TOXICITY	NA	7	Rebound				
235491	VL>1000	M184V	11	Rebound		1		
165831	VL>1000	NONE	3	Rebound				
137111	VL>1000	M184V, V106A, F227L	9	Rebound				
135311	VL>1000	I47V, M184V, K103N	17	Rebound				
135671	VL>1000	M184V, K103N, V108I	14	Rebound				
137011	VL>1000	Q58E, G190A	6	Rebound				

*Not Available

3.4. Characterisation of host polymorphisms in the CIPRA-SA cohort

A total of 1471 (1223 HIV-1 negative samples and 248 from the CIPRA-SA cohort) DNA preparations were isolated and successfully used for SNP analysis of the four polymorphisms of interest in *CYP3A4, 3A5, 2B6 and MDR-1.* A total of 248 out of the 812 CIPRA-SA participants were consented for host genetic analysis, 85 of which experienced treatment failure for the following reasons: 41 toxicities, 31 viral failure, 5 deaths, 6 defaulted on the clinical schedule, 2 lost to follow-up and 1 withdrew consent from the study. The participant that withdrew consent was removed from the analysis. The participants that failed as a result of death, default on clinical schedule or were lost to follow-up were included in the genotypic frequencies analysis, but were not linked to treatment, as the small size of each of these sub-sets would have no statistical power.

An example of the allelic discrimination plots for the CYP3A4, G1344A genotype is represented in Figure 3.14. The Y-axis represents the wild-type 1344GG genotype which occurs in the majority of samples genotyped in this study, with a wildtype genotypic frequency of 44.2% and 60.2% for the control and CIPRA-SA groups, respectively (Table 3.14). Figure 3.15 represents the allelic discrimination plots for the CYP3A5 A6986G allele. The homozygous genotype 6986GG, which occur in the majority of the samples genotyped (X-axis), were present at a genotypic frequency of 64.4% and 70.5% for control and CIPRA-SA group, respectively (Table 3.14). The allelic discrimination plots for the CYP2B6, G516T allele, are represented in Figure 3.16. The homozygous variant 516TT occurs in the minority of the samples genotyped, at a genotypic frequency of 15.78% and 13.3% for the control and CIPRA-SA groups respectively (Table 3.14). Finally, the allelic discrimination

plots for the MDR-1 C3435T are depicted in Figure 3.17. The homozygous wild-type 3435CC (Y-axis) was present at genotypic frequencies of 80.8% and 79.5% for the control and CIPRA-SA groups, respectively.

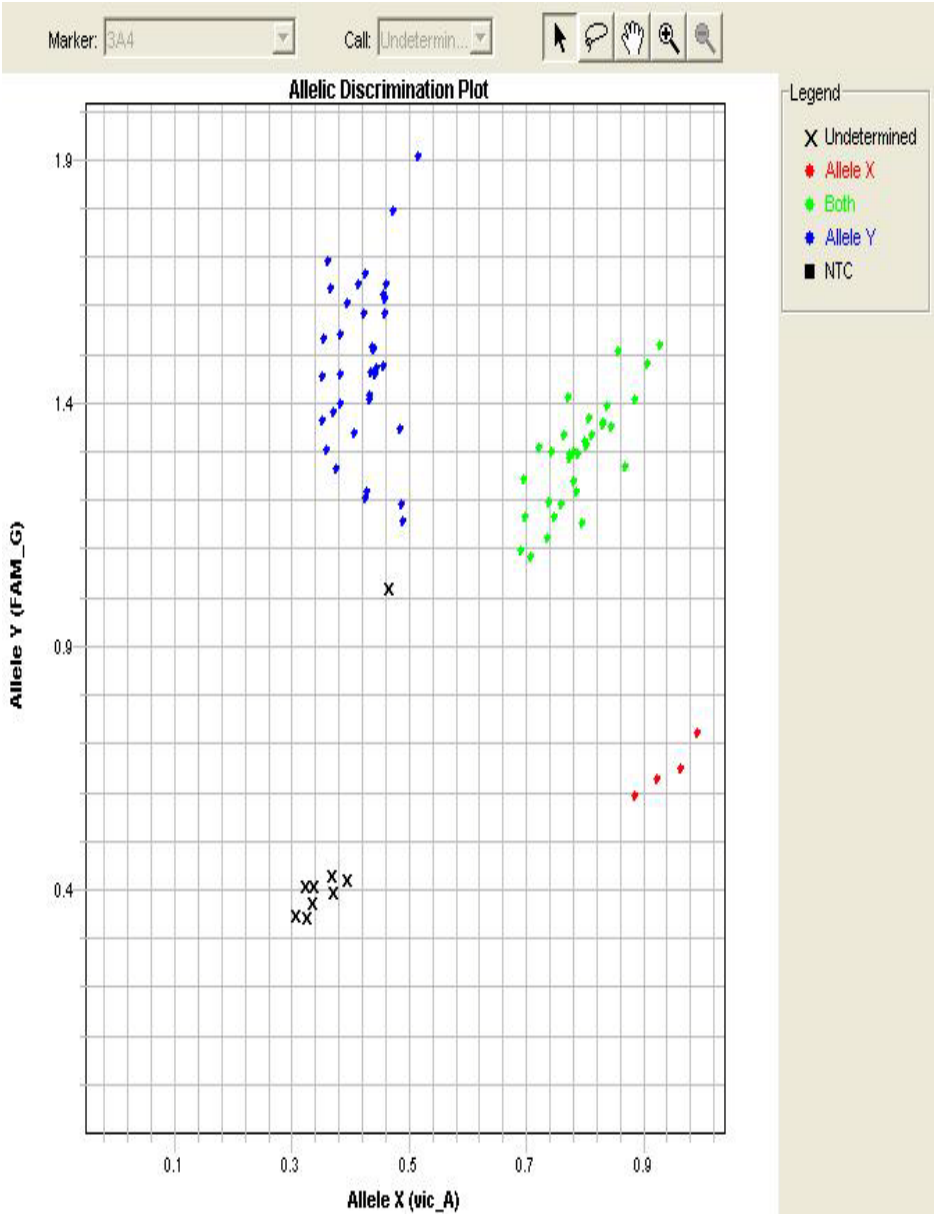


Figure 3.14: Frequency of the CYP3A4 SNP G1344A in 82 HIV-1 negative samples. Samples with the homozygous variant genotypes (A) are represented by the red dots on the x-axis, the homozygous wild-type genotypes (G) are represented by the blue dots and heterozygous genotypes (GA) are represented by the green dots. Samples that failed to amplify or no template controls are indicated by the X's.

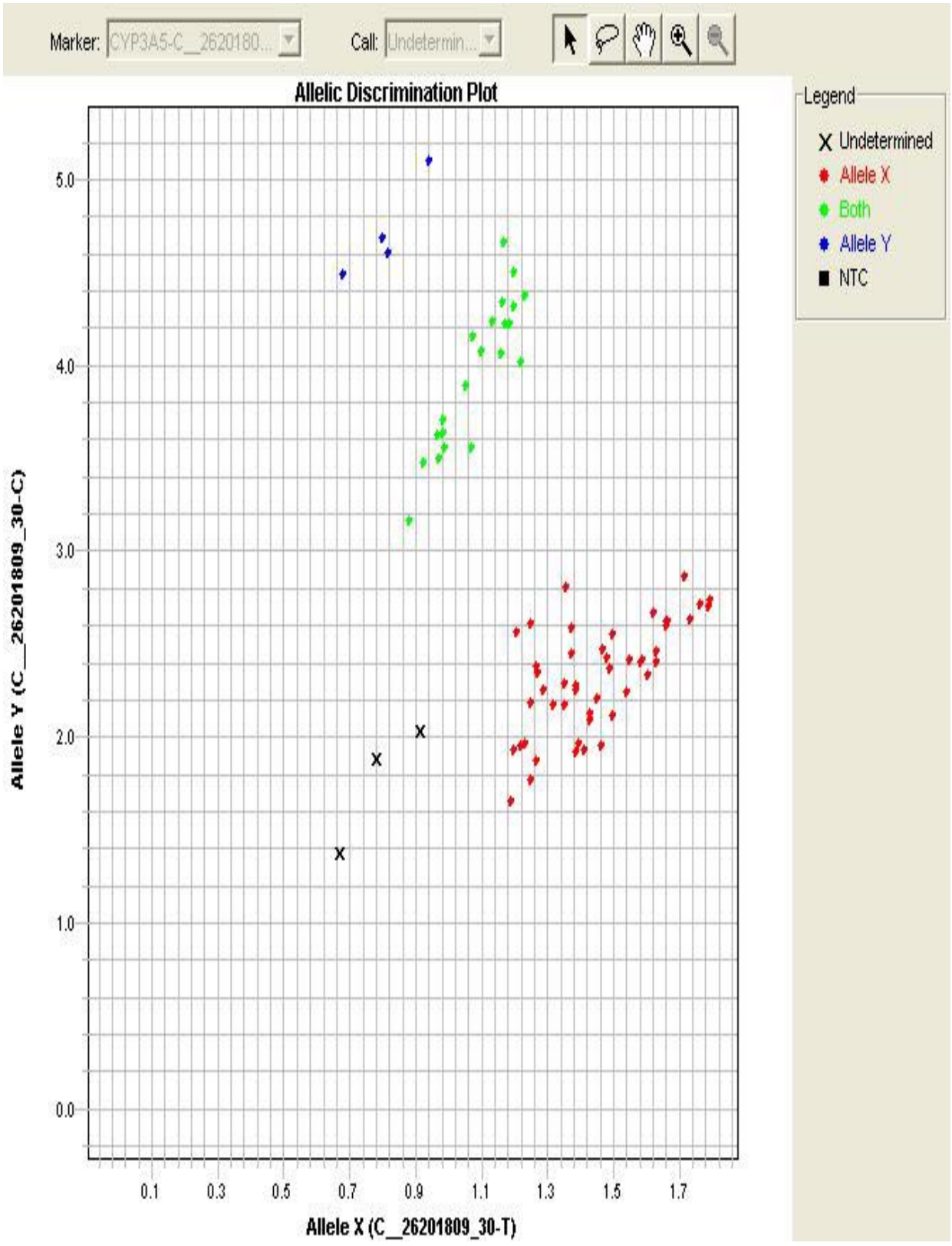


Figure 3.15: Frequency of the CYP3A5 SNP A6986G in 75 HIV-1 negative samples. Samples with the homozygous variant genotype (G) are represented by the red dots on the x-axis, the homozygous wild-type genotypes (A) are represented by the blue dots and heterozygous genotypes (AG) are represented by the green dots. No template controls are indicated by the X's.

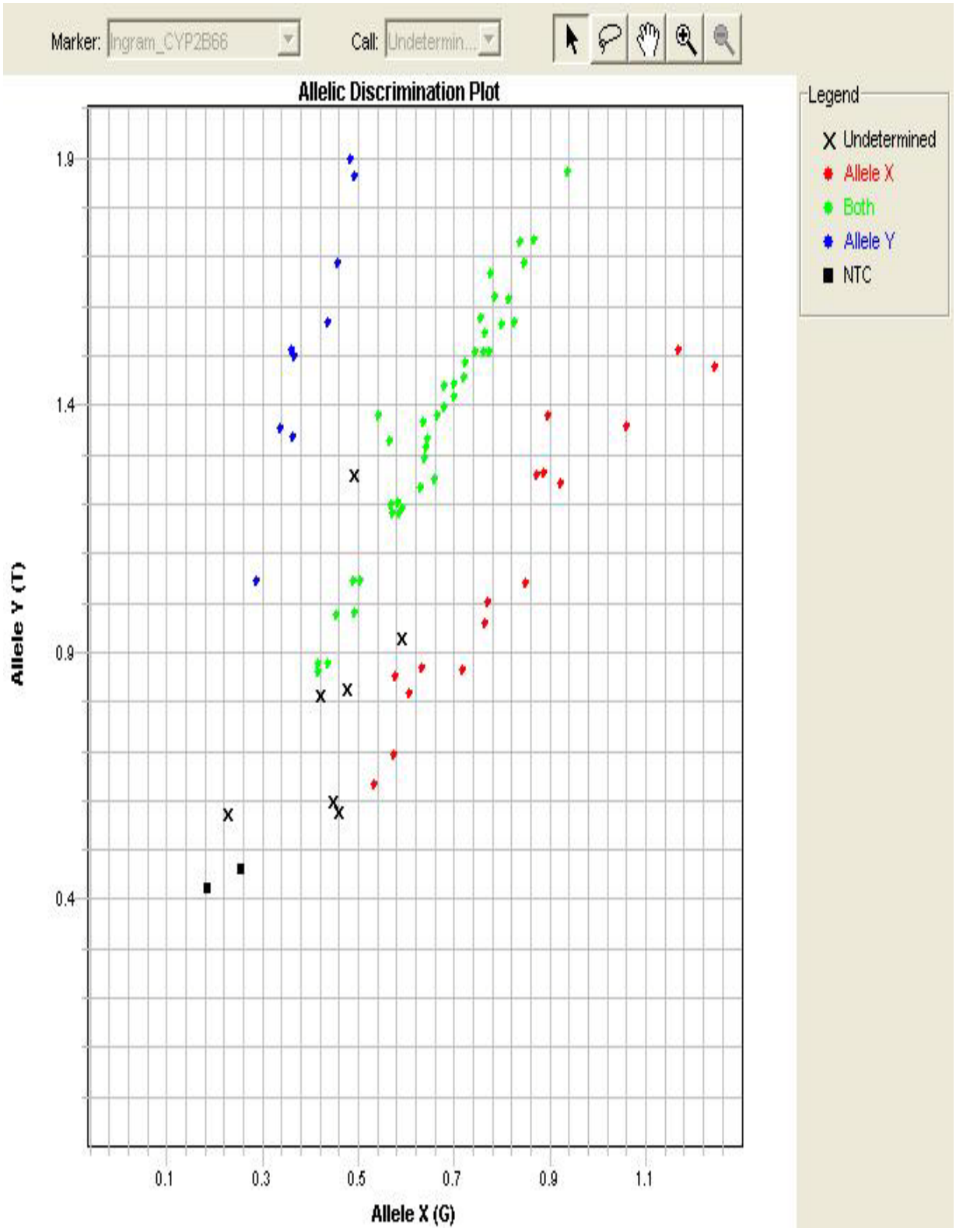


Figure 3.16: Frequency of the CYP2B6 SNP G516T in 75 HIV-1 negative samples. Samples with the homozygous variant genotypes (T) are represented by the blue dots on the y-axis, the homozygous wild-type genotypes (G) are represented by the red dots on the y-axis and heterozygous genotypes (GT) are represented by the green dots. Samples that failed to amplify and no template controls are indicated by the X and square boxes, respectively.

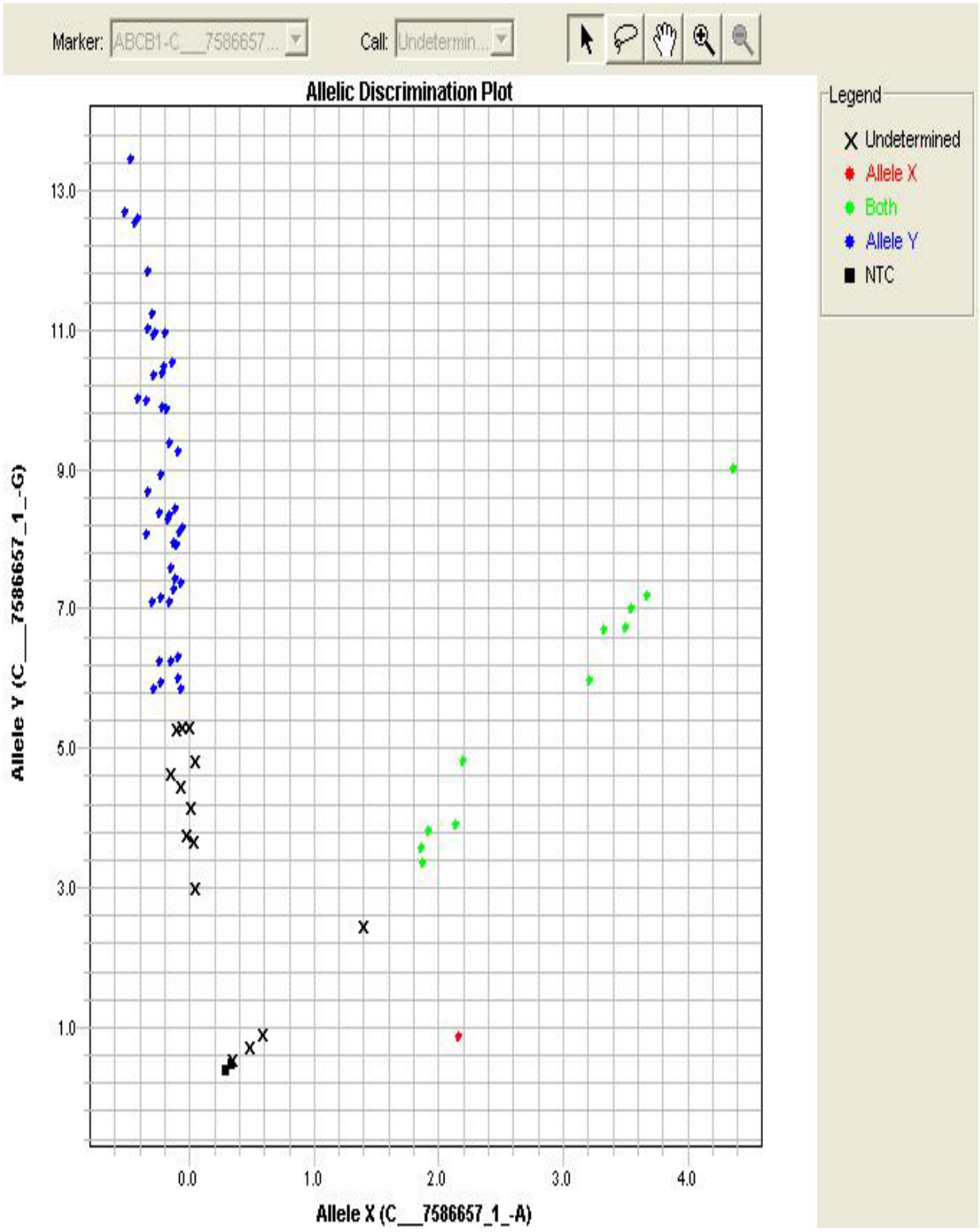


Figure 3.17: Frequency of the MDR-1 SNP C3435T, in 71 HIV-1 negative samples. Samples with the homozygous variant genotypes (T) are represented by the red dots on the x-axis, the homozygous wild-type genotypes (C) are represented by the blue dots and heterozygous genotypes (CT) are represented by the green dots. Samples that failed to amplify and no template controls are indicated by the X and square boxes, respectively.

The frequencies of the four genetic variants examined in the CIPRA-SA cohort and the HIV-1 negative control groups were compared between the two groups using a Chi-squared test (Table 3.14). Overall, there were no significant differences in SNP frequencies between the control and CIPRA-SA case population.

Table 3.14: Genotype frequencies of the 4 SNPs in the CIPRA-SA cohort and the HIV-1 negative control cohort**.**

	CYP3A4		**CYP3A5**		**CYP2B6**		**MDR-1**	
	G1344A		*A6986G*		*G516T*		*C3435T*	
	CIPRA	Control	CIPRA	Control	CIPRA	Control	CIPRA	Control
Homozygous Wild-Type	60.2%	44.2%	76.5%	64.4%	49.7%	38.9%	79.5%	80.8%
Heterozygous	37.1%	50.3%	22.5%	32.6%	37.0%	45.4%	17.1%	17.8%
Homozygous Variant	2.7%	5.5%	0.9%	3.0%	13.3%	15.7%	3.4%	1.4%
***p*-value**	0.145		0.140		0.180		0.690	

A univariate analysis was performed to determine if there was any correlation between the allelic frequencies of participants that experienced ARV-related toxicity (n=41) versus those that did not (n=206; Table 3.15). There was no statistically significant association between the four genotypes examined and development of toxicity. Similarly, univariate analysis of allelic frequencies and viral failure (n=31) showed no statistically significant differences (Table 3.16).

Table 3.15: Allele frequencies of the 4 genotypes examined from the patients that experience toxicity versus those that did not on the CIPRA-SA cohort**.**

	CYP3A4		**CYP3A5**		**CYP2B6**		**MDR-1**	
	G1344A		*A6986G*		*G516T*		*C3435T*	
	Toxicity	No Toxicity	Toxicity	No Toxicity	Toxicity	No Toxicity	Toxicity	No Toxicity
Wildtype	96%	98%	100%	99%	90%	84%	92%	97%
Variant	3%	2%	0%	1%	10%	14%	8%	3%
***p*-value**	0.6506		0.3161		0.3841		0.1210	

Table 3.16: Allele frequencies of the 4 genotypes examined from the patients that experienced viral failure versus those that were virologically suppressed on the CIPRA-SA cohort**.**

	CYP3A4		**CYP3A5**		**CYP2B6**		**MDR-1**	
	G1344A		*A6986G*		*G516T*		*C3435T*	
	Viral Failure	No Viral Failure	Viral Failure	No Viral Failure	Viral Failure	No Viral Failure	Viral Failure	No Viral Failure
Wildtype	95%	98%	100%	99%	83%	87%	96%	97%
Variant	5%	2%	0%	1%	17%	13%	4%	3%
***p*-value**	0.2484		0.3161		0.4283		0.7004	

Because CYP2B6 has been linked to EFV toxicity and or failure, a univariate analysis between participants receiving an EFV-containing regimen and outcome was performed to determine if there was any correlation. No correlation was found between the G516T genotype and outcome (p=0.9501).

4. Chapter 4: Discussion

Antiretroviral therapy has greatly improved the life-span of HIV-1 infected individuals. However, treatment failure does occur and is often a result of toxicity, poor-tolerability, non-adherence or the development of ARV drug resistance. This study focused on the development and/or establishment of assays to evaluate viral (development of HIV-1 drug resistance) and host factors (4 SNPs associated with ARV metabolism and absorption) associated with ARV treatment outcome, and the longitudinal monitoring of these viral factors in the CIPRA-SA cohort. Overall, the ARV drug resistance mutations described in a predominantly HIV-1 subtype C infected South African population were similar to those described amongst other subtype C infected cohorts throughout Africa (Marconi et al., 2008, Orrell et al., 2009, Wallis et al., 2010) and India (Kandathil et al., 2009, Vidya et al., 2009) , as well as HIV-1 subtype B infected patients. Furthermore, this study revealed that more frequent clinical monitoring and switching of ARV drug regimens when a participant had greater than 1000 RNA copies/ml resulted in less complex ARV drug resistance patterns, which is beneficial for future treatment options. Due to the lower than anticipated number of participants failing ARV treatment and the total size of the CIPRA-SA cohort, no statistically significant association could be made between the four host genetic factors examined and ARV treatment outcome.

The implementation of the South African national ARV roll-out program, and the associated emergence of HIV-1 ARV drug resistance, has driven the need for the development of an affordable HIV-1 subtype C specific ARV drug resistance assay. Results of this study confirmed the establishment of a validated; more affordable and subtype robust assay to

monitor ARV drug resistance. The use of an automated extraction method resulted in a more efficient isolation of minority quasispecies, which were detected as mixtures in the sequence chromatograms in the in-house HIV-1 ARV drug resistance assay in some patients. Detection of these extra mutations/polymorphisms did not alter the patient's ARV drug resistance profiles, however, their presence may influence future treatment options. Moreover, the automated extraction method allowed for increased sample throughput (30 test samples and 2 controls to be isolated in 90 minutes) making the test suitable for the South African ARV roll-out program (approximately 870 000 patients on ARVs by end 2009). The use of an automated extraction method is in contrast with other commercial and in-house methodologies, which use manual extraction methods (Eshleman et al., 2004, Saravanan et al., 2009).

RT-initiated amplification and sequencing was successful for 95% of all samples, and included 156 South African patient plasma samples (not dependent on viral load), 118 of the 134 patient plasma samples from 8 African sites and 10 EQA patient samples. The *pol* amplification and sequencing success rates are higher than those reported on commercially available assays (Eshleman et al., 2004), which could be attributed to the use of subtype C specific PCR primers. It should be noted that all currently available assays (commercial and in-house assays) are unable to amplify 100% of patient samples tested, mainly due to the high genetic variability of HIV-1 (Engelbrecht et al., 2007, Eshleman et al., 2004).

All of the 90 South African patient samples, with viral loads ranging from 1300 to 1.6 million RNA copies/ml, were successfully amplified using the in-house HIV-1 ARV drug resistance assay, whereas nine of the 90 samples (10%) could not be amplified using the ViroSeq

method (2300-627000 RNA copies/ml; section 3.1.4, Appendix D, Table D1). None of the samples below 1000 RNA copies/ml were tested in either assay. This is not a limitation to the study as treatment switches in patients in resource constrained countries are not conducted below this level. Of significance, seven of the nine samples that failed to amplify on ViroSeq, had mutations that resulted in resistance to both NRTIs and NNRTIs, indicating the clinical importance of analysing these samples (Table 3.4). The subtype distribution was as expected with 96.7% of the samples being classified as HIV-1 subtype C. Two of the three remaining samples were classified as HIV-1 subtype B and one as sub-subtype A1. The presence of non-C subtypes in South Africa has been reported previously (Papathanasopoulos et al., 2003). HIV-1 subtype B is prevalent in North America and Europe, while sub-subtype A1 predominates in East Africa (Papathanasopoulos et al., 2003). It is likely that these patients did not originate from South Africa, or alternatively were infected by individuals originating from countries, where these subtypes are prevalent. The use of only 5 primers to generate a fully bidirectional sequence with coverage of PR 1-99 and RT 1-240 increases the number of samples that can be sequenced on one 96 well plate (19 samples as opposed to 13 using ViroSeq), thereby improving assay cost-effectiveness.

The robustness of the in-house ARV drug resistance assay was further demonstrated by the amplification and sequencing of 118 of the 134 HIV-1 samples from newly infected patients from eight African sites. If necessary, subtype-specific or degenerate primers can be designed for implementation of this assay format for different geographic regions. Phylogenetic analysis revealed that the samples from the eight African sites were infected with different subtypes, unique or circulating recombinant forms expected in that region. For example, samples from Kigali, Rwanda were predominantly sub-subtype A1, followed by subtype C (Table 3.8; http://www.hiv.lanl.gov). This demonstrated that the newly developed

in-house ARV drug resistance assay can be used for HIV-1 subtypes other than subtype C. Future work should focus on improving the performance of this assay with non HIV-1 subtype C samples. It is highly likely that the one patient with the K103N mutation which causes high level resistance to all NNRTIs (Johnson et al., 2008) was infected with an ARV drug resistant virus, since this cohort was comprised of recently infected individuals. This reaffirms that the newly developed in-house ARV drug resistance assay can be used for surveillance studies to monitor the frequency of transmitted ARV drug resistant viruses.

The in-house ARV drug resistance assay successfully amplified all samples in the VQA panels and the external analysis revealed a high sequence homology compared to sites participating in the panels. Most importantly, the mutation patterns were comparable to other laboratories enrolled in the proficiency program, resulting in the newly established in-house ARV drug resistance assay being certified by the NIH VQA. Four of the VQA samples were subtype B and future evaluation of this subtype using the in-house assay is required.

It is becoming increasingly evident that the cost of monitoring patients on ARVs far exceeds the cost to treat, with recent studies from Uganda and Zimbabwe showing the cost to monitor one patient per year is equivalent to treating six HIV-1 infected individuals (Medina-Lara et al., 2009, Mugyenyi et al., 2009). These costs currently exclude the price of resistance testing where commercially available ARV drug resistance assays cost approximately R2700. Such prices are not prohibitive in resource rich countries and ARV drug resistance testing is used in routine patient monitoring. However, the current costs in the developing world make the ARV drug resistance monitoring of patients on HAART impossible. The newly developed assay is cheaper than the commercially available ViroSeq and costs R1300

(Appendix G). However, it should be noted that although the in-house ARV drug resistance assay costs 60% of the commercially available genotyping kits available in South Africa, the initial outlay for setting up a laboratory to perform sequencing is expensive as a result of the specialized equipment and skilled personnel required. Moreover, the ongoing costs linked to equipment maintenance may limit its widespread use.

Numerous studies worldwide have shown that the type and complexity of the emerging ARV drug resistance patterns are linked to time of initiation of treatment with respect to CD4+ T-cell count (Kitahata et al., 2009, Moore et al., 2009, Sterne et al., 2009), choice of ARV regimen, HIV-1 subtype (Morris et al., 2003), and duration on a failing regimen (Doualla-Bell et al., 2009, Hosseinipour et al., 2009, Johannessen et al., 2009, Marconi et al., 2008, Orrell et al., 2009, Wallis et al., 2010). Since one of the objectives of the CIPRA-SA study was to set-up a closely monitored cohort that was followed longitudinally from ARV initiation and throughout ARV treatment, it has provided a unique opportunity to evaluate factors linked to the development of ARV drug mutation patterns emerging from HIV-1 subtype C infected patients experiencing treatment failure. Furthermore, the data obtained from this study can be compared to data emerging from patients failing on similar drug regimens in the region, following different available guidelines (WHO- and national-guidelines throughout Africa). The in-house ARV drug resistance assay was thus used to analyse participants failing HAART on the CIPRA-SA cohort.

Overall, the CIPRA-SA study enrolled 812 participants from two sites who had CD4+ T-cell counts less than 350 cells/mm^3. The participants were representative of the demographics of patients presenting to government ARV clinics as they were predominantly female and of

childbearing age. The inclusion criteria of participants enrolled onto CIPRA-SA cohort was different from patients on the national-roll program. In particular, one major difference between the CIPRA-SA and public sector cohorts was that CIPRA-SA participants were initiated onto ARV treatment with CD4+ T-cell counts of 350 cells/mm^3 or below, unlike the public sector which initiates HAART in patients who have CD4+ T-cell counts of 200 cells/mm^3 or below. The CIPRA-SA initiation criteria at the time (2005), was in line with international practices, and subsequently several studies which have shown the sooner a patient begins ARV therapy the better the prognosis (developing AIDS and dying; Kitahata et al., 2009, Moore et al., 2009, Sterne et al., 2009). Even though the initiation criteria of CIPRA-SA was less than a CD4+ T-cell count of 350 cells/mm^3, the majority of the participants (63.7%) enrolled had CD4+ T-cell counts less than 200 cells/mm^3, and 31% had CD4+ T-cell counts between 200 and 350 cells/mm^3. This is reflective of the fact that most individuals do not know their HIV status in South Africa, and only seek treatment when presenting with opportunistic infections and their CD4+ T-cell counts are generally well below the entry criteria of this study.

The CIPRA-SA study had a mortality rate of 2.5% which is in line with studies linking mortality to treatment outcome, which have shown that, 1.4% of patients initiating ARV therapy at CD4+ T-cell counts less than 350 cells/mm^3 die. In contrast, 3.7% of patients who initiate ARV therapy at CD4+ T-cell counts less than 200 cells/mm^3 are expected to die (Sterne et al., 2009). This data provides compelling evidence for initiation of ARV treatment and higher CD4+ T-cell counts to ensure a better prognosis. The South African government recently announced (1 December 2009) that the entry criteria for the government roll-out program will be adjusted to 350 cells/mm^3 as of April 2010. This change of initiation criteria

was partly driven by results obtained from the CIPRA-SA study. The CIPRA-SA data has thus provided a baseline from which future comparisons can be made.

The choice of ARV regimen in the CIPRA-SA cohort mirrored that of the public sector and consisted of 2 NRTIs supported by an NNRTIs (first-lines) or PIs (second-line). NRTIs are the choice of backbone of these regimens as a result of their intracellular persistence allowing for constant viral inhibition. Resistance data from 55 of the 67 participants experiencing viral failure (defined as two viral loads greater than 1000 RNA copies/ml) in this study found that 82% of patients failed with known NRTIs and/or NNRTIs mutations (Figure 3.6). As expected the M184V mutation was observed at the highest frequency, followed by the K103N mutation (Figures 3.7 and 3.8). Thus, these participants were resistant to both classes of ARVS (NRTIs and NNRTIs) prescribed in the first-line regimen. The virus from participants with the M184V mutation are expected to have an increased susceptibility to AZT present in the second-line regimen. Similarly, the presence of NNRTIs mutations, such as K103N and V106M, do not impact on ARVs present in the second-line regimen. These results are similar to those observed in resource rich countries where NRTIs and NNRTIs regimens have been used (Gallant et al., 2006, Gulick et al., 2004, Margot et al., 2006). These results indicate that overall, the ARV resistance patterns emerging in HIV-1 subtype C infected individuals are in line with what was expected from this ARV drug combination and may allow for lessons learnt elsewhere to be used in South Africa.

Stavudine (d4T) was one of the two NRTIs used in the first-line regimen for participants recruited onto the CIPRA-SA study. The choice of this drug in Africa is largely based on cost considerations and not the toxic side-effects (Rosen et al., 2008). One hundred and thirty

four (16.5%) CIPRA-SA participants failed the first-line regimen as a result of toxicity (Wood et al., 2009, Appendix H), most of which were adverse events linked to d4T (Appendix A). These findings are similar to other studies in South Africa, which investigated toxicity in patients accessing the national ARV first-line regimen. (Boulle et al., 2007) found that amongst 560 of the 2679 (21%) patients that failed treatment due to toxicity, the major contributor was d4T. Furthermore, although toxicity was mainly observed early on in ARV therapy, side effects accumulated over time in participants on d4T, resulting in AZT being substituted for d4T (Boulle et al., 2007). Similarly, participants in CIPRA-SA were substituted to AZT in the event of d4T toxicities. Results from several studies (Boulle et al., 2007, Rosen et al., 2008) as well as CIPRA-SA contributed to the recommendation of removing d4T from the South African roll-out program and substituting it with a more tolerable drug like TNF.

As mentioned previously, the M184V mutation was the most frequently observed NRTIs mutation in the CIPRA-SA cohort (67%) and is linked to the presence of 3TC in the first-line regimen. Similar findings have been shown in three other studies looking at mutations emerging from the South African ARV roll-out program, in Johannesburg, Cape Town and Durban (Table 4.1; Marconi et al., 2008, Orrell et al., 2009, Wallis et al., 2010). Table 4.1 summarises published data on mutation patterns from first-line failures in the region, as compared to the CIPRA-SA cohort. Although the emergence of the M184V mutation results in decreased susceptibility to the first-line regimen, it results in increased sensitivity to AZT, one of the NRTIs present in the second-line regimen, thereby increasing the effectiveness of the second-line regimens.

In the CIPRA-SA study the NNRTIs of choice were either NVP or EFV in the first-line regimen and mirrored the national ARV roll-out program. The resistance data from this study showed that the Y181C mutation only occurred in participants on the NVP-containing regimen (40%) and was not observed in patients accessing EFV. The V106A mutation (10%) was only observed in participants on a failing NVP-containing regimen and the V106M mutation was more frequent in participants on the EFV-containing arm (18%) than the NVP-containing arm (10%) of CIPRA-SA. Furthermore, in the CIPRA-SA study, EFV was found to select for a wider range of mutations in the RT area investigated than NVP. Of interest was the frequency of the K103N mutation, which occurred in 60% of participants on EFV versus 40% of participants on NVP in the CIPRA-SA study. The difference in mutations selected by EFV and NVP could have an impact on the second generation NNRTIs, etravirine (ETR; in future treatment regimens). Etravirine is less effective in the presence of viruses with the Y181C mutation which is selected by NVP and not EFV. EFV commonly selects for the K103N mutation that does not affect responses to the more flexible second generation NNRTIs-ETR (Lazzarin et al., 2007, Madruga et al., 2007). Since ETR is being considered for incorporation into future second- or third-line regimens, this difference in mutation patterns may impact on whether EFV or NVP is used in future first-line regimens (Stevens et al., 2009). These findings of the NNRTIs mutation patterns are the same as those observed in the South African public sector (Wallis et al., 2010).

Apart from ARV regimen choice, the HIV-1 subtype plays a subtle role in the patterns of resistance mutations observed. As shown above, the CIPRA-SA resistance patterns (high frequency of M184V, K103N and V106M; Figure 3.7 and 3.8) are similar to those generated in HIV-1 subtype B (Kantor et al., 2002). However, differences were observed and are a result of different amino acid and nucleotide combinations at codons in RT or PR linked to

HIV-1 drug resistance. For example, the T74S minor PI resistance mutation was found in 13% of patients on the CIPRA-SA cohort. This mutation was observed in samples from participants at both study entry (Table 3.10) and viral failure (Appendix F, Table F.1), confirming this is a naturally occurring HIV-1 subtype C polymorphism. The presence of these polymorphisms in HIV-1 subtype C have been noted in other studies (Cane et al., 2001, Grossman et al., 2001), and may have possible effects on PI usage in second-line regimens and requires further investigation.

The V106M mutation was found to occur in 18% and 10% of patients in the CIPRA-SA cohort accessing EFV- and NVP-containing regimens, respectively. The V106A mutation was less common and only occurred in 10% of viral failure samples in the CIPRA-SA cohort. Similar results have been observed for the V106M mutation in other HIV-1 subtype C samples from patients accessing a failing EFV- or NVP-containing regimen (Morris et al., 2003). Moreover, the V106M mutation has rarely been seen in HIV-1 subtype B patients accessing EFV or NVP-based regimens (Martinez-Cajas et al., 2009). The frequency of the V106M mutation in HIV-1 subtype C is a direct result of the occurrence of a silent polymorphism at the nucleotide level in this subtype. Of importance the V106M and V106A mutations result in different levels of resistance to NNRTIs. The presence of V106M results in cross-resistance to all first-generation NNRTIs and reduced susceptibility to second-generation NNRTIs (Brenner et al., 2003). Whereas, V106A only results in a 30-fold increase in resistance to NVP and a 2.5-fold increase in resistance to EFV and DLV (Shafer, 2004). These differences could have an impact on NNRTIs used in subsequent regimens.

The K103N mutation was the most frequent NNRTIs mutation in the CIPRA-SA cohort, occurring in 50% of all participants. This high frequency is similar to a study by (Flys et al., 2006) which showed that the K103N mutation occurs in higher levels in Ugandan women infected with HIV-1 subtypes C and D versus subtype A. The reason for this difference amongst subtypes is still not completely understood and requires further investigation. As K103N does not impact on the effectiveness of ETR, the presence of this mutation may be advantageous if ETR is used in third-line regimens.

The second most frequent NRTIs mutation observed in the CIPRA-SA cohort was the K65R mutation (3%). This is in contrast to HIV-1 subtype B infected patients accessing d4T, where for example, the K65R mutation was observed in only two of 301 (0.7%) patients accessing d4T (Margot et al., 2006). Studies from countries with predominantly HIV-1 subtype C infections have confirmed that the K65R mutation commonly occurs as a consequence of ARV drug regimens containing d4T (Table 4.1; Hosseinipour et al., 2009, Orrell et al., 2009, Wallis et al., 2009b). Similarly, *in vitro* cell culture studies have revealed that the K65R mutation appears more rapidly in HIV-1 subtype C viral isolates compared to subtype B, CRF01_AE, CRF02_AG, G, and HIV-2 isolates (Brenner et al., 2006, Doualla-Bell et al., 2006, Sungkanuparph et al., 2007). Despite the more rapid appearance of K65R in HIV-1 subtype C isolates, there is no difference between the levels of resistance to TDF, abacavir (ABC), 3TC, and ddI and other subtypes (Brenner et al., 2006). Initially, the occurrence of the K65R mutation was attributed to the longer time patients were left on a failing regimen. However, recent data has linked this increase to the homopolymeric region surrounding codon 65 (Coutsinos et al., 2009). This poly-A region results in the RT enzyme pausing during cDNA synthesis and thereby increasing the chances of incorrect nucleotide incorporation. A major problem with the higher frequency of the K65R mutation seen in

HIV-1 subtype C is that it results in cross-resistance to most NRTIs (Johnson et al., 2008) and has consequences for the subsequent use of most NRTIs, especially TDF in second-line regimens. Furthermore, because TDF is being investigated for use in pre-exposure prophylaxis (PREP) the emergence of the K65R mutation and the implications this may have on PREP needs to be further investigated.

The other major difference between clinical criteria used on the CIPRA-SA study and the public sector cohorts was the definition of viral failure. In the CIPRA-SA cohort, participants were classified as experiencing virological failure by two consecutive viral loads greater than 1000 RNA copies/ml. This is unlike the South African national roll-out programme which switches at two consecutive viral loads greater than 5000 RNA copies/ml. By contrast, the WHO recommendations (prior to 1 December 2009) advised switching ARV treatment at viral loads greater than 10000 RNA copies/ml. Furthermore, some African countries which do not have access to viral load measurements use CD4+ T-cell counts (immunological failure) and/or clinical criteria to switch. The early switching in the CIPRA-SA cohort were in line with several studies which have shown that the earlier ARV therapy is switched when a patient is failing, the less complex the mutation patterns associated with HIV-1 drug resistance. Results from this study confirmed these findings, and highlight the differences between African countries using different criteria for ARV regimen switching.

Overall, 82% of participants experiencing viral failure harboured ARV drug resistance mutations and 62.7% had resistance to NRTIs and NNRTIs on the CIPRA-SA cohort. Both these percentages are lower than results from all other published studies from the region. For example, the Malawi study which accessed ARV drug resistance mutation patterns in patients

that were switched on immunological and/or clinical criteria found that 95% of the patients had mutations associated with ARV drug resistance, 94% of which had resistance to NRTIs and NNRTIs (Hosseinipour et al., 2009). Ongoing viral replication and frequency of ARV drug resistance mutations that occur as a result of different switch criteria among countries will have an impact on the use of NRTIs and second-generation NNRTIs in future ARV drug regimens.

Similarly, the M184V mutation occurred at a lower frequency in participants (67.2%) with viral failure on the CIPRA-SA cohort. This frequency is considerably lower than resistance data emerging from Malawi, with a reported M184V prevalence of 81% (Table 4.1; Hosseinipour et al., 2009). The higher percentage in Malawi is possibly attributed to the switch criteria being dependent on immunological and clinical considerations, versus virological failure. Furthermore, the M184V frequency in CIPRA-SA was lower than those reported in two other South African studies from patients on failing regimens on the South African roll-out programme. Frequencies of 78% and 74% in Cape Town (Orrell et al., 2009) and Johannesburg (Wallis et al., 2009b), respectively were reported. The higher frequency of the M184V in patients from the government sector is likely due to viral load levels of 5000 RNA copies/ml being used as the definition of virological failure. Findings from the CIPRA-SA study are supported by similar M184V frequencies (64.3%) amongst patients from the government sector in Durban, where a viral load cut-off of 1000 RNA copies/ml was used to define viral failure (Marconi et al., 2008). Moreover, analysis of longitudinal samples examined on the CIPRA-SA cohort showed that the M184V mutation is the major NRTIs mutation which is present at early ARV drug failure, or is likely to emerge in patients within the first 3 months left on a failing ARV regimen (section 3.2.4). As mentioned previously, the implications for the increased frequency of M184V are beneficial for subsequent AZT

use; however, the emergence of this mutation may have an impact on future NRTIs still in development.

Overall, the ARV drug mutation complexity, as defined by the occurrence of K65R, Q151M and TAMs was much lower than that observed in previous studies from the region. Although the predominant circulating subtype is the same for both countries, the K65R mutation occurred in 3% of the CIPRA-SA population, as compared to 19% in the Malawi study (Table 4.1; Hosseinipour et al., 2009) and the South African studies (Orrell et al., 2009, Wallis et al., 2010). This difference is most likely attributed to the length of time the Malawian patients were left on a failing ARV drug regimen (Table 4.1). Furthermore, only one CIPRA-SA participant had three TAMs on a failing first-line regimen. This is in complete contrast to all other published studies from the region which report levels of 23% to 56% (Hosseinipour et al., 2009, Marconi et al., 2008, Orrell et al., 2009, Wallis et al., 2010). The Q151M mutation did not occur in CIPRA-SA study, but has been shown to occur in up to 19% of patients on a failing first-line regimen in the region. This increase of ARV drug resistance mutations has been described in patients from the USA (Hatano et al., 2006), where participants on a failing regimen for a median of 11.3 months and viral loads greater than 1000 RNA copies/ml demonstrated an increase in both drug-associated and non-drug associated mutations, resulting in a 0.5 fold decrease in susceptibility to all NRTIs, NNRTIs and PIs. Similarly, a study by (Cozzi-Lepri et al., 2007) showed that European participants left on a failing regimen (HIV viral load > 400 copies/ml) for 6 months had on average an increased accumulation of two mutations with an average loss of 1.25 active ARV drugs. Overall, different switch criteria used amongst different countries can result in a delayed ARV regimen switching, leading to the occurrence of more complex HIV-1 ARV drug resistance mutation patterns. These complex ARV drug resistance mutation patterns result in

reduced susceptibility to all currently available FDA approved NRTIs, resulting in the PIs being the only fully effective ARV drug in the current second-line regimen in South Africa.

The longitudinal resistance data in this cohort demonstrated that the number of NNRTIs mutations increased with approximately one additional mutation for every month left on a failing ARV drug regimen (Figure 3.9). To the best of our knowledge this is the first report of the accumulation of NNRTIs ARV drug resistance mutations in HIV-1 subtype C. By contrast, resistance data for the development of TAMs in HIV-1 subtype B shows that TAMs increase at a slow rate of approximately 0.11 TAMs per year (Cozzi-Lepri et al., 2009, Goetz et al., 2006, Stevens, 2009). The more rapid accumulation of NNRTIs ARV drug resistance mutations may have an impact on the future use of second-generation NNRTIs and requires further investigation.

Eighteen percent of the CIPRA-SA participants with virological failure had no known ARV drug resistance mutations present. This is similar to what has been observed in the South African national roll-out program (16%; Wallis et al., 2010). However, this is nearly twice as high as observed in the NORA cohort (in Uganda; DART Virology Group, 2006) within the Development of Antiretroviral Therapy on Africa study (DART, 10%; full ARV drug resistance patterns for DART are not yet available). The DART study is the largest clinical trial run in Africa investigating ARV therapy in HIV-1 infected individuals, where only the CD4+ T-cell counts are measured (samples were stored for further viral investigations). This difference between South Africa and Uganda is more than likely linked to differences in switch criteria (virological versus immunological). However, there may be minority variants with known ARV drug resistance mutations that are below the lower detection limits of the

in-house ARV drug resistance assay, and thus not detected, circulating within these patients that contributed to viral failure. Analysis of the minority variants using pyrosequencing in the CIPRA-SA cohort is planned.

Overall, the subtype of the infecting virus, ARV drug choice and most importantly time of switch from the first-line regimens are essential components to ensuring there are optimal second- and third-line regimens available to patients. Furthermore, because NRTIs form the basis of most future regimens, the complexity of NRTIs ARV drug resistance mutations should be avoided. A study by (Riddler et al., 2008), has shown that NRTIs-sparing regimens are non-inferior, but patients have an increase in laboratory associated adverse events (Riddler et al., 2008). Data emerging from the South African second-line regimens indicate that failure to these regimens are more likely as a results of poor tolerability and non-adherence, as resistance to PIs is infrequently observed (Wallis et al., 2009a). Similarly, resistance patterns from the CIPRA-SA participants that accessed a failing first-line regimen indicated that they were susceptible to the NRTIs and the PIs prescribed in the second-line regimen. Of the patients that commenced the second-line, a high percentage of them never achieved full viral suppression, indicating either poor tolerability to ddI or lopinavir and/or non-adherence were the main causes of treatment failure.

The ARV drug resistance profile data emerging from the CIPRA-SA study has formed a vital component in helping determine and strategize for appropriate treatment strategies and ARV choices for second- and third-line regimens in South Africa. The CIPRA-SA study has confirmed that the earlier patients are started on ARV drug therapy, the better their chances of a favourable treatment outcome. Furthermore, baseline viral load is not a predictor of

ARV outcome, and therefore performing this test as an ARV drug treatment initiator is not required. However, the more frequent viral load monitoring and its use in determining viral failure has implications for development of less complex ARV drug resistance mutation patterns. The data also shows that the introduction of ARV drug resistance testing at a patient management level would be beneficial after first-line failure as this would identify patients with no ARV drug resistance, ensuring they are not unnecessarily switched to a more expensive second-line regimen. The high cost of ARV drug resistance testing would therefore be recouped by the reduced number of patients switched to a more expensive second-line regimen. Additionally, the patients with no ARV drug resistance would be re-counselled to increase adherence and help ensure viral suppression and reduce viral transmission.

Surveillance studies of ARV drug resistance mutation patterns like this one performed on the CIPRA-SA cohort are vital in ensuring appropriate preservation of existing and future ARV regimens, as recommended by the WHO (WHO, 2009). From this study, it is clear that the introduction of TNF into a second-line regimen after the use of d4T in the first-line regimen may be sub-optimal, as the K65R mutation occurs at a high frequency in these patients requiring a regimen switch. Therefore, the introduction of TNF into a first-line regimen would be more beneficial, from a toxicity point of view, and has recently been proposed as a change to the South African guidelines. Similarly, the use of AZT in the second-line regimen is appropriate as HIV-1 has increased susceptibility to AZT in the presence of the M184V mutation. If ARV drug regimen switching occurs when a patient has a lower viral load, the choice of NRTIs that can be used in the second-line regimen increases as there are fewer TAMs observed. Results from this study also show that second-generation NNRTIs would be more effective in patients on a failing EFV-based regimen rather than a NVP-based regimen, making it a promising option for third-line regimens. The results of the NNRTIs

resistance patterns also confirm that the earlier a patient is switched the less number of NNRTIs mutations have accumulated, ensuring second-generation NNRTIs can be used in later regimens. A small subset of second-line failures on CIPRA-SA had no resistance at the time of second-line failure, indicating that the tolerability and adherence of ARVs in the second-line is more than likely the reason for treatment failure. This highlights the need for better tolerated ARVs to be incorporated into second-line regimens, for example, the integrase inhibitor raltegravir may be a better choice than Kaletra, or a different PIs should be used to improve tolerability.

Data from the CIPRA-SA study and from other studies performed in South Africa looking at the development of HIV-1 ARV drug resistance have shown that there is a continued need for sentinel ARV drug resistance surveillance studies at ARV treatment clinics. The information obtained from such studies will help monitor the emerging ARV drug resistance patterns from current national ARV roll-out programs, and help inform the suitability of future ARV drug regimens. Furthermore, data from developed countries shows that between 3.4% to 26% of new infections have viruses harbouring ARV drug resistance (Taiwo, 2009). As more South Africans are accessing ARV therapy, it is expected that transmitted HIV-1 drug resistance will also increase, and thus will need to be monitored. Although the newly developed in-house ARV drug resistance assay is cheaper than the commercially available assays, there is still a need to further reduce the costs associated with these assays.

The CIPRA-SA data confirms that a higher CD4+ T-cell count at ARV drug treatment initiation result in a better prognosis. However, for long term monitoring of patients on ARVs, viral load testing is more beneficial than immunological monitoring. Several studies

including this study have shown that the ARV resistance patterns that emerge in patients failing therapy are less complex in patients monitored virologically than immunologically. The need for viral load monitoring is corroborated by a recent study from Malawi where 43% of patients who were classified as failing the first-line regimen on immunological or clinical grounds were in fact virologically suppressed (Hosseinipour et al., 2009) and did not require switching to a more expensive second-line regimen. This is contrary to the data emerging from the DART study which suggests that no laboratory monitoring is required for patients on ARV therapy (Medina-Lara et al., 2009). Results from the CIPRA-SA study also allude to the fact that viral monitoring should occur more frequently, however, the CIPRA-SA study was not powered to answer this question. A randomised control study needs to be performed to determine the optimal time points and frequency of viral load monitoring in patients on HAART. Having viral loads performed at three months after initiation of ARV therapy, may be beneficial in increasing adherence, as data on CIPRA-SA shows that 70% of participants with a less than a 1.5 $\log_{10}$ drop in viral load at week 12 (Figure 3.6) had no HIV-1 ARV drug resistance.

Table 4.1: A summary of the ARV drug resistance studies emerging from first-line failures in four African countries.

Site	**Malawi (Hosseinipour et al., 2009)**	**Tanzania (Johannessen et al., 2009)**	**Botswana (Doualla-Bell et al., 2009)**	**South Africa** Cape Town **(Orrell et al., 2009)**	**South Africa** Johannesburg **(Wallis et al., 2010)**	**South Africa** Durban **(Marconi et al., 2008)**	**South Africa CIPRA-SA**
Sample Size **Clinical Sites**	94 2	22 1	63 1	110 1	226 2	115 2	67 2
Indicator for treatment initiation	CD4+ T-cell count <200 cells/mm^3 or clinical staging	CD4+ T-cell count <200 cells/mm^3 or clinical staging	Unknown	CD4+ T-cell count <200 cells/mm^3	CD4+ T-cell count <200 cells/mm^3	CD4+ T-cell count <200 cells/mm^3	CD4+ T-cell count <350 cells/mm^3
Switch Criteria	CD 4 cell count decrease >30% or WHO staging	Viral load greater than 10 000 RNA copies/ml (n=9); in this study viral load > 1000 RNA copies/ml (n=14)	Viral load greater than 400 RNA copies/ml	Viral load greater than 5000 RNA copies/ml	Viral load greater than 5000 or 1000 RNA copies/ml	Viral load greater than 1000 RNA copies/ml	Viral load greater than 1000 RNA copies/ml
Frequency of Monitoring	Irregular	Unknown	Unknown	6 monthly-viral load & CD4+ T-cell	6 monthly-viral load & CD4+ T-cell	6 monthly-viral load & CD4+ T-cell	3 monthly-viral load & CD4+ T-cell
First Line Regimen	d4T, 3TC, NVP (100%)	d4T, 3TC, EFV (57.5%) AZT, 3TC, EFV (2.8%) d4T, 3TC, NVP (18.4%) AZT, 3TC, NVP (21.2%)	ddI, d4T,NVP/EFV (41%) AZT, 3TC, NVP/EFV (59%)	d4T, 3TC, EFV (67%) AZT, 3TC, EFV (16%)	d4T, 3TC, EFV (65) AZT, 3TC, EFV (24%) d4T, 3TC, NVP (10%) AZT, 3TC, NVP (1%)	d4T, 3TC, EFV (49%) AZT, 3TC, EFV (26%)	d4T, 3TC, EFV (59%) d4T, 3TC, NVP (27%) d4T, 3TC, NLF (4%) d4T, 3TC, lopinavir/r (10%)
Median Time on First-line (months)	36.5 (8-127)	22.3 (14-29.9)	9.3-26.9	Unknown	Unknown	12	15 (3-33)
% with failure & resistance	95%	82%	90%	85%	83%	83.5%	82%
Subtypes: **A** **B** **C** **D** **Other**	100%	14% 0% 32% 36% 18%	100%	98%	96.5%	98%	100%
M184V	81%	64%	50.5%	78%	72%	64.3%	67.2%
NNRTI **K103N** **V106M**	93% 28% 6%	77% 27% 0%	87% 41.6% 23.5%	86% 55% 31%	78% 38% 17%	Unknown 51% 19%	75% 50% 14%
TAMS **>3**	56%	23% 5%	TAMs I 48% TAMs II 59%	23%	11%	32.2%	1.5%
K65R	19%	5%	7.7%	9%	4.5%	2.6%	3%
Q151M	19%	0%	3.25%	Unknown	2.5%	0.9%	0%
NRTI+NNRTI	94%	64%	Unknown	83%	73%	64.3%	63%

Aside from the emergence of HIV-1 drug resistance, toxicity and/or the ability to tolerate ARVs is essential in ensuring successful ARV drug treatment outcomes. This is achieved by ensuring the bioavailability of the ARVs in the treated individual is kept at optimal plasma or intracellular levels. Changes in ARV drug plasma concentration have been linked to either toxicity when there is an increase (Marzolini et al., 2001, Stahle et al., 2004) or viral failure when there is a decrease in the plasma concentration (Langmann et al., 2002, Marzolini et al., 2001, Stahle et al., 2004). Furthermore, studies have shown that the concentration of EFV in plasma can be different between individuals that are prescribed the same fixed dose, thus affecting ARV drug treatment outcome (Gatanaga et al., 2007, Gatanaga and Oka, 2009, van Luin et al., 2009). This variation in ARV drug concentrations has been associated with DNA sequence variants in human genes involved in ARV drug metabolism and absorption.

At the time the CIPRA-SA study was initiated, four SNPs in CYP3A4, CYP3A5, CYP2B6 and MDR-1 had been associated with ARV drug metabolism and absorption (Haas et al., 2004). The NNRTI, EFV is known to be mainly metabolized by CYP2B6, with minor contributions from CYP3A4 and CYP3A5 (Desta et al., 2007, Ward et al., 2003). This study found that the 516TT genotype, linked to altered CYP2B6 function, occurred in a small percentage (13.3%; Table 3.14) of the control population examined and was similar to genotype frequencies observed elsewhere in South Africa (Parathyras et al., 2009). This is similar to other studies, which have shown that the 516TT allele occurs less frequently in African Americans, than European Americans (Ribaudo et al., 2006). Furthermore, the presence of the 516TT genotype has previously been linked to toxicity in patients accessing HAART (Rotger et al., 2005). However, dose optimisation in the presence of this genotype can reduce the central nervous system toxicities (Gatanaga et al., 2007, Gatanaga and Oka, 2009). Univariate analysis of all CIPRA-SA participants to determine if there was a link

between ARV drug treatment outcome and the presence of the 516TT genotype revealed that there was no association between the genotype and viral failure or toxicity. Studies have shown that the 516TT genotype is the major contributor to increased levels of EFV (Haas et al., 2004, Ribaudo et al., 2006, Rodriguez-Novoa et al., 2005, Rotger et al., 2005, Tsuchiya et al., 2004, Wang et al., 2006). To this end, the link between the CIPRA-SA participants accessing an EFV-based regimen and the 516TT genotype was examined. Results showed that there was no link between the 516TT genotype and treatment outcome (toxicity and viral failure) of these participants. It is possible that other SNPs present in enzymes which also metabolise EFV are responsible for treatment outcome in the CIPRA-SA participants. For example, recent studies have shown that CYP2A6 also plays a role in oxidising EFV (di Iulio et al., 2009, Kwara et al., 2009b) and that EFV undergoes glucuronidation metabolism by UGT2B7 (Kwara et al., 2008). Similarly, no association between the genotypes examined for CYP3A4 and CYP3A5 were found. This is similar to findings from another study, which has shown that the G1344A and A6986G SNPs do not link to ARV metabolism (Robertson et al., 2008).

All participants on the CIPRA-SA study were accessing d4T and 3TC as part of the first-line regimen, and as discussed above, no association was found between any of the three metabolising SNPs examined in this study and treatment outcome. This is likely due to the fact that CYP450 does not contribute to metabolism of NRTIs, except AZT (Cretton and Sommadossi, 1993), which was not available in the first-line regimen. However, 3TC and d4T are both activated by a complex process of phosphorylation and intracellular catalysis by kinases (Arner and Eriksson, 1995, Shewach et al., 1993) and 5'nucleotidases (Hunsucker et al., 2005, Zimmermann, 1992) and this study did not examine any SNPs that may alter these enzymes function, since none have been described in the literature. Furthermore, although PIs

are metabolised by CYP450 (and other enzymes), the link between this could not be examined on the CIPRA-SA study as PIs were not prescribed in the first-line regimen, except if patients were pregnant. Thus due to the small numbers, no statistically significant association could be made.

This study investigated one SNP that occurs in the gene MDR-1 (C3435T), which encodes for P-gp, a transporter linked to transportation of ARVs across the cellular membrane (Pan et al., 2007). The 3435TT genotype occurred in the minority of the CIPRA-SA participants and control group examined and is linked to a decreased level of expression on P-gp on the cellular membrane. As this genotype has previously been linked to decreased EFV and nelfinavir cellular concentrations (Fellay et al., 2002), because of a reduced transport across the cellular membrane, the association between this genotype and treatment outcome was examined. No association was found between virological failure and CIPRA-SA participants with the 3435TT genotype. Conversely, the 3435CC genotype has been associated with an increase in cellular EFV concentration and toxicity, however, no association between toxicity and the 3435CC genotype was found in the CIPRA-SA study. Studies have shown that there are over 350 transporters which are linked to the absorption of NRTIs, NNRTIs and PIs (Hediger et al., 2004) and this may be a reason why no association was found. Furthermore, other SNPs in MDR-1 (T129C and G2677A) have recently been linked to CD4+ T-cell recovery rates (Parathyras et al., 2009), which would impact on treatment outcome, however these could not be examined in this study. Future investigations into these other MDR-1 SNPs should be performed as emerging data shows that the CD4+ T-cell recovery rate in South African HIV-1 positive patients is slower than that observed in patients from Europe and the US (Lisgaris et al., 2005).

There are several limitations to the host genetic analyses performed in this study. Firstly, only 4 SNPs were examined and it has now been shown that there are a great number of pharmacogenetic and environmental factors that impact on ARV concentrations and subsequent ARV drug treatment outcome (Telenti and Zanger, 2007). Therefore, genome wide association studies of genetic variations, on microarrays for example, would undoubtedly be more beneficial and allow for linkage between different genetic variants to be made. There are several microarray technologies available which can either be used to detect up to 906600 SNPs in the human genome, or to determine differences in 949000 mRNA expression levels (Affymetrix Genome Wide Human SNP Array 6). Although, no association could be found between the host genetic variations examined in this study, it does not preclude that other SNPs can be associated with ARV drug metabolism and absorption in South African patients. Large scale genome wide association studies examining overall genetic variation and possible associations are needed to minimise the number of failure events due to adverse ARV drug reactions.

Secondly, although the background frequency from each genotype was determined for the adequately sized control South African population, the size of the CIPRA-SA clinical cohort investigated was too small to reach statistical significance because only 248 of the 812 CIPRA-SA participants consented for host genetic studies. This became apparent only after the control group frequencies were established. However, even if the total 812 participants consent for SNP analysis, standard sample size calculations to achieve an 80% power to link the gene to treatment outcome showed this would have been an insufficient number. To achieve an 80% power using a gene frequency of 10%, a tripling in the total CIPRA-SA cohort (n=812) would have been required to ensure an accurate linkage of the SNPs to viral failure or toxicity outcome. Furthermore, the CYP3A4, 3A5 and MDR-1 homozygous

variants occurred in 5.5%, 3.0% and 1.4%, of the background population examined. These frequencies are below the 10% which was used in the standard sample size calculation performed. Similarly, the homozygous variant for CYP2B6 was found to occur in 15.7% of the background population and this would have required a 2.3 increase in the overall CIPRA-SA sample size, which equated to approximately 1868 participants. The background frequencies described here, however, provide a framework for better planned studies in the future.

In conclusion, the study has shown that ARV drug treatment outcome in South Africa is dependent on the combination of time of initiation of treatment, ARV drug regimen chosen, HIV-1 subtype, duration on a failing regimen and individual genetic background. This is similar to other international studies. The higher the CD4+ T-cell count when commencing ARV therapy, the greater the chance of viral suppression. The overall resistance patterns are less complex when viral load is monitored more frequently and viral failure determined at 1000 RNA copies/ml. When viral failure is determined at a lower level, it is likely that patients who virologically fail due to non-adherence will be detected earlier. These patients can be re-counselled to prevent the emergence of ARV drug resistance attributed to non-adherence. This will ensure fewer patients are switched to the more expensive second-line regimen. In addition, the CIPRA-SA cohort has shown that the usage of d4T is linked to high levels of toxicity, and the development of the K65R mutation which impacts on most second-line NRTIs choices. HIV-1 subtype C natural sequence variation causes the unique development of mutations such as K65R and V106M, which result in different levels of ARV drug resistance and may impact on future treatment options. Overall, the results highlight the importance of frequent monitoring of virological parameters such as viral loads and ARV drug resistance in patients on ARV therapy, to preserve future ARV treatment options.

APPENDIX A

APPENDIX A
DIVISION OF AIDS TABLE FOR GRADING THE SEVERITY OF ADULT AND PEDIATRIC ADVERSE EVENTS
VERSION 1.0, DECEMBER, 2004; CLARIFICATION AUGUST 2009

The Division of AIDS Table for Grading the Severity of Adult and Pediatric Adverse Events ("DAIDS AE Grading Table") is a descriptive terminology which can be utilized for Adverse Event (AE) reporting. A grading (severity) scale is provided for each AE term.

This clarification of the DAIDS Table for Grading the Severity of Adult and Pediatric AE's provides additional explanation of the DAIDS AE Grading Table and clarifies some of the parameters.

I. Instructions and Clarifications

Grading Adult and Pediatric AEs

The DAIDS AE Grading Table includes parameters for grading both Adult and Pediatric AEs. When a single set of parameters is not appropriate for grading specific types of AEs for both Adult and Pediatric populations, separate sets of parameters for Adult and/or Pediatric populations (with specified respective age ranges) are given in the Table. If there is no distinction in the Table between Adult and Pediatric values for a type of AE, then the single set of parameters listed is to be used for grading the severity of both Adult and Pediatric events of that type.

> **Note:** In the classification of adverse events, the term "**severe**" is not the same as "**serious**." Severity is an indication of the intensity of a specific event (as in mild, moderate, or severe chest pain). The term "**serious**" relates to a participant/event outcome or action criteria, usually associated with events that pose a threat to a participant's life or functioning.

Addenda 1-3 Grading Tables for Microbicide Studies

For protocols involving topical application of products to the female genital tract, male genital area or rectum, strong consideration should be given to using Appendices I-III as the primary grading scales for these areas. The protocol would need to specifically state that one or more of the Appendices would be primary (and thus take precedence over the main Grading Table) for items that are listed in both the Appendix and the main Grading Table.

- Addendum 1 - Female Genital Grading Table for Use in Microbicide Studies - PDF
- Addendum 2 - Male Genital Grading Table for Use in Microbicide Studies - PDF
- Addendum 3 - Rectal Grading Table for Use in Microbicide Studies - PDF

Grade 5

For any AE where the outcome is death, the severity of the AE is classified as Grade 5.

Estimating Severity Grade for Parameters Not Identified in the Table

In order to grade a clinical AE that is not identified in the DAIDS AE grading table, use the category "Estimating Severity Grade" located on Page 3.

Determining Severity Grade for Parameters "Between Grades"

If the severity of a clinical AE could fall under either one of two grades (e.g., the severity of an AE could be either Grade 2 or Grade 3), select the higher of the two grades for the AE. If a laboratory value that is graded as a multiple of the ULN or LLN falls between two grades, select the higher of the two grades for the AE. For example, Grade 1 is 2.5 x ULN and Grade 2 is 2.6 x ULN for a parameter. If the lab value is 2.53 x ULN (which is between the two grades), the severity of this AE would be Grade 2, the higher of the two grades.

Values Below Grade 1

Any laboratory value that is between either the LLN or ULN and Grade 1 should not be graded.

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Determining Severity Grade when Local Laboratory Normal Values Overlap with Grade 1 Ranges
In these situations, the severity grading is based on the ranges in the DAIDS AE Grading Table, even when there is a reference to the local lab LLN.

For example: Phosphate, Serum, Low, Adult and Pediatric > 14 years (Page 20) Grade 1 range is 2.50 mg/dL - < LLN. A particular laboratory's normal range for Phosphate is 2.1 – 3.8 mg/dL. A participant's actual lab value is 2.5. In this case, the value of 2.5 exceeds the LLN for the local lab, but will be graded as Grade 1 per DAIDS AE Grading Table.

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II. Definitions of terms used in the Table:

Basic Self-care Functions	Adult Activities such as bathing, dressing, toileting, transfer/movement, continence, and feeding. Young Children Activities that are age and culturally appropriate (e.g., feeding self with culturally appropriate eating implement).
LLN	Lower limit of normal
Medical Intervention	Use of pharmacologic or biologic agent(s) for treatment of an AE.
NA	Not Applicable
Operative Intervention	Surgical OR other invasive mechanical procedures.
ULN	Upper limit of normal
Usual Social & Functional Activities	Adult Adaptive tasks and desirable activities, such as going to work, shopping, cooking, use of transportation, pursuing a hobby, etc. Young Children Activities that are age and culturally appropriate (e.g., social interactions, play activities, learning tasks, etc.).

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PARAMETER	GRADE 1 MILD	GRADE 2 MODERATE	GRADE 3 SEVERE	GRADE 4 POTENTIALLY LIFE-THREATENING
ESTIMATING SEVERITY GRADE				
Clinical adverse event NOT identified elsewhere in this DAIDS AE Grading Table	Symptoms causing no or minimal interference with usual social & functional activities	Symptoms causing greater than minimal interference with usual social & functional activities	Symptoms causing inability to perform usual social & functional activities	Symptoms causing inability to perform basic self-care functions OR Medical or operative intervention indicated to prevent permanent impairment, persistent disability, or death
SYSTEMIC				
Acute systemic allergic reaction	Localized urticaria (wheals) with no medical intervention indicated	Localized urticaria with medical intervention indicated OR Mild angioedema with no medical intervention indicated	Generalized urticaria OR Angioedema with medical intervention indicated OR Symptomatic mild bronchospasm	Acute anaphylaxis OR Life-threatening bronchospasm OR laryngeal edema
Chills	Symptoms causing no or minimal interference with usual social & functional activities	Symptoms causing greater than minimal interference with usual social & functional activities	Symptoms causing inability to perform usual social & functional activities	NA
Fatigue Malaise	Symptoms causing no or minimal interference with usual social & functional activities	Symptoms causing greater than minimal interference with usual social & functional activities	Symptoms causing inability to perform usual social & functional activities	Incapacitating fatigue/ malaise symptoms causing inability to perform basic self-care functions
Fever (nonaxillary)	37.7 – 38.6°C	38.7 – 39.3°C	39.4 – 40.5°C	> 40.5°C
Pain (indicate body site) DO NOT use for pain due to injection (See Injection Site Reactions: Injection site pain) See also Headache, Arthralgia, and Myalgia	Pain causing no or minimal interference with usual social & functional activities	Pain causing greater than minimal interference with usual social & functional activities	Pain causing inability to perform usual social & functional activities	Disabling pain causing inability to perform basic self-care functions OR Hospitalization (other than emergency room visit) indicated

Basic Self-care Functions – Adult: Activities such as bathing, dressing, toileting, transfer/movement, continence, and feeding.

Basic Self-care Functions – Young Children: Activities that are age and culturally appropriate (e.g., feeding self with culturally appropriate eating implement).

Usual Social & Functional Activities – Adult: Adaptive tasks and desirable activities, such as going to work, shopping, cooking, use of transportation, pursuing a hobby, etc.

Usual Social & Functional Activities – Young Children: Activities that are age and culturally appropriate (e.g., social interactions, play activities, learning tasks, etc.).

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PARAMETER	GRADE 1 MILD	GRADE 2 MODERATE	GRADE 3 SEVERE	GRADE 4 POTENTIALLY LIFE-THREATENING
Unintentional weight loss	NA	5 – 9% loss in body weight from baseline	10 – 19% loss in body weight from baseline	≥ 20% loss in body weight from baseline OR Aggressive intervention indicated [e.g., tube feeding or total parenteral nutrition (TPN)]
INFECTION				
Infection (any other than HIV infection)	Localized, no systemic antimicrobial treatment indicated AND Symptoms causing no or minimal interference with usual social & functional activities	Systemic antimicrobial treatment indicated OR Symptoms causing greater than minimal interference with usual social & functional activities	Systemic antimicrobial treatment indicated AND Symptoms causing inability to perform usual social & functional activities OR Operative intervention (other than simple incision and drainage) indicated	Life-threatening consequences (e.g., septic shock)
INJECTION SITE REACTIONS				
Injection site pain (pain without touching) Or Tenderness (pain when area is touched)	Pain/tenderness causing no or minimal limitation of use of limb	Pain/tenderness limiting use of limb OR Pain/tenderness causing greater than minimal interference with usual social & functional activities	Pain/tenderness causing inability to perform usual social & functional activities	Pain/tenderness causing inability to perform basic self-care function OR Hospitalization (other than emergency room visit) indicated for management of pain/tenderness
Injection site reaction (localized)				
Adult > 15 years	Erythema OR Induration of 5x5 cm – 9x9 cm (or 25 cm^2 – 81cm^2)	Erythema OR Induration OR Edema > 9 cm any diameter (or > 81 cm^2)	Ulceration OR Secondary infection OR Phlebitis OR Sterile abscess OR Drainage	Necrosis (involving dermis and deeper tissue)
Pediatric ≤ 15 years	Erythema OR Induration OR Edema present but ≤ 2.5 cm diameter	Erythema OR Induration OR Edema > 2.5 cm diameter but < 50% surface area of the extremity segment (e.g., upper arm/thigh)	Erythema OR Induration OR Edema involving ≥ 50% surface area of the extremity segment (e.g., upper arm/thigh) OR Ulceration OR Secondary infection OR Phlebitis OR Sterile abscess OR Drainage	Necrosis (involving dermis and deeper tissue)

Basic Self-care Functions – Adult: Activities such as bathing, dressing, toileting, transfer/movement, continence, and feeding.

Basic Self-care Functions – Young Children: Activities that are age and culturally appropriate (e.g., feeding self with culturally appropriate eating implement).

Usual Social & Functional Activities – Adult: Adaptive tasks and desirable activities, such as going to work, shopping, cooking, use of transportation, pursuing a hobby, etc.

Usual Social & Functional Activities – Young Children: Activities that are age and culturally appropriate (e.g., social interactions, play activities, learning tasks, etc.).

APPENDIX A
DIVISION OF AIDS TABLE FOR GRADING THE SEVERITY OF ADULT AND PEDIATRIC ADVERSE EVENTS
VERSION 1.0, DECEMBER, 2004; CLARIFICATION AUGUST 2009

PARAMETER	GRADE 1 MILD	GRADE 2 MODERATE	GRADE 3 SEVERE	GRADE 4 POTENTIALLY LIFE-THREATENING
Pruritis associated with injection See also Skin: Pruritis (itching - no skin lesions)	Itching localized to injection site AND Relieved spontaneously or with < 48 hours treatment	Itching beyond the injection site but not generalized OR Itching localized to injection site requiring ≥ 48 hours treatment	Generalized itching causing inability to perform usual social & functional activities	NA
SKIN – DERMATOLOGICAL				
Alopecia	Thinning detectable by study participant (or by caregiver for young children and disabled adults)	Thinning or patchy hair loss detectable by health care provider	Complete hair loss	NA
Cutaneous reaction – rash	Localized macular rash	Diffuse macular, maculopapular, or morbilliform rash OR Target lesions	Diffuse macular, maculopapular, or morbilliform rash with vesicles or limited number of bullae OR Superficial ulcerations of mucous membrane limited to one site	Extensive or generalized bullous lesions OR Stevens-Johnson syndrome OR Ulceration of mucous membrane involving two or more distinct mucosal sites OR Toxic epidermal necrolysis (TEN)
Hyperpigmentation	Slight or localized	Marked or generalized	NA	NA
Hypopigmentation	Slight or localized	Marked or generalized	NA	NA
Pruritis (itching – no skin lesions) (See also Injection Site Reactions: Pruritis associated with injection)	Itching causing no or minimal interference with usual social & functional activities	Itching causing greater than minimal interference with usual social & functional activities	Itching causing inability to perform usual social & functional activities	NA
CARDIOVASCULAR				
Cardiac arrhythmia (general) (By ECG or physical exam)	Asymptomatic AND No intervention indicated	Asymptomatic AND Non-urgent medical intervention indicated	Symptomatic, non-life-threatening AND Non-urgent medical intervention indicated	Life-threatening arrhythmia OR Urgent intervention indicated
Cardiac-ischemia/infarction	NA	NA	Symptomatic ischemia (stable angina) OR Testing consistent with ischemia	Unstable angina OR Acute myocardial infarction

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PARAMETER	GRADE 1 MILD	GRADE 2 MODERATE	GRADE 3 SEVERE	GRADE 4 POTENTIALLY LIFE-THREATENING
Hemorrhage (significant acute blood loss)	NA	Symptomatic AND No transfusion indicated	Symptomatic AND Transfusion of ≤ 2 units packed RBCs (for children ≤ 10 cc/kg) indicated	Life-threatening hypotension OR Transfusion of > 2 units packed RBCs (for children > 10 cc/kg) indicated
Hypertension				
Adult > 17 years (with repeat testing at same visit)	140 – 159 mmHg systolic OR 90 – 99 mmHg diastolic	160 – 179 mmHg systolic OR 100 – 109 mmHg diastolic	**≥** 180 mmHg systolic OR **≥** 110 mmHg diastolic	Life-threatening consequences (e.g., malignant hypertension) OR Hospitalization indicated (other than emergency room visit)
Correction: in Grade 2 to 160 - 179 from > 160-179 (systolic) and to ≥ 100 -109 from > 100-109 (diastolic) and in Grade 3 to ≥ 180 from > 180 (systolic) and to ≥ 110 from > 110 (diastolic).				
Pediatric ≤ 17 years (with repeat testing at same visit)	NA	91st – 94th percentile adjusted for age, height, and gender (systolic and/or diastolic)	≥ 95th percentile adjusted for age, height, and gender (systolic and/or diastolic)	Life-threatening consequences (e.g., malignant hypertension) OR Hospitalization indicated (other than emergency room visit)
Hypotension	NA	Symptomatic, corrected with oral fluid replacement	Symptomatic, IV fluids indicated	Shock requiring use of vasopressors or mechanical assistance to maintain blood pressure
Pericardial effusion	Asymptomatic, small effusion requiring no intervention	Asymptomatic, moderate or larger effusion requiring no intervention	Effusion with non-life threatening physiologic consequences OR Effusion with non-urgent intervention indicated	Life-threatening consequences (e.g., tamponade) OR Urgent intervention indicated
Prolonged PR interval				
Adult > 16 years	PR interval 0.21 – 0.25 sec	PR interval > 0.25 sec	Type II 2nd degree AV block OR Ventricular pause > 3.0 sec	Complete AV block
Pediatric ≤ 16 years	1st degree AV block (PR > normal for age and rate)	Type I 2nd degree AV block	Type II 2nd degree AV block	Complete AV block

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PARAMETER	GRADE 1 MILD	GRADE 2 MODERATE	GRADE 3 SEVERE	GRADE 4 POTENTIALLY LIFE-THREATENING
Prolonged QTc				
Adult > 16 years	Asymptomatic, QTc interval 0.45 – 0.47 sec OR Increase interval < 0.03 sec above baseline	Asymptomatic, QTc interval 0.48 – 0.49 sec OR Increase in interval 0.03 – 0.05 sec above baseline	Asymptomatic, QTc interval ≥ 0.50 sec OR Increase in interval ≥ 0.06 sec above baseline	Life-threatening consequences, e.g. Torsade de pointes or other associated serious ventricular dysrhythmia
Pediatric ≤ 16 years	Asymptomatic, QTc interval 0.450 – 0.464 sec	Asymptomatic, QTc interval 0.465 – 0.479 sec	Asymptomatic, QTc interval ≥ 0.480 sec	Life-threatening consequences, e.g. Torsade de pointes or other associated serious ventricular dysrhythmia
Thrombosis/embolism	NA	Deep vein thrombosis AND No intervention indicated (e.g., anticoagulation, lysis filter, invasive procedure)	Deep vein thrombosis AND Intervention indicated (e.g., anticoagulation, lysis filter, invasive procedure)	Embolic event (e.g., pulmonary embolism, life-threatening thrombus)
Vasovagal episode (associated with a procedure of any kind)	Present without loss of consciousness	Present with transient loss of consciousness	NA	NA
Ventricular dysfunction (congestive heart failure)	NA	Asymptomatic diagnostic finding AND intervention indicated	New onset with symptoms OR Worsening symptomatic congestive heart failure	Life-threatening congestive heart failure
GASTROINTESTINAL				
Anorexia	Loss of appetite without decreased oral intake	Loss of appetite associated with decreased oral intake without significant weight loss	Loss of appetite associated with significant weight loss	Life-threatening consequences OR Aggressive intervention indicated [e.g., tube feeding or total parenteral nutrition (TPN)]
Comment: Please note that, while the grading scale provided for Unintentional Weight Loss may be used as a guideline when grading anorexia, this is not a requirement and should not be used as a substitute for clinical judgment.				
Ascites	Asymptomatic	Symptomatic AND Intervention indicated (e.g., diuretics or therapeutic paracentesis)	Symptomatic despite intervention	Life-threatening consequences

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PARAMETER	GRADE 1 MILD	GRADE 2 MODERATE	GRADE 3 SEVERE	GRADE 4 POTENTIALLY LIFE-THREATENING
Cholecystitis	NA	Symptomatic AND Medical intervention indicated	Radiologic, endoscopic, or operative intervention indicated	Life-threatening consequences (e.g., sepsis or perforation)
Constipation	NA	Persistent constipation requiring regular use of dietary modifications, laxatives, or enemas	Obstipation with manual evacuation indicated	Life-threatening consequences (e.g., obstruction)
Diarrhea				
Adult and Pediatric ≥ 1 year	Transient or intermittent episodes of unformed stools OR Increase of ≤ 3 stools over baseline per 24-hour period	Persistent episodes of unformed to watery stools OR Increase of 4 – 6 stools over baseline per 24-hour period	Bloody diarrhea OR Increase of ≥ 7 stools per 24-hour period OR IV fluid replacement indicated	Life-threatening consequences (e.g., hypotensive shock)
Pediatric < 1 year	Liquid stools (more unformed than usual) but usual number of stools	Liquid stools with increased number of stools OR Mild dehydration	Liquid stools with moderate dehydration	Liquid stools resulting in severe dehydration with aggressive rehydration indicated OR Hypotensive shock
Dysphagia-Odynophagia	Symptomatic but able to eat usual diet	Symptoms causing altered dietary intake without medical intervention indicated	Symptoms causing severely altered dietary intake with medical intervention indicated	Life-threatening reduction in oral intake
Mucositis/stomatitis (clinical exam) Indicate site (e.g., larynx, oral) See Genitourinary for Vulvovaginitis See also Dysphagia-Odynophagia and Proctitis	Erythema of the mucosa	Patchy pseudomembranes or ulcerations	Confluent pseudomembranes or ulcerations OR Mucosal bleeding with minor trauma	Tissue necrosis OR Diffuse spontaneous mucosal bleeding OR Life-threatening consequences (e.g., aspiration, choking)
Nausea	Transient (< 24 hours) or intermittent nausea with no or minimal interference with oral intake	Persistent nausea resulting in decreased oral intake for 24 – 48 hours	Persistent nausea resulting in minimal oral intake for > 48 hours OR Aggressive rehydration indicated (e.g., IV fluids)	Life-threatening consequences (e.g., hypotensive shock)

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PARAMETER	GRADE 1 MILD	GRADE 2 MODERATE	GRADE 3 SEVERE	GRADE 4 POTENTIALLY LIFE-THREATENING
Pancreatitis	NA	Symptomatic AND Hospitalization not indicated (other than emergency room visit)	Symptomatic AND Hospitalization indicated (other than emergency room visit)	Life-threatening consequences (e.g., circulatory failure, hemorrhage, sepsis)
Proctitis (functional-symptomatic) Also see Mucositis/stomatitis for clinical exam	Rectal discomfort AND No intervention indicated	Symptoms causing greater than minimal interference with usual social & functional activities OR Medical intervention indicated	Symptoms causing inability to perform usual social & functional activities OR Operative intervention indicated	Life-threatening consequences (e.g., perforation)
Vomiting	Transient or intermittent vomiting with no or minimal interference with oral intake	Frequent episodes of vomiting with no or mild dehydration	Persistent vomiting resulting in orthostatic hypotension OR Aggressive rehydration indicated (e.g., IV fluids)	Life-threatening consequences (e.g., hypotensive shock)
NEUROLOGIC				
Alteration in personality-behavior or in mood (e.g., agitation, anxiety, depression, mania, psychosis)	Alteration causing no or minimal interference with usual social & functional activities	Alteration causing greater than minimal interference with usual social & functional activities	Alteration causing inability to perform usual social & functional activities	Behavior potentially harmful to self or others (e.g., suicidal and homicidal ideation or attempt, acute psychosis) OR Causing inability to perform basic self-care functions
Altered Mental Status For Dementia, see Cognitive and behavioral/attentional disturbance (including dementia and attention deficit disorder)	Changes causing no or minimal interference with usual social & functional activities	Mild lethargy or somnolence causing greater than minimal interference with usual social & functional activities	Confusion, memory impairment, lethargy, or somnolence causing inability to perform usual social & functional activities	Delirium OR obtundation, OR coma
Ataxia	Asymptomatic ataxia detectable on exam OR Minimal ataxia causing no or minimal interference with usual social & functional activities	Symptomatic ataxia causing greater than minimal interference with usual social & functional activities	Symptomatic ataxia causing inability to perform usual social & functional activities	Disabling ataxia causing inability to perform basic self-care functions

Basic Self-care Functions – Adult: Activities such as bathing, dressing, toileting, transfer/movement, continence, and feeding.

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PARAMETER	GRADE 1 MILD	GRADE 2 MODERATE	GRADE 3 SEVERE	GRADE 4 POTENTIALLY LIFE-THREATENING
Cognitive and behavioral/attentional disturbance (including dementia and attention deficit disorder)	Disability causing no or minimal interference with usual social & functional activities OR Specialized resources not indicated	Disability causing greater than minimal interference with usual social & functional activities OR Specialized resources on part-time basis indicated	Disability causing inability to perform usual social & functional activities OR Specialized resources on a full-time basis indicated	Disability causing inability to perform basic self-care functions OR Institutionalization indicated
CNS ischemia (acute)	NA	NA	Transient ischemic attack	Cerebral vascular accident (CVA, stroke) with neurological deficit
Developmental delay – **Pediatric ≤ 16 years**	Mild developmental delay, either motor or cognitive, as determined by comparison with a developmental screening tool appropriate for the setting	Moderate developmental delay, either motor or cognitive, as determined by comparison with a developmental screening tool appropriate for the setting	Severe developmental delay, either motor or cognitive, as determined by comparison with a developmental screening tool appropriate for the setting	Developmental regression, either motor or cognitive, as determined by comparison with a developmental screening tool appropriate for the setting
Headache	Symptoms causing no or minimal interference with usual social & functional activities	Symptoms causing greater than minimal interference with usual social & functional activities	Symptoms causing inability to perform usual social & functional activities	Symptoms causing inability to perform basic self-care functions OR Hospitalization indicated (other than emergency room visit) OR Headache with significant impairment of alertness or other neurologic function
Insomnia	NA	Difficulty sleeping causing greater than minimal interference with usual social & functional activities	Difficulty sleeping causing inability to perform usual social & functional activities	Disabling insomnia causing inability to perform basic self-care functions
Neuromuscular weakness (including myopathy & neuropathy)	Asymptomatic with decreased strength on exam OR Minimal muscle weakness causing no or minimal interference with usual social & functional activities	Muscle weakness causing greater than minimal interference with usual social & functional activities	Muscle weakness causing inability to perform usual social & functional activities	Disabling muscle weakness causing inability to perform basic self-care functions OR Respiratory muscle weakness impairing ventilation

Basic Self-care Functions – Adult: Activities such as bathing, dressing, toileting, transfer/movement, continence, and feeding.

Basic Self-care Functions – Young Children: Activities that are age and culturally appropriate (e.g., feeding self with culturally appropriate eating implement).

Usual Social & Functional Activities – Adult: Adaptive tasks and desirable activities, such as going to work, shopping, cooking, use of transportation, pursuing a hobby, etc.

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PARAMETER	GRADE 1 MILD	GRADE 2 MODERATE	GRADE 3 SEVERE	GRADE 4 POTENTIALLY LIFE-THREATENING
Neurosensory alteration (including paresthesia and painful neuropathy)	Asymptomatic with sensory alteration on exam or minimal paresthesia causing no or minimal interference with usual social & functional activities	Sensory alteration or paresthesia causing greater than minimal interference with usual social & functional activities	Sensory alteration or paresthesia causing inability to perform usual social & functional activities	Disabling sensory alteration or paresthesia causing inability to perform basic self-care functions
Seizure: (new onset) **– Adult ≥ 18 years** See also Seizure: (known pre-existing seizure disorder)	NA	1 seizure	2 – 4 seizures	Seizures of any kind which are prolonged, repetitive (e.g., status epilepticus), or difficult to control (e.g., refractory epilepsy)
Seizure: (known pre-existing seizure disorder) **– Adult ≥ 18 years** For worsening of existing epilepsy the grades should be based on an increase from previous level of control to any of these levels.	NA	Increased frequency of pre-existing seizures (non-repetitive) without change in seizure character OR Infrequent break-through seizures while on stable medication in a previously controlled seizure disorder	Change in seizure character from baseline either in duration or quality (e.g., severity or focality)	Seizures of any kind which are prolonged, repetitive (e.g., status epilepticus), or difficult to control (e.g., refractory epilepsy)
Seizure **– Pediatric < 18 years**	Seizure, generalized onset with or without secondary generalization, lasting < 5 minutes with < 24 hours post ictal state	Seizure, generalized onset with or without secondary generalization, lasting 5 – 20 minutes with < 24 hours post ictal state	Seizure, generalized onset with or without secondary generalization, lasting > 20 minutes	Seizure, generalized onset with or without secondary generalization, requiring intubation and sedation
Syncope (not associated with a procedure)	NA	Present	NA	NA
Vertigo	Vertigo causing no or minimal interference with usual social & functional activities	Vertigo causing greater than minimal interference with usual social & functional activities	Vertigo causing inability to perform usual social & functional activities	Disabling vertigo causing inability to perform basic self-care functions

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Basic Self-care Functions – Young Children: Activities that are age and culturally appropriate (e.g., feeding self with culturally appropriate eating implement).

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RESPIRATORY				
Bronchospasm (acute)	FEV1 or peak flow reduced to 70 – 80%	FEV1 or peak flow 50 – 69%	FEV1 or peak flow 25 – 49%	Cyanosis OR FEV1 or peak flow < 25% OR Intubation
Dyspnea or respiratory distress				
Adult ≥ 14 years	Dyspnea on exertion with no or minimal interference with usual social & functional activities	Dyspnea on exertion causing greater than minimal interference with usual social & functional activities	Dyspnea at rest causing inability to perform usual social & functional activities	Respiratory failure with ventilatory support indicated
Pediatric < 14 years	Wheezing OR minimal increase in respiratory rate for age	Nasal flaring OR Intercostal retractions OR Pulse oximetry 90 – 95%	Dyspnea at rest causing inability to perform usual social & functional activities OR Pulse oximetry < 90%	Respiratory failure with ventilatory support indicated
MUSCULOSKELETAL				
Arthralgia See also Arthritis	Joint pain causing no or minimal interference with usual social & functional activities	Joint pain causing greater than minimal interference with usual social & functional activities	Joint pain causing inability to perform usual social & functional activities	Disabling joint pain causing inability to perform basic self-care functions
Arthritis See also Arthralgia	Stiffness or joint swelling causing no or minimal interference with usual social & functional activities	Stiffness or joint swelling causing greater than minimal interference with usual social & functional activities	Stiffness or joint swelling causing inability to perform usual social & functional activities	Disabling joint stiffness or swelling causing inability to perform basic self-care functions
Bone Mineral Loss				
Adult ≥ 21 years	BMD t-score -2.5 to -1.0	BMD t-score < -2.5	Pathological fracture (including loss of vertebral height)	Pathologic fracture causing life-threatening consequences
Pediatric < 21 years	BMD z-score -2.5 to -1.0	BMD z-score < -2.5	Pathological fracture (including loss of vertebral height)	Pathologic fracture causing life-threatening consequences
Myalgia (non-injection site)	Muscle pain causing no or minimal interference with usual social & functional activities	Muscle pain causing greater than minimal interference with usual social & functional activities	Muscle pain causing inability to perform usual social & functional activities	Disabling muscle pain causing inability to perform basic self-care functions

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Osteonecrosis	NA	Asymptomatic with radiographic findings AND No operative intervention indicated	Symptomatic bone pain with radiographic findings OR Operative intervention indicated	Disabling bone pain with radiographic findings causing inability to perform basic self-care functions
GENITOURINARY				
Cervicitis (symptoms) (For use in studies evaluating topical study agents) For other cervicitis see Infection: Infection (any other than HIV infection)	Symptoms causing no or minimal interference with usual social & functional activities	Symptoms causing greater than minimal interference with usual social & functional activities	Symptoms causing inability to perform usual social & functional activities	Symptoms causing inability to perform basic self-care functions
Cervicitis (clinical exam) (For use in studies evaluating topical study agents) For other cervicitis see Infection: Infection (any other than HIV infection)	Minimal cervical abnormalities on examination (erythema, mucopurulent discharge, or friability) OR Epithelial disruption < 25% of total surface	Moderate cervical abnormalities on examination (erythema, mucopurulent discharge, or friability) OR Epithelial disruption of 25 – 49% total surface	Severe cervical abnormalities on examination (erythema, mucopurulent discharge, or friability) OR Epithelial disruption 50 – 75% total surface	Epithelial disruption > 75% total surface
Inter-menstrual bleeding (IMB)	Spotting observed by participant OR Minimal blood observed during clinical or colposcopic examination	Inter-menstrual bleeding not greater in duration or amount than usual menstrual cycle	Inter-menstrual bleeding greater in duration or amount than usual menstrual cycle	Hemorrhage with life-threatening hypotension OR Operative intervention indicated
Urinary tract obstruction (e.g., stone)	NA	Signs or symptoms of urinary tract obstruction without hydronephrosis or renal dysfunction	Signs or symptoms of urinary tract obstruction with hydronephrosis or renal dysfunction	Obstruction causing life-threatening consequences

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Vulvovaginitis (symptoms) (Use in studies evaluating topical study agents) For other vulvovaginitis see Infection: Infection (any other than HIV infection)	Symptoms causing no or minimal interference with usual social & functional activities	Symptoms causing greater than minimal interference with usual social & functional activities	Symptoms causing inability to perform usual social & functional activities	Symptoms causing inability to perform basic self-care functions
Vulvovaginitis (clinical exam) (Use in studies evaluating topical study agents) For other vulvovaginitis see Infection: Infection (any other than HIV infection)	Minimal vaginal abnormalities on examination OR Epithelial disruption < 25% of total surface	Moderate vaginal abnormalities on examination OR Epithelial disruption of 25 - 49% total surface	Severe vaginal abnormalities on examination OR Epithelial disruption 50 - 75% total surface	Vaginal perforation OR Epithelial disruption > 75% total surface
OCULAR/VISUAL				
Uveitis	Asymptomatic but detectable on exam	Symptomatic anterior uveitis OR Medical intervention indicated	Posterior or pan-uveitis OR Operative intervention indicated	Disabling visual loss in affected eye(s)
Visual changes (from baseline)	Visual changes causing no or minimal interference with usual social & functional activities	Visual changes causing greater than minimal interference with usual social & functional activities	Visual changes causing inability to perform usual social & functional activities	Disabling visual loss in affected eye(s)
ENDOCRINE/METABOLIC				
Abnormal fat accumulation (e.g., back of neck, breasts, abdomen)	Detectable by study participant (or by caregiver for young children and disabled adults)	Detectable on physical exam by health care provider	Disfiguring OR Obvious changes on casual visual inspection	NA

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Diabetes mellitus	NA	New onset without need to initiate medication OR Modification of current medications to regain glucose control	New onset with initiation of medication indicated OR Diabetes uncontrolled despite treatment modification	Life-threatening consequences (e.g., ketoacidosis, hyperosmolar non-ketotic coma)
Gynecomastia	Detectable by study participant or caregiver (for young children and disabled adults)	Detectable on physical exam by health care provider	Disfiguring OR Obvious on casual visual inspection	NA
Hyperthyroidism	Asymptomatic	Symptomatic causing greater than minimal interference with usual social & functional activities OR Thyroid suppression therapy indicated	Symptoms causing inability to perform usual social & functional activities OR Uncontrolled despite treatment modification	Life-threatening consequences (e.g., thyroid storm)
Hypothyroidism	Asymptomatic	Symptomatic causing greater than minimal interference with usual social & functional activities OR Thyroid replacement therapy indicated	Symptoms causing inability to perform usual social & functional activities OR Uncontrolled despite treatment modification	Life-threatening consequences (e.g., myxedema coma)
Lipoatrophy (e.g., fat loss from the face, extremities, buttocks)	Detectable by study participant (or by caregiver for young children and disabled adults)	Detectable on physical exam by health care provider	Disfiguring OR Obvious on casual visual inspection	NA

Basic Self-care Functions – Adult: Activities such as bathing, dressing, toileting, transfer/movement, continence, and feeding.

Basic Self-care Functions – Young Children: Activities that are age and culturally appropriate (e.g., feeding self with culturally appropriate eating implement).

Usual Social & Functional Activities – Adult: Adaptive tasks and desirable activities, such as going to work, shopping, cooking, use of transportation, pursuing a hobby, etc.

Usual Social & Functional Activities – Young Children: Activities that are age and culturally appropriate (e.g., social interactions, play activities, learning tasks, etc.).

APPENDIX A
DIVISION OF AIDS TABLE FOR GRADING THE SEVERITY OF ADULT AND PEDIATRIC ADVERSE EVENTS
VERSION 1.0, DECEMBER, 2004; CLARIFICATION AUGUST 2009

LABORATORY				
PARAMETER	**GRADE 1 MILD**	**GRADE 2 MODERATE**	**GRADE 3 SEVERE**	**GRADE 4 POTENTIALLY LIFE-THREATENING**
HEMATOLOGY	***Standard International Units are listed in italics***			
Absolute CD4+ count **– Adult and Pediatric > 13 years** (HIV NEGATIVE ONLY)	300 – 400/mm^3 *300 – 400/µL*	200 – 299/mm^3 *200 – 299/µL*	100 – 199/mm^3 *100 – 199/µL*	< 100/mm^3 *< 100/µL*
Absolute lymphocyte count **– Adult and Pediatric > 13 years** (HIV NEGATIVE ONLY)	600 – 650/mm^3 *0.600 x 10^9 – 0.650 x 10^9/L*	500 – 599/mm^3 *0.500 x 10^9 – 0.599 x 10^9/L*	350 – 499/mm^3 *0.350 x 10^9 – 0.499 x 10^9/L*	< 350/mm^3 *< 0.350 x 10^9/L*
Comment: Values in children ≤ 13 years are not given for the two parameters above because the absolute counts are variable.				
Absolute neutrophil count (ANC)				
Adult and Pediatric, > 7 days	1,000 – 1,300/mm^3 *1.000 x 10^9 – 1.300 x 10^9/L*	750 – 999/mm^3 *0.750 x 10^9 – 0.999 x 10^9/L*	500 – 749/mm^3 *0.500 x 10^9 – 0.749 x 10^9/L*	< 500/mm^3 *< 0.500 x 10^9/L*
Infant*†, 2 – ≤ 7 days	1,250 – 1,500/mm^3 *1.250 x 10^9 – 1.500 x 10^9/L*	1,000 – 1,249/mm^3 *1.000 x 10^9 – 1.249 x 10^9/L*	750 – 999/mm^3 *0.750 x 10^9 – 0.999 x 10^9/L*	< 750/mm^3 *< 0.750 x 10^9/L*
Infant*†, ≤1 day	4,000 – 5,000/mm^3 *4.000 x 10^9 – 5.000 x 10^9/L*	3,000 – 3,999/mm^3 *3.000 x 10^9 – 3.999 x10^9/L*	1,500 – 2,999/mm^3 *1.500 x 10^9 – 2.999 x 10^9/L*	< 1,500/mm^3 *< 1.500 x 10^9/L*
Comment: Parameter changed from "Infant, < 1 day" to "Infant, ≤1 day"				
Fibrinogen, decreased	100 – 200 mg/dL *1.00 – 2.00 g/L* OR 0.75 – 0.99 x LLN	75 – 99 mg/dL *0.75 – 0.99 g/L* OR 0.50 – 0.74 x LLN	50 – 74 mg/dL *0.50 – 0.74 g/L* OR 0.25 – 0.49 x LLN	< 50 mg/dL *< 0.50 g/L* OR < 0.25 x LLN OR Associated with gross bleeding

* Values are for term infants. Preterm infants should be assessed using local normal ranges.

† Use age and sex appropriate values (e.g., bilirubin).

APPENDIX A
DIVISION OF AIDS TABLE FOR GRADING THE SEVERITY OF ADULT AND PEDIATRIC ADVERSE EVENTS
VERSION 1.0, DECEMBER, 2004; CLARIFICATION AUGUST 2009

LABORATORY				
PARAMETER	**GRADE 1 MILD**	**GRADE 2 MODERATE**	**GRADE 3 SEVERE**	**GRADE 4 POTENTIALLY LIFE-THREATENING**
Hemoglobin (Hgb)				
Comment: The Hgb values in mmol/L have changed because the conversion factor used to convert g/dL to mmol/L has been changed from 0.155 to 0.6206 (the most commonly used conversion factor). For grading Hgb results obtained by an analytic method with a conversion factor other than 0.6206, the result must be converted to g/dL using the appropriate conversion factor for that lab.				
Adult and Pediatric ≥ 57 days (HIV POSITIVE ONLY)	8.5 – 10.0 g/dL *5.24 – 6.23 mmol/L*	7.5 – 8.4 g/dL *4.62–5.23 mmol/L*	6.50 – 7.4 g/dL *4.03–4.61 mmol/L*	< 6.5 g/dL *< 4.03 mmol/L*
Adult and Pediatric ≥ 57 days (HIV NEGATIVE ONLY)	10.0 – 10.9 g/dL *6.18 – 6.79 mmol/L* OR Any decrease 2.5 – 3.4 g/dL *1.58 – 2.13 mmol/L*	9.0 – 9.9 g/dL *5.55 - 6.17 mmol/L* OR Any decrease 3.5 – 4.4 g/dL *2.14 – 2.78 mmol/L*	7.0 – 8.9 g/dL *4.34 - 5.54 mmol/L* OR Any decrease ≥ 4.5 g/dL *> 2.79 mmol/L*	< 7.0 g/dL *< 4.34 mmol/L*
Comment: The decrease is a decrease from baseline				
Infant*†, 36 – 56 days (HIV POSITIVE OR NEGATIVE)	8.5 – 9.4 g/dL *5.24 – 5.86 mmol/L*	7.0 – 8.4 g/dL *4.31 – 5.23 mmol/L*	6.0 – 6.9 g/dL *3.72 – 4.30 mmol/L*	< 6.00 g/dL *< 3.72 mmol/L*
Infant*†, 22 – 35 days (HIV POSITIVE OR NEGATIVE)	9.5 – 10.5 g/dL *5.87 - 6.54 mmol/L*	8.0 – 9.4 g/dL *4.93 – 5.86 mmol/L*	7.0 – 7.9 g/dL *4.34 – 4.92 mmol/L*	< 7.00 g/dL *< 4.34 mmol/L*
Infant*†, ≤ 21 days (HIV POSITIVE OR NEGATIVE)	12.0 – 13.0 g/dL *7.42 – 8.09 mmol/L*	10.0 – 11.9 g/dL *6.18 – 7.41 mmol/L*	9.0 – 9.9 g/dL *5.59- 6.17 mmol/L*	< 9.0 g/dL *< 5.59 mmol/L*
Correction: Parameter changed from "Infant < 21 days" to "Infant ≤ 21 days"				
International Normalized Ratio of prothrombin time (INR)	1.1 – 1.5 x ULN	1.6 – 2.0 x ULN	2.1 – 3.0 x ULN	> 3.0 x ULN
Methemoglobin	5.0 – 10.0%	10.1 – 15.0%	15.1 – 20.0%	> 20.0%
Prothrombin Time (PT)	1.1 – 1.25 x ULN	1.26 – 1.50 x ULN	1.51 – 3.00 x ULN	> 3.00 x ULN
Partial Thromboplastin Time (PTT)	1.1 – 1.66 x ULN	1.67 – 2.33 x ULN	2.34 – 3.00 x ULN	> 3.00 x ULN
Platelets, decreased	100,000 – 124,999/mm^3 *100.000 x 10^9 – 124.999 x 10^9/L*	50,000 – 99,999/mm^3 *50.000 x 10^9 – 99.999 x 10^9/L*	25,000 – 49,999/mm^3 *25.000 x 10^9 – 49.999 x 10^9/L*	< 25,000/mm^3 *< 25.000 x 10^9/L*
WBC, decreased	2,000 – 2,500/mm^3 *2.000 x 10^9 – 2.500 x 10^9/L*	1,500 – 1,999/mm^3 *1.500 x 10^9 – 1.999 x 10^9/L*	1,000 – 1,499/mm^3 *1.000 x 10^9 – 1.499 x 10^9/L*	< 1,000/mm^3 *< 1.000 x 10^9/L*

* Values are for term infants. Preterm infants should be assessed using local normal ranges.

† Use age and sex appropriate values (e.g., bilirubin).

APPENDIX A
DIVISION OF AIDS TABLE FOR GRADING THE SEVERITY OF ADULT AND PEDIATRIC ADVERSE EVENTS
VERSION 1.0, DECEMBER, 2004; CLARIFICATION AUGUST 2009

LABORATORY				
PARAMETER	**GRADE 1 MILD**	**GRADE 2 MODERATE**	**GRADE 3 SEVERE**	**GRADE 4 POTENTIALLY LIFE-THREATENING**
CHEMISTRIES	***Standard International Units are listed in italics***			
Acidosis	NA	pH < normal, but ≥ 7.3	pH < 7.3 without life-threatening consequences	pH < 7.3 with life-threatening consequences
Albumin, serum, low	3.0 g/dL – < LLN *30 g/L – < LLN*	2.0 – 2.9 g/dL *20 – 29 g/L*	< 2.0 g/dL *< 20 g/L*	NA
Alkaline Phosphatase	1.25 – 2.5 x ULN†	2.6 – 5.0 x ULN†	5.1 – 10.0 x ULN†	> 10.0 x ULN†
Alkalosis	NA	pH > normal, but ≤ 7.5	pH > 7.5 without life-threatening consequences	pH > 7.5 with life-threatening consequences
ALT (SGPT)	1.25 – 2.5 x ULN	2.6 – 5.0 x ULN	5.1 – 10.0 x ULN	> 10.0 x ULN
AST (SGOT)	1.25 – 2.5 x ULN	2.6 – 5.0 x ULN	5.1 – 10.0 x ULN	> 10.0 x ULN
Bicarbonate, serum, low	16.0 mEq/L – < LLN *16.0 mmol/L – < LLN*	11.0 – 15.9 mEq/L *11.0 – 15.9 mmol/L*	8.0 – 10.9 mEq/L *8.0 – 10.9 mmol/L*	< 8.0 mEq/L *< 8.0 mmol/L*
Comment: Some laboratories will report this value as Bicarbonate (HCO_3) and others as Total Carbon Dioxide (CO_2). These are the same tests; values should be graded according to the ranges for Bicarbonate as listed above.				
Bilirubin (Total)				
Adult and Pediatric > 14 days	1.1 – 1.5 x ULN	1.6 – 2.5 x ULN	2.6 – 5.0 x ULN	> 5.0 x ULN
Infant*†, ≤ 14 days (non-hemolytic)	NA	20.0 – 25.0 mg/dL *342 – 428 µmol/L*	25.1 – 30.0 mg/dL *429 – 513 µmol/L*	> 30.0 mg/dL *> 513.0 µmol/L*
Infant*†, ≤ 14 days (hemolytic)	NA	NA	20.0 – 25.0 mg/dL *342 – 428 µmol/L*	> 25.0 mg/dL *> 428 µmol/L*
Calcium, serum, high				
Adult and Pediatric ≥ 7 days	10.6 – 11.5 mg/dL *2.65 – 2.88 mmol/L*	11.6 – 12.5 mg/dL *2.89 – 3.13 mmol/L*	12.6 – 13.5 mg/dL *3.14 – 3.38 mmol/L*	> 13.5 mg/dL *> 3.38 mmol/L*
Infant*†, < 7 days	11.5 – 12.4 mg/dL *2.88 – 3.10 mmol/L*	12.5 – 12.9 mg/dL *3.11 – 3.23 mmol/L*	13.0 – 13.5 mg/dL *3.245 – 3.38 mmol/L*	> 13.5 mg/dL *> 3.38 mmol/L*
Calcium, serum, low				
Adult and Pediatric ≥ 7 days	7.8 – 8.4 mg/dL *1.95 – 2.10 mmol/L*	7.0 – 7.7 mg/dL *1.75 – 1.94 mmol/L*	6.1 – 6.9 mg/dL *1.53 – 1.74 mmol/L*	< 6.1 mg/dL *< 1.53 mmol/L*
Infant*†, < 7 days	6.5 – 7.5 mg/dL *1.63 – 1.88 mmol/L*	6.0 – 6.4 mg/dL *1.50 – 1.62 mmol/L*	5.50 – 5.90 mg/dL *1.38 – 1.51 mmol/L*	< 5.50 mg/dL *< 1.38 mmol/L*
Comment: Do not adjust Calcium, serum, low or Calcium, serum, high for albumin				

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APPENDIX A
DIVISION OF AIDS TABLE FOR GRADING THE SEVERITY OF ADULT AND PEDIATRIC ADVERSE EVENTS
VERSION 1.0, DECEMBER, 2004; CLARIFICATION AUGUST 2009

LABORATORY					
PARAMETER		**GRADE 1 MILD**	**GRADE 2 MODERATE**	**GRADE 3 SEVERE**	**GRADE 4 POTENTIALLY LIFE-THREATENING**
Cardiac troponin I (cTnI)		NA	NA	NA	Levels consistent with myocardial infarction or unstable angina as defined by the manufacturer
Cardiac troponin T (cTnT)		NA	NA	NA	≥ 0.20 ng/mL OR Levels consistent with myocardial infarction or unstable angina as defined by the manufacturer
Cholesterol (fasting)					
	Adult ≥ 18 years	200 – 239 mg/dL *5.18 – 6.19 mmol/L*	240 – 300 mg/dL *6.20 – 7.77 mmol/L*	> 300 mg/dL *> 7.77 mmol/L*	NA
	Pediatric < 18 years	170 – 199 mg/dL *4.40 – 5.15 mmol/L*	200 – 300 mg/dL *5.16 – 7.77 mmol/L*	> 300 mg/dL *> 7.77 mmol/L*	NA
Creatine Kinase		3.0 – 5.9 x ULN†	6.0 – 9.9 x ULN†	10.0 – 19.9 x ULN†	≥ 20.0 x ULN†
Creatinine		1.1 – 1.3 x ULN†	1.4 – 1.8 x ULN†	1.9 – 3.4 x ULN†	≥ 3.5 x ULN†

LABORATORY					
PARAMETER		**GRADE 1 MILD**	**GRADE 2 MODERATE**	**GRADE 3 SEVERE**	**GRADE 4 POTENTIALLY LIFE-THREATENING**
Glucose, serum, high					
	Nonfasting	116 – 160 mg/dL *6.44 – 8.88 mmol/L*	161 – 250 mg/dL *8.89 – 13.88 mmol/L*	251 – 500 mg/dL *13.89 – 27.75 mmol/L*	> 500 mg/dL *> 27.75 mmol/L*
	Fasting	110 – 125 mg/dL *6.11 – 6.94 mmol/L*	126 – 250 mg/dL *6.95 – 13.88 mmol/L*	251 – 500 mg/dL *13.89 – 27.75 mmol/L*	> 500 mg/dL *> 27.75 mmol/L*
Glucose, serum, low					
	Adult and Pediatric ≥ 1 month	55 – 64 mg/dL *3.05 – 3.55 mmol/L*	40 – 54 mg/dL *2.22 – 3.06 mmol/L*	30 – 39 mg/dL *1.67 – 2.23 mmol/L*	< 30 mg/dL *< 1.67 mmol/L*
	Infant*†, < 1 month	50 – 54 mg/dL *2.78 – 3.00 mmol/L*	40 – 49 mg/dL *2.22 – 2.77 mmol/L*	30 – 39 mg/dL *1.67 – 2.21 mmol/L*	< 30 mg/dL *< 1.67 mmol/L*

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† Use age and sex appropriate values (e.g., bilirubin).

APPENDIX A
DIVISION OF AIDS TABLE FOR GRADING THE SEVERITY OF ADULT AND PEDIATRIC ADVERSE EVENTS
VERSION 1.0, DECEMBER, 2004; CLARIFICATION AUGUST 2009

Lactate		ULN - < 2.0 x ULN without acidosis	≥ 2.0 x ULN without acidosis	Increased lactate with pH < 7.3 without life-threatening consequences	Increased lactate with pH < 7.3 with life-threatening consequences
Comment: Added ULN to Grade 1 parameter					
LDL cholesterol (fasting)					
	Adult ≥ 18 years	130 – 159 mg/dL *3.37 – 4.12 mmol/L*	160 – 190 mg/dL *4.13 – 4.90 mmol/L*	≥ 190 mg/dL *≥ 4.91 mmol/L*	NA
	Pediatric > 2 - < 18 years	110 – 129 mg/dL *2.85 – 3.34 mmol/L*	130 – 189 mg/dL *3.35 – 4.90 mmol/L*	≥ 190 mg/dL *≥ 4.91 mmol/L*	NA
Lipase		1.1 – 1.5 x ULN	1.6 – 3.0 x ULN	3.1 – 5.0 x ULN	> 5.0 x ULN
Magnesium, serum, low		1.2 – 1.4 mEq/L *0.60 – 0.70 mmol/L*	0.9 – 1.1 mEq/L *0.45 – 0.59 mmol/L*	0.6 – 0.8 mEq/L *0.30 – 0.44 mmol/L*	< 0.60 mEq/L *< 0.30 mmol/L*
Pancreatic amylase		1.1 – 1.5 x ULN	1.6 – 2.0 x ULN	2.1 – 5.0 x ULN	> 5.0 x ULN
Phosphate, serum, low					
	Adult and Pediatric > 14 years	2.5 mg/dL – < LLN *0.81 mmol/L – < LLN*	2.0 – 2.4 mg/dL *0.65 – 0.80 mmol/L*	1.0 – 1.9 mg/dL *0.32 – 0.64 mmol/L*	< 1.00 mg/dL *< 0.32 mmol/L*
	Pediatric 1 year – 14 years	3.0 – 3.5 mg/dL *0.97 – 1.13 mmol/L*	2.5 – 2.9 mg/dL *0.81 – 0.96 mmol/L*	1.5 – 2.4 mg/dL *0.48 – 0.80 mmol/L*	< 1.50 mg/dL *< 0.48 mmol/L*
	Pediatric < 1 year	3.5 – 4.5 mg/dL *1.13 – 1.45 mmol/L*	2.5 – 3.4 mg/dL *0.81 – 1.12 mmol/L*	1.5 – 2.4 mg/dL *0.48 – 0.80 mmol/L*	< 1.50 mg/dL *< 0.48 mmol/L*
Potassium, serum, high		5.6 – 6.0 mEq/L *5.6 – 6.0 mmol/L*	6.1 – 6.5 mEq/L *6.1 – 6.5 mmol/L*	6.6 – 7.0 mEq/L *6.6 – 7.0 mmol/L*	> 7.0 mEq/L *> 7.0 mmol/L*
Potassium, serum, low		3.0 – 3.4 mEq/L *3.0 – 3.4 mmol/L*	2.5 – 2.9 mEq/L *2.5 – 2.9 mmol/L*	2.0 – 2.4 mEq/L *2.0 – 2.4 mmol/L*	< 2.0 mEq/L *< 2.0 mmol/L*
Sodium, serum, high		146 – 150 mEq/L *146 – 150 mmol/L*	151 – 154 mEq/L *151 – 154 mmol/L*	155 – 159 mEq/L *155 – 159 mmol/L*	≥ 160 mEq/L *≥ 160 mmol/L*
Sodium, serum, low		130 – 135 mEq/L *130 – 135 mmol/L*	125 – 129 mEq/L *125 – 129 mmol/L*	121 – 124 mEq/L *121 – 124 mmol/L*	≤ 120 mEq/L *≤ 120 mmol/L*
Triglycerides (fasting)		NA	500 – 750 mg/dL *5.65 – 8.48 mmol/L*	751 – 1,200 mg/dL *8.49 – 13.56 mmol/L*	> 1,200 mg/dL *> 13.56 mmol/L*

* Values are for term infants. Preterm infants should be assessed using local normal ranges.

† Use age and sex appropriate values (e.g., bilirubin).

APPENDIX A
DIVISION OF AIDS TABLE FOR GRADING THE SEVERITY OF ADULT AND PEDIATRIC ADVERSE EVENTS
VERSION 1.0, DECEMBER, 2004; CLARIFICATION AUGUST 2009

LABORATORY				
PARAMETER	**GRADE 1 MILD**	**GRADE 2 MODERATE**	**GRADE 3 SEVERE**	**GRADE 4 POTENTIALLY LIFE-THREATENING**
Uric acid	7.5 – 10.0 mg/dL *0.45 – 0.59 mmol/L*	10.1 – 12.0 mg/dL *0.60 – 0.71 mmol/L*	12.1 – 15.0 mg/dL *0.72 – 0.89 mmol/L*	> 15.0 mg/dL *> 0.89 mmol/L*
URINALYSIS	***Standard International Units are listed in italics***			
Hematuria (microscopic)	6 – 10 RBC/HPF	> 10 RBC/HPF	Gross, with or without clots OR with RBC casts	Transfusion indicated
Proteinuria, random collection	1 +	2 – 3 +	4 +	NA
Proteinuria, 24 hour collection				
Adult and Pediatric ≥ 10 years	200 – 999 mg/24 h *0.200 – 0.999 g/d*	1,000 – 1,999 mg/24 h *1.000 – 1.999 g/d*	2,000 – 3,500 mg/24 h *2.000 – 3.500 g/d*	> 3,500 mg/24 h *> 3.500 g/d*
Pediatric > 3 mo - < 10 years	201 – 499 mg/m^2/24 h *0.201 – 0.499 g/d*	500 – 799 mg/m^2/24 h *0.500 – 0.799 g/d*	800 – 1,000 mg/m^2/24 h *0.800 – 1.000 g/d*	> 1,000 mg/ m^2/24 h *> 1.000 g/d*

* Values are for term infants. Preterm infants should be assessed using local normal ranges.

† Use age and sex appropriate values (e.g., bilirubin).

APPENDIX B

Appendix B: Informed consent for: Impact of Viral and Host Genetic Factors on Antiretroviral Treatment Outcome in South African HIV-1 subtype C infected AIDS patients

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Wallis

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M061025**
PROJECT	Impact of Viral and Host Genetic Factors on Antiretroviral Treatment Outcome in South African HIV-1 Sub....
INVESTIGATORS	Dr C Wallis
DEPARTMENT	Molecular Med. & Haematology
DATE CONSIDERED	06.10.27
DECISION OF THE COMMITTEE*	Approved unconditionally

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

DATE 06.10.30 **CHAIRPERSON** .....
(Professor P E Cleaton Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Prof W Stevens

DECLARATION OF INVESTIGATOR(S)

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

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix B: Informed consent for: Impact of Viral and Host Genetic Factors on Antiretroviral Treatment Outcome in South African HIV-1 subtype C infected AIDS patients

INFORMED CONSENT CIPRA PROJECT 1

Information Sheet and Consent From

Title of Research Project: Impact of Viral and Host Genetic Factors on Antiretroviral Treatment Outcome in South African HIV-1 subtype C infected AIDS patients

GENOTYPING OF HOST GENETIC FACTORS

We would like to perform genotyping (studying your genes) on a sample of your blood. We will tell you what this involves so you can decide if you will consent to genotyping.

1. What is the purpose of this genetic research?

You are currently taking antiretroviral therapy as part of CIPRA Project 1. There are enzymes (proteins in the body, usually in the liver and bowel) that help to break down any drug that you take, including antiretrovirals. These enzymes differ in different groups of people and have not been well studied in South Africans. It is important to understand if these enzymes are different in our population as they affect how our bodies handle different antiretroviral drugs and may impact on interactions with other medications or on the side-effects your experience.

We would like to study the following enzymes:

- Cytochrome P450
- P-glycoprotein
- HLA expression
- Any published gene linked to HIV/AIDS, which will help in further understanding of the virus and its treatment and prevention.

All genetic results will be treated confidentially and identified only by your study number (and not your name).

2. What do I gain from this research?

You will not receive any direct benefit from this genetic research.

3. What are the risks associated with the procedure?

You may experience some mild discomfort and minor bruising at the site of blood sampling.

4. What will happen to my blood sample?

No extra blood will be required. A small amount of blood (about 4ml) taken at the week 4 visit will be used to study your genes.

5. **What will happen if I change my mind?**

You are free to change your mind at any time. If you change your mind about participating, you can withdraw your sample by making a request to the trial doctor/nurse. This will not effect your treatment on CIPRA in any way.

6. **Will the information be kept confidential?**

All the findings of this research will be kept confidential in accordance with the standards followed by medical researchers in compliance with International Good Clinical Practice.

7. **Will I know the results of the research project?**

This project is not performed in real-time, but as the results become available you may request your result from your doctor/nurse. These results may not be useful to you, as combined information from many people is needed to see if the enzymes in South Africans make a difference to how we break down our antiretrovirals. The results of the whole study will be made known to you once it is complete.

8. **What are my rights?**

The trial sponsor will not sell or transfer ownership to other parties. The samples will be used only by CIPRA-SA employees and/or researchers working with CIPRA-SA, and only for the research described above.

YOUR PARTICIPATION IN THIS RESEARCH PROJECT IS VOLUNTARY. YOU MAY REFUSE TO PARTICIPATE IN THIS RESEARCH PROJECT IF YOU WISH.

Appendix B: Informed consent for: Impact of Viral and Host Genetic Factors on Antiretroviral Treatment Outcome in South African HIV-1 subtype C infected AIDS patients

DOCUMENTATION OF THIS CONSENT:

One copy of this consent document will be given to you and one copy will be kept as part of the records of this research project, separate from the data resulting from the research project.

Study ID:	**Initials:**	**Date of Birth:**
Trial Number: **CIPRAZA001**	**Title of Research Project:** Impact of Viral and Host Genetic Factors on Antiretroviral Treatment Outcome in South African HIV-1 subtype C infected AIDS patients	

Consent Form
Agreement to participate in a clinical trial

Your Consent	**Subject Initials**
1. I confirm I have read and understand the genotyping information sheet and have the opportunity to ask questions.	
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without given reason, without my medical care or legal rights being affected	
3. I agree to take part in the above project	

Name (print): _____ **Date:** _____

Signed by Subject: _____ **Date:** _____

Study staff Name: _____ **Date:** _____

Signed by Study staff: _____ **Date:** _____

Witness: _____ **Date:** _____

IMVUME ESEKELWE ELWAZINI YEPROJEKTHI YOKU-1 YE-CIPRA

Iphepha loLwazi neFomu yeMvume

Isihloko seProjekthi yoPhando: Impembelelo yeeMeko zeVayirasi neSondli soFuzo kwiZiphumo zoNyango ngeAntiretroviral eMzantsi Afrika kubaguli beAIDS abanosulelo lweHIV-1 yohlobo olungaphantsi lwe-C

UKUHLELA IIJINI ZOFUZO ZEEMEKO ZESONDLI SOFUZO

Sinqwenela ukuqhuba uhlelo lweejini zofuzo (ukuphononongа iijini zakho) kwisampulu yegazi lakho. Siza kukuxelela ukuba oku kubandakanya ntoni ukuze wenze isigqibo sokuba uya kuvuma ukuba kuphononongwe iijini zofuzo.

1. Yintoni injongo yolu phando lweejini zofuzo?

Njengangoku uthatha unyango nge-antiretroviral njengenxalenye yeProjekthi yoku-1 yeCIPRA. Kukho ii-enzyme (iiprotini emzimbeni, ngamaxa amaninzi kwisibindi nasemathunjini) ezincedisa ukwahlukanisa (ukucalucalula) nawuphi umchiza owuthathayo, kuquka nee-antiretroviral. Ezi enzyme zahlukile kumaqela ahlukeneyo abantu yaye azikaphononongwa ngokukholisayo eMzantsi Afrika. Kubalulekile ukuqonda ukuba ngaba ezi enzyme zahlukile kwisizwe sethu njengoko zichaphazela indlela imizimba yethu iphatha ngayo imichiza ehlukeneyo ye-antiretroviral yaye inokuba nempembelelo ekusebenzeni namanye amayeza okanye kwiziphumo ezingalindelekanga onazo.

Sinqwenela ukuphononongа ii-enzyme ezilandelayo:

- I-Cytochrome P450
- I-P-glycoprotein
- Imbonakalo yeHLA
- Nayiphi ijini epapashiweyo enxulumene neHIV/AIDS, eya kunceda ekuqondeni ngokongezelelweyo ngevayirasi nonyango lwayo nothintelo.

Zonke iziphumo zofuzo ziya kugcinwa ziyimfihlo yaye zichongwe kuphela ngenombolo yakho yophononongo (hayi ngegama lakho).

2. Yintoni endiya kuyizuza kolu phando?

Awunakufumana naluphi uncedo olungqalileyo kolu phando lofuzo.

3. Zeziphi iingxaki ezayamaniswa nenkqubo?

Unokuba nokungonwabi okuthile okuzolileyo nokugruzuka okuncinci kwindawo yokuthatha isampulu yegazi.

4. Kuya kwenzeka ntoni kwisampulu yam yegazi?

Alikho igazi elongezelelweyo eliya kufunwa. Ubungakanani obuncinci begazi (malunga ne-4ml) obuthathwe kutyelelo lweveki yesi- 4 luya kusetyenziswa ukuphononongа iijini zakho zofuzo.

5. Kuya kwenzeka ntoni xa ndijika ingqondo yam?

Ukhululekile ukujika ingqondo yakho nanini na. Ukuba ujika ingqondo yakho malunga nokuthatha inxaxheba, ungarhoxisa isampulu yakho ngokwenza isicelo kugqirha wolingo/ umongikazi. Oku akunakuchaphazela unyango lwakho kwiCIPRA nangeyiphi na indlela.

6. Ingaba ulwazi luya kugcinwa luyimfihlo?

Zonke iziphumo zolu phando ziya kugcinwa ziyimfihlo ngokwemigangatho elandelwayo ngabaphandi bezonyango ukuthobela i-International Good Clinical Practice (ukuziPhatha okuLungileyo kwezoNyango kweZizwe ngezizwe).

7. Ingaba ndiza kuzazi iziphumo zeprojekthi yophando?

Le projekthi ayiqhutywa ngexesha lokwenyani, kodwa xa iziphumo zimane zifumaneka ungenza isicelo seziphumo zakho kugqirha/umongikazi. Kungenzeka ukuba ezi ziphumo zingabinalo uncedo kuwe, njengoko ulwazi oludityanisiweyo lwabantu abaninzi lufuneka ukubona ukuba ingaba ii-enzyme kubemi baseMzantsi Afrika zenza umhluko kwindlela ii-antiretroviral zethu zahlukaniswa ngayo. Uya kwaziswa ngeziphumo zophononongo lonke xa lugqityiwe.

8. Ayintoni amalungelo am?

Umxhasi wolingo akanakuthengisa okanye adlulisele ubumnini kwamanye amaqela. Iisampulu ziya kusetyenziswa ngabasebenzi bakwa-CIPRA-SA kuphela kunye/ okanye abaphandi abasebenza no-CIPRA-SA, yaye malunga nophando oluchazwe ngentla kuphela.

UKUTHATHA KWAKHO INXAXHEBA KULE PROJEKTHI YOPHANDO KUNGOKUZITHANDELA. UNGALANDULA UKUTHATHA INXAXHEBA KULE PROJEKTHI YOPHANDO XA UTHANDA.

AMAXWEBHU ALE MVUME:

Ikopi enye yeli xwebhu lemvume iya kunikwa wena kuthi ikopi enye igcinwe njengenxenye yeerekhodi zale projekthi yophando, ngokwahlukeneyo nedata evela kwiprojekthi yophando.

I-ID yoPhononongo:	**Oonobumba bokuqala:**	**Umhla wokuZalwa:**
Inombolo yoLingo: **CIPRAZA001**	**Isihloko seProjekthi yoPhando:** Impembelelo yeeMeko zeVayirasi neSondli soFuzo kwiZiphumo zoNyango ngeAntiretroviral eMzantsi Afrika kubaguli beAIDS abanosulelo lweHIV-1 yohlobo olungaphantsi lwe-C	

Ifomu yeMvume
Imvumelwano yokuthatha inxaxheba kulingo lwezonyango

Imvume Yakho	**Oonobumba bokuqala boMlingwa**
1. Ndingqina ukuba ndilifundile yaye ndaliqonda iphepha lolwazi lophononongo lwejini yofuzo yaye ndilifumene ithuba lokubuza imibuzo.	
2. Ndiyaqonda ukuba ukuthatha kwam inxaxheba kungokuzithandela yaye ndikhululekile ukurhoxa nanini na, ngaphandle kokunika isizathu, ngaphandle kokuchaphazeleka kwenkathalelo yam yezonyango okanye amalungelo am asemthethweni	
3. Ndiyavuma ukuthatha inxaxheba kwiprojekthi engentla	

Igama (ungadibanisi): _____ **Umhla:** _____

Isayinwe ngumLingwa: _____ **Umhla:** _____

Igama lelungu lesiTafu soPhononongo: _____ **Umhla:** _____

Isayinwe lilungu lesiTafu soPhononongo: _____ **Umhla:** _____

Ingqina: _____ **Umhla:** _____

IMVUME EYAZISIWE YECIPRA PROJECT 1

Ipheshana lemininingwane nefomu lemvume

Isihloko sephrojekthi yocwaningo: Amandla emiphumela yokwelashwa ngemithi elwa namagcinwe ezimpawini zofuzo egciwaneni nakulowo onegciwane ezigulini zaseNingizimu Afrika ezineHIV-1 subtype C ezihlaselwe yiNgculaza

UKUHLOLWA KWEZIMPAWU ZOFUZO KULOWO ONEGCIWANE

Sithanda ukuhlola izimpawu zakho zofuzo esampuleni legazi lakho. Sizokutshela ukuthi lokhu kuphathelene nani ukuze ukwazi ukunquma uma ufuna ukunikeza imvume yokuhlola izimpawu zofuzo.

1. Yini inhloso yalolu cwaningo lwezimpawu zofuzo?

Njengamanje usebenzisa imithi elwa neHIV njengengxenye yeCIPRA Project 1. Kunama-enzayimu (amaphrotheni emzimbeni, avame esibindini nasesiswini) asiza ukuncozulula noma yimuphi umuthi owusebenzisayo, okubandakanya nemithi elwa neHIV. La ma-enzayimu ayehluka emaqenjini ehlukene abantu futhi awakaze acutshungulwe kahle eNingizimu Afrika. Kubalulekile ukuthi uqondisise ukuthi la ma-enzayimu ehlukile esibalweni sethu sabantu njengoba anomthelela ekuthini imizimba yethu iyisebenzisa kanjani imithi ehlukile elwa neHIV nokuthi ingaba namandla ekuhlanganeni neminye imithi noma ezifeni eziqhamuka eceleni oba nazo.

Sithanda ukucwaninga ama-enzayimu alandelayo:

- Cytochrome P450
- P-glycoprotein
- HLA expression
- Noma yiluphi uphawu lofuzo oluxhumene neHIV/AIDS, olungasiza ekuqondisiseni kabanzi ngaleli gciwane nokwelashwa kwalo kanye nokuvinjelwa kwalo.

Yonke imiphumela ephathelene nezimpawu zofuzo izophathwa ngokuyimfihlo futhi izokwaziwa kuphela ngenombolo yakho yocwaningo (hhayi ngegama lakho).

2. Ngizozuzani ngalolu cwaningo?

Ungeke uthole ukusizakala okuqondile kulolu cwaningo lwezimpawu zofuzo.

3. Yiziphi izingozi ezihambisana nokuzokwenziwa?

Ungaba nokuphatheka kabi okuncane kanye nokuhuzuka okuncane kuleyo ndawo okuthethwe kuyo isampula legazi.

4. Kuzokwenzekani kusampula legazi lami?

Alikho elinye igazi elizodingeka. Igazi elincane (elingaba ngu-4 ml) elizothathwa ekuzeni kwakho emtholampilo ngeviki 4 lizosetshenziselwa ukucwaninga izimpawu zofuzo.

5. Kuzokwenzekani uma ngiguqula umqondo wami?

Ukhululekile ukuthi ungaguqula umqondo wakho noma nini. Uma uguqula umqondo wakho ngokuhlanganyela, ungahoxisa isampula legazi lakho ngokwenza isicelo kudokotela/umhlengikazi obhekene nocwaningo. Lokhu kungeke kukhinyabeze ukwelashwa kwakho kuCIPRA noma ngayiphi indlela.

6. Imininingwane izogcinwa iyimfihlo yini?

Konke okutholakele kulolu cwaningo kuzogcinwa kuyimfihlo ngokwemigomo elandelwa ngabacwaningi kwezokwelapha ehambisana ne-International Good Clinical Practice.

7. Ngizoyazi yini imiphumela yephrojekthi yocwaningo?

Le phrojekthi ayenziwa ngesikhathi sangempela, kodwa uma imiphumela itholakala ungayicela kudokotela/umhlengikazi wakho. Le miphumela ingahle ingabi wusizo kuwe, ngenxa yokuthi ulwazi okuxutshiwe lwabantu abaningi luyadingeka ukubona uma ama-enzayimu eNingizimu Afrika enza umehluko ekuthini siyicozulula kanjani imithi yethu elwa neHIV. Uzokwaziswa ngayo yonke imiphumela yalo lonke ucwaningo uma seluphelile.

8. Yimaphi amalungelo enginawo?

Abaxhasi bocwaningo bangeke bathengise noma bedlulisele ukuba ngabanikazi kwabanye abantu. Amasampula azosetshenziswa ngabasebenzi beCIPRA-SA kuphela kanye/noma abacwaningi abasebenzisana neCIPRA-SA, futhi lokho kuzokwenziwa mayelana nocwaningo oluchazwe ngenhla kuphela.

UKUHLANGANYELA KWAKHO KULE PHROJEKTHI YOCWANINGO KUNGOKUZIKHETHELA. UNGENQABA UKUHLANGANYELA KULE PHROJEKTHI YOCWANINGO UMA UTHANDA.

INCWADI ENIKA UBUFAKAZI BALE MVUME:

Uzonikezwa ikhophi eyodwa yale ncwadi yemvume bese kuthi enye ikhophi igcinwe njengengxenye yamarekhodi ale phrojekthi yokucwaninga, endaweni ehlukile kunemininingwane ezotholakala kuphrojekthi yokucwaninga.

I-ID yocwaningo:	**Amanishali:**	**Usuku lokuzalwa:**
Inombolo yocwaningo: **CIPRAZA001**	**Isihloko sePhrojekthi yocwaningo:** Amandla emiphumela yokwelashwa ngemithi elwa namagcinwe ezimpawini zofuzo egciwaneni nakulowo onegciwane ezigulini zaseNingizimu Afrika ezineHIV-1 subtype C ezihlaselwe yiNgculaza	

Ifomu lemvume
Isivumelwano sokuhlanganyela ocwaningweni lokwelapha

Imvume yakho	**Amanishali esiguli**
1. Ngiyaqinisekisa ukuthi ngilifundile, ngaliqondisisa ipheshana lemininingwane yokuhlola izimpawu zofuzo futhi ngibe nethuba lokubuza imibuzo.	
2. Ngiyaqondisisa ukuthi ukuhlanganyela kwami kungokuzikhethela nokuthi ngikhululekile ukuphuma noma nini, ngaphandle kokunika isizathu, nangaphandle kokuthi ukwelashwa kwami noma amalungelo ami ngokusemthethweni akhinyabezeke.	
3. Ngiyavuma ukuhlanganyela ephrojekthini engenhla.	

Igama (phrinth): _____ **Usuku**_____

Kusayina Isiguli: _____ **Usuku:**_____

Igama lomsebenzi ocwaningweni:_____ **Usuku:**_____

Kusayina umsebenzi ocwaningweni :_____ **Usuku**_____

Ufakazi:_____ **Usuku:**_____

PROJEKE 1 YA CIPRA YA TUMELLO E BONTSHANG KUTLWISISO

Leqephe la Lesedi Foromo ya Tumello

Sehlooho sa Projeke ya Diphuputso: Tshushumetso ya Kokwanahloko le Diphatsa tsa Lefutso Diphethong tsa Kalafo ka Meriana e Lwantshang Kokwanahloko ya HIV ho bakudi ba Afrika Borwa ba nang le HIV-1 subtype C ba nang le tshwaetso AIDS

PHUPUTSO YA DIPHATSA TSA LEFUTSO

Re lakatsa ho etsa *genotyping* (phuputso ya diphatsa tsa lefutso) ka disampole tsa hao tsa madi. Re tla o bolella hore na ho ameha eng e le hore o ka etsa qeto ya hore na o ka dumela ho etswa diphuputso tsena tsa diphatsa tsa lefutso (*genotyping)*.

1. Morero wa phuputso ena ya diphatsa tsa lefutso ke ofe?

Hona jwale o ntse o nwa meriana e lwantshang kokwanahloko ya HIV e le karolo ya Projeke 1 ya CIPRA. Ho na le di-enzyme (diprotheine mmeleng, hangata di sebeteng kapa ka maleng) tse thusang ho sila moriana leha e le ofe oo o o nwang, ho akarelletswa le meriana e lwantshang kokwanahloko ya HIV. Di-enzyme tsena di a fapana bathong ba mefuta e sa tshwaneng mme ha ho e so etswe dipatlisiso tse batsi ka tsona ho Maafrika Borwa. Ke habohlokwa hore re utlwisise haeba di-*enzyme* tsena di fapane ho baahi ba habo rona kaha di ama tsela eo mmele ya rona e sebetsanang le meriana e sa tshwaneng e lwantshang kokwanahloko ya HIV mme di ka nna tsa ama kamoo meriana ena e kena kenanang le meriana e meng kapa ditlamorao tseo o ka bang le tsona.

Re lakatsa ho fuputsa di-enzyme tse latelang:

- Cytochrome P450
- P-glycoprotein
- HLA expression
- Phatsa ya lefutso efe kapa efe eo ho seng ho hatisitswe ditlaleho ka yona e amanang le HIV/AIDS, e tla thusa ho utlwisisa kokwanahloko ena haholwanyane le kalafo le thibelo.

Diphetho tsohle tse amanang le diphatsa tsa lefutso di tla bolokwa e le lekunutu mme di tla tsejwa feela ka nomoro ya hao ya patlisiso (mme e seng ka lebitso la hao).

2. Ke una molemo ofe ka ho nka karolo phuputsong ena?

O ke ke wa una melemo ka kotlololoho ka ho nka karolo phuputsong ena ya diphatsa tsa lefutso.

3. Ke dikotsi dife tse teng tse amanang le mekgwa e sebediswang?

O ka nna wa utlwa bohloko bo bonyenyane kapa wa petla hanyenyane moo disampole tsa madi di nkuweng teng.

4. Ho tla etsahalang ka disampole tsa madi a ka?

Ha ho na madi a mang a tla hlokahala. Ketelong ya beke 4 ho tla nkuwa madi a manyenyane (hoo e ka bang 4 ml) a tla sebedisetswa ho hlahloba diphatsa tsa hao tsa lefutso.

5. Ho tla etsahalang haeba ke fetola mohopolo?

O na le bolokolohi ba ho fetola mohopolo wa hao neng kapa neng. Haeba o fetola mohopolo wa hao mabapi le ho nka karolo dipatlisisong tsena, o ka hula disampole tsa madi a hao ka ho di kopa ho ngaka/mooki wa dipatlisiso. Ho hang sena se ke ke sa ama kalafo ya hao ka CIPRA.

6. Na lesedi leo le tla bolokwa e le lekunutu?

Diphetho tsohle tse sibollwang ha ho etswa patlisiso ena di tla bolokwa e le lekunutu ho latela melao e latelwang ke bafuputsi ba meriana ba tsamaisanang le International Good Clinical Practice.

7. Na ke tla tseba diphetho tsa projeke ena phuputso?

Patlisiso ena ha e etswe ka nako e itseng ka kotloloho, empa ha diphetho di ntse di fumaneha, o ka di kopa ho ngaka/mooki wa hao. Diphetho tsena di ka nna tsa se o thuse ka letho, kaha ho hlokahala lesedi le kopaneng la batho ba bangata ho bona haeba di-enzyme ho Maafrika Borwa di fapane tseleng eo di silang ka teng meriana e lwantshang kokwanahloko ya HIV. Hang ha patlisiso ena e fedile o tla tsebiswa diphetho tsa yona kaofela.

8. Ditokelo tsa ka di dife?

Motshehetsi wa patlisiso ena a ke ke a rekisa kapa a fetisetsa ditokelo tsa ho ba le disampole tsena ho batshehetsi ba bang. Ke basebetsi le/kapa bafuputsi ba CIPRA-SA feela ba tla sebedisa disampole tsa hao, mme e tla ba feela bakeng sa diphuputso tse hlalositsweng ka hodimo mona.

HO NKA KAROLO HA HAO PHUPUTSONG ENA HO ETSWA KA BOITHAOPO. O KA NNA WA HANA HO KOPANELA PHUPUTSO ENA HAEBA O BATLA.

TOKOMANE YA TUMELLO ENA:

O tla fuwa kopi e nngwe ya tokomane ena ya tumello mme kopi e nngwe e tla bolokwa e le karolo ya ditlaleho tsa projeke ena ya phuputso, e arohane le lesedi le bokellwang projekeng ena ya phuputso.

ID ya Patlisiso:	**Ditlhaku tsa pele tsa mabitso:**	**Letsatsi la Tswalo:**
Nomoro ya Phuputso: **CIPRAZA001**	**Sehlooho sa Morero wa Patlisiso:** Tshushumetso ya Kokwanahloko le Diphatsa tsa Lefutso Diphethong tsa Kalafo ka Meriana e Lwantshang Kokwanahloko ya HIV ho bakudi ba Afrika Borwa ba nang le HIV-1 subtype C ba nang le tshwaetso AIDS	

Foromo ya Tumello
Tumellano ya ho nka karolo phuputsong ya tliliniki

Tumello ya Hao	**Ditlhaku tsa pele tsa mabitso a Mohlahlobuwa**
1. Ke tiisa hore ke badile mme ke utlwisisa leqephe la lesedi le mabapi le diphuputso tsena tsa diphatsa tsa lefutso (genotyping) mme ke bile le monyetla wa ho botsa dipotso.	
2. Ke a utlwisisa hore ke nka karolo patlisisong ena ka boithaopo le hore ke na le bolokolohi ba ho ikgula neng kapa neng, ho sa hlokahale hore ke fane ka mabaka, mme ho sa amehe tlhokomelo ya ka ya tsa bongaka kapa ditokelo tsa ka tsa molao	
3. Ke a dumela hore ke nka karolo projekeng e ka hodimo	

Lebitso (printa): _____ Letsatsi: _____

E saennwe ke Mohlahlobuwa: _____ Letsatsi: _____

Lebitso la moifo wa Dipatlisiso _____ Letsatsi: _____

E saennwe ke moifo wa Dipatlisiso: _____ Letsatsi: _____

Paki _____ Letsatsi: _____

APPENDIX C

Update of the Drug Resistance Mutations in HIV-1: December 2008

Victoria A. Johnson, MD, Françoise Brun-Vézinet, MD, PhD, Bonaventura Clotet, MD, PhD, Huldrych F. Günthard, MD, Daniel R. Kuritzkes, MD, Deenan Pillay, MD, PhD, Jonathan M. Schapiro, MD, and Douglas D. Richman, MD

The International AIDS Society–USA (IAS–USA) Drug Resistance Mutations Group reviews new data on HIV-1 drug resistance that have been published or presented at recent scientific meetings to maintain a current list of mutations associated with antiretroviral drug resistance. This December 2008 version of the IAS–USA drug resistance mutations figures updates those published in this journal in March/April 2008 (Johnson VA, Brun-Vézinet F, Clotet B, et al, *Top HIV Med*, 2008;16:62-68). The compilation includes mutations that may contribute to a reduced virologic response to HIV-1 drugs. It should not be assumed that the list presented here is exhaustive. Drugs that have been approved by the US Food and Drug Administration (US FDA) as well as any drugs available in expanded access programs are included and listed in alphabetical order by drug class. The figures are designed for practitioners to use in identifying key mutations associated with viral resistance to antiretroviral drugs and in making therapeutic decisions. Updates are posted periodically at www.iasusa.org. For more in-depth reading and an extensive reference list, see the 2008 IAS–USA panel recommendations for resistance testing (Hirsch MS, Gunthard HF, Schapiro JM, et al, *Clin Infect Dis*, 2008:47:266-285).

The mutations listed have been identified by 1 or more of the following criteria: (1) in vitro passage experiments or validation of contribution to resistance by using site-directed mutagenesis; (2) susceptibility testing of laboratory or clinical isolates; (3) nucleotide sequencing of viruses from patients in whom the drug is failing; (4) correlation studies between genotype at baseline and virologic response in patients exposed to a drug. The availability of more recently approved drugs that cannot be tested as monotherapy precludes assessment of the impact of resistance on antiretroviral activity that is not seriously confounded by activity of other drug components in the background regimen. Readers are encouraged to consult the literature and experts in the field for clarification or more information about specific mutations and their clinical impact. Polymorphisms associated with impaired treatment responses that occur in wild-type viruses should not be used in epidemiologic analyses to identify transmitted HIV-1 drug resistance.

In the context of making clinical decisions regarding antiretroviral therapy, evaluating the results of HIV-1 genotypic testing includes: (1) assessing whether the pattern or absence of a pattern in the mutations is consistent with the patient's antiretroviral therapy history; (2) recognizing that in the absence of drug (selection pressure), resistant strains may be present at levels below the limit of detection of the test (analyzing stored samples, collected under selection pressure, could be useful in this setting); and (3) recognizing that virologic failure of the first regimen typically involves HIV-1 isolates with resistance to only 1 or 2 of the drugs in the regimen (in this setting, resistance most commonly develops to lamivudine or the nonnucleoside analogue reverse transcriptase inhibitors [NNRTIs]). The absence of detectable viral resistance after treatment failure may result from any combination of the following factors: the presence of drug-resistant minority viral populations, nonadherence to medications, laboratory error, lack of current knowledge of the association of certain mutations with drug resistance, the occurrence of relevant mutations outside the regions targeted by routine resistance assays, drug-drug interactions leading to subtherapeutic drug levels, and possibly compartmental issues, indicating that drugs may not reach optimal levels in specific cellular or tissue reservoirs.

Current Revision

This December 2008 update includes several changes to the list of drug resistance mutations for HIV-1, as shown on the figure bars. For etravirine, 3 new mutations were added—K101H, E138A, and M230L—and the mutations at positions L100, K101, and Y181 were changed to boldface to indicate their newer categorization as more important mutations because they are sufficient on their own to confer partial

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reduction in virologic response based on weighting factors identified through correlations with phenotype (see User Note m). Changes to the figure bar for ritonavir-boosted darunavir include the removal of G73S and addition of L74P. For ritonavir-boosted tipranavir, the representations for 3 existing mutations—at positions I47, Q58, and T74—were changed to boldface. Finally, the mutations Y143R/H/C were added to the raltegravir figure bar.

The IAS–USA Drug Resistance Mutations Group also undertook a comprehensive revision of the user notes. The information in each note was reviewed and updated as necessary. The references were updated as needed; citations to full papers replaced those to abstract presentations whenever possible.

Mutations Panel

The authors comprise the IAS–USA Drug Resistance Mutations Group, an independent, volunteer panel of experts charged with the goal of delivering accurate, unbiased, and evidence-based information on these mutations to practitioners. As for all IAS–USA panels, a rotation procedure is in place whereby 1 or 2 panel members periodically step down from panel participation and new members join. These rotations are designed to ensure that all IAS–USA expert panels remain diverse in member affiliations and areas of expertise.

Comments

The IAS–USA Drug Resistance Mutations Group welcomes comments on the mutations figures and user notes.

Please send your evidence-based comments, including relevant reference citations, to the IAS–USA at **resistance2009''at''iasusa.org** or by fax at 415-544-9401. Please include your name and institution.

Reprint Requests

The Drug Resistance Mutations Group welcomes interest in the mutations figures as an educational resource for practitioners and encourages dissemination of the material to as broad an audience as possible. However, permission is required to reprint the figures and **no alterations in the content can be made**. If you wish to reprint the mutations figures, please send your request to the IAS–USA via e-mail or fax (see above).

To ensure the integrity of the mutations figures, IAS–USA policy is to grant permission for only minor, preapproved adaptations of the figures (eg, an adjustment in size). Minimal adaptations only will be considered; no alterations of the content of the figures or user notes will be permitted. Please note that permission will be granted only for requests to reprint or adapt the most current version of the mutations figures as they are posted on the Web site (www.iasusa.org). Because scientific understanding of HIV drug resistance evolves rapidly and the goal of the Drug Resistance Mutations Group is to maintain the most up-to-date compilation of mutations for HIV clinicians and researchers, publication of out-of-date figures is counterproductive. If you have any questions about reprints or adaptations, please contact us.

Financial Disclosures: The authors disclose the following affiliations with commercial organizations that may have interests related to the content of this article: Dr Brun-Vézinet has received grants and research support from GlaxoSmithKline, Tibotec Therapeutics, has served as a consultant to Merck & Co, Inc, Monogram Biosciences, Inc, and Tibotec Therapeutics, and has served as a paid lecturer for Bristol-Myers Squibb, GlaxoSmithKline, and Tibotec Therapeutics. Dr Clotet has served on scientific and marketing advisory boards and has received honoraria for lectures from Abbott Laboratories, Boehringer Ingelheim Pharmaceuticals, Inc, Merck Sharp & Dohme, Bristol-Myers Squibb, Gilead Sciences, Inc, GlaxoSmithKline, Panacos Pharmaceuticals, Inc, Pfizer Inc, Roche Pharmaceuticals, and Tibotec Therapeutics. Dr Günthard has served as a scientific and medical advisor for Abbott Laboratories, Bristol-Myers Squibb, Boehringer Ingelheim Pharmaceuticals, Inc, GlaxoSmithKline, Novartis Pharmaceuticals Corp, and Tibotec Therapeutics and has received unrestricted research and travel grants from Abbott Laboratories, Boehringer Ingelheim Pharmaceuticals, Inc, Bristol-Myers Squibb, Gilead Sciences, Merck Sharp & Dohme, and Roche Pharmaceuticals. Dr Johnson has received grant and research support from Agouron Pharmaceuticals, Bristol-Myers Squibb, GlaxoSmithKline, Monogram Biosciences, Inc, and Visible Genetics, Inc (later Bayer, now Siemens Medical Solutions Diagnostics); has served on the speaker's bureaus or received honoraria from Abbott Laboratories, GlaxoSmithKline, and Monogram Biosciences, Inc; and has served on medical or clinical advisory boards of Bristol-Myers Squibb, GlaxoSmithKline, Monogram Biosciences, Inc, and Virco Lab, Inc. Dr Kuritzkes has served as a consultant to and has received honoraria from Abbott Laboratories, Avexa Ltd, Boehringer Ingelheim Pharmaceuticals, Inc, Bristol-Myers Squibb, Gilead Sciences, Inc, GlaxoSmithKline, Human Genome Sciences, Inc, Idenix Pharmaceuticals, Inc, Merck & Co, Inc, Monogram Biosciences, Inc, Pfizer Inc, Roche Pharmaceuticals, Schering-Plough Corp, Siemens, and Trimeris, Inc and has received research grant support from GlaxoSmithKline, Human Genome Sciences, Inc, Merck & Co, Inc, and Schering-Plough Corp. Dr Pillay has served as a consultant to Boehringer Ingelheim Pharmaceuticals, Inc, Bristol-Myers Squibb, Gilead Sciences, Inc, and Roche Pharmaceuticals. Dr Schapiro has served as a consultant, advisor, or speaker for Abbott Laboratories, Ambrilia Biopharma, Inc, Bristol-Myers Squibb, Boehringer Ingelheim Pharmaceuticals, Inc, Gilead Sciences, Inc, GlaxoSmithKline, Merck & Co, Inc, Monogram Biosciences, Inc, Pfizer Inc, Roche Pharmaceuticals, Siemens, Tibotec Therapeutics, Virco Lab, Inc, and Virology Education; and has received research support from GlaxoSmithKline, Monogram Biosciences, Inc, Roche Pharmaceuticals, and Tibotec Therapeutics. Dr Richman has served as a consultant to Anadys Pharmaceuticals, Inc, Biota, Bristol-Myers Squibb, Gilead Sciences, Inc, Idenix Pharmaceuticals, Inc, Merck & Co, Inc, Monogram Biosciences, Inc, Pfizer Inc, Roche Pharmaceuticals, and Tobira Therapeutics, Inc. The International AIDS Society–USA has received grants in the past 3 years for selected continuing medical education activities that are pooled (ie, no single company supports any single effort) from Abbott Laboratories, Boehringer Ingelheim Pharmaceuticals, Inc, Bristol-Myers Squibb, Gilead Sciences, Inc, GlaxoSmithKline, Merck & Co, Inc, Roche Pharmaceuticals, Tibotec Therapeutics, and Pfizer Inc.

Funding/Support: This work was funded by the IAS–USA. No private sector or government funding was used to support the effort. Panel members are not compensated.

MUTATIONS IN THE REVERSE TRANSCRIPTASE GENE ASSOCIATED WITH RESISTANCE TO REVERSE TRANSCRIPTASE INHIBITORS

Nucleoside and Nucleotide Analogue Reverse Transcriptase Inhibitors (nRTIs)[a]

Multi-nRTI Resistance: 69 Insertion Complex[b] (affects all nRTIs currently approved by the US FDA)

M	A	▼	K	L	T	K
41	**62**	**69**	**70**	**210**	**215**	**219**
L	V	Insert	R	W	Y F	Q E

Multi-nRTI Resistance: 151 Complex[c] (affects all nRTIs currently approved by the US FDA except tenofovir)

A	V	F	F	Q
62	**75**	**77**	**116**	**151**
V	I	L	Y	M

Multi-nRTI Resistance: Thymidine Analogue-associated Mutations[d,e] (TAMs; affect all nRTIs currently approved by the US FDA)

M	D	K	L	T	K
41	**67**	**70**	**210**	**215**	**219**
L	N	R	W	Y F	Q E

Abacavir[f,g]

K	L	Y	M
65	**74**	**115**	**184**
R	V	F	V

Didanosine[g,h]

K	L
65	**74**
R	V

Emtricitabine

K	M
65	**184**
R	V I

Lamivudine

K	M
65	**184**
R	V I

Stavudine[d,e,i,j]

M	D	K	L	T	K
41	**67**	**70**	**210**	**215**	**219**
L	N	R	W	Y F	Q E

Tenofovir[k]

K	K
65	**70**
R	E

Zidovudine[d,e,i,j]

M	D	K	L	T	K
41	**67**	**70**	**210**	**215**	**219**
L	N	R	W	Y F	Q E

Nonnucleoside Analogue Reverse Transcriptase Inhibitors (NNRTIs)[a,l]

Efavirenz

L	K	V	V	Y	Y	G	P
100	**103**	**106**	**108**	**181**	**188**	**190**	**225**
I	N	M	I	C I	L	S A	H

Etravirine[m]

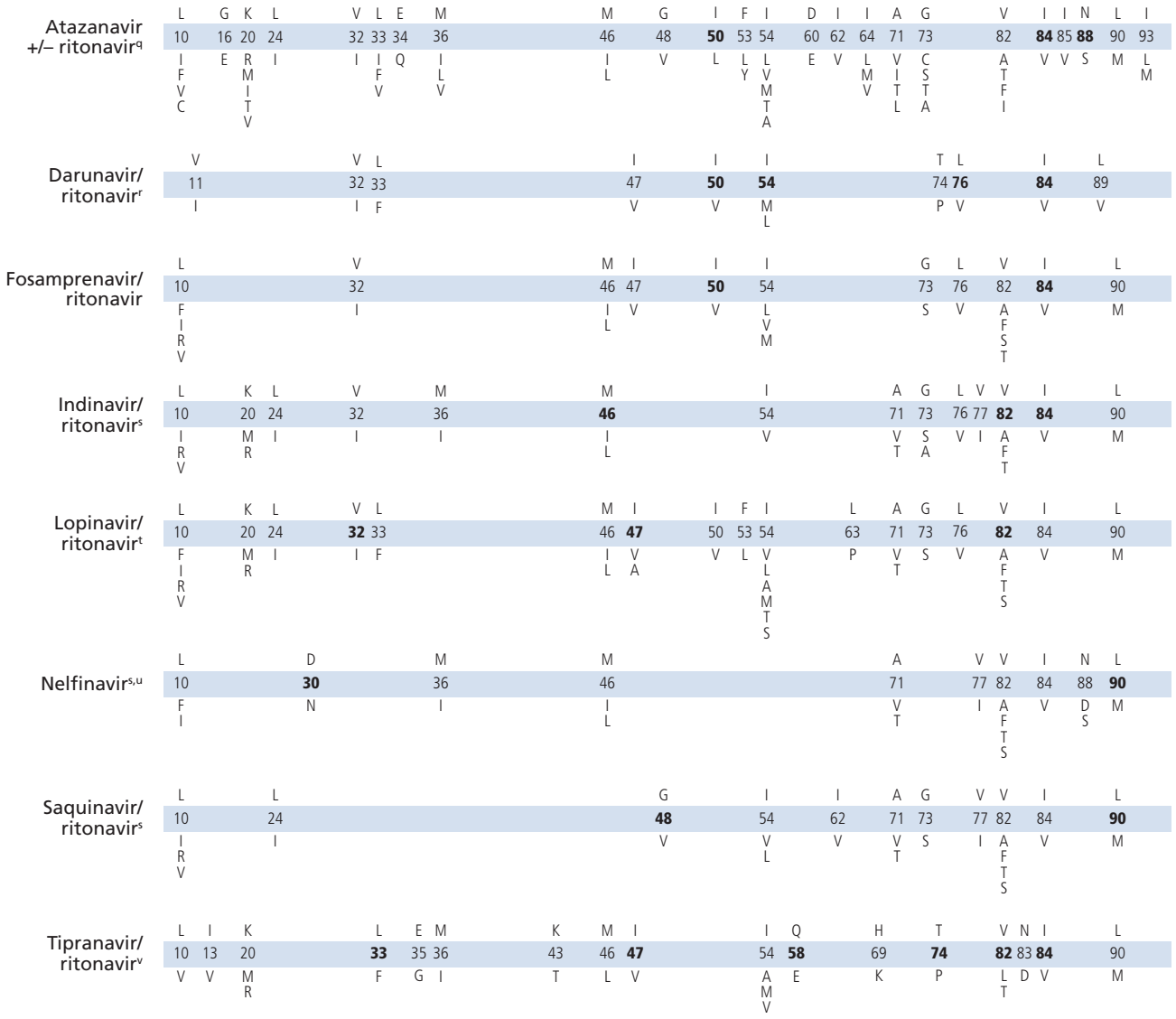
V	A	L	K	V	E	V	Y	G	M
90	98	**100**	**101**	106	138	179	**181**	190	230
I	G	I	E H P	I	A	D F T	C I V	S A	L

Nevirapine

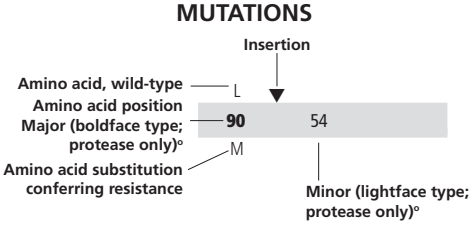
L	K	V	V	Y	Y	G
100	**103**	**106**	**108**	**181**	**188**	**190**
I	N	A M	I	C I	C L H	A

Appendix C

MUTATIONS IN THE PROTEASE GENE ASSOCIATED WITH RESISTANCE TO PROTEASE INHIBITORS[n,o,p]



Amino acid abbreviations: A, alanine; C, cysteine; D, aspartate; E, glutamate; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.



Appendix C

The International AIDS Society–USA (IAS–USA) Drug Resistance Mutations Group reviews new data on HIV-1 drug resistance that have been published or presented at recent scientific meetings to maintain a current list of mutations associated with antiretroviral drug resistance. The compilation includes mutations that may contribute to a reduced virologic response to HIV-1 drugs. It should not be assumed that the list presented here is exhaustive. Drugs that have been approved by the US Food and Drug Administration (FDA) as well as any drugs available in expanded access programs are included and listed in alphabetic order by drug class.

The mutations listed have been identified by 1 or more of the following criteria: (1) in vitro passage experiments or validation of contribution to resistance by using site-directed mutagenesis; (2) susceptibility testing of laboratory or clinical isolates; (3) nucleotide sequencing of viruses from patients in whom the drug is failing; (4) correlation studies between genotype at baseline and virologic response in patients exposed to a drug. The availability of more recently approved drugs that cannot be tested as monotherapy precludes assessment of the impact of resistance on antiretroviral activity that is not seriously confounded by activity of other drug components in the background regimen. Readers are encouraged to consult the literature and experts in the field for clarification or more information about specific mutations and their clinical impact. Polymorphisms associated with impaired treatment responses that occur in wild-type viruses should not be used in epidemiologic analyses to identify transmitted HIV-1 drug resistance.

User Notes

a. Numerous nucleoside (or nucleotide) analogue reverse transcriptase inhibitor (nRTI) mutations, like M41L, L210W, and T215Y, may lead to viral hypersusceptibility to the nonnucleoside analogue reverse transcriptase inhibitors (NNRTIs), including etravirine,[1] in nRTI-treated individuals. The presence of these mutations may improve subsequent virologic response to NNRTI-containing regimens (nevirapine or efavirenz) in NNRTI-naive individuals[2-6] or with etravirine in some NNRTI-experienced individuals.

b. The 69 insertion complex consists of a substitution at codon 69 (typically T69S) and an insertion of 2 or more amino acids (S-S, S-A, S-G, or others). The 69 insertion complex is associated with resistance to all nRTIs currently approved by the US FDA when present with 1 or more thymidine analogue–associated mutations (TAMs) at codons 41, 210, or 215.[7] Some other amino acid changes from the wild-type T at codon 69 without the insertion may be associated with broad nRTI resistance.

c. Tenofovir retains activity against the Q151M complex of mutations.[7]

d. Mutations known to be selected by thymidine analogues (M41L, D67N, K70R, L210W, T215Y/F, and K219Q/E, termed TAMS) also confer reduced susceptibility to all approved nRTIs.[8] The degree to which cross-resistance is observed depends on the specific mutations and number of mutations involved.[9-12] Mutations at the C-terminal reverse transcriptase domains (amino acids 293–560) outside of regions depicted on the figure bars may prove to be important for HIV-1 drug resistance. The clinical relevance of these in vitro findings remains unclear, and there is yet no evidence that they have a substantial impact in the absence of other, established mutations. Thus, they are not depicted on the figure bars.

e. The E44D and the V118I mutations increase the level of resistance to zidovudine and stavudine in the presence of TAMs and correspondingly increase cross-resistance to other nRTIs. Their presence in the absence of other key mutations does not substantially alter resistance.[13,14] Furthermore, V118I alone does not compromise response to nRTI-containing regimens.[15]

f. The M184V mutation alone does not appear to be associated with a reduced virologic response to abacavir in vivo.[16,17] When present with 2 or 3 TAMs, M184V contributes to reduced susceptibility to abacavir and is associated with impaired virologic response in vivo.[17] The M184V mutation plus 4 or more TAMs results in no virologic response to abacavir in vivo.[17] Slightly increased treatment responses to tenofovir are observed if M184V is present.[7]

g. The K65R mutation may be selected by didanosine or abacavir and is associated with decreased susceptibility to these drugs.[16,18,19] The impact of K65R on clinical response to didanosine-containing triple-drug regimens remains unclear.

h. The presence of 3 of the following mutations—M41L, D67N, L210W, T215Y/F, K219Q/E—is associated with resistance to didanosine.[20] The presence of K70R or M184V alone does not decrease virologic response to didanosine.[21]

i. The presence of M184V appears to delay or prevent emergence of TAMs.[22] This effect may be overcome by an accumulation of TAMs or other mutations.

j. The T215A/C/D/E/G/H/I/L/N/S/V substitutions are revertant mutations at codon 215 that confer increased risk of virologic failure of zidovudine or stavudine in antiretroviral-naive patients.[23-25] The T215Y mutant may emerge quickly from 1 of these mutations in the presence of zidovudine or stavudine.[26,27]

k. The presence of K65R is associated with a reduced virologic response to tenofovir.[7] A reduced response also occurs in the presence of 3 or more TAMs inclusive of either M41L or L210W.[7] Slightly increased treatment responses to tenofovir are observed when M184V is present.[7]

l. The sequential use of nevirapine and efavirenz (in either order) is not recommended because of cross-resistance between these drugs.[28]

m. Resistance to etravirine has been extensively studied only in the context of coadministration with darunavir/ritonavir. In this context, mutations associated with virologic outcome have been assessed and their relative weights (or magnitudes of impact) assigned. In addition, phenotypic cutoff values have been calculated, and assessment of genotype-phenotype correlations from a large clinical database have determined relative importance of the various mutations. These 2 approaches are in agreement for many, but not all, mutations and weights.[29-31] The single mutations Y181C/I/V, K101P, and L100I reduce but do not preclude clinical utility. The presence of K103N does not affect etravirine response.[32] Accumulation of several mutations results in greater reductions in susceptibility and virologic response than do single mutations.[33]

n. Often, numerous mutations are necessary to substantially impact virologic response to a ritonavir-boosted protease inhibitor (PI).[34] When used as unboosted

Appendix C

agents, atazanavir, fosamprenavir, and saquinavir generally select the same mutations as the ritonavir-boosted drug regimen, although the relative frequency of mutations may differ.

o. Resistance mutations in the protease gene are classified as "major" or "minor."

> Major mutations in the protease gene are defined as those selected first in the presence of the drug or those substantially reducing drug susceptibility. These mutations tend to be the primary contact residues for drug binding.
>
> Minor mutations generally emerge later than major mutations and by themselves do not have a substantial effect on phenotype. They may improve replication of viruses containing major mutations. Some minor mutations are present as common polymorphic changes in HIV-1 nonsubtype-B clades.

p. Ritonavir is not listed separately, as it is currently used only at low dose as a pharmacologic booster of other PIs.

q. Many mutations are associated with atazanavir resistance. Their impacts differ, with I50L, I84V, and N88S having the greatest effect. Higher atazanavir levels obtained with ritonavir boosting increase the number of mutations required for loss of activity. The presence of M46I + L76V might increase susceptibility to atazanavir.[35]

r. Ritonavir-boosted darunavir correlates with baseline susceptibility and the presence of several specific PI mutations. Reductions in response are associated with increasing numbers of the mutations indicated in the figure bar. Some of these mutations appear to have a greater effect on susceptibility than others (eg, I50V vs V11I). A median darunavir phenotypic fold-change greater than 10 (low clinical cutoff) occurs with 3 or more of the 2007 IAS–USA mutations listed for darunavir[36] and is associated with a diminished virologic response.[37]

s. The mutations depicted on the figure bar cannot be considered comprehensive because little relevant research has been reported in recent years to update the resistance and cross-resistance patterns for this drug.

t. In PI-experienced patients, the accumulation of 6 or more of the mutations indicated on the figure bar is associated with a reduced virologic response to lopinavir/ritonavir.[38,39] The product information states that accumulation of 7 or 8 mutations confers resistance to the drug.[40] However, there is emerging evidence that specific mutations, most notably I47A (and possibly I47V) and V32I, are associated with high-level resistance.[41-43] The addition of L76V to 3 PI resistance–associated mutations substantially increases resistance to lopinavir/ritonavir.[35]

u. In some nonsubtype-B HIV-1, D30N is selected less frequently than are other PI mutations.[44]

v. Clinical correlates of resistance to tipranavir are limited by the paucity of clinical trials and observational studies of the drug. Lists of mutations associated with accumulating resistance have been presented, with some conflicting results. In vitro studies and initial analysis of clinical data show mutations L33F, V82L/T, and I84V as having substantial contributions. Confirmatory studies are pending. A number of mutations (L24I, I50L/V, I54L, and L76V) are associated with decreased resistance in vitro and improved short-term virologic response if 2 or more are present.

w. Resistance to enfuvirtide is associated primarily with mutations in the first heptad repeat (HR1) region of the gp41 envelope gene. However, mutations or polymorphisms in other regions of the envelope (eg, the HR2 region or those yet to be identified) as well as coreceptor usage and density may affect susceptibility to enfuvirtide.[45-47]

x. Maraviroc activity is limited to patients with virus that uses only the CC chemokine receptor 5 (CCR5) for entry (R5 virus); viruses that use both CCR5 and the CXC chemokine receptor 4 (CXCR4) (termed dual/mixed or D/M) or only CXCR4 (X4) do not respond to maraviroc treatment. Virologic failure with maraviroc therapy frequently is associated with outgrowth of X4 virus that preexisted as a minority population below the level of assay detection. Mutations in the HIV-1 gp120 molecule that allow the virus to bind to the maraviroc-bound form of CCR5 have been described in viruses from some patients whose virus remained R5 at the time of virologic failure. The resistance profile for maraviroc is too complex to be depicted on the figure bar. The frequency and rate at which maraviroc resistance mutations emerge are not yet known.

y. Raltegravir failure is associated with integrase mutations in at least 3 distinct genetic pathways defined by 2 or more mutations including (1) a signature (major) mutation at Q148H/K/R, N155H, or Y143R/H/C; and (2) 1 or more additional minor mutations. Minor mutations described in the Q148H/K/R pathway include L74M + E138A, E138K, or G140S. The most common mutational pattern in this pathway is Q148H + G140S, which also confers the greatest loss of drug susceptibility. Mutations described in the N155H pathway include this major mutation plus either L74M, E92Q, T97A, E92Q + T97A, Y143H, G163K/R, V151I, or D232N.[48] The Y143R/H/C mutation is uncommon.[49-53]

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APPENDIX D

Table D.1: Viral Loads, Sequence Similarity, Mutation Patterns and Subtypes of the 90 samples used in the HIV-1 ARV drug resistance in-house validation. The yellow highlighted samples have different mutation patterns for ViroSeq and the in-house assay. The purple highlighted samples failed to amplify on ViroSeq.

Patient Name	Viral Load	Sequence Similarity	Mutation Pattern	PI-Major	PI-Minor	NRTI	NNRTI	Subtype
VAL001	1500	93.65	same			A62V, M184V	K103N, G190A	C
VAL002	10000	98.92	VS T215I/T			D67G,K70R, M184V, K219Q	V106M, V179D	C
VAL003	36000	99.45	VS V108I/V			D67N, K70R, M184V, K219E	A98G, K103N, V106M, Y188C	C
VAL004	77000	98.5	VS V108I/V		T74S	M184V	K103N, P225H	C
VAL005	300000	99.11	VS L100I/L		L10I		K103N, V106M	C
VAL006	1300	99.28	same			D67N, K70R, K219E	K103N, V106M	C
VAL007	1600	99.77	same				K103N	C
VAL008	1700	98.81	same			D67N, K70R, M184V	K103N, G190A	C
VAL009	2000	99.13	same		T74S	M184V	K103N, V106M	C
VAL010	2500	99.2	same			M184V	V106M, Y188L	C
VAL011	3900	98.53	same					C
VAL012	3900	100	same	M46I, I84V	L10F			C
VAL013	3900	99.38	same			M184V	K103N	C
VAL014	4300	99.3	same		L10V, T74S	M184V	K101E, K103R, V106M, G190A	C
VAL015	5200	99.13	same			A62V,M184V	K101E, V106M	C
VAL016	5400	99.92	same			M184V	Y188L	C
VAL017	5500	99.61	same			M184V, T215Y	G190S, M230L	C
VAL018	5900	99.45	same			M184V	V90I, K101E, Y181C	C
VAL019	6800	99.76	same			D67N, M184V	V106M, Y188C	C
VAL020	8000	99.23	same			D67N, T69N, K70R, V118I, M184V, T215F, K219Q	A98G, K101E, K103R, V106M, F227L	C
VAL021	8700	99.84	same			M184V	K103N	C
VAL022	9700	99.21	same			L74V, M184V	K103N, P225H	C
VAL023	10000	99.45	same			M184V	K103N, Y181C	C
VAL024	11000	99.34	same			M184V	K103N, V108I	C
VAL025	11000	99.68	same			M184V, T215Y	Y181C	C

Table D.1: Viral Loads, Sequence Similarity, Mutation Patterns and Subtypes of the 90 samples used in the HIV-1 ARV drug resistance in-house validation. The yellow highlighted samples have different mutation patterns for ViroSeq and the in-house assay. The purple highlighted samples failed to amplify on ViroSeq.

Patient Name	Viral Load	Sequence Similarity	Mutation Pattern	PI-Major	PI-Minor	NRTI	NNRTI	Subtype
VAL026	12000	99.45	same			D67N, K70E, M184V	V106M, H221Y, F227L	C
VAL027	12000	99.77	same			K70R, M184V	K103R, V106M, V179D	C
VAL028	13000	99.75	same		L23F	M184V	K103N, V108I	C
VAL029	14000	99.84	same			M184V	V108I, Y181C, H221Y	C
VAL030	15000	98.72	same			M184V	V106M, Y188L	C
VAL031	19000	99.53	same			M184V, T215F	K103N, V108I	C
VAL032	19000	99.29	same					C
VAL033	21000	99.36	same				K103N	C
VAL034	21000	99.06	same			A62V, M184V, T215S	V106M, G190A, M230L	C
VAL035	23000	98.99	same			M41L, D67N, K70R, M184V, T215F/Y	V106M, Y188L	C
VAL036	38000	99.61	same					C
VAL037	49000	99.92	same			M184V	K101E, V106M, F227L	C
VAL038	51000	99.1	same			M184V	K101P, K103N	C
VAL039	62000	99.68	same			V118I	K103N, E138A	C
VAL040	67000	99.76	same			V118I, M184V	V106M, Y188L	C
VAL041	72000	97.79	same			M184V	K103N, P225H	C
VAL042	76000	100	same			K65R, M184V	L100I, K103N, P225H	C
VAL043	77000	99.13	same		Q58E		K103N	C
VAL044	77000	99.69	same				K103N, V106M, Y188C	C
VAL045	78000	99.19	same		V11I	M184V	V106M, V179D, F227L	C
VAL046	85000	99.2	same			M41L, D67N, K70R, V75M, M184V, L210W, T215F, K219E	V106M, V179D, F227L	C
VAL047	91000	99.61	same		L10I	A62V, K65R, Y115F, M184V	V106M, Y188C	C
VAL048	95000	99.1	same		A71T	M184V	K101E, K103R, V179E, G190A, P225H	C

Table D.1: Viral Loads, Sequence Similarity, Mutation Patterns and Subtypes of the 90 samples used in the HIV-1 ARV drug resistance in-house validation. The yellow highlighted samples have different mutation patterns for ViroSeq and the in-house assay. The purple highlighted samples failed to amplify on ViroSeq.

Patient Name	Viral Load	Sequence Similarity	Mutation Pattern	PI-Major	PI-Minor	NRTI	NNRTI	Subtype
VAL049	108000	99.14	same					C
VAL050	110000	99.06	same	M46I, I54V,L76V, V82A	A71V, T74S	M184V		C
VAL051	130000	99.21	same			M184V	K103N	C
VAL052	140000	99.38	same					C
VAL053	160000	98.99	same		Q58E			C
VAL054	210000	99.6	same			D67N, M184I, T215F	K103N	C
VAL055	300000	99.36	same				K103N	C
VAL056	310000	99.29	same				K103N	C
VAL057	320000	99.69	same			D67N, T69S, M184V	K101E, V106M, F227L	C
VAL058	350000	97.85	same		T74S			C
VAL059	710000	98.67	same			M184V	K103N, G190A	C
VAL060	750000	99.53	same					C
VAL061	920000	99.84	same			M41L, K65R, V75I	K103N, V106M, V179D, Y188C	C
VAL062	1200000	99.12	same			M41L, E44D, D67N, T69D, L74V, M184V, L210W, T215Y	K103N, V179E, M230L	C
VAL063	1600000	99.84	same			T69N	Y181C, G190A	C
VAL064	9700000	99.29	same				K103N	C
VAL065	unknown	99.13	same			M41L, D67N, M184V, T215Y	K101E/V, Y181C, G190A	C
VAL066	unknown	99.43	same		A71T	M184V	K103N	B
VAL067	unknown	97.79	same		A71T			C

Table D.1: Viral Loads, Sequence Similarity, Mutation Patterns and Subtypes of the 90 samples used in the HIV-1 ARV drug resistance in-house validation. The yellow highlighted samples have different mutation patterns for ViroSeq and the in-house assay. The purple highlighted samples failed to amplify on ViroSeq.

Patient Name	Viral Load	Sequence Similarity	Mutation Pattern	PI-Major	PI-Minor	NRTI	NNRTI	Subtype
VAL068	unknown	99.92	same			M184V		C
VAL069	unknown	98.38	same			M184V	K103R, V106M, E138A, V179E, F227L	C
VAL070	unknown	99.21	same					C
VAL071	unknown	99.68	same		A71T	A62V, M184V	K101E, V106M, G190A	C
VAL072	11000	98.17	same			M184V	K103S, V106M	C
VAL073	unknown	99.2	same			M184V	K103N, Y188L	C
VAL074	unknown	98.39	same					C
VAL075	2300	VS failed	VS failed			D67N, M184V	K101E, K103N, V108I, G190A	C
VAL076	2400	VS failed	VS failed			D67N, K70R, M184V	Y188L, G190A, K238T	C
VAL077	7400	VS failed	VS failed			M184V	K103R, V106M, V179D, M230L	C
VAL078	14000	VS failed	VS failed			K65R, M184V		C
VAL079	32000	VS failed	VS failed			V118I, M184V		C
VAL080	42000	VS failed	VS failed					C
VAL081	150000	VS failed	VS failed			K65R, D67N, T69I, K70R, F116W, M184V	K101P, K103N, Y318F	C
VAL082	627000	VS failed	VS failed			V75I, M184V	V106M, F227L	C
VAL083	unknown	VS failed	VS failed					C
VAL084	17000	100	same			A62V, M184V	V106M, Y188C	C
VAL085	84000	99.05	No clinical difference IN K70R &T215F VS P225H			D67N, M184V, T215F	L100I, K103N, H221I	C

Table D.1: Viral Loads, Sequence Similarity, Mutation Patterns and Subtypes of the 90 samples used in the HIV-1 ARV drug resistance in-house validation. The yellow highlighted samples have different mutation patterns for ViroSeq and the in-house assay. The purple highlighted samples failed to amplify on ViroSeq.

Patient Name	Viral Load	Sequence Similarity	Mutation Pattern	PI-Major	PI-Minor	NRTI	NNRTI	Subtype
VAL086	230000	99.3	same	M46I, I54V, L76V, V82A	A71V, T74S	M184V		C
VAL087	unknown	99.21	same	L90M	A71V, T74S	D67N, T69D, K70R, V75T, V118I, K219Q	E138A	A
VAL088	unknown	99.29	same					C
VAL089	unknown	99.13	same					C
VAL090	unknown	99.43	same					B

APPENDIX E

Table E.1: Baseline CIPRA-SA demographics of the 83 patients that experience viral failure.

Patient Name	Initials	Reference Number	Date Of Birth	Gender	Viral Load	Age	CD4	PI*-major	PI-minor	NRTI^	NNRTI#	Comments
135011	K-M	CP0505B44480	30-Apr-76	Female	8,990	29	260					No Storage
135101	M-K	CP0505B44531	14-Aug-60	Female	28,500	45	262					
135141	MTM	CP0505B44476	16-Jun-76	Female	49,800	29	146					
135241	B-S	CP0505B48418	12-Aug-05	Female	30,500	0	198					
135311	PMM	CP0505B48405	01-Feb-57	Male	65,400	48	115		T74S			
135371	J-Q	CP0505B49004	12-May-80	Male	12,500	25	318		T74S			
135381	IZM	CP0505B49009	19-Jul-73	Male	21,600	32	313					No amplification
135431	C-M	CP0505B49001	12-May-76	Female	39,400	29	201		I85VI			
135641	ITR	CP0505B49925	16-Jul-77	Female	110,000	28	181		T74S			
135671	RTM	CP0505B49904	15-May-67	Female	74,400	38	123		T74S		K103N	
135771	HAT	CP0505B50128	07-Jul-56	Male	16,600	50	208					
135851	RSZ	CP0505B48657	19-Apr-63	Male	122,000	43	235					
135891	MPS	CP0505B48672	15-Aug-65	Female	ND[1]	41	311				E138A	
135941	Q-S	CP0505B48670	23-May-81	Female	455,000	25	214		T74S			
136101	NPM	CP0505B50446	24-Dec-75	Female	5,180	31	137					No amplification
136151	MSM	CP0505B50688	24-Jun-77	Female	93,000	29	219					
136251	DUS	CP0505B49933	11-Jul-74	Female	448,000	32	55					
136351	NMB	CP0505B51801	12-May-77	Female	33,100	29	119					
136611	BMR	CP0505B52516	22-May-69	Male	168,000	37	191					No Storage
136801	MAH	CP0505B52858	01-Feb-71	Female	24,500	35	214					
136841	MEL	CP0505B53087	15-May-75	Female	135,000	31	148		A71T			
136851	PTG	CP0505B52490	17-Jul-82	Female	298,000	24	91					

Table E.1: Baseline CIPRA-SA demographics of the 83 patients that experience viral failure.

Patient Name	Initials	Reference Number	Date Of Birth	Gender	Viral Load	Age	CD4	PI*-major	PI-minor	NRTI^	NNRTI#	Comments
136911	GTG	CP0505B52503	16-Jan-67	Female	293,000	39	191					
137011	LPR	CP0505B52876	06-Mar-79	Female	308,000	27	129	Q58E				
137111	TJM	CP0505B52869	21-Mar-71	Female	468,000	35	213					
165121	A-M	CP0505B44479	02-Oct-69	Female	678,000	36	224	M46V Q58E			K101E K103R G190A	
165131	J-N	CP0505B44532	22-Dec-65	Male	80,400	40	168					
165341	MPM	CP0505B48288	02-Aug-75	Female	37,000	30	148					No Storage
165351	NCS	CP0505B49010	28-Jan-85	Female	58,700	20	164		T74S			
165461	MSD	CP0505B49362	19-Jul-67	Female	18,900	38	354					
165531	BTM	CP0505B49524	07-Sep-69	Female	399	36	256					No amplification
165621	TQQ	CP0505B49553	12-Dec-70	Female	155,000	35	124				V106M/V E138A K103N	
165781	ILT	CP0505B50133	13-Jun-71	Female	249,000	35	123					
165831	LQC	CP0505B48673	07-May-78	Female	2,890	28	162					
165881	RMP	CP0505B48659	12-Jan-84	Female	204,000	22	106					
165961	JTT	CP0505B50421	22-Sep-70	Male	58,200	36	194		A71T			
166141	MLS	CP0505B49921	29-Jan-73	Male	218,000	33	118		L10I			
166151	P-M	CP0505B51190	07-Feb-81	Female	70,300	25	61					
166161	NHP	CP0505B51193	15-May-86	Female	609,000	20	177					
166311	NTS	CP0505B50453	10-Oct-70	Female	152,000	36	87					No amplification
166321	ANK	CP0505B51802	25-Apr-62	Male	215,000	44	168					
166351	EKP	CP0505B51798	28-Jun-82	Female	293,000	24	109					

Table E.1: Baseline CIPRA-SA demographics of the 83 patients that experience viral failure.

Patient Name	Initials	Reference Number	Date Of Birth	Gender	Viral Load	Age	CD4	PI*-major	PI-minor	NRTI^	NNRTI#	Comments
166591	FNM	CP0505B52534	25-Jan-71	Female	111,000	35	159					
166811	TMA	CP0505B52494	12-Mar-78	Female	28,700	28	147		T74S			
166831	SAL	CP0505B53096	28-Jun-54	Male	201,000	52	171					
166871	M-F	CP0505B51805	04-Feb-81	Female	162,000	25	284					
166891	WSM	CP0505B52510	02-Jun-62	Female	138,000	44	101					
166911	NHM	CP0505B53079	08-Aug-74	Female	25,200	32	188				V179D E138A	
166931	SJM	CP0505B52758	23-Sep-79	Male	71,400	27	114				M46I	
166941	LRM	CP0505B52764	06-Dec-70	Female	750,001	36	19					
166951	NMQ	CP0505B52511	07-Jun-84	Female	637,000	22	77					
166961	NJO	CP0505B52761	14-Nov-71	Female	15,100	35	181					
166981	PSM	CP0505B52852	02-Nov-77	Female	347,000	29	140					
167041	FJM	CP0505B52886	09-Jan-76	Female	266,000	30	159					
235141	Z-M	CP0505A00249	16-May-77	Female	4,120,000	28	122					
235211	Z-M	CP0505A00398	11-Jun-74	Female	94,800	31	216					
235241	P-T	CP0505A00407	25-Dec-77	Female	854,000	28	138					
235251	S-K	CP0505A00404	15-Mar-70	Male	78,700	35	251					No Storage
235281	SEN	CP0505A00274	12-Feb-68	Male	7,500,001	37	65					
235491	K-S	CP0505A48034	20-Jun-67	Male	1,680,000	38	54					
235681	P-M	CP0505A49126	25-Jul-69	Female	287,000	36	153					
235722	N-M	CP0505A48030	26-Oct-70	Female	750,001	36	ND[1]					
235801	I-G	CP0505A48029	21-Jan-75	Female	210,000	31	127					No amplification
236081	F-W	CP0505A50739	28-Feb-79	Female	292,000	27	251					No Storage

Table E.1: Baseline CIPRA-SA demographics of the 83 patients that experience viral failure.

Patient Name	Initials	Reference Number	Date Of Birth	Gender	Viral Load	Age	CD4	PI*-major	PI-minor	NRTI^	NNRTI[#]	Comments
236281	M-B	CP0505A50764	25-Apr-76	Male	261,000	30	75					
236411	L-G	CP0505A52353	04-Apr-74	Male	99,900	32	90		T74S		V179D	
236611	NVZ	CP0505A55924	05-May-79	Female	379,000	27	16					
236641	G-R	CP0505A55707	12-Oct-72	Female	478,000	34	135					
265241	KVM	CP0505A00402	09-Jul-72	Female	945,000	33	20					
265251	Z-L	CP0505A00394	03-Jun-69	Male	623,000	36	146					No Storage
265451	C-N	CP0505A00472	31-May-66	Female	306,000	39	34					
265551	B-G	CP0505A00480	25-Jul-85	Female	751,000	20	85					
265641	M-V	CP0505A49113	25-Dec-58	Female	221,000	47	135					
265651	BBK	CP0505A49132	21-Jan-71	Female	24,800	34	225		T74S			
265771	N-N	CP0505A50341	15-Jun-76	Male	30,400	30	70					
265791	N-K	CP0505A00471	28-Apr-68	Female	107,000	38	183					No Storage
265811	N-M	CP0505A00396	05-Mar-80	Female	139,000	26	122					
265951	D-M	CP0505A50740	19-Mar-53	Male	188,000	53	62		T74S			
266011	N-M	CP0505A51408	08-Apr-78	Female	2,260,000	28	25					
266261	P-X	CP0505A50750	01-Jan-76	Male	460,000	30	178					
266262	N-X	CP0505A57668	15-Sep-87	Female	95,500	19	94				K103R	
266571	B-M	CP0505A55218	20-Jul-76	Female	153,000	30	143				M46L	
266661	NEM	CP0505A57662	15-Sep-80	Female	707,000	26	162		T74S			

*PI=Protease Inhibitor; ^NRTI=Nucleoside Reverse Transcriptase Inhibitor; [#]NNRTI=Non-NRTI; [1]ND=Not Done

APPENDIX F

Table F.1: 83 CIPRA-SA patients with viral failure.

Patient Name	Initials	Regimen	Date Of Birth	Gender	Viral load	PI-major	PI-minor	NRTI	NNRTI	Comments
135011	K-M	d4T-3TC-EFV	30-Apr-76	Female	13,000				K103N	
135101	M-K	d4T-3TC-EFV	14-Aug-60	Female	9,040					
135141	MTM	d4T-3TC-NVP	16-Jun-76	Female	11,400					
135241	B-S	d4T-3TC-NLF	12-Aug-81	Female	1,310			M184V	K103N, Y188H	
135311	PMM	d4T-3TC-EFV	01-Feb-57	Male	8,240	I47V	T74S	M184V	K103N	
135371	J-Q	d4T-3TC-EFV	12-May-80	Male	3,060		T74S	M184V		
135381	IZM	d4T-3TC-EFV	19-Jul-73	Male	9,810					
135431	C-M	d4T-3TC-NVP	12-May-76	Female	4,610			M184V	K103N, G190A	
135641	ITR	d4T-3TC-NLF	16-Jul-77	Female	15,900		T74S	M184V	K103N, V106M	
135671	RTM	d4T-3TC-EFV	15-May-67	Female	1,294		T74S	M184V	K103N, V108I, P225H	
135771	HAT	d4T-3TC-EFV	02-Jul-56	Male	50,800					
135851	RSZ	d4T-3TC-EFV	19-Apr-63	Male	1,110		V90I	M184V	K103N, K101E, K238N	
135891	MPS	d4T-3TC-EFV		Female					E138A, K103N	
135941	Q-S	d4T-3TC-EFV	23-May-81	Female	27,300		T74S		K103N	
136101	NPM	d4T-3TC-LPVr	24-Dec-75	Female	2,900		V118I		K103N/K, V106A/V, Y188C/Y	

Table F.1: 83 CIPRA-SA patients with viral failure.

Patient Name	Initials	Regimen	Date Of Birth	Gender	Viral load	PI-major	PI-minor	NRTI	NNRTI	Comments
136151	MSM	d4T-3TC-NVP	24-Jun-77	Female	40,600			M184V	Y181C	
136251	DUS	d4T-3TC-NVP	11-Jun-74	Female	6,840					No amplification
136351	NMB	d4T-3TC-NVP	12-May-77	Female	1,350			M184V	Y181C	
136611	BMR	d4T-3TC-EFV	22-May-69	Male	14,700				V106M, Y188C	
136801	MAH	d4T-3TC-LPVr	01-Feb-71	Female	8,160					
136841	MEL	d4T-3TC-EFV	15-May-75	Female	100,001		A71T			
136851	PTG	d4T-3TC-EFV	17-Jul-82	Female	100,001			D67A, T69L		
136911	GTG	d4T-3TC-EFV	16-Jan-67	Female	42,600				K103N	
137011	LPR	d4T-3TC-NVP	06-Mar-77	Female	7,400	Q58E			G190A	
137111	TJM	d4T-3TC-NVP	21-Mar-71	Female	96,700			M184V	V106A, F227L	
165121	A-M	d4T-3TC-EFV	02-Oct-69	Female	4,670	M46V, Q58E		M184V	K103S, G190A	
165131	J-N	d4T-3TC-EFV	22-Dec-65	Male	1,030			M184V	K103N, P225H	
165341	MPM	d4T-3TC-NVP	02-Aug-75	Female	49					No amplification
165351	NCS	d4T-3TC-NVP	28-Jan-85	Female	25,200					
165461	MSD	d4T-3TC-EFV	19-Jul-67	Female	46,800		L10V/L		K103N/K, V106M/V	

Table F.1: 83 CIPRA-SA patients with viral failure.

Patient Name	Initials	Regimen	Date Of Birth	Gender	Viral load	PI-major	PI-minor	NRTI	NNRTI	Comments
165531	BTM	d4T-3TC-EFV	07-Sep-69	Female	3,610					
165621	TQQ	d4T-3TC-NVP	12-Dec-70	Female	1,220			M184V	K103N, E138A	
165781	ILT	d4T-3TC-EFV	13-Jun-71	Female	100,001				V106M, E138A	
165831	LQC	d4T-3TC-LPVr	07-May-78	Female	23,800		A71V			
165881	RMP	d4T-3TC-NVP	12-Jan-84	Female	100,001			M184V	K103N, Y181C	
165961	JTT	d4T-3TC-EFV	22-Sep-70	Male	50,600		A71T			
166141	MLS	d4T-3TC-EFV	29-Jan-73	Male	67,400		L10I			
166151	P-M	d4T-3TC-NVP	07-Feb-81	Female	6,620			M184V	K103N, Y181C	
166161	NHP	d4T-3TC-LPVr	15-May-86	Female	609,000					
166311	NTS	d4T-3TC-LPVr	10-Oct-70	Female	17,800					No amplification
166321	ANK	d4T-3TC-EFV	25-Apr-62	Male	2,360				K103N	
166351	EKP	d4T-3TC-LPVr	28-Jun-82	Female	100,001					
166591	FNM	d4T-3TC-NVP	25-Jan-71	Female	3,690			M184V	K103N	
166811	TMA	d4T-3TC-NVP	12-Mar-78	Female	55,800		T74S	M184V	V108I, Y181C, H221Y	
166831	SAL	d4T-3TC-EFV	28-Jun-54	Male	100,001			M184V	K103N	

Table F.1: 83 CIPRA-SA patients with viral failure.

Patient Name	Initials	Regimen	Date Of Birth	Gender	Viral load	PI-major	PI-minor	NRTI	NNRTI	Comments
166871	M-F	d4T-3TC-EFV	04-Feb-81	Female	100,001			M184V	K101E, P225H, K103N, V108I	
166891	WSM	d4T-3TC-EFV	02-Jun-62	Female	31,800				K103N	
166911	NHM	d4T-3TC-EFV	08-Aug-74	Female	100,001					NO STORAGE
166931	SJM	d4T-3TC-EFV	23-Sep-79	Male	40,300	M46I			K103N	
166941	LRM	d4T-3TC-NVP	06-Dec-70	Female	100,001			M184V	K101E, K103N, V106M	
166951	NMQ	d4T-3TC-NVP	07-Jun-84	Female	3,460			M184V	K103N, K238N	
166961	NJO	d4T-3TC-NVP	14-Nov-71	Female	20,600			M184V	Y181C	
166981	PSM	d4T-3TC-NVP	02-Nov-77	Female	1,590					
167041	FJM	d4T-3TC-EFV	09-Jan-76	Female	100,001		T74S, V75I	M184V	K101E, K103N, G190A	
235141	Z-M	d4T-3TC-NVP	16-May-77	Female	1,500			M184V	V106M, M230L	
235211	Z-M	d4T-3TC-NVP	11-Jun-74	Female	6,100			M184V	A98G, Y181C	
235241	P-T	d4T-3TC-EFV	25-Dec-77	Female	695			M184V	F227L, G190A, V106M	
235251	S-K	d4T-3TC-EFV	15-Mar-70	Male	55,100					
235281	SEN	d4T-3TC-EFV	12-Feb-68	Male	100,001			M184V	K101E, K103N, G190A	
235491	K-S	d4T-3TC-EFV	20-Jun-67	Male	78,200			M184V		

Table F.1: 83 CIPRA-SA patients with viral failure.

Patient Name	Initials	Regimen	Date Of Birth	Gender	Viral load	PI-major	PI-minor	NRTI	NNRTI	Comments
235681	P-M	d4T-3TC-EFV	25-Jul-69	Female	2,290				K103N	
235722	N-M	d4T-3TC-EFV	26-Oct-70	Female	29,400			M184V	K103N, G190S	
235801	I-G	d4T-3TC-EFV	21-Jan-75	Female	1,770			M184V	K103N, P225H	
236081	F-W	d4T-3TC-EFV	28-Feb-79	Female	10,700			M184V	K103N, V108I, P225H	
236281	M-B	d4T-3TC-EFV	25-Apr-76	Male	8,710			M184V	K103N, V108I, P225H	
236411	L-G	d4T-3TC-EFV	04-Apr-74	Male	5,590		T74S		V179D	
236611	NVZ	d4T-3TC-EFV	05-May-79	Female	2,370			K65R, V62A	K103N	
236641	G-R	d4T-3TC-EFV	12-Oct-72	Female	3,910			M184V, a62v	V106M, Y188H	
265241	KVM	d4T-3TC-EFV	09-Jul-72	Female	100,001			M184V	V106M, G190A	
265251	Z-L	d4T-3TC-EFV	03-Jun-69	Male	100,001				E138A	
265451	C-N	d4T-3TC-EFV	31-May-66	Female	6,060			D67N, K70R, M184V, K219E	K101E, V106M	
265551	B-G	d4T-3TC-NVP	25-Jul-85	Female	100,001				V106A	
265641	M-N	d4T-3TC-EFV	25-Dec-58	Female	51,700			M184V		
265651	BBK	d4T-3TC-NLF	21-Jan-71	Female	1,910					No amplification
265771	N-N	d4T-3TC-EFV	15-Jun-76	Male	24,700			M184V	K103N, P225H	

Table F.1: 83 CIPRA-SA patients with viral failure.

Patient Name	Initials	Regimen	Date Of Birth	Gender	Viral load	PI-major	PI-minor	NRTI	NNRTI	Comments
265791	N-K	d4T-3TC-LPVr	28-Apr-68	Female	63,600					
265811	N-M	d4T-3TC-LPVr	05-Mar-80	Female	2,260			M184V	E138A	
265951	D-M	d4T-3TC-EFV	19-Mar-53	Male	3,480		T74S	M184V	K103N, M230L	
266011	N-M	d4T-3TC-EFV	08-Apr-78	Female	21,000			K65R, M184V	V106M, V179D	
266261	P-X	d4T-3TC-EFV	01-Jan-76	Male	1,700			M184V, D67G	G190Q	
266262	N-X	d4T-3TC-NVP	15-Sep-87	Female	8,890			M184V	K103E, V108I, Y181C	
266571	B-M	d4T-3TC-LPVr	20-Jul-76	Female	7,530					No amplification
266661	NEM	d4T-3TC-EFV	15-Sep-80	Female	6,520		T74S	M184V	K103N	

APPENDIX G

Table G1: ViroSeq versus in-house cost analysis

ViroSeq

Assuming Batch of 8 samples and 2 controls

RNA Extraction	Cost per Run
1.5ml Startec Tubes	R 11.20
Tips-1ml	R 25.83
Tips-100ul	R 19.38
Tips-10ul	R 25.83
Isopropanol	R 2.28
Centrifuge tubes (ppt, 15 ml)	8.76
Ethanol	R 2.80
Rnase Free Water	R 10.00
Labels	R 2.50
Pasteur pippete (10ml)	R 11.57
	R 108.58

RT-PCR	
Tips-100ul	R 32.29
Tips-10ul	R 32.29
0.2ml PCR tubes	R 5.15
1.5ml Startec Tubes	R 2.24
	R 71.97

Purification and Quantification	
Tips-1ml	R 0.00
Tips-100ul	R 32.29
Tips-10ul	R 51.67
0.2ml PCR strips	R 3.71
Agarose	R 30.16
50xTAE	R 40.20
Ethidium bromide	R 0.48
Rnase Free Water	R 10.00
Polaroid	R 30.00
Labels	R 2.50
1.5ml Startec Tubes	R 11.20
	R 212.21

Cycle Sequencing and Purifcation	
Tips-1ml	R 0.00
Tips-100ul	R 258.33
Tips-10ul	R 0.00
Isopropanol	R 4.56
Rnase Free Water	R 5.00
Centrifuge tubes (ppt, 15 ml)	8.76
Reagent reservoir (50ml)	R 22.78
Hi-Di Formamide	R 8.00
1.5ml Startec Tubes	R 28.00
96 well plate	R 71.75
Pasteur pippete (10ml)	R 11.57
	R 418.75

Running Sequencer	
Cappillary 50cm	R 336.25
Buffer	R 30.56
POP-6	R 706.00
Speta	R 60.00
Retainer	R 100.00
Rnase Free Water	R 5.00
Centrifuge tubes (ppt, 15 ml)	R 8.76
	R 1,232.81

General Running Costs of the Gentyping Laboratory (divided by 104 runs/year)	
Powder-free, (inner coated, latex)	R 9.90
Tissue	R 6.42
RNAse away/250ml	R 1.13
De-ionised water	R 41.00
Ethanol	R 350.00
Laboratory coat (blue, disposable)	R 22.26
Laboratory coat [Large, white, cotton]	R 22.26
Laboratory marker [wet and dry surfaces]	R 10.95
Timer	R 66.00
Graduated Measuring cylinder	R 26.75
Conical fask	R 48.00
Tube racks	R 48.90
70% Ethanol in spray bottle	R 350.00
10 % bleach (0,5 % sodium hypochlorite in spray bottle	R 42.00
Bottle spray	R 34.29
	R 1,079.86

Consumable Costs	**R 3,124.19**
ViroSeq Kit (Pack 1 and 2)	**R 13,000.00**
Total for 10 reactions	**R 16,124.19**
Labour	**R 1,380.00**

Cost for one sample	**R 2,188.02**
20% failure rate	**R 2,625.63**
Cost per test	**R 2,625.63**

In-house

Assuming Batch of 6 samples and 2 controls

RNA Extraction	Cost per Run
MagNaPure Kit	R 422.19
MagNaPure Consumables/Run	R 641.52
2ml Startec Tubes	R 8.80
SABEX	R 4.00
	R 1,076.51

RT-PCR	
PCR tube	R 5.98
RT Expand	R 297.67
Rnase Inhibitor	R 30.89
dNTPs	R 12.51
Primers	R 1.56
Tips	R 70.00
	R 418.61

PCR	
PCR plus kit	R 43.27
dNTPs	R 6.26
Primers	R 3.12
Tips	R 140.00
SABEX	R 0.40
	R 193.04

Purification and Quantification	
Tips-1ml	R 0.00
Tips-100ul	R 32.29
Tips-10ul	R 51.67
0.2ml PCR strips	R 3.71
Agarose	R 30.16
50xTAE	R 40.20
Ethidium bromide	R 0.48
Rnase Free Water	R 10.00
Polaroid	R 30.00
Labels	R 2.50
1.5ml Startec Tubes	R 11.20
	R 212.21

Cycle Sequencing and Purifcation	
Primers	R 12.48
Big Dye Terminator	R 1,200.00
ViroSeq Pack 2	R 166.67
Tips-1ml	R 0.00
Tips-100ul	R 258.33
Tips-10ul	R 0.00
Isopropanol	R 4.56
Rnase Free Water	R 5.00
Centrifuge tubes (ppt, 15 ml)	8.76
Reagent reservoir (50ml)	R 22.78
Hi-Di Formamide	R 8.00
1.5ml Startec Tubes	R 28.00
96 well plate	R 71.75
Pasteur pippete (10ml)	R 11.57
	R 1,797.90

Running Sequencer	
Cappillary 50cm	R 336.25
Buffer	R 30.56
POP-6	R 706.00
Speta	R 60.00
Retainer	R 100.00
Rnase Free Water	R 5.00
Centrifuge tubes (ppt, 15 ml)	R 8.76
	R 1,232.81

Powder-free, (inner coated, latex)	R 9.90
Tissue	R 6.42
RNAse away/250ml	R 1.13
De-ionised water	R 41.00
Ethanol	R 350.00
Laboratory coat (blue, disposable)	R 22.26
Laboratory coat [Large, white, cotton]	R 22.26
Laboratory marker [wet and dry surfaces]	R 10.95
Timer	R 66.00
Graduated Measuring cylinder	R 26.75
Conical fask	R 48.00
Tube racks	R 48.90
70% Ethanol in spray bottle	R 350.00
10 % bleach (0,5 % sodium hypochlorite in spray bottle	R 42.00
Bottle spray	R 34.29
	R 1,079.86

Total for 8 reaction	**R 6,010.95**
Labour	**R 1,035.00**

Cost of one sample	R 1,174.32
10% repeats	**R 117.43**
Cost per test	**R 1,291.76**

APPENDIX H

The NEW ENGLAND JOURNAL of MEDICINE

Please review the Supplemental Files folder to review documents not compiled in the PDF.

Nurse management is not inferior to doctor management of antiretroviral patients: The CIPRA South Africa randomized trial.

Journal:	*New England Journal of Medicine*
Manuscript ID:	Draft
Article Type:	Special Article
Date Submitted by the Author:	
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Keywords:	HIV/AIDS < Infectious Disease, Health Care Delivery < Public Health, Policy, and Training
Abstract:	Introduction Combination antiretroviral therapy administered by experienced physicians is highly effective. However, due to the scarcity of doctors, continued expansion of antiretroviral therapy (ART) in resource-poor settings will require large-scale task shifting from doctors to other health providers. Methods A randomised comparative strategy trial of treatment outcomes of doctor-initiated and monitored ART and doctor-initiated and nurse-monitored ART was performed at two South African primary-care clinics using regimens of the South African national ART program. Results 812 HIV-positive adults were randomised to either doctor (n=408) or nurse monitored ART (n=404). At baseline patients were 70% female, 34.7% had prior AIDS diagnoses and the median CD4 cell count was 164 cells/mm3. After a median follow-up of 24.3 months, deaths (10 vs 11), virological failures (44 vs 39) and CD4 cell count gain (270 vs 248 cells/mm3), toxicity failures (68 vs 66) and program losses (70 vs 63) were similar in nurse and doctor arms respectively. 371 (46%) patients reached a protocol pre-defined composite endpoint of treatment failure incorporating mortality, viral failure, treatment-limiting toxicities and visit schedule adherence; 192 (47.5%) and 179 (43.9%) in the nurse and doctor arms respectively. The hazard ratio for composite failure was 1.09 (95%CI 0.89-1.33) which lay within the protocol-defined limits for non-inferiority. Conclusions Survival was similar in both strategy arms. Using a comprehensive composite endpoint of treatment outcomes, nurse-monitored ART was shown to be non-inferior to doctor monitored therapy. This study supports task shifting to appropriately trained nurses for monitoring antiretroviral therapy in low income settings.

Nurse management is not inferior to doctor management of antiretroviral patients: The CIPRA South Africa randomized trial

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Acknowledgements: Funding for the CIPRA-SA trial was by the Division of AIDS (DAIDS) of the National Institutes of Allergy and Infectious Diseases, the National Institutes of Health, through grant No 1U19AI53217-01. Funding was also provided by the United States Agency for International Development (USAID) under the terms of agreement 674-A-00-08-00007-00 with Right to Care (RTC). The project was also supported by Awards K01AI083097 and P30-AI50410 from the National Institute of Allergy And Infectious Diseases. The content is solely the responsibility of the authors and does not necessarily represent the official views of the Division of AIDS, the National Institute of Allergy and Infectious Diseases, the National Institutes of Health, USAID, or other parties.

We would like to acknowledge the support of the Gauteng and Western Cape Provincial Health Authorities, the work of the CIPRA-SA Study Team (Sharlaa Badal-Faesen, Mildred Botile, Nastassja Choonilal, Jennipher Gelant, Janet Grab,Veronica Graham, Najma Hafejee, Lynda Hamber, Sindesh Harduth, Johean Hendricks, Colleen Herman, Mellissa Hero, Richard Kaplan, Nicola Killa, Daniella Klemp, Faisel Laher, Thandi Mabiletsa, Zanele Madlala, Ntombekaya Mafukuzela, Bontle Mahlatsi, Helgard Marias, Nomakhaya Mfundisi, Buang Motloba, Cindy Moyo, Mcebisi Mtshizana, Lundi Ncana, Kevin Newell, Sean Palmer, Deborah Pearce, Mary-Ann Potts, Daphne Radebe, Anne Reyneke, Anna Segeneco, Jennifer Sekgale, Jan Steyn, Pinky Thebe, Handre Truter, Diederik van Niekerk, Frieda Verheye-Dua, Karlien Voges, Helen Woolgar), the Endpoint Review Committee (Gary Maartens, David Spencer; Charles van der Horst), and especially the contribution of our patients.

Abstract (words 249)

Introduction

Combination antiretroviral therapy administered by experienced physicians is highly effective. However, due to the scarcity of doctors, continued expansion of antiretroviral therapy (ART) in resource-poor settings will require large-scale task shifting from doctors to other health providers.

Methods

A randomised comparative strategy trial of treatment outcomes of doctor-initiated and monitored ART and doctor-initiated and nurse-monitored ART was performed at two South African primary-care clinics using regimens of the South African national ART program.

Results

812 HIV-positive adults were randomised to either doctor (n=408) or nurse monitored ART (n=404). At baseline patients were 70% female, 34.7% had prior AIDS diagnoses and the median CD4 cell count was 164 cells/mm^3. After a median follow-up of 24.3 months, deaths (10 *vs* 11), virological failures (44 *vs* 39) and CD4 cell count gain (270 *vs* 248 cells/mm^3), toxicity failures (68 *vs* 66) and program losses (70 *vs* 63) were similar in nurse and doctor arms respectively. 371 (46%) patients reached a protocol pre-defined composite endpoint of treatment failure incorporating mortality, viral failure, treatment-limiting toxicities and visit schedule adherence; 192 (47.5%) and 179 (43.9%) in the nurse and doctor arms respectively. The hazard ratio for composite failure was 1.09 (95%CI 0.89-1.33) which lay within the protocol-defined limits for non-inferiority.

Conclusions

Survival was similar in both strategy arms. Using a comprehensive composite endpoint of treatment outcomes, nurse-monitored ART was shown to be non-inferior to doctor monitored therapy. This study supports task shifting to appropriately trained nurses for monitoring antiretroviral therapy in low income settings.

INTRODUCTION (words 3010)

Combination drug therapy has had a remarkable impact in reducing AIDS-related morbidity and mortality [1-3]. In industrialised countries antiretroviral management is administered by specialist physicians who prescribe from the full range of available antiretroviral drugs, supported by frequent laboratory monitoring including resistance testing [4,5]. Several studies in industrialised settings have demonstrated that outpatients cared for by a physician with HIV expertise have better outcomes, including quality of care and survival, [6-12] which may reflect the complexities of HIV infection and its management [4]. In contrast to the relatively small epidemic in resource rich countries, there are 22.4 million people living with HIV in sub-Saharan Africa [13] with an estimated 3.8 million in urgent need to treatment [14]. Globally, there is a shortage of 4.3 million health workers [15] and in South Africa there are only 17.4 medical practitioners per 100,000 people, largely concentrated in urban areas [16, 17]

In contrast to the individualised approach to HIV care in developed countries, the World Health Organisation (WHO) has proposed a public-health approach to antiretroviral therapy (ART) to enable scaling-up access to treatment for large numbers of HIV-positive adults and children in developing countries [18]. An approach using standardised simplified treatment protocols and decentralised service delivery was developed to enable lower level health-care workers to deliver care [19]. Models of care have explored task shifting to clinical officers [20] and a combination of nurses and community workers [21]; however, nurse-led models of antiretroviral delivery have been one of the most widely implemented models of HIV care in poor-resourced African settings [22-24]. The HIV/AIDS strategic plan of South Africa, a medium income country with the world's largest national antiretroviral therapy programme, envisions increasing reliance on nurses for monitoring of antiretroviral therapy [25]. With increasing deployment of nurses for HIV care there is an urgent need for operational research to determine if nurse-led models of care are safe and effective.

We therefore conducted a prospective randomised controlled strategy trial comparing "doctor-initiated-nurse-monitored" to the current standard of care, "doctor-initiated-doctor-monitored" ART. Efficacy was assessed

using a composite endpoint which reflected both treatment outcomes and patient management. Endpoint criteria included death, loss to follow up, viral suppression, drug interruptions due to toxicity and adherence to schedule of visits.

METHODS

The study was a community-based ART strategy trial performed as part of the Comprehensive International Program for Research in AIDS in South Africa (CIPRA-SA - NCT00255840) [26]. The trial was conducted at two primary healthcare sites in South African townships: Masiphumelele in Cape Town and Soweto in Johannesburg.

Study Population

The eligible study population consisted of HIV-1 infected antiretroviral drug naïve adults (< 6 weeks) over 16 years with a CD4+ count <350 cells/mm^3 or a prior AIDS-defining illness [27] and not in first trimester of pregnancy. Women who had received short course ART for prevention of mother to child transmission were not excluded. Screening laboratory investigations for renal function, liver enzymes and hematology were required to be less than grade 3 by the National Institutes of Health Division of AIDS toxicity grading scale [28]. An active opportunistic infection was exclusionary if the patient's treatment status was not considered stable (i.e. treatment for > 7 days) or in the case of tuberculosis (TB) if the treatment had been for less than eight weeks (amended in October 2005 to < 2 weeks of TB treatment). Other exclusion criteria included; concomitant treatment with systemic myelosuppressive, neurotoxic, pancreatotoxic, hepatotoxic, or cytotoxic treatment within 30 days of randomization; acute hepatitis, intractable diarrhea (lasting > 6 weeks), bilateral peripheral neuropathy of grade 2 or higher and drug or alcohol abuse considered by the investigator to potentially interfere with study compliance.

The study was approved at the institutional review boards of the University of Cape Town and University of the Witwatersrand, and written informed consent was obtained from all participants prior to the initiation of study procedures.

Study Design

The CIPRA-SA study was a prospective, unblinded, randomized controlled trial comparing two treatment monitoring strategies. Subjects were allocated to either receive their primary care from doctors (hereafter referred to as doctor arm) or from primary health care nurses (hereafter referred to as nurse arm). Participants were randomized 1:1 within sites in pre-defined blocks of six.

The standard of care strategy (doctor) was consistent with the routine management of patients in the current South African ART program which is based on doctor initiated and monitored treatment [19]. The experimental nurse monitoring strategy utilized doctor-initiated primary health care nurse-monitored ART with participants aware of their randomized treatment assignment. To ensure that all procedures and overall study management conformed to the national [28], and NIH guidelines for research on human subjects [29], a clinical safety team was established consisting of research experienced clinicians. The clinical safety team was responsible for the recruitment of participants including consent, screening processes, initiating therapy and providing ongoing telephonic consultation support to study nurses and doctors.

At each site the experimental arm (nurse) utilized two experienced primary health care nurses. Primary health care nurses are a nationally registered cadre of nurses who have undergone further training in primary health care. The control arm (doctor) consisted of two doctors at each site. Primary-care providers in both arms had limited or no prior experience with antiretroviral therapy. Both arms were supported by lay community counselors trained in treatment adherence counseling and a clinic nurse scheduled patient visits and performed

routine clinic procedures. In order to limit contamination between randomized arms, routine visits were scheduled on different days. A pharmacist oversaw ordering and dispensing of antiretroviral drugs at each site.

The primary study outcome was a composite end-point of possible treatment limiting events that may occur on first-line therapy. These included the following: 1) all-cause mortality, 2) loss-to-follow-up, 3) virologic failure, 4) toxicity failure, 5) withdrawn consent, 6) defaulting clinic schedule, and 7) HIV-disease progression.

Virologic failure was defined as either a decline of < 1.5 $\log_{10}$ in viral load from baseline to 12 weeks of treatment (early failure), or two consecutive viral loads 4 weeks apart of >1000 copies/ml (late failure).

Toxicity failure was defined as Grade 3 and 4 adverse events or other events requiring treatment interruption for more than 42 days [30]. However, single drug substitution as a result of drug related toxicity was not considered failure if treatment was interrupted for less than 42 days.

Patients who missed three consecutive study visits and were not able to be contacted by the study team were defined as lost to follow-up. Defaulting clinic schedule was defined as missing three or more consecutive scheduled clinic appointments with a study visit window of seven days but able to be traced.

Disease progression was defined by new AIDS-defining clinical events, as defined in the revised case definition of the Centers for Disease Control and Prevention [27]. Tuberculosis (TB) is hyperendemic in South Africa and therefore pulmonary TB was not included in the composite endpoint but was analyzed separately.

Throughout the study, a data monitoring team reviewed data from all study visits to identify any default or loss-to-follow-up. An end-point review committee reviewed all events classified as death and toxicity failure to ascertain if the correct assignment to study regimen and procedure was undertaken. An independent data and safety monitoring board reviewed the safety and efficacy of the CIPRA-SA study at six monthly intervals.

Antiretroviral therapy

ART was provided and specified by the South African Department of Health. Regimens initially prescribed by the Clinical Safety Team [31] included a nucleoside backbone of stavudine and lamivudine, with a choice of efavirenz, nevirapine or lopinavir/ritonavir. The initial dose of stavudine was 40 mgs daily for individuals over 60 kgs, which was reduced to 30 mgs for all patients from mid 2007 in line with WHO recommendations [32]. Efavirenz was the preferred non-nucleoside for men and women not wishing to become pregnant and willing to maintain both barrier and hormonal contraception throughout the study. Nevirapine and lopinavir/ritonavir were prescribed to women of child bearing potential depending on whether their CD4+ lymphocyte count was above or less than 250 cells/mm^3 respectively. Pregnant women, who were allowed to enroll after their first trimester, were prescribed either nelfinavir or lopinavir/ritonavir.

Data collection and monitoring

After consenting and randomization by the Clinical Safety Team, the primary-care provider of the assigned arm undertook responsibility for treatment initiation, adherence counseling and follow up visits. Patients were scheduled for study visits at baseline and then at weeks 2, 4, 8, 12, and 12 weekly thereafter. Clinical records were maintained by the primary-care providers in each arm. Study coordinators at each site extracted relevant study data into case report forms which were relayed to a central database using Datafax®.

Statistical analysis and sample size

The sample size was calculated based on an 18-month accrual and 96 weeks follow-up period with 80% power and alpha of 0.05. Non-inferiority of the nurse arm over the doctor arm for cumulative treatment failure was pre-specified as an upper 95% confidence limit for the hazard ratio that was below 1.40. An initial sample size of 850 subjects accounted for potential clustering of multiple enrolled subjects within households. As

significant household clustering was not observed, enrollment was able to be discontinued after 812 subjects with no compromise of pre-established study power.

Baseline differences in randomization groups were described using simple proportions for categorical variables and means and standard deviations for continuous variables. The primary analysis was an intention-to-treat analysis of any treatment failure using Cox proportional hazards regression. Differences in specific reasons for treatment failure (e.g. lost to follow-up, toxicity, death, etc.) were compared by treatment group using hazard ratios and 95% confidence intervals. Finally, differences in time to failure used Kaplan-Meier analyses. Group comparisons using the log-rank statistic, were considered significant if p-values were < 0.05.

RESULTS

The study enrolment flow diagram is shown in figure 1. Between February 2005 and January 2007, 917 subjects were screened for study enrolment, of whom 828 met eligibility criteria and 812 consented and were randomized. Of the 89 patients excluded from the study, 32 did not meet the ART initiation criteria, 22 had acute medical conditions, 18 were considered unsuitable by investigators or failed to return, 8 had laboratory results out of eligible range and 9 were unable to take oral medication or were on excluded medications. Study subjects were 99% black African and 70% were female. Four hundred and eight individuals were randomized to the nurse arm and 404 to the doctor arm. The median follow-up was 120 weeks (IQR 60-144) with no difference between the nurse and doctor arms (median 119 *vs* 120 weeks respectively).The total follow-up period was 815.7 patient years and 830.9 patient years for the nurse and doctor arms, respectively.

Baseline characteristics together with prior antiretroviral exposure and initial regimens are shown for each study arm in table 1. The study cohort had a median age of 32 years and had advanced HIV-disease as manifested by 35% with prior AIDS, 57% with viral loads >100,000 copies per millilitre (cpm) and a median of 164 CD4 cell/mm^3. Patients in the nurse arm were slightly more likely to be female (73% vs 68%) and be in CDC stage A (40% vs 35%) than patients in the doctor arm but differences were small and non-significant. Despite the slight

preponderance of females in the nurse arm, prior exposure to antiretroviral prophylaxis as part of mother-to-child prophylaxis was evenly distributed between study arms.

Most subjects commenced with non-nucleoside based therapy (92%) together with a nucleoside backbone of stavudine and lamivudine which reflected the prevailing South African national treatment guidelines.

Cumulative treatment failure

The primary study end-point of cumulative treatment failure was reached by 371 (45.7%) patients after a total of 1647 patient-years of follow-up. One hundred and ninety two (48%) of the subjects in the nurse arm reached the composite treatment failure endpoint and 179 (44%) in the doctor's arm. Using proportional hazards regression there was a nine percent increased risk of failure in the nurse arm (Hazard Ratio = 1.09 (95% confidence interval 0.89-1.33). The hazard ratio and 95% confidence limits lie below the pre-defined study criterion for inferiority (1.4). The Kaplan-Meier estimated time to composite failure was similar for each arm (Figure 2).

The hazard ratios for individual treatment failure parameters of the composite end-point are shown in Table 2. Deaths contributed 5.7% of the total events, viral failure 22.4%, toxicity failure 36% and protocol defined lost to follow up failure 36% of end-points. The subcategories of the composite endpoint hazard ratios are all closely distributed to 1.0 (0.92-1.15). There were no significant differences between study arms for Kaplan-Meier estimates of time to death, viral failure, toxicity failure and lost to follow up (Figure 2).

Deaths

A total of 21 deaths, 10 in nurse arm and 11 in the doctor arms, were included in the analysis. One further death was not included in the analysis as the participant had met a protocol defined end-point of toxicity failure before the death occurred. All the deaths were reviewed by a blinded end-point review committee to assign the cause

of death as study related, treatment related, disease related or non-study related. Two deaths were assessed as due to lactic acidosis, related lactic acidosis and 2 deaths were considered to be non-study and non-HIV related.

Immunology

Immune response was not a component of the primary endpoint; however, CD4 cell count increases from base line were 155 cells (IQR 119-193) and 158 cells (IQR 125-169) at 1 year and 239 cells (IQR 217-290) and 220 cells (IQR 174-274) at 2 years for the nurse and doctor arms, respectively. Cumulative treatment failure was not impacted significantly by either low baseline CD4+ count <200 cells/mm^3 nor high viral load > 100,000 cpm. However, there were a non-significant trends for increased failure in the nurse arm for individuals with CD4 cell count <200cells/mm^3 (p=0.14) and with viral loads >100,000 copies/ml (p=0.09).

Safety monitoring and toxicity:

Grade 3 and 4 toxicities which occurred during the study are shown in table 3. The most frequent laboratory abnormalities were anemia and neutropenia, raised lactate and abnormal hepatic enzymes occurring at 10.2, 10.1 and 7.0 per 100 patient years respectively. The high frequency of hyperlactatemia resulted in a data safety monitoring board recommendation in 2007, for additional training and management of raised lactate. Grade 3 and 4 and dose limiting toxicities were more commonly reported in the doctor arm compared to the nurse arm (incidence rate of 55.5 *vs* 45.5 respectively). However, a retrospective review by the CIPRA clinical safety team of all laboratory investigations performed throughout the study was consistent with the equal distribution of laboratory defined adverse events between arms.

Discussion:

This study reports the findings of the first prospective, randomized, controlled study comparing of nurse versus doctor managed ART. Mortality, viral suppression, CD4 cell count response and a composite end-point reflecting multiple aspects of ART delivery, demonstrated that nurse monitored therapy was not inferior to

doctor monitored therapy. These findings support observational data from other treatment programmes reporting successful use of task shifting in HIV care [33-41] and also for other disease management [42].

Expansion of ART services is urgently required in resource-poor countries in order to achieve universal access targets by 2010 [43] and further expansion will be needed with initiation of universal testing and treating strategies [44]. There was no difference in mortality, viral failure or immune recovery between the study arms although there was a non-significant trend in increased failure in the nurse arm for patients with advanced HIV disease. This study therefore strongly supports the strategy of "task shifting" and indicates that HIV management by nurses can be safe and effective even for those patients commencing therapy with advanced HIV infection.

Although both study strategies successfully managed drug related toxicities, the study does highlight a high frequency of lipomorphologic changes and lactate elevation associated with use of regimens including stavudine. Recent WHO guidelines have moved away from reliance on stavudine [45] however, it remains widely used in resource-poor HIV therapy programs [18]. In our study the overall drug toxicity frequency appeared to be lower than earlier reports of stavudine based toxicities which resulted in drug substitutions in excess of 20% after three year [46]. The dose reduction of stavudine to 30 mgs after the first year of the study which was in line with WHO recommendations [30] may have reduced drug limiting toxicities somewhat. However, two of the study deaths were due to hyperlactatemia, a recognised complication of stavudine use.

Randomised controlled studies are frequently considered the "gold standard" on which treatment policies should be based. However, there may be some caveats in applying trial findings to non-study settings and other populations. A strength of our study was that it was performed at urban and peri-urban primary care clinics in South African high-burdened communities where large scale task shifting will be required. Additionally, in order to limit the "contamination" between the arms of the study, once the participants were randomised scheduled visits were booked for different days of the week,

The study, however, did not necessarily replicate the typical conditions under which therapy is presently delivered in resource-poor settings. For instance, all the clinical staff in the study received protocol specific training in the conduct of ethical research including didactic clinical management and ongoing telephonic clinical support if required. However, widespread "task shifting" will require increased training, a redefinition of scope of practice for nurses and a clinical support structure.

It should be noted that the study design did not address "nurse initiated" antiretroviral therapy because the prescription of licensed medication in South Africa is restricted to doctors. Implementation of nurse initiated therapy would therefore require additional changes to the existing legislation. However, the new national HIV strategic plan does envisage initiation of therapy by doctors together with wide scale task shifting to nurses for ongoing patient management [25].

In conclusion, nurses were shown to be non-inferior to doctors in monitoring a public health ART program in South Africa. The results of this study strongly support the expanded access to treatment using models of task shifting in primary health care.

Figure 1: CONSORT study flow chart and participant disposition for the CIPRA-SA trial, a randomized trial of doctor vs. nurse monitored antiretroviral therapy in South Africa.

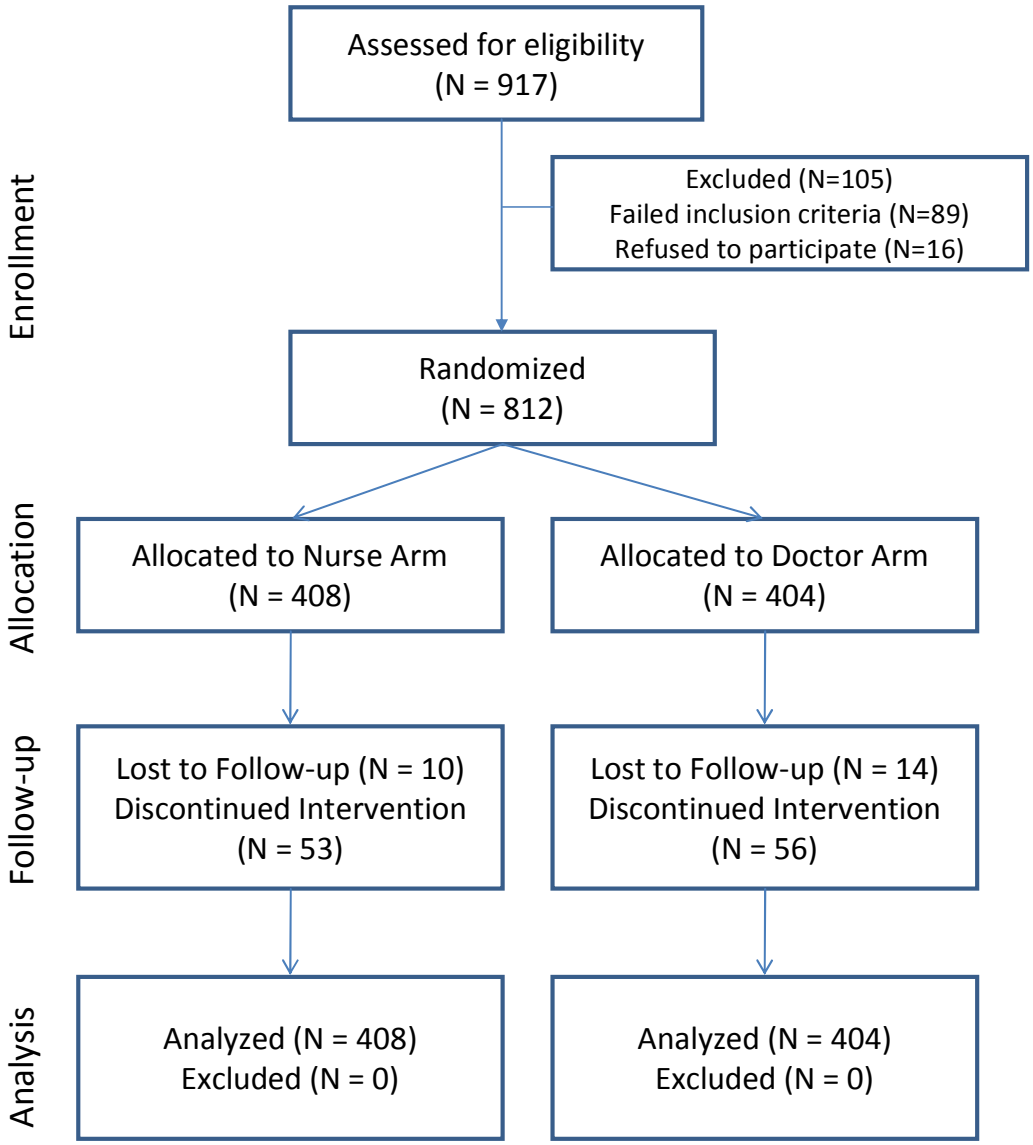
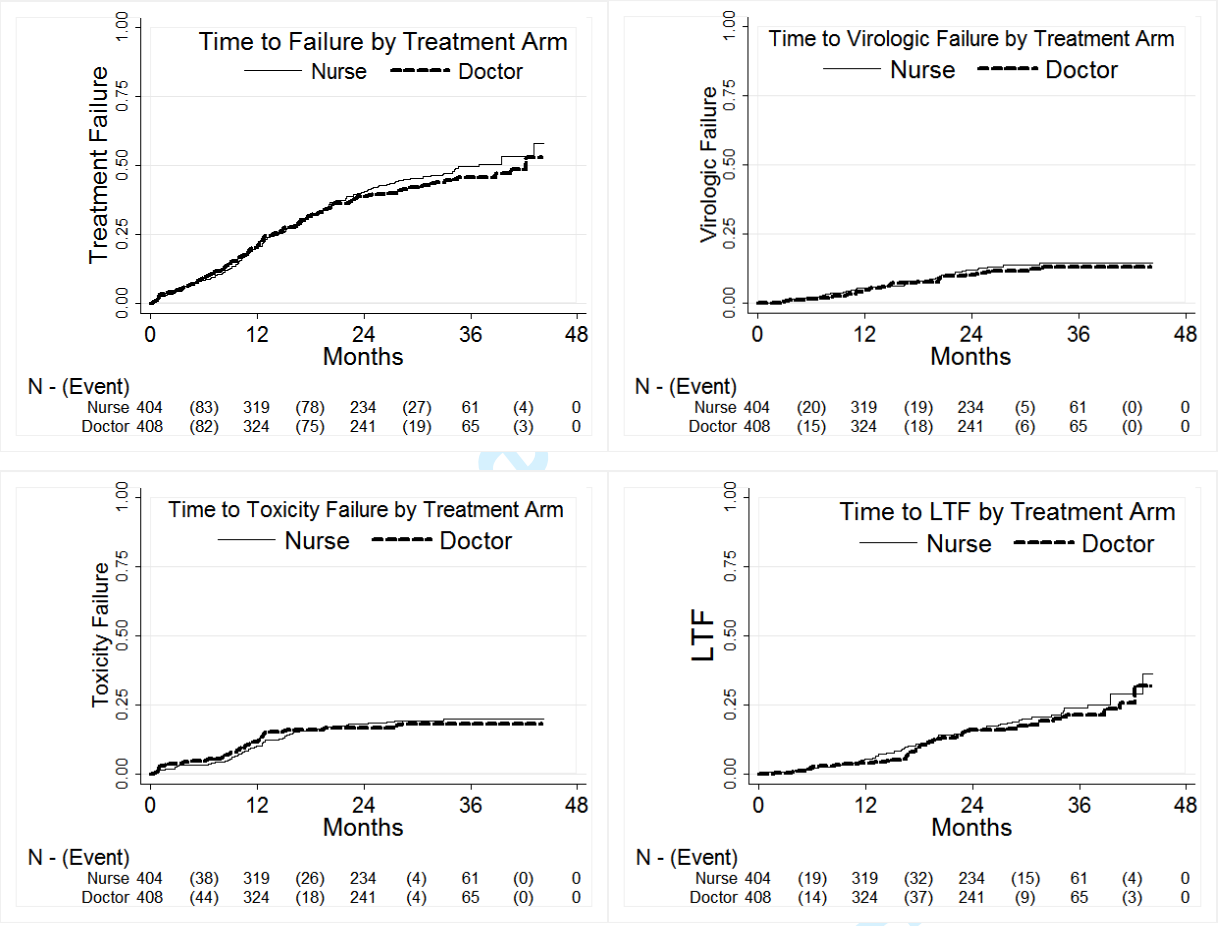


Figure 2a: Kaplan-Meier curves of time to cumulative treatment failure by study arm among 812 subjects randomized to doctor or nurse monitored antiretroviral therapy in the CIPRA-SA trial in South Africa; Figure 2b-d: Kaplan-Meier curves of time to specific reasons for treatment failure by study arm among 812 subjects randomized to doctor or nurse monitored antiretroviral therapy in the CIPRA-SA trial in South Africa*



* a) a Kaplan-Meier curve demonstrating the composite end-point of cumulative treatment failure. The primary health care nurse arm of the study is non-inferior to the doctor arm (log-rank p-value 0.4238). b) shows time to virologic failure stratified by treatment ARM (log-rank p-value = 0.5340); c) shows time to toxicity failure stratified by treatment ARM (log-rank p-value = 0.4678); d) shows time to loss to follow-up stratified by treatment ARM (log-rank p-value = 0.8358);

Table 1: Baseline demographic and clinical characteristics of 812 subjects randomized to doctor or nurse monitored antiretroviral therapy in the CIPRA-SA trial in South Africa

	Nurse Arm (N=404)		Doctor Arm (N=408)		Total (N=812)	
Female	297	(73.5%)	276	(67.7%)	573	(70.6%)
Age years median (IQR)	32.3	(28.0-36.6)	32.2	(28.0-37.4)	32.3	(28.0-37.1)
BMI (kg/m^2) (IQR)	23.5	(21.3-27.6)	23.5	(20.4-26.8)	23.5	(20.8-27.2)
CDC Classification						
Class A (%)	160	(39.6%)	141	(34.6%)	301	(37.1%)
Class B (%)	111	(27.5%)	118	(28.9%)	229	(28.2%)
Class C (%)	133	(32.9%)	149	(36.5%)	282	(34.7%)
CD4+ Count (cells/ml)						
<200 (%)	260	(64.4%)	257	(63.0%)	517	(63.7%)
200-350 (%)	119	(29.5%)	131	(32.1%)	250	(30.8%)
350-500 (%)	23	(5.7%)	18	(4.4%)	41	(5.1%)
> 500 (%)	2	(0.5%)	2	(0.5%)	4	(0.5%)
Median (IQR)	165	(105-235)	164	(110-225)	164	(109-229)
Viral load (copies/ml)						
≤ 100,000 (%)	181	(44.8%)	170	(41.7%)	351	(43.2%)
> 100,000 (%)	223	(55.2%)	238	(58.3%)	461	(56.8%)
Log_{10} mean viral load (std)	4.99	(0.75)	5.09	(0.73)	5.04	(0.74)
Baseline regimen prescribed						
Stavudine, Lamivudine, Efavirenz	293	(72.5%)	304	(74.5%)	597	(73.5%)
Stavudine, Lamivudine, Nevirapine	72	(17.8%)	81	(19.9%)	153	(18.8%)
Stavudine, Lamivudine, Lopinavir/rtv †	35	(8.7%)	20	(4.9%)	55	(6.8%)
Stavudine, Lamivudine, Nelfinavir‡	4	(1%)	3	(0.7%)	7	(0.9%)
Prior exposure to antiretrovirals*						
Single Dose Nevirapine (%)	81	(20%)	86	(21.1%)	167	(20.6%)
Zidovudine (%)	2	(0.5%)	4	(1.0%)	6	(0.7%)
Nevirapine, Zidovudine (%)	14	(3.5%)	15	(3.7%)	29	(3.6%)
Triple drug therapy (%)	1	(0.2%)	0	(0%)	1	(0.1%)

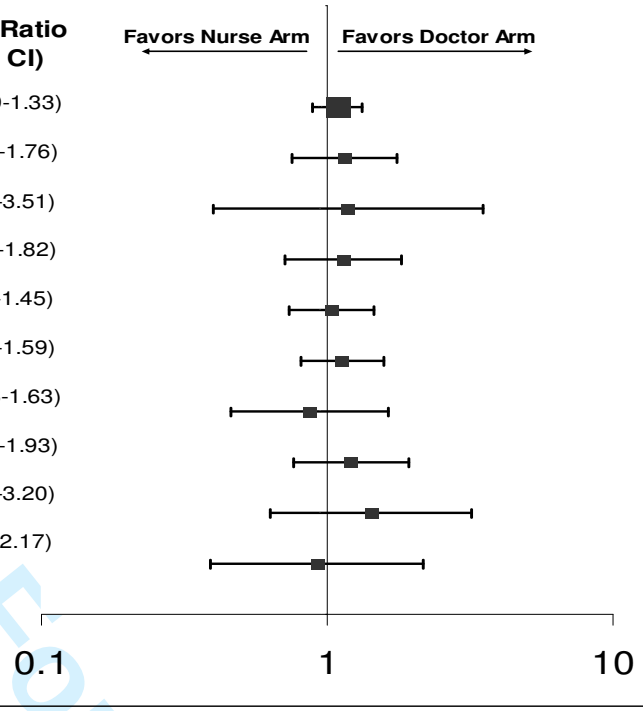
† ‡Protease inhibitor containing regimens were prescribed to pregnant women or women of childbearing potential with CD4+ count >250 who were unable or unwilling to use both a barrier contraceptive and a progesterone contraceptive. These women could not receive either a Nevirapine or Efavirenz containing regimen

* Prior exposure to antiretroviral therapy for prevention of transmission, either from mother-to-child or in post-sexual exposure prophylaxis was permitted by the protocol for up to 6 weeks of treatment.

Table 2: Cumulative treatment failure (primary end-point) and accompanying reasons by study arm for 812 subjects randomized to doctor or nurse monitored antiretroviral therapy in the CIPRA-SA trial in South Africa†

†The two arms of the study were compared using a composite end-point or cumulative treatment failure. The composite consisted of each of the reasons listed below. ^ Early virologic failure was defined when a participant failed to demonstrate a serologic viral load decline of more than 1.5 logarithm within 12 weeks of initiating treatment. * Late virologic failure was defined as rebound in viral load from undetectable to more than 1000 copies/ml confirmed within one month.

	Nurse (N=404)	Doctor (N=408)	Hazard Ratio (95% CI)
CUMULATIVE FAILURE	192 (48%)	179 (44%)	1.09 (0.89-1.33)
All Virologic Failure	44 (11%)	39 (10%)	1.15 (0.75-1.76)
<1.5 Log drop VL^	7 (2%)	6 (2%)	1.18 (0.40-3.51)
2 VL > 1000*	37 (9%)	33 (8%)	1.14 (0.71-1.82)
Toxicity Failure	68 (17%)	66 (16%)	1.04 (0.74-1.45)
All Loss**	70 (17%)	63 (15%)	1.13 (0.81-1.59)
Withdrew Consent	18 (5%)	21 (5%)	0.87 (0.46-1.63)
Default Clinic Schedule	38 (9%)	32 (8%)	1.21 (0.76-1.93)
Lost to follow up	14 (4%)	10 (3%)	1.42 (0.63-3.20)
Death	10 (3%)	11 (3%)	0.92 (0.39-2.17)



**Any loss was defined as: 1) withdrawn consent was if the patient withdrew from participating in the study for whatever reason and represents in most cases a transfer away from the site to another geographic location and clinic; 2) defaulting clinic schedule was a protocol defined measure of adherence to the clinic schedule, any participant who missed three consecutive visits was considered to be defaulting; (3) Loss to follow-up was consider if a participant did not return to the clinic for three consecutive clinic visits and could not be traced.

Table 3: Rate of laboratory and clinical dose limiting or Aids Clinical Trial Group grade three and four defined toxicities [30] by study arm among 812 subjects randomized to doctor or nurse monitored antiretroviral therapy in the CIPRA-SA trial in South Africa*†

	Nurse arm		Doctor arm			
TOXICITY	**Event in 815.74 py**	**Rate/ 100 py**	**Event in 830.88 py**	**Rate/ 100 py**	**IRR (95% CI)**	**p-value**
Laboratory:						
Haematology	62	7.6	106	13	0.60 (0.44 - 0.82)	0.0011
Biochemistry	1	0.1	2	0.2	0.51 (0.05 - 5.62)	0.5745
Liver	44	5.4	72	8.7	0.62 (0.43 - 0.91)	0.0124
Hyperlactataemia	80	9.8	87	10	0.94 (0.69 - 1.27)	0.6724
Pancreatic	4	0.5	4	0.5	1.02 (0.25 - 4.07)	0.9793
Renal	3	0.4	1	0.1	3.06 (0.32 - 29.4)	0.3085
Drug related rash	4	0.5	5	0.6	0.81 (0.22 - 3.03)	0.7598
Clinical HIV events:						
Tuberculosis	28	3.4	31	3.7	0.92 (0.55 - 1.53)	0.7490
Cervical dysplasia	1	0.1	4	0.5	0.25 (0.03 - 2.28)	0.1865
Neurologic	10	1.2	32	3.9	0.32 (0.16 - 0.65)	0.0009
Intestinal	8	1.0	7	0.8	1.16 (0.42 - 3.21)	0.7689
Skin	1	0.1	2	0.2	0.51 (0.05 - 5.62)	0.5745
Lipodystrophy, lipoatrophy	45	5.5	51	6.1	0.90 (0.60 - 1.34)	0.6015
Miscellaneous	23	2.8	23	2.8	1.02 (0.57 - 1.82)	0.9503
Clinical Non-HIV events:						
CNS	5	0.6	13	1.6	0.39 (0.14 - 1.10)	0.0648
Obstetric/gynaecology	10	1.2	7	0.8	1.46 (0.55 - 3.82)	0.4439
Miscellaneous	34	4.2	37	4.5	0.94 (0.59 - 1.49)	0.7806

* PHCN arm had 815.7 total person-years while the MO arm had 830.9

† Active reporting of adverse events was undertaken by the primary care giving nurse or doctor, and a retrospective review by the study team of all laboratory adverse events greater than or equal to grade three was undertaken.

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