An Investigation of Virologic Failure and the Spectrum of Drug Resistance Mutations in a Paediatric ART Programme in Rural KZN, SA

Submitted in fulfillment of the requirements for the degree of Masters Medical Science at the Department of Virology, Nelson R Mandela School of Medicine, College of Health Sciences, University of KwaZulu – Natal, Durban, South Africa

By

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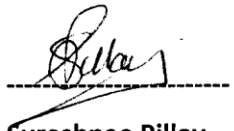
AUTHOR'S DECLARATION

This study represents original work by the author. It has not been submitted previously to this or any other University. Where use was made of the work of others, it has been duly acknowledged in the text. This study was approved by the UKZN IRB (Ethics Clearance # BF052/010)



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PUBLICATIONS

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An Affordable HIV-1 Drug Resistance Monitoring Method for Resource Limited Settings. *JOVE*. In review.

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AIDS Research and Therapy. In review.

Johannes Viljoen, Sandrine Gampini, Siva Pragashini, Dian Valea, Sureshnee Pillay, Dramane Kania, Nicolas Meda, Marie-Louise Newell, Philippe Van de Perre and Francois Rouet for the World Health Organisation/ ANRS 1289 Kesho Bora Study Group. Dried Blood Spot HIV-1 RNA Quantification Using Open Real-Time Systems in South Africa and Burkina Faso. *J Acquir Defic Syndr.* 55:3

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LIST OF ABBREVIATIONS

3TC	Lamivudine
ABC	Abacavir
ACVL	Africa Centre Virology Laboratory
AIDS	Acquired Immunodeficiency Syndrome
ALT	Alanine aminotransferase
ANRS	French AIDS National Agency
ART	Antiretroviral therapy
ARV	Antiretroviral
AZT	Zidovudine
cDNA	Complementary deoxyribonucleic acid
CHER	Children with HIV Early Antiretroviral Therapy
CPR	Calibrated Population Resistance
d4T	Stavudine
ddI	Didanosine
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
DoH	Department of Health
DRMs	Drug resistance mutations
EDTA	Ethylenediaminetetraacetic acid
EFV	Efavirenz
EI	Entry inhibitors
FBC	Full blood count
FDA	Food and Drug Administration
FI	Fusion Inhibitors
FTC	Emtricitabine
GSS	Genetic susceptibility scores
HAART	Highly Active Antiretroviral Therapy
HDAPs	HIV drug resistance programmes
HFC	Hlabisa Failure Clinic
HIV	Human Immunodeficiency Virus

IAS	International Aids Society
II	Intergrase inhibitors
IQR	Interquartile range
KZN	KwaZulu- Natal
LDL	Low density lipoprotein
LIMS	Laboratory information management system
LPV/r	Lopinavir/ritonavir
LTR	Long terminal repeat
MVC	Maraviroc
MDR	Multi Drug Resistant
Mgcl	Magnesium chloride
ML	Maximum likelihood
MM1	Master Mix #1
MM2	Master Mix #2
mRNA	Messenger ribonucleic acid
NNRTI	Non-Nucleoside Reverse Transcriptase Inhibitor
NRTI	Nucleoside Reverse Transcriptase Inhibitor
NVP	Nevirapine
PCR	Polymerase chain reaction
PHC	Primary Health Care
PI	Protease inhibitors
pMTCT	prevention of mother –to-child transmission
qPCR	quantitative polymerase chain reaction
RAL	Raltegravir
RLS	Resource limited setting
RNA	Ribonucleic acid
RT	Reverse transcriptase
RTV	Ritonavir
SA DoH	South African Department of Health
SATuRN	South Africa Treatment Resistance Network
SSIII	Superscript III
TAMs	Thymidine analogue mutations
TDF	Tenofovir

TR	Transmitted Resistance
VL	Viral load
WHO	World Health Organisation
XDR TB	Extreme Drug Resistant Tuberculosis

ABSTRACT

Background

Better understanding of drug resistance patterns in HIV-infected children on antiretroviral therapy (ART) is required to inform public health policies in high prevalence settings. The aim of this study was to characterise the acquired drug resistance in HIV-infected children failing first-line ART in a decentralised rural HIV programme.

Methods

Plasma samples were collected from 101 paediatric patients (<15 years of age) identified as failing ART. RNA was extracted from the plasma, reverse transcribed and a 1.3kb region of the protease gene was amplified and sequenced using Sanger sequencing protocols. Sequences were edited in Geneoius and drug resistance mutations were identified using the RegaDB and the Stanford, Rega and ANRS resistance algorithms. The prevalence and frequency of mutations were analysed together with selected clinical and demographic data in STATA v11.

Results

A total of 101 children were enrolled and 89 (88%) were successfully genotyped; 73 on a non-nucleoside reverse-transcriptase inhibitor (NNRTI)-based regimen and 16 on a protease inhibitor (PI)-based regimen at the time of genotyping. The majority of patients on an NNRTI regimen (80%) had both nucleoside reverse-transcriptase inhibitor (NRTI) and NNRTI resistance mutations. M184V and K103N were the most common mutations amongst children on NNRTI-based and PI-based regimens. 23% had one or more thymidine analogue mutation (TAM) and 6% had ≥3 TAMs. Only one child on a PI-based regimen harboured a major PI resistance mutation.

Conclusions

Whilst the patterns of resistance were largely predictable, the few complex resistance patterns seen with NNRTI-based regimens and the absence of major PI mutations in children failing PI-based regimens suggest the need for wider access to genotypic resistance testing in this setting.

CHAPTER ONE

1. INTRODUCTION

By the end of 2009 there were an estimated 2.5 million (range = 1.34million) children living with the human immunodeficiency virus (HIV) around the world[1]; and approximately 340 000 children younger than 15 years of age were living in South Africa[2]. In the same year approximately 1,000 babies, per day, were newly infected with HIV through one of three transmission routes, namely mother-to-child transmission (MTCT) in-utero, during labor or delivery, and during breastfeeding (UNICEF, 2010). This translated to an estimated 370 000 [230 000–510 000] new paediatric HIV infections, a decline of 24% from the estimate of five years previously[1].

Without antiretroviral (ARV) treatment life expectancy among HIV-infected infants is reduced to just 2 years[3]. However, greater access to anti-retroviral therapy (ART) globally has already had a significant impact on survival rates and clinical outcomes[4-8]. In 2010, an estimated 250 000 (range=220 000-290 000) children less than 15 years of age, died from AIDS-related causes; 20% fewer than in 2005[9]. In addition, infection rates have declined. Early ART initiation results in an up to five-fold reduced mortality rate and a 90% increase in survival rates among infants[10]. Violari et al[10] in their randomized trial of 377 infants (6-12 weeks of age) from two major South African centre's, namely Gauteng and Western Cape, (the Children with HIV Early Antiretroviral Therapy (CHER) study) reported a 76% and 75% reduction in mortality and disease progression rates respectively following early diagnosis and treatment initiation. The authors proposed ART coupled with treatment "holidays" as a feasible and practical paediatric management strategy, which they suggest, may become the norm in future[11]. Based on this and similar

studies, the WHO, in 2010, amended their treatment guidelines[10, 12-14] recommending ART for all HIV-positive infants irrespective of CD4 count or clinical stage.

Once children are on treatment, the cumulative risk of failure, defined as the inability to achieve or maintain suppression of viral replication, increases over time[15] as does the likelihood of developing drug resistance mutations[13] Drug resistance is the consequence of mutations that emerge in the viral proteins targeted by antiretroviral agents[16]. Without careful management, patients on failing regimens will develop not only drug resistance mutations at high prevalence rates but also accumulate resistance mutations if in failure for long periods. The unfortunate consequence is that future drug choices are severely compromised[17]. Current ARVs primarily target specific functional proteins of HIV; namely the reverse transcriptase enzyme targeted by RT inhibitors and the protease enzyme targeted by protease inhibitors, or PIs. Mutations in these genomic regions result in conformational and functional adaptations that confer drug resistance to these viral variants. ART prophylaxis as part of prevention of mother-to-child transmission (pMTCT) programmes, in addition, may expose infants to sub-therapeutic levels of ARVs such as nevirapine[18]. Sub-therapeutic drug levels have been associated with virological failure and the development of drug resistance among treated infants[14].

Resource–limited settings, such as South Africa pose unique challenges to the implementation of effective and sustainable ART programmes. The most notable restrictions remain the limited treatment options available in this country, the un-availability of paediatric-friendly drug formulations and limited laboratory infrastructure to monitor treatment efficacy and virologic failure[19]. In addition, socio-economic and psychosocial[18] factors impede optimal patient management, delay access to ARVs and accelerate the development of drug resistance. Given that children have a higher risk of developing drug resistance, it is clear that interventions within coordinated surveillance strategies are vital to long-

term clinical success. As a result there is an urgent need to identify patients in virological failure, and classify drug resistance patterns as well as levels, in a timely manner.

Either genotypic or phenotypic tests to detect drug resistance mutations can guide therapeutic management of HIV-infected patients failing ART[20]. Data on paediatric ARV resistance in South Africa, particularly from rural primary health care programmes in the public sector, remains limited. This study, therefore, aimed to redress this imbalance in current knowledge of paediatric drug resistance in these resource-limited settings as a model for patient management. Towards the goal of implementing resistance testing on a large scale, our study investigated the spectrum and prevalence of drug resistance among paediatric patients within a South African Department of Health (DoH) primary health care facility in rural KwaZulu-Natal and the efficacy of implementing routine drug resistance testing for patient management as well as for research purposes. Resistance testing of patients failing ART has been successfully used in adult patients in our setting[21] as the system facilitates regular patient monitoring and routine resistance genotyping. This initiative has, in addition, yielded a cheaper, more accessible in-house genotyping method that may be applied at a larger public health level[22, 23], as well as the use of resistance genotyping as both a research and clinical management tool. The same system was applied to the analysis and management of paediatric patients failing ART in this project.

1.1 JUSTIFICATION

Despite increased prevention strategies there will always be a proportion of children who will acquire HIV. Since these children as well as those that are already on treatment will require ART for the rest of their lives there exists a high risk of virologic failure and the development of drug resistance over time[13]. South Africa has the largest ART programme in the world with approximately 152 000 children receiving ARVs in 2011[24] This number is growing annually yet infrastructure to monitor their response to treatment remains limited. Paediatric patients, under the current South African Department of Health guidelines (SA DoH) (Table 1), receive two nucleoside reverse transcriptase inhibitors (NRTI)[22] and a non-nucleoside reverse transcriptase inhibitor (NNTRI) or a protease inhibitor (PI). Thus the breadth of drugs available in this setting is severely limited. In addition, there is currently no 3rd line option available to children failing both 1st and 2nd line options. This is of particular significance given the newly revised South African guidelines recommending ART initiation of all HIV-positive infants, at diagnosis, irrespective of their CD4 count or clinical stage[23]. The number of children on life-long ART is thus expected to increase significantly while children already on ART will be ageing, on treatment, into adolescence and adulthood.

The number of current publications related to paediatric HIV drug resistance from sub-Saharan Africa is scant (Table 2). There is some evidence that outcomes for children in rural areas of South Africa are poorer than those in urban areas[25]. The data on ARV resistance in children in South Africa are relatively limited and have largely been restricted to urban hospital-based programmes, which may not be representative of all programmes[26-32].We found only nine studies, to date, conducted in South Africa that used genotyping to determine the prevalence of drug resistance among children at a population level[26-34]. All these studies included HIV-1 subtype C only. Unfortunately, none of these studies consist of sample size greater than 50 children with the result that no reliable conclusions on resistance patterns

can be drawn from these data sets (Table 2). Of these studies two [28, 30] of them used in-house genotyping methods to assess resistance patterns in their cohorts.

Our aim was therefore to add to this knowledge pool by describing the profile of drug resistance in a rural public health programme. There are 1653 children currently active in the Hlabisa HIV Treatment and Care Programme (Personal communication: Dr James Ndirangu*) in Northern KwaZulu-Natal (KZN), a rural, decentralized HIV treatment programme partnered by the Africa Centre and the SA Department of Health[35]. It has been previously reported that approximately 25% of children[4], within this programme are vireamic i.e. do not display optimal viral suppression (VL <25 copies/ml), after 6-12 months of first-line ART[4] as has also been reported by Davies et al[12] in a larger (n=6078) multi-cohort South African study. Since maintaining individuals on failing ARV regimens promotes the accumulation of drug resistance mutations and a high level of cross-resistance, it is vital that emerging resistance patterns in paediatric cohorts are identified early in order to safe-guard future ART regimens and ensure that paediatric patients will reach adulthood. Therefore the primary objective of this prospective descriptive study was to determine the prevalence and spectrum of resistance mutations in children failing the current South African DoH ARV guidelines.

Dr James Ndirangu* is a demographer and biostatistician at the Africa Centre for Health and Population studies who has been actively involved in monitoring the epidemiology of HIV in the Hlabisa Treatment and Care Program.

Table 1. Standardised national ART regimens for infants and children[22]

First Line Regimen	
All infants and children under 3 years (or < 10kg)	ABC + 3TC + LPV/r
Children ≥ 3 years (or ≥ 10kg)∞	ABC + 3TC + EFV
Currently on d4T-based regimen	Change d4T to ABC if viral load is undetectable If viral load >1000 copies/ml manage as treatment failure If viral load between 50 – 1000 copies/ml – consult with expert for advice
Second Line Regimen	
Failed first line Protease Inhibitor (PI)-based regimen	
Failed first line PI-based regimen	**Recommended second line regimen**
ABC + 3TC + LPV/r	**Consult with expert for advice***
D4T + 3TC + LPV/r	
Unboosted PI-based regimen	
Failed First line NNRTI based regimen (discuss with expert before changing)	
Failed first line NNRTI-based regimen	**Recommended second line regimen**
ABC +3TC + EFV (or NVP)	AZT + 3TC +LPV/r
d4T +3TC + EFV (or NVP)	AZT + ABC + LPV/r
Third line regimens	
Failing any 2nd line regimen	Refer for specialist opinion – Regimen based on genotype resistance testing, expert opinion and supervised care Access to third line ART will be managed centrally by the National Department of Health

∞ Children ≥ 3 years and exposed to NVP for 6 weeks or longer (PMTCT) should be initiated on ABC + 3TC + LPV/r

Table 2. Prevalence of drug resistance mutations in South African paediatric cohorts

Site	*n*	HIV RNA criteria for genotyping	PI	NNRTI	M184V	TAMs	Subtype
Pretoria[34]	33	>1000copies/ml*	21%	39%	76%	18%	C
Johannesburg[30]	41	>1000copies/ml*	36%	10%	71%	N/A	C
Johannesburg[33]	20	>1000copies/ml*	N/A	65%	65%	N/A	C
Johannesburg[26]	41	>5000copies/ml*	44%	98%	82%	N/A	C
Cape Town[28]	39	>4000copies/ml*	43%	N/A	83%	26%	C
Elandsdoorn, Limpopo[27]	23	Above detection (> 1000copies/ml)*	N/A	87%	78%	13%	C
Gauteng[29]	39	N/A	N/A	N/A	N/A	N/A	C
Durban[31]	41	N/A	N/A	N/A	70.7%	58.5%	C
Cape Town[32]	37	>1000copies/ml**	15%	23%	59%	19%	C

Key: * one viral load greater than the indicated HIV copy number

****Two viral loads greater than the indicated HIV copy number**

N/A = data not available

1.3 AIMS OF THE STUDY

1. To investigate and describe the prevalence and patterns of drug resistance mutations in paediatric patients in virological failure receiving first line ART at the Hlabisa Treatment and Care Programme (HTC) in rural KwaZulu-Natal, South Africa
2. To implement and determine the utility of prospective resistance genotyping as part of patient management within a decentralized rural public health program.

1.4 OBJECTIVES

1. To determine the drug resistance profiles of patients within the programme using genotyping and bioinformatics tools.
2. To determine the prevalence of first and second line ART failure in the Hlabisa Treatment and Care Programme in rural KZN, and compare it to the prevalence in other national and international settings.

CHAPTER TWO

2. Review of literature

2.1. Human Immunodeficiency Virus -1 (HIV-1)

The Human immunodeficiency virus-1, responsible for >95% of the HIV epidemic worldwide belongs to the subclass of lentiviruses or "slow viruses", which are characterized by a long interval between infection and disease development. Its 9kb RNA genome encodes nine functional proteins, Gag, Pol, Env, Tat, Rev, Nef, Vif, Vpu and Vpr[36], capable of generating 19 gene products. These products can be divided into three major categories, structural and enzymatic (Pol, Gag and Env), immediate early regulatory (Tat, Rev and Nef), and late regulatory (Vif, Vpu and Vpr)[37]. The coding regions are flanked by long terminal repeats[12] that are important in the replication of the virus. The persistent form of the HIV-1 genome is proviral double stranded DNA (dsDNA), which integrates into the host genome within infected cells[38] (Fig. 1).

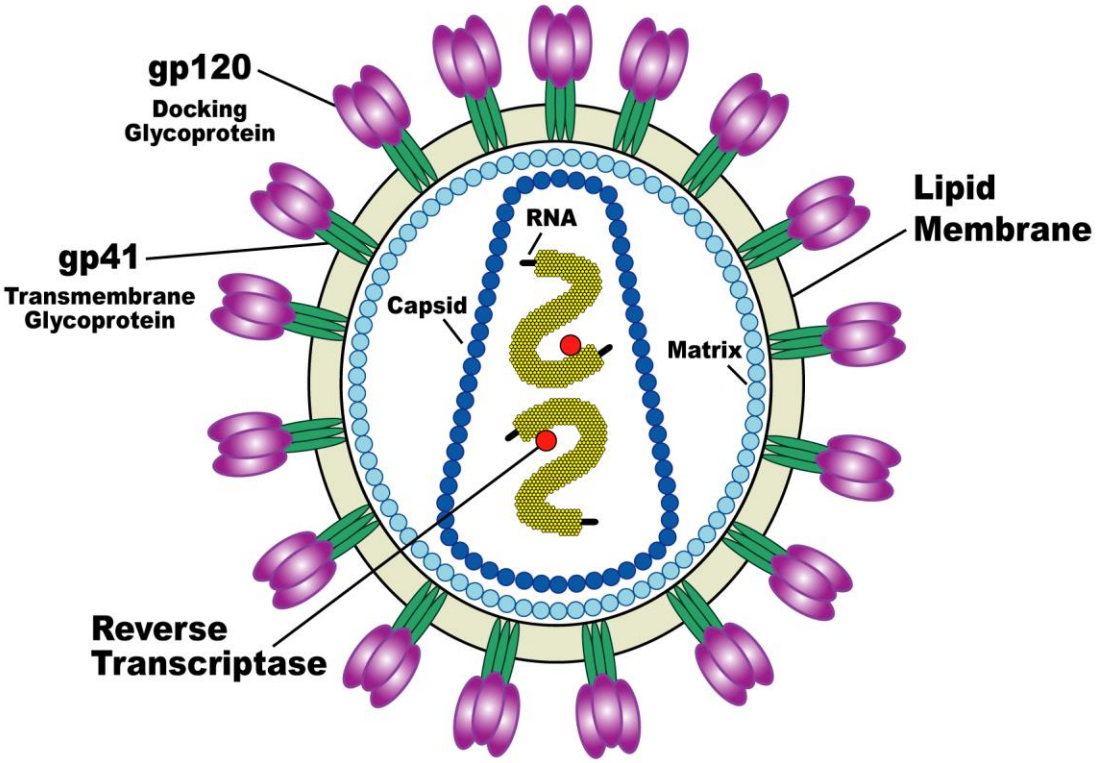


Figure 1. Structure of Human Immunodeficiency Virus[39]

2.2. HIV-1 Replication

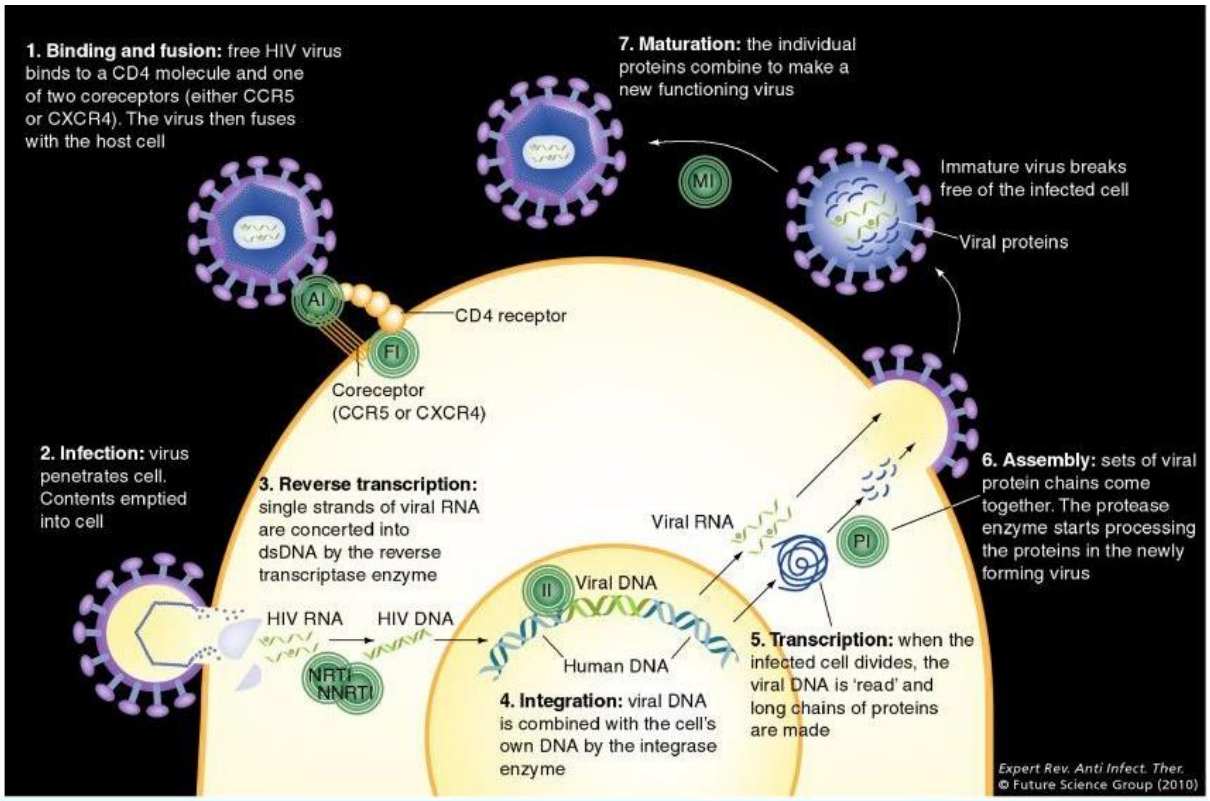


Figure 2. Illustration of the HIV life cycle as it occurs in the human body[40]

The viral life cycle as depicted in Figure 2 begins when the viral membrane glycoproteins attach to the CD4 receptors and CCR5 or CXCR4 co-receptors of target cells. Following attachment, there is fusion of the viral envelope with the cell membrane and release of the HIV capsid into the cell. Shortly after the viral capsid enters the cell, reverse transcriptase (RT) converts the single-stranded HIV RNA to double-stranded HIV DNA. The double-stranded viral DNA is carried into the host cell's nucleus where the viral enzyme integrase splices the viral DNA into the host cell's chromosomal DNA. During viral replication, the synthesis of the viral genome begins with the transcription of proviral DNA into mRNA. This mRNA is then spliced into smaller fragment, exported from the nucleus back into the cytoplasm, and translated into regulatory and structural proteins such as the envelope glycoprotein's gp41 and gp120. These are

then transported to the plasma membrane of the host cell. Once all components of a newly synthesised viral particle, including a copy of the full-length 9kb HIV genome are assembled, it buds off the host cell incorporating part of the cell's membrane into its own outer membrane. The virus then enters the maturation stage, where the HIV protease enzyme processes viral proteins producing a mature infectious virion.

2.3. Antiretroviral Drugs (ARVs)

Currently, there is no cure for or vaccine against HIV infection; the only means of managing the disease is through the use of antiretroviral (ARV) drugs. ARVs have been used to successfully manage and treat HIV-infected individuals since the late 1980s[41] following FDA approval for the use of the first RT inhibitor, Zidovudine (AZT) in 1987[42]. Eight years later, the FDA approved PI inhibitors for use as antiretroviral drugs thus expanding the options for HIV treatment and management significantly. Later, with the advent of highly active antiretroviral therapy (HAART), a standard 3-drug regimen, HIV-1 management could be approached as a chronic disease in patients who have access to medication and who achieve durable virologic suppression[42].

In recent years, significant advances in ARV therapy have been made. Antiretroviral drugs are classified according to the step they inhibit in the viral life cycle. A milestone in the history of HIV disease has been the discovery of new classes of drugs in 1995-96 and thus introducing combination ARV therapy (HAART) and the gradual evolution of HIV infection into a chronic, usually non-fatal condition[43]. There are six distinct classes of drugs that target various stages of the HIV life cycle (Fig. 3). They are the reverse transcriptase inhibitors, which are the nucleoside, and nucleotide reverse transcriptase inhibitors (NRTIs, NtRTIs) and the non-nucleoside reverse transcriptase inhibitors (NNRTIs). In addition, ARVs

include the protease inhibitors (PIs), the fusion inhibitors (FIs), the entry inhibitors also known as CCR-5 co-receptor antagonists, and the integrase inhibitors[42]. The current standard for a highly active ART regimen recommends the use of either a PI or an NNRTI in combination with two NRTIs. This is because the combination of multiple drugs from the same drug class decreases the potency of the regimen, for example three NRTIs or two NRTIs and NNRTIs will not result in optimal viral suppression.

2.4. ARVs and HIV replication

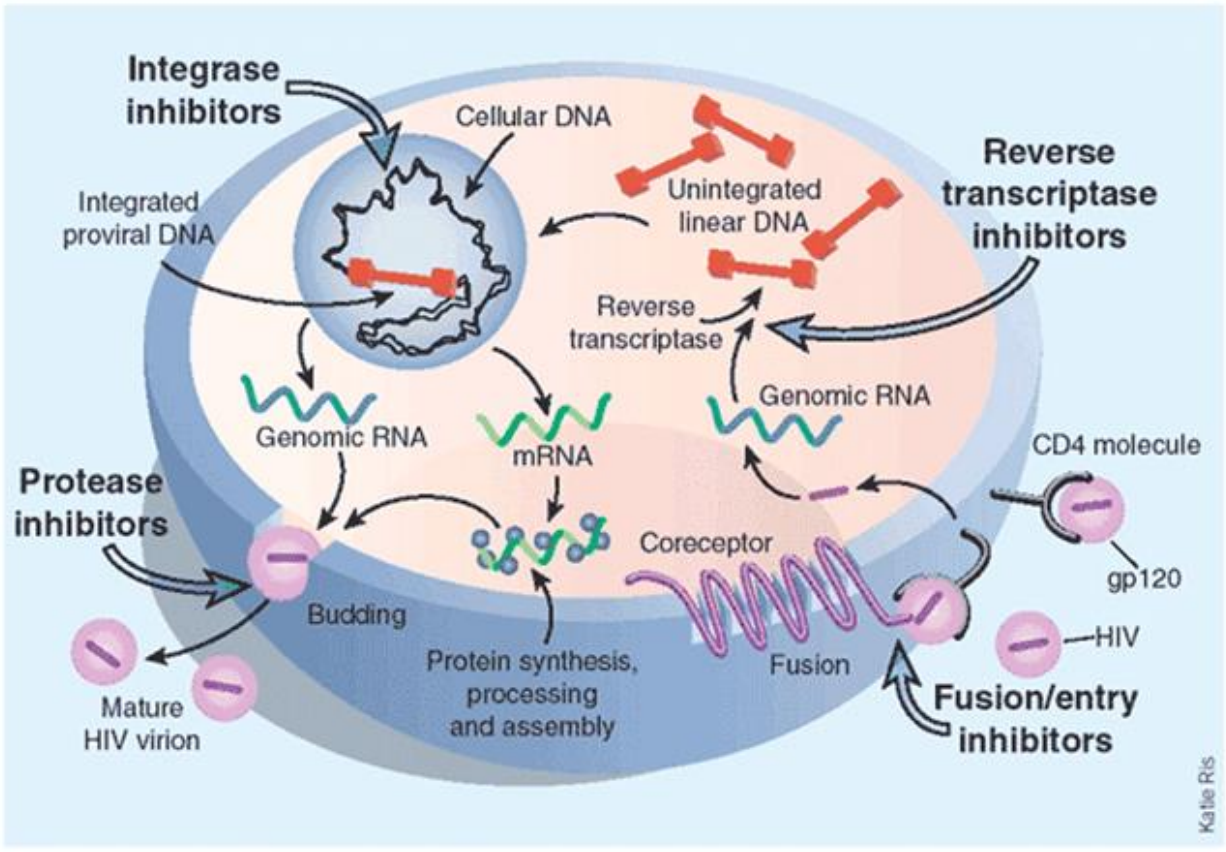


Figure 3. The stages at which the different ARVs block HIV replication in the human body[44]

2.4.1 Reverse transcriptase inhibitors

Nucleoside analog reverse transcriptase inhibitors were the first antiretroviral drugs to be approved for the treatment of HIV. The designation nucleoside analog refers to the structural similarity of these drugs to the building blocks (nucleotides) of nucleic acids (RNA, DNA). While structurally similar, these drugs differ in that the hydroxyl (-OH) group at the 3' position of its structure is replaced by another group that is unable to form the 5'to 3' phosphodiester linkage essential for DNA elongation. Thus, NRTIs interfere with reverse transcriptase activity by competing with the natural substrates, become incorporated into viral DNA and thereby act as chain terminators in the synthesis of proviral DNA (Fig. 4)[45]. Viral reverse transcriptase (RT) is the first crucial enzyme involved in HIV replication and an important target of antiretroviral therapy. Similar to NRTIs the NNRTIs also inhibit the synthesis of viral DNA. Rather than acting as false nucleotides, NNRTIs act by binding to and altering the structure of the RT enzyme (Fig. 3), so that it is unable to function properly[46]. Of the twelve RT inhibitors currently in use worldwide[47], six are available in the public health sector of South Africa[23] and 5 are distributed as child-friendly formulations (stavudine (d4T), lamivudine (3TC), zidovudine (AZT), ritonavir (RTV), abacavir (ABC), nevirapine (NVP), lopinavir/ritonavir (LPV/r)

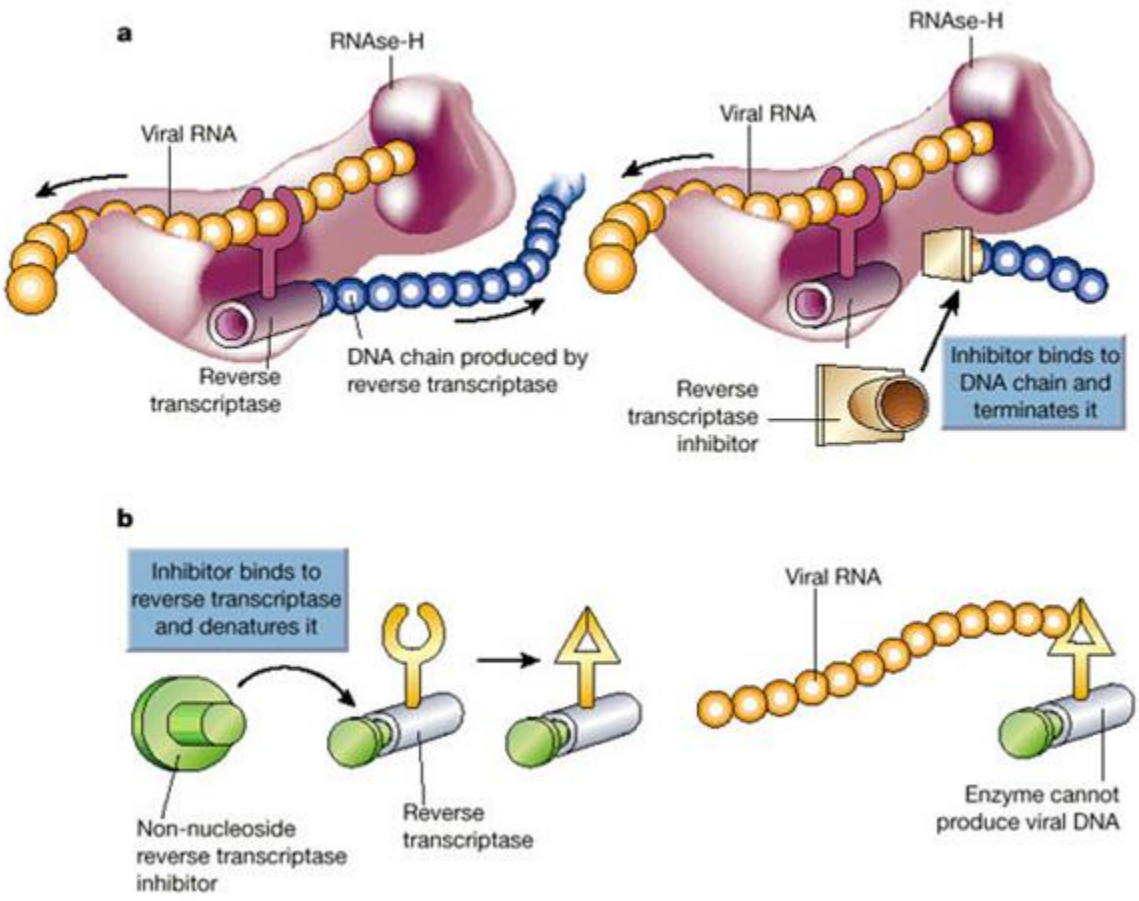


Figure 4: An illustration of the mechanism of action of NRTI (a) and NNRTI (b) drugs showing how RT is disabled and thus prevents HIV from replicating

http://www.nature.com/nature/journal/v410/n6831/fig_tab/410995a0_F3.html

2.4.2 **Protease inhibitors** (PIs)

HIV protease is a 99-amino-acid, aspartic acid protein and is responsible for maturation of virus particles into infectious virions late in the viral life cycle. HIV protease systematically cleaves individual proteins from the *gag* and *gag -pol* polypeptide precursors into functional subunits for viral capsid formation during or shortly after viral budding from an infected cell. HIV protease inhibitors function as

competitive inhibitors that directly bind to HIV protease and prevent subsequent cleavage of polypeptides (Fig. 5). They exhibit activity against clinical isolates of both HIV-1 and HIV-2[42] . Currently, there are 9 PIs approved for use in HIV management[47] and of these 5 (LPV/r and duranavir, fosampreanvir, ritonavir and tripanavir)[48] are available in child-friendly formulations.

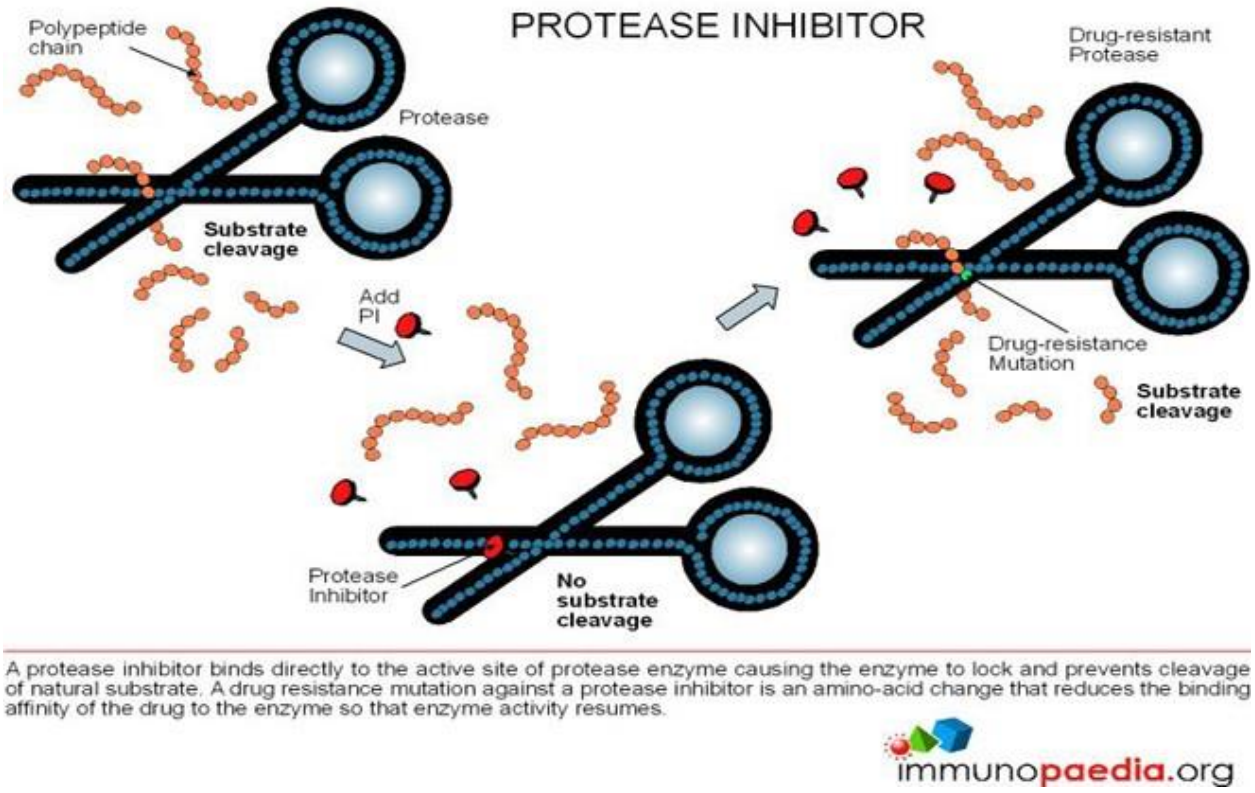


Figure 5. An illustration of how a protease inhibitor attaches to the cleavage site and thus rendering it inactive. This figure also illustrates how an amino acid change in the protease inhibitor can reduce the affinity of the drug to the enzyme and thus the function of the enzyme is restored[49]

2.4.3 Fusion / entry inhibitors

Fusion inhibitors (FIs) were the first class of antiretroviral medications to target the HIV replication cycle extracellularly (before entry into a target cell) and received accelerated FDA approval in 2003. Their unique mechanism of action provides additional options for therapy in patients who are highly

treatment resistant. The use of fusion inhibitors has been limited, however, because of the production time and costs, limited coverage from insurance companies and HIV drug-assistance programs (HDAPs), complex/inconvenient drug administration (subcutaneous injection), and adverse side-effect profiles. Currently, enfuvirtide (Fuzeon) is the only product marketed in this class[42].

2.4.4 Intergrase inhibitors (IIs)

Integrase inhibitors impede the strand transfer activity of the HIV integrase protein. Integrase catalyzes the process of incorporating the viral DNA into the host's chromosomes. Integrase inhibitors show remarkable suppression of HIV replication in treatment-naive and treatment-experienced adults, even if the latter is infected with an extensively drug-resistant virus. Raltegravir (RAL) is the only approved drug in this class[40]. It is recommended for use in children from of age. RAL is also available in a chewable form suitable for children from the ages of 2-11[50] it has been shown that RAL together with LPV/r may be a good second line option rather than LPV/r and two NRTI's. However at its current price, it is not cost effective for low-middle-income countries[51]. There are two more IIs (elvitegravir and dolutegravir)[51] that will be entering the developed world market soon. Appendix 3 lists all the different drug classes and drugs that are available in these classes.

2.4.5 Chemokine receptor 5 (CCR5) antagonists

The first CCR5 antagonist maraviroc (MVC) was approved in 2007. It is the first ARV drug that does not bind to a viral protein but rather binds to a host protein[52]. The CCR5 antagonists interact with the host co receptor by altering its structure and thus hindering the recognition and binding of the viral gp120. Due to co receptor specificity the use of CCR5 antagonists are limited to patients lacking X4[53]. Since 50%-62% of patients that carry resistant virus, carry R5 viruses only, MVC will benefit majority of the

treatment experienced HIV-1 infected population. CCR5 inhibitors could be effectively used against HIV-1 subtype C, which accounts for >50% of infections worldwide. Subtype C rarely switches to CXCR4 co receptor use[53].

2.5 Antiretroviral therapy

Patients living with HIV are treated with a combination of three antiretroviral drugs from at least two drug classes i.e. NRTI/NNRTI/PI/II/entry inhibitors (EI). Since 1996 combination antiretroviral therapy has altered the course of the HIV epidemic among those living with HIV in high-income countries. However ART has only reached a fraction of people in low and middle-income countries, which bear 90% of the global HIV burden. Access to antiretroviral therapy in low–and middle income countries increased from 400 000 in 2003 to 6.65 million HIV-infected individuals in 2010, equating to 47% coverage of people eligible for treatment[54]. This has resulted in substantial declines in the number of people dying from AIDS related causes during the past decade (Fig.6)[54]. The number of children receiving antiretroviral therapy increased from 71 500 at the end of 2005 to 456 000 in 2010[54]. Mounting scientific evidence suggests that increased access to antiretroviral therapy is also contributing substantially to declines in the number of people acquiring HIV infection[54] – a strategy termed “Treatment as Prevention”. The number of new HIV infections globally declined 19% over the past decade. In 15 high burden countries HIV prevalence declined more than 25% among young people aged 15-24 years. These declines are largely attributable to expanded, improved HIV programmes[55].

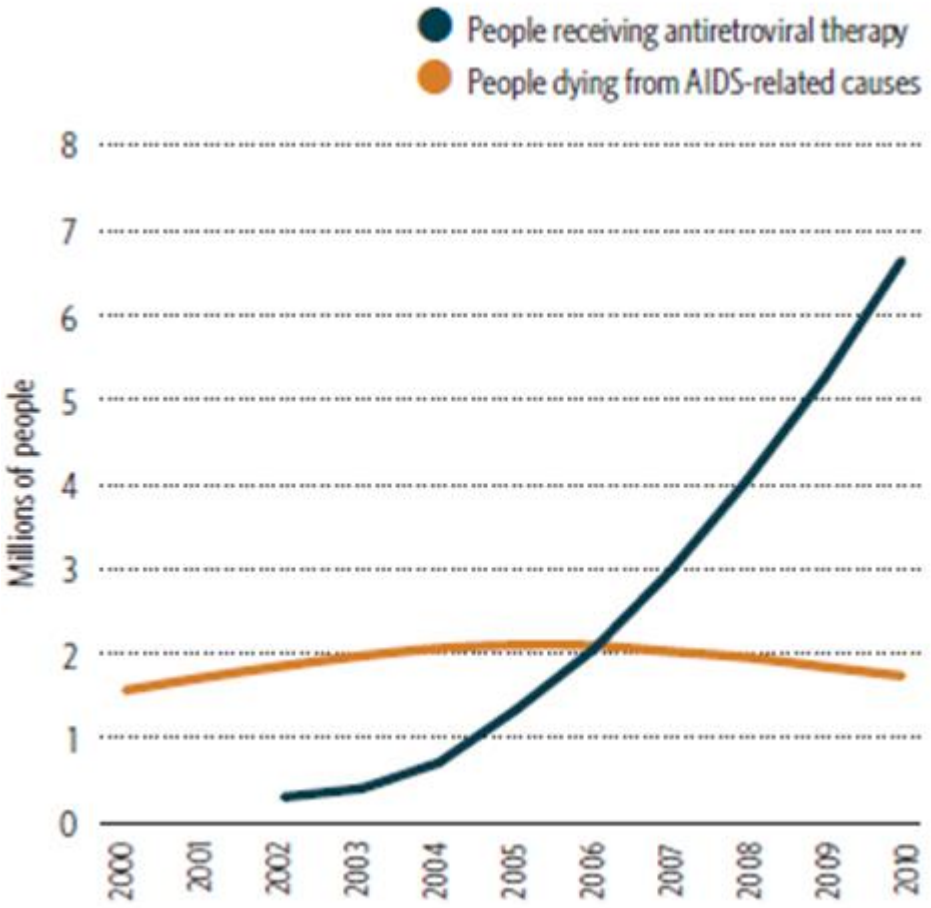


Figure 6. The UNAIDS estimates of the number of people with access to ARV therapy and the number of people dying from AIDS-related causes in low and middle-income countries[54]

2.6 Paediatric ART in South Africa

South Africa has the largest peadiatric HIV epidemic and the largest paediatric antiretroviral treatment (ART) programme in the world[2]. According to the DoH's most recent guidelines (2013), first-line paediatric ART must include two NRTIs plus a NNRTI for children older than three years or greater than 10 kg in weight[22]. Younger children (<3yrs of age) or those with a body mass <10kg require the addition of a protease inhibitor (PI) to the NRTI and NNRTI regimen. Unfortunately the guidelines do not specify recommendations for children at 3 years of age or 10kg in weight. In these instances, the general practice[56] is the use of body weight rather than age to guide drug choices as this ensures accurate dosing and prevents delivery of sub-therapeutic drug doses, which may lead to virologic failure and the development of drug resistance mutations[14] . As illustrated in Table 1, the choice of drugs for children failing 1st line ART are limited and options for those in 2nd failure are doubly scant. This highlights the urgent need to maintain patients on 1st line therapies for as long as possible[23].

Table 3. Standardised national eligibility criteria for starting ART regimens for infants and children[22]

Eligible to start ART
• All children less than 5 years of age, irrespective of CD4 • Children 5 years to 15b years with WHO clinical stage 3 or 4 or CD4 ≤350 cells/µl
Require fast- track (i.e. start ART within 7 days of being eligible)
• Children less than 1 year of age • WHO clinical Stage 4 • MDR or XDR-TB • CD4 Count <200 cells/µl or <15%

Since 2010 DoH recommends that all HIV positive children under the age of five are eligible for ART[22]. In addition, those between 5-15 years of age at WHO clinical stage 3 or 4 or with suppressed immunity (CD4 count ≤350cells/µl) must be initiated immediately[22] (Table 3).

2.7 HIV drug resistance

2.7.1 How does Drug Resistance develop?

While studies have indicated that the introduction of HAART has altered the natural history of the HIV epidemic worldwide[57], there remains a subset of individuals who do not recover immunologically nor do they suppress viral replication. In the majority of these instances, incomplete suppression invariably leads to the development of HIV drug resistance with significantly decreased antiretroviral efficacy[57]. While in others, virologic failure as a result of poor adherence, leads to the development of drug resistance mutations if not identified, and interventions initiated in a timely manner.

High levels of virus production and turnover characterize HIV infection. In most untreated patients, the total number of productively infected cells in the lymphoid tissue has been estimated to be 10^7 to 10^8 cells. The viral population in a patient is diverse due to the highly error prone reverse transcriptase enzyme during the process of converting viral RNA to DNA. There is on average, one mutation for each viral genome that is produced, which ensures that each patient has a complex and diverse mixture of quasispecies, each differing by one or more mutations from another[16]. In addition to this rapid accumulation of minor genotypic changes, different HIV-1 strains can also recombine at a high rate generating significant genetic and phenotypic alterations. Long-term suppression requires that a therapeutic regimen be potent enough to suppress viral replication sufficiently to prevent rapid escape of mutants. However, if drug levels fluctuate or are at sub-therapeutic levels selection of drug resistant variants becomes inevitable in this setting of continued viral replication or insufficient suppression. Over time, resistance mutations may accumulate or a single mutation may develop that confer cross-resistance to all drugs within a drug class. The consequence is that treatment options become severely limited[57].

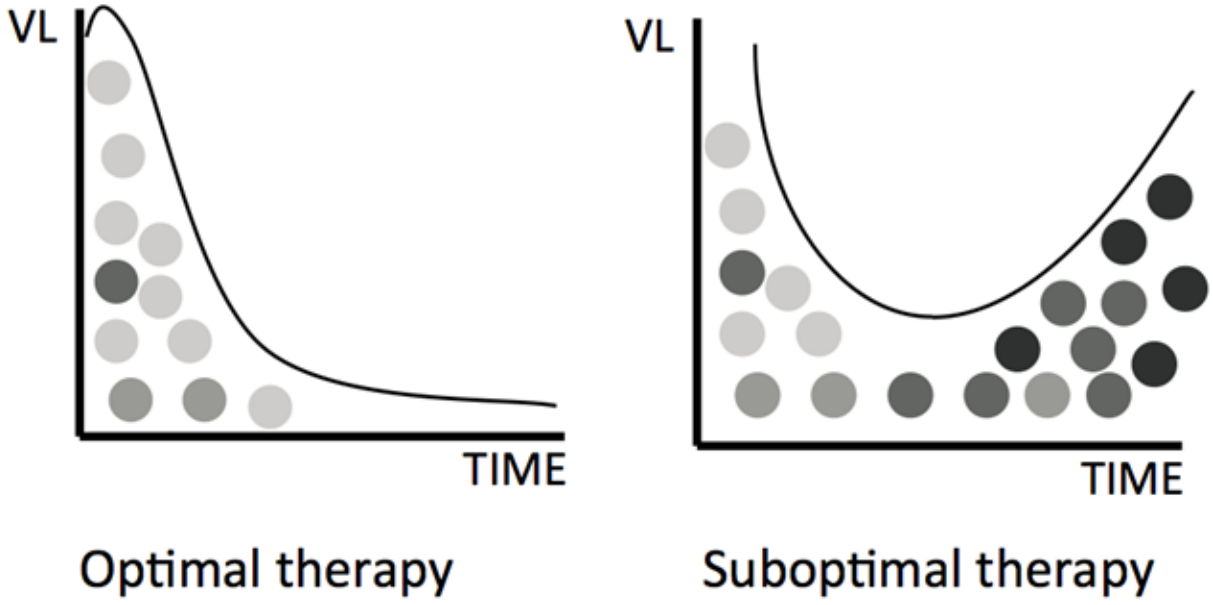


Figure 7. Selection of resistant quasispecies by sub-optimal antiretroviral therapy[58]

2.7.2 Types of Drug Resistance

There are two types of drug resistance, primary or transmitted drug resistance and secondary or acquired drug resistance (Fig 8). Transmitted drug resistance involves infection of a patient with an already resistant virus either from a partner or maternally (from mother to child). Acquired drug resistance is the most common type of drug resistance, which occurs as a result of mutations in the viral genome when HIV continues to replicate in the presence of ART[58]. Resistance mutations, which occur in response to either drug selection pressure or genetic drift (Fig. 7), allow the virus to overcome the activity of ARVs to halt its replication cycle[59]. There are two factors that are essential for resistance to occur: firstly, the virus must be actively replicating so that a specific mutation can be generated and

allowed to outgrow the wild-type; and secondly the ARV must be present in sufficient concentrations in order to exert inhibitory pressure and allow replication of the resistant mutant. Sub-therapeutic drug levels may lead to the development of drug resistance in treatment experienced patients and has also been associated with virologic failure.

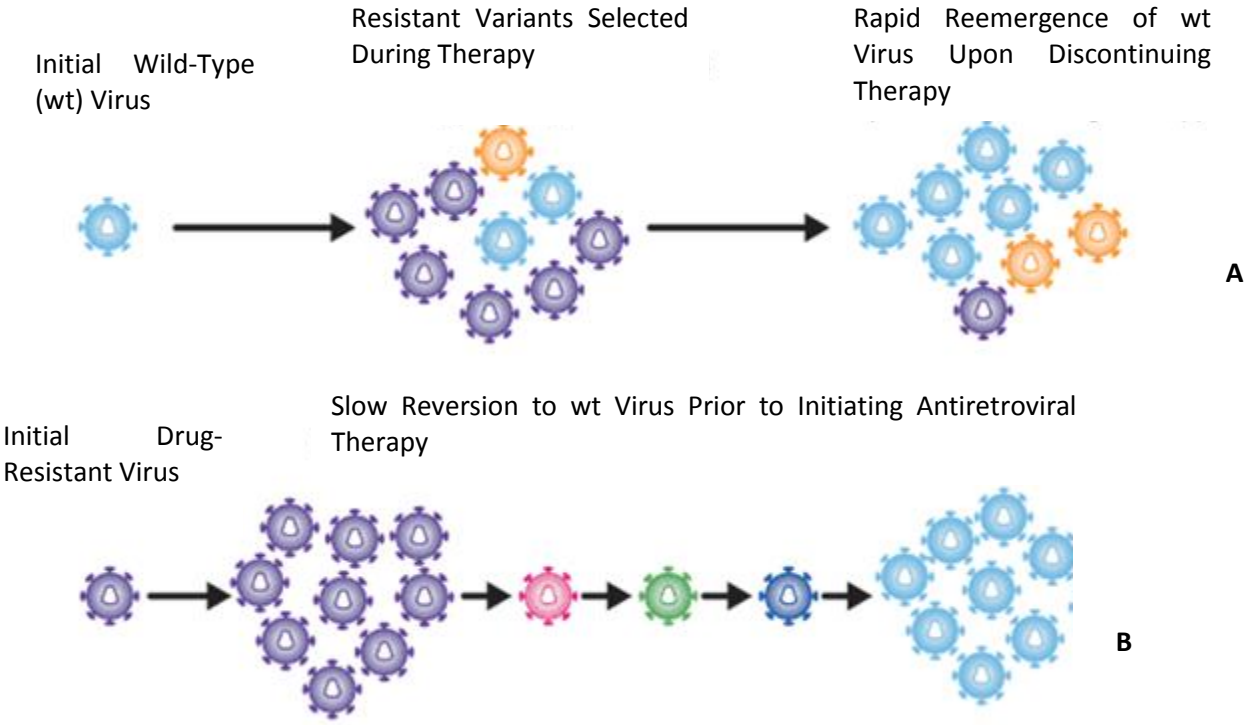


Figure 8: An illustration of the mechanism of development of acquired drug resistance (A) and transmitted drug resistance (B) . Adapted from Kuritzkes (2004)[60]

2.7.3 Risk factors for drug resistance

Drug resistance has been associated with a number of risk factors that can be divided into virus related, drug related and host related.

2.7.4 Virus related

Virus related factors relate to the high replication and error rate characteristic of HIV and its proteins. HIV produces 10 billion virions daily and during its replication cycle frequent errors are made. This high mutation rate makes the virus particularly prone to the development of resistance to HAART[58]. Maintaining adequate therapeutic drug levels is thus crucial to controlling or inhibiting HIV replication. Several factors determine intra-patient drug levels and these include the following:

2.7.5 Host related

2.7.5.1 Adherence

ART has been strongly correlated with HIV viral suppression, reduced rates of resistance, an increase in survival, and improved quality of life. Since HIV treatment is lifelong, adherence poses a significant challenge and requires commitment from the patient and the health care team[61]. Some contributing factors are poor understanding of the disease and the treatment regimen, problems of getting the medication from the clinic, lack of social support and alcohol and other drug issues[62]. Other factors that affect children are difficulty in swallowing tablets and poor palatability of medication. Side effects also play a major role in non-adherence causing the patient to stop taking the medication. Some of the side effects are diarrhea, nausea and vomiting when taking LPV/r, AZT causes hyper pigmentation of the skin and nails, and ABC causes hypersensitivity syndrome and a rash[63]. When ARV doses are skipped or when

only two out of three drugs are taken at a time, this leads to inadequate drug levels in the body [64]which is not enough to suppress the virus and thus enables it to multiply in suboptimal drug levels.

2.7.5.2 Dose

Sub therapeutic doses of ARVs can lead to drug resistance. This is of particular importance in children given their unique physiologies and complex pharmacokinetic profiles[65]. Despite the need for careful and accurate dosing, information on the pharmokinetics in young children is insufficient at present[66]. Dosing in infants is challenging because drug concentrations are highly variable[65]. Accurate measurement of the dose is challenging for many caregivers as regimens for children are complicated involving small drug volumes, which must be adjusted as the child grows. In addition, large volume liquid doses can be problematic as children do not always swallow the entire dose and sometimes spit some of it out[65, 67]. Since children cannot swallow tablets, liquid formulations are a more favorable option. Unfortunately, the number of liquid formulations is limited; they require refrigeration, which many high burden settings have limited access to and they are often not well tolerated[68]. Furthermore the caregiver rather than the patient is responsible for adherence to treatment, which has been shown to be a problem in South Africa with a high number of orphans being raised by grandparents[69]. Elderly caregivers may have poor eye-sight and have difficulty drawing up the correct volumes required. Liquid formulations need to be shaken well before administration to ensure that the correct dose is administered[70].

2.7.5.3 Absorption

Adequate levels of ARVs in the bloodstream are required for the suppression of HIV replication. Inadequate levels allow the virus to multiply and results in the development of mutations and an accumulation of mutations over time[71]. The main problem in children is vomiting and diarrhea causing malabsorption of drugs.

2.7.6 Drug related

2.7.6.1 Drug-drug interactions

Most ARVs, especially the NNTRIs and particularly the PIs have a high potential to interact with other medication[58]. There are pharmokinetic drug-drug interactions between the TB drug class of rifamycins and the NNRTIs and the PIs, causing decreased plasma levels of ARVs in co-infected patients[72].

ARV's can be classified by their potency which is their ability to prevent HIV from replicating. Their genetic barrier to resistance is a measure of how many mutations it takes for the virus to become resistant to that drug[64]. NVP and EFV are low genetic barrier drugs and the virus only needs a single mutation in order to replicate[73]. High genetic barrier drugs such as LPV/r requires at least six mutations before the virus becomes resistant to it[64]. There is also the matter of using the CCR5 antagonist MVC that only target the R5 (those using exclusively CCR5 as a co receptor) tropic viruses. Using this drug could lead to treatment failure if there is a large percentage of X4 (those using exclusively CXCR4 as a co receptor) and R5X4 (able to use both receptors) or dual tropic viruses[52].

2.7.6.2 Patient related

The widespread use of ARVs has greatly increased the level and duration of suppression of HIV replication. The long-term clinical and immunological success of ARVs is dependent upon strict adherence to the prescribed regimen. Poor drug adherence not only compromises the health of the patient but also threatens the health of the public with multidrug resistant HIV and widespread transmission of drug resistant virus[18]. Adherence levels of above 95% are required in order to prevent the emergence and spread of drug resistant HIV variants[58]. It is important to note that very slim margins for missed doses prior to development of resistance exist (Table 4). In addition, drug intolerance, toxicity, not taking drugs properly (with food, adequate liquid, etc.), the inconvenience of having to take ARVs, skipping doses and drug holidays may increase the chances of developing resistance. These risks are amplified in resource-poor settings that hold the added challenge of proximity to primary health care centres, cost of transportation, drug stock outs and other socio-economic factors.

Table 4. Maximum number of doses that can be missed to still achieve ≥95% adherence[62]

Time Interval	Once daily ART regimen	Twice daily ART regimen
Last week	0 (out of 7 doses)	0-1 (out of 14 doses)
Last 10 days	0 (out of 10 doses)	1 (out of 20 doses)
Last month	1-2 (out of 30 doses)	3 (out of 60 doses)

2.7.7 Drug resistance in children

It is clear that ART for infants and children must be individualized to account for specific growth and developmental patterns especially in those initiated early and who survive to adolescence and adulthood[6]. ARVs are often sub-optimally dosed because of a failure to adjust for ongoing growth[66]. As is often the case, adult doses are erroneously extrapolated to children without considering potential differences in drug handling, age or dose requirements for effectiveness with the inevitable consequence of the development of drug resistance and virologic failure.

Children have higher rates of virological failure than adults, often associated with more extensive resistance and resulting in severely limited 2nd line options[6]. This is of particular concern in resource-limited settings, such as South Africa, where the range of ARV drugs available is restricted to half the number of FDA approved drugs available in developed countries[23, 74]. There are a number of socioeconomic and psycho-social factors, including stigma, that also affect adherence to medication throughout childhood and into adolescence[6]. Adherence is often further compromised during adolescence. These problems highlight the importance of population-based surveys of drug resistance during childhood and adolescence. Currently only the routine tests described in Table 5 are done.

Table 5. Routine monitoring tests in children with ART[75]

Test	Timing
CD4 count and percentage	At initiation After six months, after one year, then annually
VL	At initiation After six months, after one year, then annually
FBC	For all children-baseline

	If child on Zidovudine, then-baseline, 1mo, 2mo, 3mo and then annually
LDL cholesterol triglycerides	Children on Lopinavir/ritonavir Annually
ALT	For a child on nevaripine, baseline, and repeat if child develops rash or jaundice

Management of HIV-1-infected children will become increasingly complex as more children are initiated on ART and more develop extensive multiclass drug resistance. It is recommend that, rather than delaying until the patient is failing ART, resistance testing is performed at diagnosis and where feasible during treatment[76]. This would include all newly diagnosed children regardless of age at diagnosis and whether or not their mother received ART during pregnancy for pMTCT. In addition, testing should be conducted promptly while the patient is in virological failure[76]. According to the South African DoH guidelines, the action taken when a patient's viral load is greater than a thousand copies/ml is to reinforce adherence and monitoring but does not include resistance testing (Table 6). Resistance mutations decrease the fitness of the virus to replicate therefore; removal of the selective drug pressure results in a rapid reversion of the resistant variant to become a minor variant in the viral population. These resistant variants may not be detected by current resistance assays. Testing is therefore recommended while a patient is still on therapy, while selection pressure is still being maintained[77].

Table 6. Viral load monitoring and recommended action[75]

Viral load (VL)	**Response**
<400 copies/mL	6 monthly viral load monitoring and routine adherence support
400-1000copies/mL	Repeat viral load in 6 months

	Begin step-up adherence package if VL still between 400-1000	
>1000 copies/mL	Begin step-up adherence package Repeat viral load in 3 months	-If <400, return to 6-monthly monitoring -If between 400 and 1000, continue step up adherence and repeat VL after 6 months -If > 1000, despite stepped up adherence support, AND child is on a NNRTI-based regimen, switch to second-line therapy only if adherence is >80%. - If >1000 and child is on a PI-based regimen: • Reinforce adherence (it is very difficult to fail a PI-based regimen unless the child ever received an unboosted PI) • Switch to second-line therapy if VL > 5000, only if adherence is >80% and consider drug resistance testing if available -If child received an unboosted PI (eg. ritonavir alone) in the past, do resistance testing if available and change to second line if VL >1000

2.8 HIV-1 Drug Resistance Testing

HIV resistance testing has become an essential component of HIV patient management especially in selecting appropriately active ARVs in the setting of treatment failure. There are two types of tests for HIV Drug Resistance, namely phenotyping and genotyping.

2.8.1 Phenotyping

Phenotyping is an *in vitro* assay that measures the drug susceptibility of the viral population derived from an HIV-positive patient to all available ARVs. It provides a resistance index value for each drug which tells us the concentration of the drug that is required to inhibit viral replication by 50% (IC_{50})[57]. Phenotypic assays are recombinant viral assays where relevant amplified sections of the HIV genome are inserted into a laboratory vector to make a replicating recombinant virus. This recombinant virion is then allowed to grow following exposure to different concentrations of antiretrovirals[57]. Phenotypic testing has some advantages such as easier interpretation but it is very expensive and requires a high safety laboratory[58] and is time consuming.

2.8.2 Genotyping

Genotypic testing involves the identification of drug resistance-associated mutations along the relevant segments of the viral genome. The process involves amplification of the regions of interest (example HIV pol, rt, env, integrase) followed by sequencing to obtain the specific nucleic acid sequence of the amplified products. An example is the 99 amino acids of the protease gene and the first 300 to 400 codons of the reverse transcriptase gene when analysed will inform on resistance mutations to PIs and

RT inhibitors, both drug classes currently predominantly in use[77, 78]. Commercial genotyping assays are available but are prohibitively costly particularly to resource-poor settings. In-house assays are a more cost effective alternative and allow sequencing of any region of the genome in both HIV-1 and 2 and in different subtypes[79]. It is equally important to use reference sequences to take into account emerging resistance mutations. Therefore, the International AIDS Society (IAS-USA) compiles a consensus list of mutations annually which can be accessed from their website: http://www.iasusa.org/resistancemutations/mutations figures.pdf (Appendices 1 and 2).

2.8.3 Limitations of resistance testing

The HIV -1 population within an individual consists of multiple variants. Often minor variants remain undetected while only viral variants that represent a significant percentage of the population (>10% to 20%) will be detected by traditional sequencing techniques. In addition, current resistance genotyping protocols are unable to detect "archives" or "viral reservoirs" since genotyping assays generally target viral RNA and not viral DNA[58]. When patients failing therapy are switched to an alternate regimen, the mutations associated with their first failure may no longer be detectable in plasma, which is a source of productive viremia[58]. However, these archived mutations within infected cells will rebound or re-emerge if patients are not maintained on an appropriately suppressive regimen[58].

Unlike current viral load assays which are sensitive to 50 copies/ml, resistance genotyping assays are generally only sensitive to 500 to 1000 HIV copies /mL[77]. Resistance tests are therefore not useful in determining the presence of resistance in patients with low-level viraemia[58].

CHAPTER THREE

MATERIALS AND METHODS

For this study, we used the affordable and open access Southern African Treatment Resistance Network (SATuRN) HIV-1 drug resistance genotyping system (Manasa et al. in preparation). Briefly, this is an in-house method that costs approximately 50 US$ at reagent price. In order to keep the costs low, no viral load was performed before drug resistance genotyping. A 1.3kb fragment of the protease gene was targeted in this assay and sequenced using Sanger sequencing and multiple primers as will be detailed further in this Chapter and as is illustrated in Figure 8. This study was designed to link the rural primary health care clinics in the Hlabisa Treatment and Care Program with the laboratory where genotypes were generated to be used not only for research purposes but also to inform patient management (Fig 9 study design).

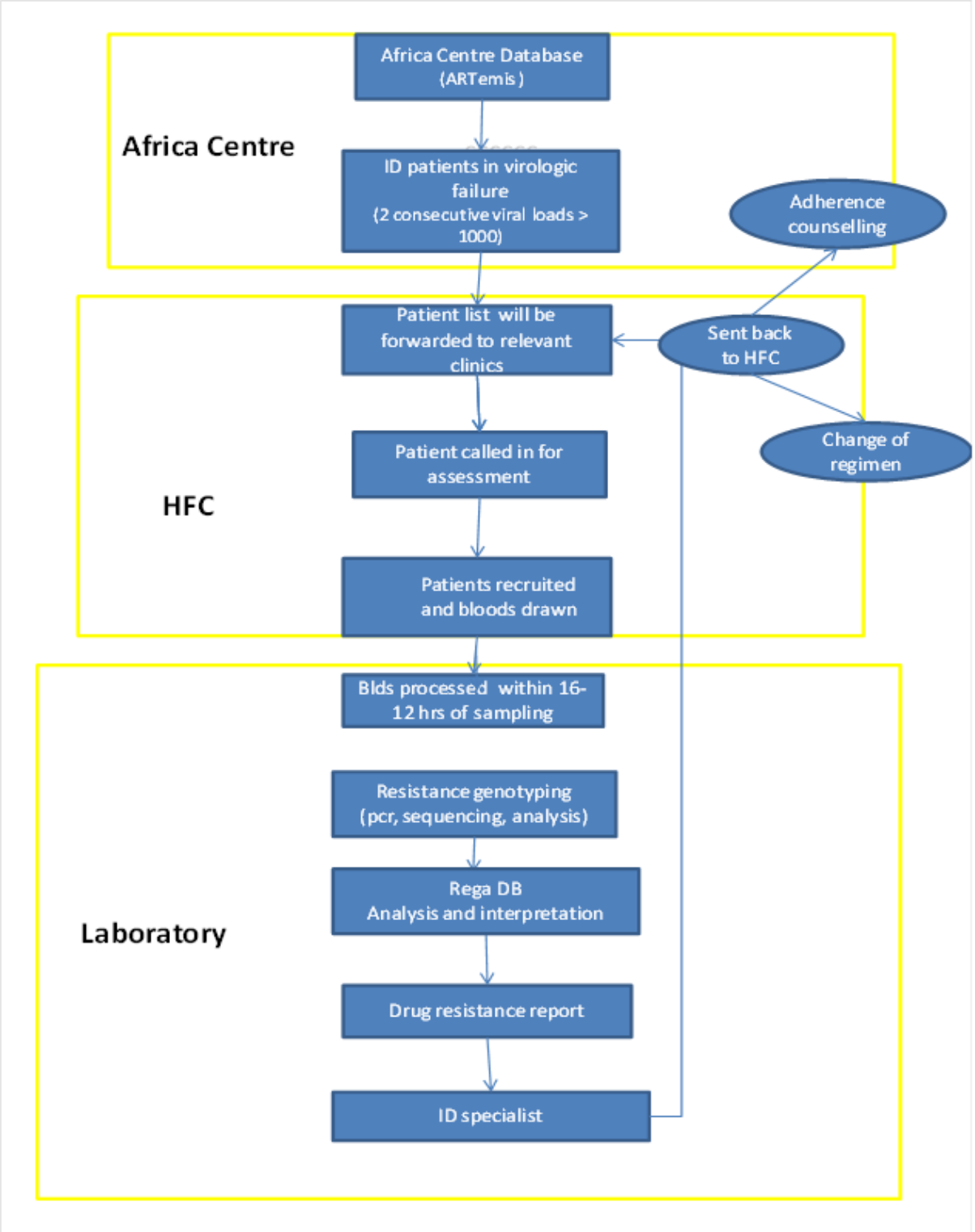


Figure 9: A flow diagram of the study design illustrating the interaction between the clinics where samples are collected and the laboratory where sequences are generated and analysed.

ID specialist= infectious disease specialist and HFC=Hlabisa Failure Clinic

3.1 Setting

This study was conducted in the predominantly rural Hlabisa sub-district within the uMkhanyakhude District of northern KwaZulu-Natal (Fig 10). The programme, delivered by the Department of Health with support from the Africa Centre (www.africacentre.com), has been described previously[4, 24, 35]. HIV treatment and care is fully devolved to 16 primary health care (PHC) clinics (Fig. 10) and is delivered largely by nurses and counsellors, with medical officers visiting clinics on a weekly or fortnightly basis.

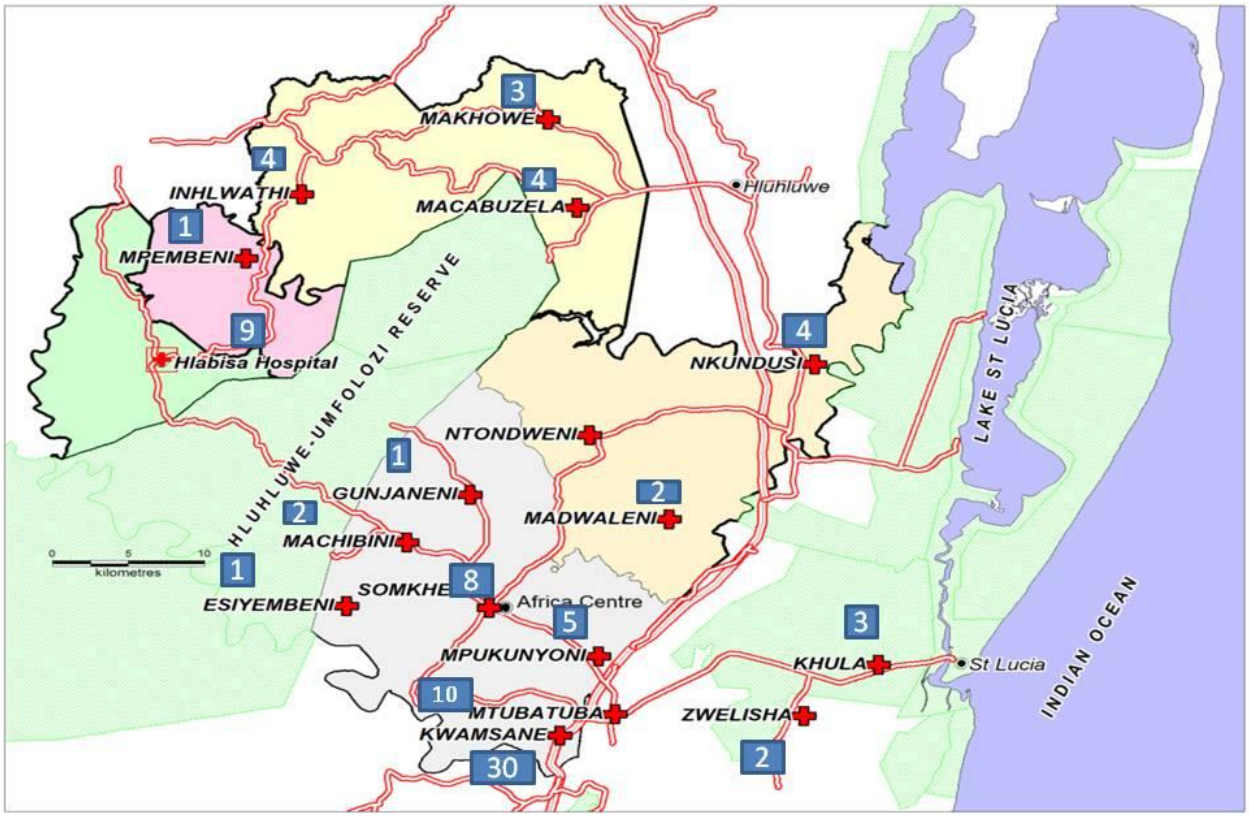


Figure 10: Shows the 17 clinics (red cross) in the Hlabisa sub District where patient recruitment and sample collection was conducted ((http://www.africacentre.ac.za/Portals/0/Monograph%201.pdf**), The number of patients genotyped from each clinic are indicated (blue blocks). Twelve patients that failed genotyping are not indicated on the map.**

3.2 Inclusion and Exclusion criteria

This descriptive cross-sectional study enrolled children (≤ 15 years of age) who had been receiving ART for more than 12 months with evidence of virological failure, defined as two consecutive viral loads >1000 copies/ml. Children with virological failure were identified from all 16 clinics (Fig. 10) both passively, during routine clinic visits, and actively, through interrogation of the ARTemis programme database (Fig 9), which is housed at the Africa Centre. Children who have had either one or two drug switches in their regimen prior to their enrollment in the study were included. A medical officer enrolled all children.

Children 16yrs and older were excluded from the study and if found to be in virologic failure were deferred to the adult resistance cohort, also managed by the Africa Centre. Our analysis excluded children on 2nd Line ART i.e. those who have had all three drugs that they were previously on, switched.

3.3 Study population:

Basic demographic, clinical and laboratory data on all individuals initiating ART within the Africa Centre surveillance area are stored in the ARTemis database at the Africa Centre (Fig. 9). Children recruited into this study were identified in two ways:

1. Actively, where the ARTeMIS database of the Africa Centre was interrogated and children presenting with two successive viral loads >1000cp/ml were contacted for a follow-up visit.

2. Passively, where at their routine follow-up visits, and following an assessment of their charts and their current clinical status, children identified as failing ART were flagged and recruited into the study

A multidisciplinary team, consisting of a peadiatrician, a social worker, dietician and two members of the full-time resistance team, evaluated each HIV-infected patient ≤15 years, and that have been on treatment for more than a year, attending the primary health clinics within the sub-district, individually (Fig 9). Patients living near the primary family health clinic in Kwamasane (Fig 10) were transported to the site on a Friday. The aforementioned team of medical and clinical experts assessed and recruited children at their local clinics in more distant and remote locations within the sub-district. The following took place at the first visit, but not necessarily in this order:

1. The counselor informed the caregivers accompanying the child, as well as the child, about the resistance study and gave them an information sheet (Appendix 6).
2. The caregivers provided written informed consent (Appendices 4 and 5) and the children older than 12 years of age also provided their own written consent
3. A clinical information sheet (appendix 8), including details of ART drugs, a record of each viral load and CD4 count and possible barriers to adherence (clinical and social) was completed for each child recruited into the study.
4. An infectious disease peadiatrician examined the child clinically, for routine follow-up and to rule out co- morbidities.
5. A professional nurse drew a blood sample (4.5ml EDTA tube) for resistance testing.
6. A social worker provided support to each child and their caregiver/s in order to identify any socio-economic factors that may be contributing to treatment failure.
7. A dietician examined and conversed with each child and their caregiver in order to identify whether the household experienced food insecurity, to advise on and provide nutritional support and to explore the child's normal diet and its insufficiencies as an impediment to treatment success.

8. A follow-up date was provided for the child and his/her caregiver to receive the results of the resistance genotyping.

A social worker together with the clinician gathered details of treatment compliance from all study participants at each visit and adherence counseling was provided. This is in line with current national clinical guidelines and to determine whether treatment failure is a result of poor adherence or drug resistance. The information gathered at this stage afforded aid in informing on appropriate interventions should no resistance mutations be identified. There was no alteration in ART or interventions apart from those recommended by the South African DoH guidelines for paediatric HIV management[23].

3.4 Sample Size

For this prospective study we recruited all children who were experiencing virologic failure within the HIV Treatment and Care Program in the uMkhanyakude sub-district. As of September 2013, the Africa Centre recorded a total of 28 447 patients ever initiated in the program; 8.4% of which are children less than or equal to 15 years of age. The total number of children ever initiated in the program thus stands at 2390 but 1653 of these are currently active in the program. Of the, 1000 children that are currently active at 16 clinics within the sub district (Fig. 10), 101 children were included in this study. The majority of the children were recruited at the Kwamsane Family Clinic, the largest and busiest clinic within the program. Other centrally located clinics were the Somkhele and Mtubatuba clinics.

3.4 Africa Centre Virology Laboratory (ACVL)

The ACVL is a molecular laboratory serving the Africa Centre in addition to conducting laboratory-driven research projects. The laboratory boasts long- standing (12 years) experience in serology, HIV viral loads (qPCR), polymerase chain reactions (PCR) and Sanger sequencing. The ACVL has a genetic analyser (ABI 3130xl, Life Technologies) that has been used for various applications including resistance genotyping in adults, TB geno-identification and sequencing of HIV env, LTR, gag and full length HIV. As such this facility provided the technological requirements to advance this research project.

3.5 Laboratory assays

3.5.1 Sample collection and transport

Blood specimens, collected at the primary health care clinics, were transported from the Africa Centre in Mtuba to the ACVL in Durban (~200km away) on the same day of collection. Samples were received at the ACVL, recorded in the Laboratory information management system (LIMS) and the plasma was separated and stored at -80°C within 24 hours of collection, until use.

3.5.2 Resistance genotyping

3.5.2.1 RNA extraction

Ribonucleic acid (RNA) was extracted using the Qiagen RNA Mini kit (Qiagen N.V., Venlo, Netherlands) according to the manufacturer's recommendations but with the following modifications; 200µl of plasma was added to 800µl of lysis and 800µl Ethanol was used to precipitate the RNA prior to application of the mixture to the spin column. Due to the increased volume of sample+lysis buffer+ethanol, addition and centrifugation of this mix to the column for RNA binding was repeated

twice in order to allow for maximum RNA binding. Two washes with Wash Buffer 2 were performed and RNA was eluted in 60µl of Buffer AVE.

3.5.2.2 HIV Reverse transcription

HIV-1 was reverse transcribed using the SuperScript® III First-Strand Synthesis kit (Life Technologies, Carlsbad, CA) and RT21 gene specific primer as per the protocol outlined in Table 7.

Table 7. Master Mix for cDNA synthesis

MM1		
Reagent	**Volume/Sample**	**Final concentration**
Sterile Water	0.0	0
RT21 (20mM)	0.5	0.2mM
dNTP Mix (10 mM)	0.5	0.4mM
Volume/Sample	**1.0**	
Add 6ul of RNA to the MM1for each of the samples		
Prepare MM2 as per table below. **DO NOT Aliquot this mix**		
MM2		
Reagent	**Volume/Sample**	**Final concentration**
10 x Buffer	1.0	1X
MgCl (25mM)	2.0	4mM
DTT (0.1M)	1.0	0.008M
RNAse Out (40U/µl)	0.5	1.6U/µl
SuperScriptIII	0.5	
Volume/Sample	**5.0**	

Table 8. **Cycling conditions for reverse transcription**

Temperature (°C)	Time (min)
65	5
4	2
Pause to add MM2	
50	60
85	5
Pause to add 1ul of RNaseH	
37	20
4	∞

3.5.2.3 In-House Nested PCR Method

The cDNA generated was then PCR amplified using an optimized in-house protocol. A 1315bp fragment of the *pol* gene, covering all the 99 protease codons and the first 300 codons of the reverse transcriptase (RT) gene was amplified with the Platinum® Taq DNA polymerase (Life Technologies, Carlsbad, CA) and primers MAW26 and RT21 (Table 9) for the first step and PRO1 and RT20 (Table 9) for the second step polymerase chain reaction (PCR). Master mixes and thermo cycling conditions are shown in Table 10.

Table 9. Summary of primer sequences used for 1st and 2nd round PCR amplification of pol gene

Stage	Primer name	Sequence	Length	Direction
1st Round	MAW-26	TTGGAAATGTGGAAAGGAAGGAC	23	Forward
	RT-21	CTGTATTTCAGCTATCAAGTCCTTTGATGGG	31	Reverse
2nd Round	Pro-1	TAGAGCCAACAGCCCCACCA	20	Forward
	RT-20	CTGCCAATTCTAATTCTGCTTC	22	Reverse

Table 10. Master Mix 1 and 2 for the in-house nested PCR

1ST Round PCR		
	Vol/Sample (ul)	final concentration
Water	18.4	
10 x Buffer	2.5	1
MgCl (50mM)	1	2
dNTP (10mM)	0.5	0.2
MAW26 (5 pmol/ul)	0.25	0.1
RT21 (5 pmol/ul)	0.25	0.1
Platinum Taq	0.1	0.02
Volume/Sample	**23**	
Sample	2	
Total Rxn Vlolume	**25**	
2nd Round PCR		
	Vol/Sample (ul)	Conc/reaction
Water	18.4	
10 x Buffer	2.5	4
MgCl (50mM)	1	2
dNTP (10mM)	0.5	0.2
PRO1 (5 pmol/ul)	0.25	0.1
RT20 (5 pmol/ul)	0.25	0.1
Platinum Taq (5U/ul)	0.1	0.02
Volume/Sample	**23**	
Sample	2	
Total Reaction Volume	**25**	
Cycling Conditions		
Temperature (˚C)	**Time**	**#cycles**
94	2min	Hold
95	30sec	X 30
58	20sec	
72	2min	
72	10min	Hold

3.6 HIV Genotyping

HIV genome

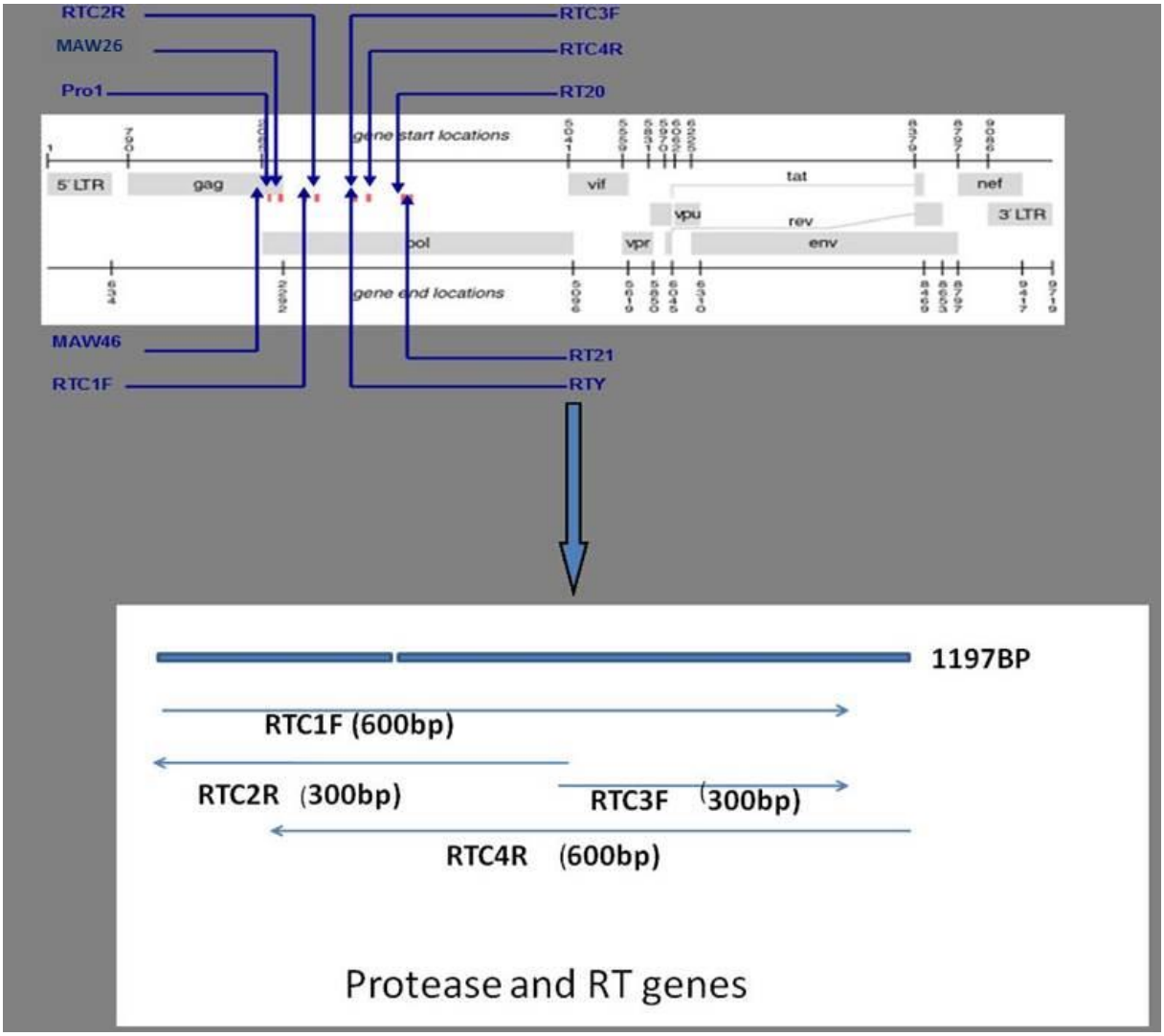


Figure 11. Diagram showing the primers covering the protease and reverse transcriptase regions

Sanger sequencing was performed on successfully amplified population PCR products that were identified by gel electrophoresis and visualized as a 1.3kb fragment under ultraviolet (UV) light. The PCR products were cleaned using the PureLink® PCR purification kit (Life Technologies, Carlsbad, CA), eluted

in 35µl of elution buffer and were sequenced using the Sanger BigDye® terminator V3.1 sequencing protocol (Table 11), (Life Technologies, Carlsbad, CA) and four bidirectional primers (RTC1F, RTC2R, RTC3F, and RTC4R (Table 12), and run on an ABI 3130xl genetic analyzer (Life Technologies, Carlsbad, CA), finally generating a consensus sequence spanning 300 amino acids of RT and 99 amino acids of the protease gene. The first 240 codons of the RT gene cover all currently recognized RT mutations associated with resistance to available RT inhibitors.

Table 11. Master Mix for the big dye sequencing reactions

Master Mix	
	x1
Water	6.10
Big Dye Ready Reaction mix	0.40
Primer (3.20pmol/µl)	0.50
5X sequencing buffer	2.00
Template (20pmol/µl)	1.00
Volume per sample	
Total reaction volume	**10.00**

Table 12. Summary of primers used to sequence the pol gene

stage	**Primer name**	**Sequence**	**Length**	**Direction**
1st round	**RTC1F**	ACCTACACCTGTCAACATAATTG	23	Forward
	RTC2R	TGTCAATGGCCATTGTTTAACCTTTGG	27	Reverse
2nd round	**RTC3F**	CACCAGGGATTAGATATCAATATAATGTGC	30	Forward
	RTC4R	CTAAATCAGATCCTACATACAAGTCATCC	29	Reverse

3.7 Confirmatory HIV viral load

Samples that failed to amplify by PCR were submitted for viral load testing using the Biocentric HIV RNA Charge Virale method (Biocentric, Bandol, France) with a detection limit of 50 copies/ml. A separate aliquot stored at -80°C is used for the viral load quantification. Briefly this assay uses re-extracted RNA and is a quantitative real-time reverse transcription PCR technique targeting a conserved region within the LTR gene[80] using MGB probes. An internal control included in the kit confirms extraction efficiency and positive and negative controls are co-extracted and assayed in parallel with samples.

3.8 Sequence Datasets and bioinformatics

Sequence datasets comprised of 101 paediatric sequences, which were genotyped at the ACVL. This involved the steps as explained in detail below.

3.8.1 Sequence editing, assembling and FASTA file construction.

Electropherograms generated by sequencing were imported into Geneious version 6.1.2 (Biomatters Ltd, Auckland, New Zealand), an integrated and extendable software platform for the organization and analysis of genomic and sequence data. The quality of the reads was manually assessed and the ends trimmed to improve the quality. The trimmed reads, 4 sequences for each sample (RTC1F, RTC2R, and RTC3F AND RTC4R) were assembled against a Southern African subtype C reference sequence (Accession# 1005313). The contiguous sequence obtained from this assembly was manually edited checking for possible base mixtures at the major drug resistance sites. Once sequence editing was complete, the consensus sequence was extracted, appropriately labeled and saved in a separate folder.

The quality of the sequences was assessed using the HIV quality analysis tool hosted on BioAfrica.net. These programs provide a report of the quality of the sequence and contains information regarding the HIV subtype, similarities to sequences already submitted to the Genbank, the presence of stop codons and /or ambiguous amino acids in the sequence. Prior to the detection of DRMs using bioinformatics software applications, each consensus was submitted to the calibrated Population Resistance Tool (CPR) (http://hivdb.stanford.edu) for a final quality check. Sequences were deemed high quality if they had no ambiguities or insertions/deletions.

In order to test for contamination we Blast searched our sequences against the public dataset using NCBI blast (http://www.ncbi.nlm.nih.gov/blast) and against our local database using the BlastServer application. Sequences were deemed acceptable and not contaminants if the identity was lower than 98%. Finally, we constructed a maximum likelihood (ML) phylogenetic tree using phyML with GTR, gamma and percentage of invariable sites estimated from the dataset. Trees were visualized in FigTree (http://tree.bio.ed.ac.uk/software/figtree/) in order to identify contamination indicated by sequences that cluster together with low genetic diversity.

3.8.2 Adding sequences with clinical information into RegaDB.

After quality assessment the sequences were loaded onto the SATuRN database. This is a database that combines sequence data and clinical/patient monitoring measurements such as CD4 and Viral load as well as details of the patient's ART history. The database uses three leading online drug resistance algorithms (ANRS 2009.07, HIVDB6.0.5, and REGAv8.0.2) to interpret the drug resistance data from the submitted sequence. These resistance algorithms and their mutation lists are regularly updated and curated. The amino acid positions on the RT and PI genes relevant to drug resistance mutations are

tabulated in Table 13. Mutations were coded as DRMs at these positions based on the IAS mutation list of 2013[81].

Table 13. Amino acid positions at the RT and PI genes relevant to drug resistant mutations

Wild type	Amino acid position	Mutant
Nucleoside Reverse Transcriptase inhibitors (NRTI)		
M	41*	**L**
A	62**	**V**
K	65	**R**
D	67*	**N**
T	69	**S**
K	70*	**R**
L	74	**V**
V	75**	**I**
F	77**	**L**
Y	115	**F**
F	116**	**Y**
Q	151**	**M**
M	184	**V**
L	210*	**W**
T	215*	**F/Y**
K	219*	**Q/E**
Non-nucleoside Reverse Transciptase Inhibitors (NNRTI)		
L	100	**I**
K	101	**P**
K	103	**N**
V	106	**M**
V	108	**I**
E	138	**A/G/K/Q/R**
V	179	**L**
Y	181	**C**
Y	188	**L**
G	190	**A**
H	221	**Y**
P	225	**H**

F	227	**C**
M	230	**L**
Protease Inhibitors (PI)		
D	30	**N**
V	32	**I**
M	46	**I/L**
I	47	**V/A**
G	48	**V**
I	50	**V**
I	54	**V/L/A/M/T/S**
Q	58	**E**
T	74	**P**
L	76	**V**
V	82	**A/F/T/S**
N	83	**D**
N	88	**DS**
L	90	**M**

Key: *Mutations associated with TAMS; **Mutations associated with Q151 complex

3.8.3 Drug Resistance Mutation (DRMs) Interpretation

The effect of DRMs on treatment failure was determined using three independent drug resistance interpretation algorithms, which were developed at Stanford University, ANRS (French AIDS National Research Agency) and the REGA Institute. All of the three previous mentioned algorithms were accessed from RegaDB (http://www.bioafrica.net/regadb/).

3.8.4 Interpretation of resistance results and clinical tests (CD4 +VLs)

The drug resistance profile for each sequence together with the clinical data and treatment history (Appendix 8) of the patient's was used to generate a drug resistance report (Appendix 7). This report contains drug resistance mutations identified from the patient virus and drug resistance interpretation based on the HIVDB version 6.0 algorithm. The report also contains genotypic susceptibility scores (GSS) where a GSS of 1.0 indicates drugs that are still active against the virus, 0.5 indicates drugs that have intermediate resistance and 0.0 is indicative of high-level resistance. A total GSS of 3 for all three drugs in the patient's regimen is thus most desirable. In addition to the drug resistance information the report also provided the patient's ART history, (start and stop dates for ART, ARV combinations (regimens) used) and CD4 and viral load histories in the form of a clinical chart.

Once the reports were generated, they were sent to an Infectious Disease Specialist[#] who evaluated the report together with the patient's clinical data and provided treatment recommendations based on the current South African guidelines.

ID specialists that advised over the duration of this study were: Dr Theressa Rossouw (University of Pretoria) and Dr Richard Lessells (Wellcome Trust Fellow at the Africa Centre)

3.8.5 Querying the data from RegaDB

The REGA database was queried using in-built parameters such as dataset names, viral isolate, treatment regimens and other measurements. This allowed for the compilation of smaller datasets with certain common characteristics. These datasets could then be analyzed using statistical programs such as Stata v10.0.

3.9 Statistical Analysis

In order to investigate the patterns of mutations in this cohort, the drug resistance mutations, clinical measurements and demographic data were exported for all patients genotyped from RegaDB for further statistical analysis. Descriptive statistics were used to summarize the demographic and clinical characteristics. Medians and the interquartile range (IQR) were calculated for continuous data and are quoted unless otherwise stated; in which case means (range) are presented. For analysis of drug resistance mutations, frequency distributions of the major DRMs were derived. The following definitions were used in this study:

- Duration on ART was defined as the number of months between the date of ART initiation and the date of genotyping.
- Duration of ART failure was the period in months between the date of the first viral load >1000 copies/ml to date of genotyping, unless there was a viral load <50 copies/ml in between, in which cases the duration was estimated from the time of the next subsequent viral load >1000 copies/ml. If there was no viral load ≤1000 copies/ml then duration was calculated from date of ART initiation.

CHAPTER FOUR
RESULTS

4.1 Study population

This section presents the descriptive and basic statistics of paediatric patients recruited from 16 clinics within the uMkhanyakude (Fig 10). Of the approximately 1653 children (≤15 years) who were initiated in and are currently active in the ART program, a total of 101 children (≤15 years of age), were identified as failing therapy, were enrolled between August 2011 and December 2012. These patients had been on ART for > 1year and presented with two or more raised viral loads (>1000cp/ml). Of the 101 patients, 89 patients were successfully genotyped using the SATuRN genotyping method (Fig 9). The twelve samples that failed to amplify by PCR were subsequently submitted for viral load quantification. Viral loads measured indicated that seven patients had a viral load of >1000cp/ml (mean= 3.77log cp/ml; range=3.0-4.4 log cp/ml) at the time of genotyping but failed to amplify, while five were suppressed with viral loads <1000cp/ml. There was insufficient sample to perform viral load quantification for one patient's sample (Fig 12).

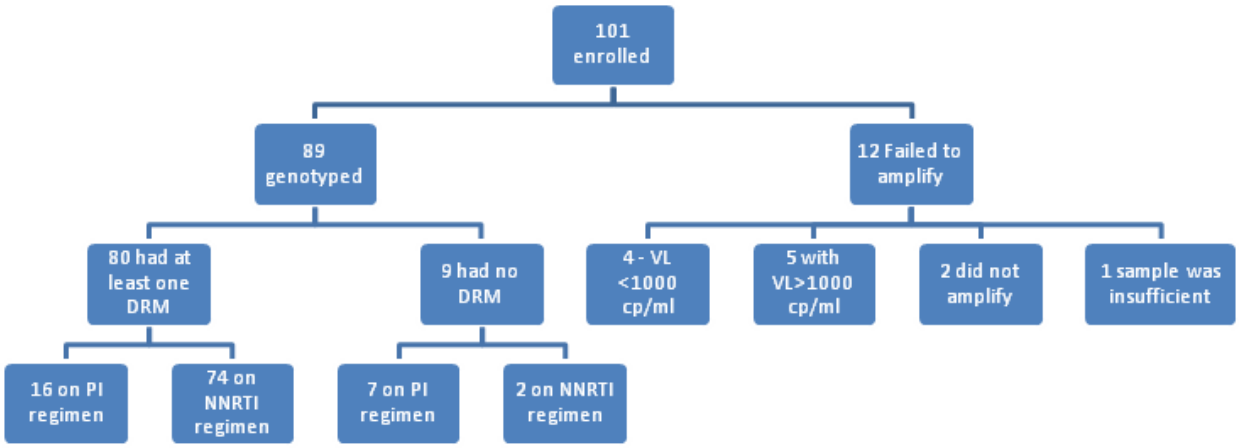


Figure 12: An illustration of the distribution of patients that were successfully genotyped compared to those that failed to amplify and the subsequent measures performed on the latter subset.

The median duration of time between last VL result and genotyping was 3.1 months (1.4-7.0). The median log viral load prior to genotyping was 4.92 log cp/ml ± 5.3 (2.35-6.18log cp/ml. Of the successful genotypes the median VL was 4.0 (4.7) (table 14) and the median time between last viral and sampling was 3.1 (1.4 – 7.0) (Table 14).

Table 14. Demographic and clinical characteristics of the 89 children with a genotype

Characteristic	**Outcome**
Gender, male, *n* (%)	53 (59.6)
At ART initiation	
Age, years, median (IQR)	7 (5.9)
0-3 (%)	17 (23)
4-9 (%)	37 (50)
10-15 (%)	20 (27)
Viral load, $\log_{10}$ copies/ml, median (IQR)	4.0 (4.7)
CD4+ cell count, cells/µl, median (IQR)	286 (448)
By Age category:	
0 - 2yrs (n= 3)	817 (1533)
>2 – 5yrs (n= 29)	469 (425)
>5yrs (n= 38)	200 (294)

ART regimen, *n* (%)	
d4T/3TC/EFV	64 (71.9)
d4T/3TC/LPVr	12 (13.5)
ABC/3TC/EFV	8 (8.9)
ABC/3TC/LPVr	4 (4.5)
AZT/3TC/EFV	1 (1.1)
At genotyping	
Age, years, median (IQR)	10.2 (7.7 – 12.9)
0-3 (%)	4 (4.5)
4-9 (%)	38 (42.7)
10-15 (%)	47 (52.8)
Viral load prior to genotyping*, $\log_{10}$ copies/ml, median (IQR)	10.0(5.5)
CD4+ cell count prior to genotyping*, cells/µl, median (IQR)	460 (541)
By Age category:	

ART regimen at time of genotyping, *n* (%)	
d4T/3TC/EFV	58 (65.2)
d4T/3TC/LPVr	8 (8.9)
ABC/3TC/EFV	14 (15.7)
ABC/3TC/LPVr	8 (8.9)
AZT/3TC/EFV	1 (1.1)
Duration of ART, years, median (IQR)	3.3 (1.9))
Duration of ART failure[†], years, median (IQR)	1.8 (1.6)
Time between last viral load and genotype, months, median (IQR)	3.1 (5.6)
History of ART substitution[#], yes, n (%)	12 (13.5)

Key:

d4T, stavudine; 3TC, lamivudine; EFV, efavirenz; LPVr, lopinavir/ritonavir; ABC, abacavir; AZT, zidovudine; ART, antiretroviral therapy; IQR, interquartile range

* Last measurements recorded prior to date of genotype

† Duration of antiretroviral failure was estimated from the date of the first viral load >1000 copies/ml to date of genotype, unless there was a viral load <50 copies/ml in-between, in which case the time was estimated from the next viral load >1,000 copies/ml. If there was no viral load ≤1,000 copies/ml then time was calculated from date of ART initiation

Substitutions included changes of single drugs due to toxicity or guideline changes

4.2 Characteristics of patients genotyped

This cohort was primarily male (59.6%) with a median age of 10.2 years (7.7-12.9 years) (Table 14). Patients had been on ART for a median time of 3.3 years (2.5-4.4). At initiation, patients were predominantly on NNRTI based regimens with only 4 (4.4%) patients on the currently recommended (DoH, 2010) regimen for children <3yrs of age (3TC, ABC, LPV/r) and 8 (8.9%) on the currently recommended regimen for children over 3yrs of age (ABC, 3TC, EFV) (Table1). We noted that most patients were on a failing regimen for approximately half this period of time (1.75; 03-3.4 years). Twelve patients had either one or two drug substitutions in their regimens (Table 14). Of the 89 children genotyped, 50 (56.2%) had, on at least one occasion, successfully suppressed viraemia to <1000 copies/ml. This would indicate that 44% of patients were unable to successfully suppress viral replication while on ART. The median duration on ART was slightly longer for those children on an NNRTI-based regimen compared to those on a PI-based regimen (3.4 years vs. 2.7 years) but the duration of ART failure was broadly similar between the same two groups (Table 15).

4.3. Characteristics of patients stratified according to regimens

Patients on PI regimen were younger (1.5; 4.9 years) at initiation and similarly, the median age at genotyping was almost double in the NNRTI group (11.4; 4.4 years) than that in the PI group (5.0; 4.6 years). We noted that age at initiation of ART ($p<0.01$) and at genotyping ($p<0.01$) was significantly higher in the group of children on an NNRTI-based regimen as compared to those on a PI-based regimen (Table 15). In addition, CD4 counts were higher in children on a PI-based regimen at initiation ($p<0.05$) and at genotyping ($p<0.01$) but these clinical measurements are likely a reflection of the age, at imitation and genotyping, of children on a PI-based regimen (Table 16).

There was no significant difference in the duration on ART between patients in the NNRTI and PI groups despite a higher duration on treatment in the NNRTI group (Table 15).

Table 15. Characteristics of children on NNRTI-based and PI-based regimens (based on regimen at time of genotype)

	NNRTI (*n* = 73)	**PI (*n* = 16)**
At ART initiation		
Age, years, median (IQR)**	7.6 (3.1)	1.5 (4.9)
CD4+ cell count, cells/µl, median (IQR)*	256 (369)	647 (705)
Viral load, $\log_{10}$ copies/ml, median (IQR)	3.9 (4.1)	4.7 (3.6)
At Genotyping		
Age at genotyping, years, median (IQR)	11.4 (4.4)	5.0 (4.6)
CD4+ cell count prior to genotyping, cells/µl, median (IQR)**	379 (501)	762 (520)
Viral load prior to genotyping, $\log_{10}$ copies/ml, median (IQR)	4.3 (4.6)	4.3 (5.2)
Comparative measures		
Duration of ART, years, median (IQR)	3.4 (1.9)	2.7 (2.4)
Duration of ART failure, years, median (IQR)	1.8 (1.6)	1.6 (1.5)
Time between last viral load and genotyping, months, median (IQR)	3.3 (5.5)	3.0 (3.8)

Key: Difference in means at a 1% (**) or 5 %(*) level of significance using the Student's T-Test

4.4 Resistance mutations

4.4.1 Phylogenetic reconstruction

Our cohort of 89 patients who had been genotyped consisted of 73 (81.1%) patients who were on an NNRTI-based regimen while 16 (14.2%) were on a PI-based regimen (Table 16). All of the sequences accepted for analysis were deemed of high quality having passed all quality and contamination tests as described previously (Chapter Three). The HIV isolates from all patients successfully genotyped were identified as HIV-1 subtype C variants (Fig. 13). There was no evidence of contamination as each sequence resolved independently. 70/89 (79%) clustered with strains from South Africa (ZA). Two sequences clustered with strains from Malawi (PRES064 and PRES065) and 17 others clustered with strains from Tanzania (Fig 13). All 89 sequences that were genotyped were not homogenous within the community. Sequences from each clinic did not cluster together in a discrete monophyletic branch but instead sequences from each clinic were intermixed.

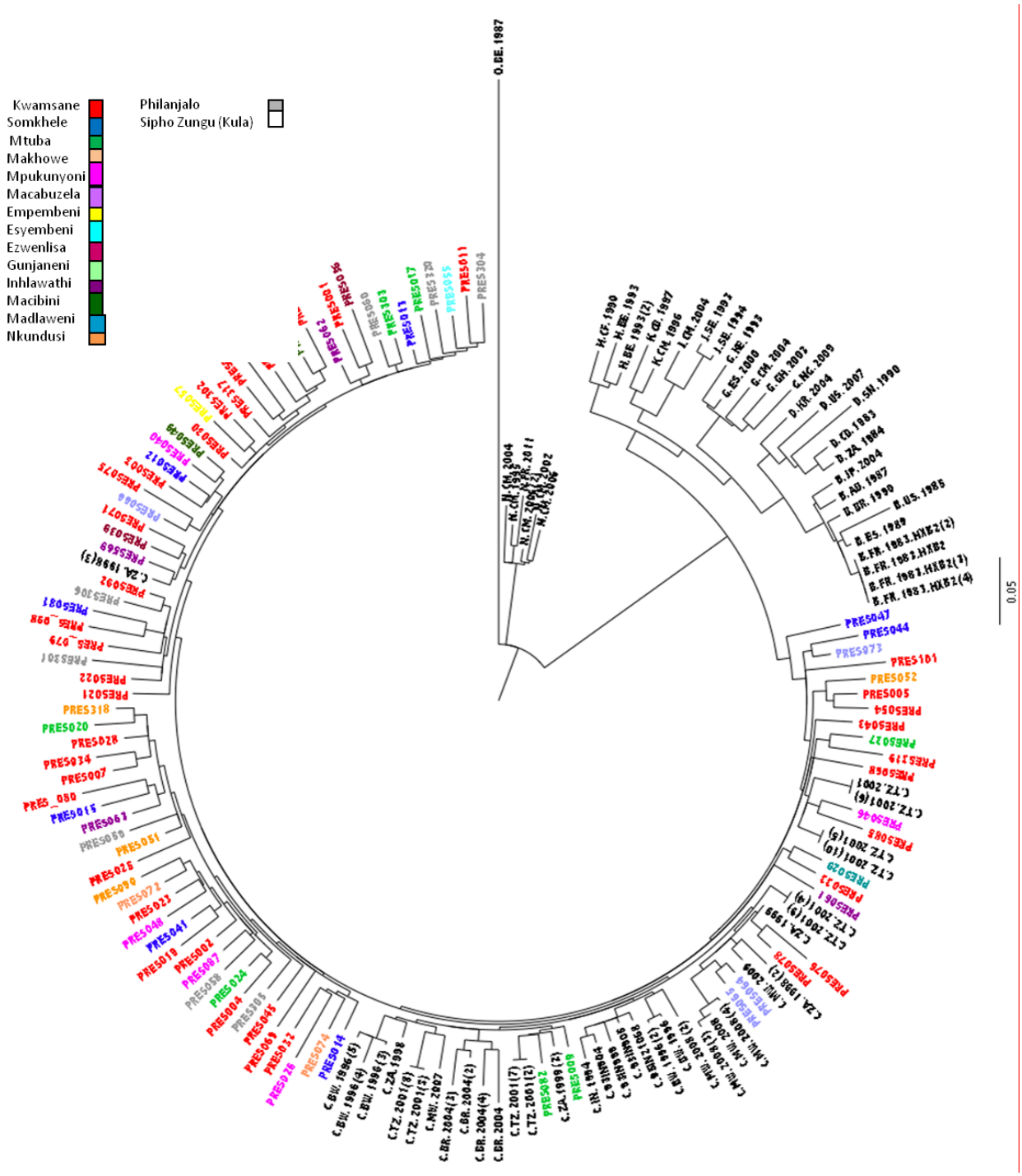


Figure 13. Maximum likelihood tree of the 89 patients that were genotyped and HIV-1 Group M reference sequences. Also included are representative Group N and Group P sequences. Sequences generated from patients in our cohort are colour –coded according to the clinics they attended as per the key attached.

4.4.2 Overall patterns of Drug resistance mutations

Of the 89 genotypes, 81 (91.0%) demonstrated at least one DRM while 8 (9.0%) had no DRM (Table 16). For those on an NNRTI-based regimen, the majority of genotypes had both NRTI and NNRTI mutations Thymidine analogue mutations (TAMs) were detected in 22 (23.4%) genotypes, while multiple TAMS of three or more were detected in four genotypes (4.5%). The Q151M complex (a multi-nucleoside resistance mutation) was present in two genotypes, one from a child on an NNRTI-based regimen and one from a child on a PI-based regimen.

4.4.3 Patterns of Drug Resistance mutation of Patients on NNRTI-based regimens (n=73)

Of those on an NNRTI-based regimen, 60(81.08%) had NNRTI mutations and 63 (84.14%) patients had NRTI mutations (Table 16). M184V was the most common NRTI mutation occurring in 60(81.08%) patients and K103N was the most common NNRTI mutation detected in 46 (62.16%) patients. Thirty-nine of the 73 patients on NNRTI-based regimens (43.3%) had both the M184V and K103N mutations indicating resistance across drug classes. In two patients, the viral isolate sequenced harbored the Q151 complex (Table 16). Thymidine analogue mutations (TAMs) were detected in 22 (23.40%) children of whom 4 had 3 or more TAMS. The D67N and T215F mutations occurred most frequently. Multiple TAMS indicative of a longer duration on a failing regimen was detected in four children. Of the 22 children that presented with TAMs, 18 harbored the TAM pathway 2 mutations (D67N, K70R, T215F, and K219EQ).

4.4.4 Patterns of Drug Resistance mutation of Patients on PI-based regimens (n=17)

Of the 16 children that were on a PI-based regimen, only one patient harbored a PI mutation (V82A). Seven of the nine patients that had no DRMs were on a PI based regimen. The most common mutation for children on a PI regimen was the NRTI-specific mutation, M184V (Table 16). Despite the absence of

PI mutations among patients on a PI-based regimen, patients in this group had a high frequency of NRTI (62.5%) mutations (Table 16).

Table 16: The frequency of major drug resistance mutations as well as resistance complexes associated with PIs, NRTIs and NNRTIs by regimen in the 89 genotyped patients prior to genotyping.

	NNRTI-based regimen (n=73)		**PI-based regimen (n=16)**	
NNRTI mutations	n	%	n	%
Any NNRTI DRM	60	82.2	4	25.0
L100I	5	6.9	0	0
K101EP	6	8.2	0	0
K103NRS	46	63.0	2	12.5
V106M	23	31.5	2	12.5
V108I	7	9.6	1	6.3
Y181C	2	2.7	0	0
Y188HCL	7	9.6	1	6.3
ll	9	12.3	1	6.3
P225H	14	19.2	0	0
M230L	3	4.1	0	0
NRTI mutations				
Any mutation	63	86.3	10	62.5

M41L	7	9.6	0	0
K65NR	4	5.5	1	6.3
D67NG	8	11.0	0	0
K70ER	7	9.6	0	0
L74VI	4	5.5	1	6.3
Y115F	3	4.1	2	12.5
M184VI	60	82.2	10	62.5
L210W	1	1.4	0	0
T215FYI	9	12.3	0	0
K219QREN	5	6.9	0	0
Any TAMS	22	30.1	0	0
1 TAM	11	15.1	0	0
2 TAMs	7	9.6	0	0
≥3 TAMs	4	5.5	0	0
Q151M complex	1	1.4	1	6.3
PI mutations				
Any PI mutation	0	0	1	6.3
V82A	0	0	1	6.3

Table 17. GSS scores of the 89 patients that were genotyped

GSS score	Number of patients	Percentage
0	**8**	**9**
0.5	**12**	**13.5**
1.0	**45**	**51**
1.5	**2**	**2.0**
2.0	**10**	**11**
3.0	**12**	**13.5**

Key: GSS score of 0= high level resistance; GSS score of 0.5= intermediate resistance; GSS score of 1.0= susceptible

There were 65 patients that had GSS scores ≤ 1 (Table 17). This shows that almost half of the children in our cohort were on suboptimal regimens with perhaps 1 or two effective drugs. Thirty-seven children that had GSS scores ≤ 1 were on 3TC, d4T and EFV, showing that majority of the children in this regimen were failing ART.

Of the 89 patients we have outcomes data for 73 (82%) children. The mean time between genotyping and follow-up was 11.6 months (range=0.06-26months). A total of 72 children received adherence counseling, while 17 children were switched to a new regimen. Of the 17 children that were switched, 16 were switched to a PI regimen and one was switched to an NNRTI regimen. A total of 39 children managed to achieve viral suppression, 23 children suppressed to <25 cp/ml and 16 suppressed to < 1000cp/ml. Fifty children were unable to achieve viral suppression following genotyping and either adherence counseling or treatment switch. The median VL, after a mean of 11.6months following genotyping (range=<1mth to 26mths), of the children that were switched was 2.4 $\log_{10}$ cp/ml (IQR=0.3$\log_{10}$ cp/ml) and the median CD4 was 402cells/µl (IQR=82 cells/µl). In comparison, the median

VL of the children that received adherence counseling was higher at 3.2 $3\log_{10}$ cp/ml (IQR=$0.23\log_{10}$ cp/ml) and the median CD4 count was also elevated at 624 cells/µl (IQR=68.7 cells/µl).

CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.1 General comments

This descriptive study presented genotyping data from one of the largest paediatric cohorts in South Africa. A further novelty of the study was that all patients in this cohort were from a rural setting where health services are decentralized, rather than an urban setting. This study has enabled us to determine the prevalence and patterns of drug resistance mutations in children failing first-line ART at the Hlabisa Treatment and Care Programme which is a primary healthcare ART programme, in rural KwaZulu-Natal, South Africa. The population of Hlabisa in 2011 was 220 000, of which 83 160 were children aged 1 to ≤ 15 years[24]. The area has a high burden of both HIV and tuberculosis with an estimated HIV prevalence in 2010 of 23%[24]. This study was unique because it was done in a rural decentralized PHC programme where the delivery of ART to adults and children has scaled up rapidly. Our study was conducted at 16 PHC clinics, where a doctor and social worker saw all paediatric patients in order to use the genotyping for clinical management, as was done with adult patients (Manasa in preparation).

This study has enabled us to determine the prevalence and patterns of drug resistance mutations in children failing first-line ART in the Hlabisa Treatment and Care Programme. The care in this facility is largely nurse and counsellor driven. Eighty-nine patients were genotyped in this study. We detected high levels of DRMs in our cohort. A total of 81 patients had at least one DRM suggesting that almost 91% of our cohort were failing ART due to resistance and not as a result of poor adherence. Thirteen patients, failing their current regimen, were initiated on a previous regimen and had drug substitutions suggesting that they had been in virologic failure for a long period of time. Efavirenz was the only NNRTI

used in this cohort. It was not established if these patients were previously exposed to any NNRTI. However we can deduce that the older children would not have been exposed to pMTCT since the roll out only started In 2003[82]. Seventy-four children were on an NNRTI-based regimen, with the majority of older children on these regimens harboring drug resistance mutations. In most cases the mutations would be unlikely to significantly compromise a second line regimen based on a ritonavir-boosted PI.

5.2 Patterns of Drug Resistance Mutations

M184V was the most commonly occurring NRTI resistance mutation causing high-level resistance to 3TC and FTC, low-level resistance to ddI and ABC and increased susceptibility to AZT and d4T. A high percentage (82.2%) of patients in our cohort harboured this mutation. This indicates that there was adherence, granted poor adherence at the time of genotyping since the M184V mutation is rapidly overgrown by wild-type strains in the absence of 3TC therapy. M184V is usually the first mutation to occur when regimens contain 3TC[83]. Four patients had ≥3 TAMs indicating that they were on failing regimens for a prolonged period. It has been shown that the length of time spent on a failing regimen leads to the development of complex resistance patterns[84]. The factors that drive the selection of the TAM1 and TAM2 pathways are currently unknown. Some of these factors may include the genetic background of HIV-1, immune system pressure, the antiretroviral used, the sequence of drugs and possible pharmacokinetic parameters. Alternatively, which TAM is the first to appear might be purely due to chance[85]. The prevalence of TAM1 seems to be higher than TAM2 in patients with less than three TAMs. We should also note that TAM-1 mutations are associated with an increase in phenotypic resistance and cross resistance to ddl and TDF. TAM-2 remains susceptible to these two drugs[86].

5.3 Previous studies

One patient had the Q151M complex. These complex mutation patterns would significantly compromise the future activity of the NRTI class of drugs. An earlier study, done in an urban setting in South Africa, showed similar resistance patterns[31]. This study was done in Durban, KwaZulu Natal. They had a cohort of 94 patients. However only 41 patients were genotyped in this study. The most prevalent mutations that they detected were M184V (70.1%), K103N (39%) and V106M (41.5%). These mutations were also the most prevalent in our study where their frequencies were 78.6%, 72% and 28% respectively. We found a 24.7% prevalence of TAMs on our cohort, which was much lower as compared to the study done by Green et al where 39% of patients had three or more TAMs as well as to other previously reported investigations in other setting[27, 28, 31, 34]. The high prevalence of three or more TAMs was noted even though patients in this cohort were on ART for a shorter duration of time (median=2.8; IQR=1.9-2.3) as compared with the patients in our cohort (median=3.3yrs; IOR=2.5- 4.4yrs). In comparison to these previous studies, the prevalence of mutations in our study was similar (Table 2). Majority of these studies were carried out in urban settings and only one was done in a rural setting. Comparing our results to this rural study done in Elandshoorn, Limpopo, the prevalence of NNRTI and M184V was much higher than ours. However we should take into account that the number of patients in this cohort was much lower (n=23) as compared to ours (n=89). An additional consideration is that we may not have identified and recruited all patients at failure within the Africa Centre surveillance area.

Our data however indicates that patients failing first line ART in our cohort should therefore remain susceptible to a second line regimen consisting of two alternative NRTI's and a ritonavir-boosted PI. Majority of our patients (65, 74%) had GSS scores ≤1. This is an indication that they were on a suboptimal regimen. It is of great concern that 8 patients had a GSS score of 0 which means that none of the drugs in their regimens were effective. They were fully resistant to all three drugs that they were on.

We can speculate that this occurred due to the poor tolerability of d4T and its toxic side effect[87]. It was also noted that majority of the children that had low GSS scores were older than 3 years of age. Older children would not have been exposed to pMTCT and would not have been put on a PI regimen. Most of the older children would have initiated ART on 3TC, d4T, EFV which was the standard starting regimen of the DoH before 2010[2]. It would seem that this regimen is not ideal to start with in children given the high failure rates. Given that these are children who will have to remain on ART for longer compared to adults, it is vitally important to preserve future drug regimens especially in resource-poor setting where access to 3rd line drugs is limited.

5.4 NNRTI's and pMTCT

In South Africa the first line ART is largely NNRTI-based. It is challenging since drug options such as NVP and EFV have low genetic barriers and a single mutation can confer resistance to the entire class of drugs[73]. Both cross-resistance and high-level resistance severely compromises future ART options. A high percentage (72%) of patients failing ART was on NNRTI-based regimens. In the majority of cases (80%) had drug resistance to both the NRTI's and the NNRTI's alluding to very poor outcomes. K103N was the most common NNRTI mutation occurring in 63% of patients, followed by V106M, which confers broad cross-resistance. Exposure to NVP in pMTCT increases the risk of infection with drug resistant virus in infants and of treatment failure with first line regimens containing NNRTI's[82]. Exposure to NVP increases the risk of HIV drug resistance development in resource-limited settings[73]**.** This in turn leads to treatment failure of first line regimens containing NNRTI's[83, 88]. It is unclear how many of the patients in our cohort were exposed to pMTCT however given the timing of pMTCT in the South African National ART program, older children in our cohort may not have been exposed to pMTCT. The K103N mutation appeared in 72% of our patients. HIV positive pregnant women that are exposed to suboptimal ART

often leads to selection of resistance, which is inevitably transmitted to the child[89]. It has been suggested that DRMs resulting from single dose NVP may pre-exist at low levels, even in the absence of NNRTI exposure. These mutants become integrated in the virus reservoir and can re- surface during subsequent exposure to NNRTI's. The occurrence of NVP resistance is of particular concern because it confers resistance to EFV as well. Both NVP and EFV are part of the first line regimens that are used globally to treat both children and adults[88]. In this study we have not explored this and therefore cannot associate NNRTI mutations with single dose NVP or MTCT.

5.5 PI regimens

Only one of seventeen patients on a PI-based regimen had a major PI resistance mutation. Of the 16 children that were on a PI based regimen, only one patient harbored a PI mutation (V82A) which was low as compared to previous reporting on PI mutations[26, 28, 30]. Since LPV/r has a high genetic barrier, this would explain the absence of drug resistance mutations in these patients. The younger children on PI-based regimens more often had no drug resistance mutations (six out of 16 cases) and only one child had a major protease mutation. This suggests that adherence in these children was very poor such that there was not enough drug pressure to allow for resistance to occur. The advantage of PI's is their high genetic barrier to resistance and this is especially important when adherence is a great concern[73]. This does raise the possibility of differential adherence to different components of the ART regimen (LPV/r syrup can be poorly tolerated), to problems with dosing of LPV/r syrup or possibly to drug-drug interactions, all issues we were unable to explore in detail for this study but which are subject to on-going research.

5.6 d4T- Containing regimens

The majority of our patients in this cohort were on a d4T- containing regimen. Since the initial ARV scale-up, d4t- containing regimens have been the most commonly used ARV regimens used in many national treatment programmes particularly in RLS due to its efficacy, short- term tolerability, low cost and its availability in a co-formulated form suitable for paediatric patient. 3TC and d4T were the first drugs used as the NRTI backbone in the treatment of children. In 2010, the World Health Organisation (WHO)[90] recommended phasing out d4T due its potential for serious toxicity associated with long term use. This could explain the high rate of virologic failure in our cohort since the poor tolerability of d4T limits therapeutic durability and encourages poor adherence. It was noted that 52 of 58 patients on a d4T regimen had GSS scores ≤ 1. It is critical that first line drugs are as tolerable as possible since it is a patient's best chance of achieving optimal treatment and extending treatment efficacy[91]. Patients will adhere to treatment if the drugs are tolerable and the dosing is simple eg. once daily rather that twice daily[91].

In 2010 due to the World Health Organisation recommendations, South African Guidelines replaced d4T with ABC due to concerns around toxicity[87]. Twenty-two children in our cohort who were on an ABC containing regimen include those that have been switched from a d4T-containing regimen. Technau[87] et al in a recent study compared the outcomes of ABC/ 3TC in combination with either EFV or LPV/r as compared to ABC/ d4T in combination with either EFV or LPV/r. Their results showed that ABC containing regimens had a lower probability of achieving viral suppression and higher probability of virological rebound than the d4T containing regimens. Adherence is a huge concern especially in children and since d4T has a higher genetic barrier to resistance than ABC[87] , d4T is better able to deal with suboptimal adherence than ABC. This is of particular concern since children over three years of age

are being treated with ABC under the SA National HIV treatment guidelines. This may pose a problem if children on ABC fail to suppress within the first year of treatment and this may compromise future treatment options.

5.7 Adherence

Nine patients in this cohort had no DRMs yet were viraemic, strongly suggesting that they were non-adherent or poorly adherent. The success of optimal antiretroviral therapy is directly dependent on the level of adherence achieved. It has been shown that in order for ART to be successful, adherence should be ≥95%[92]. Drug resistance develops very rapidly when drugs are not maintained within a therapeutic range. It is therefore critical that patients adhere to their complex ART regimens[93]. There are several reasons why children don't adhere to their regimens. These include inability to swallow tablets, unpalatable medications and privacy issues when medication has to be administered at school or daycare[93]. There is also a problem of side effects where a child has side effects and the caregiver stops treatment without consulting the doctor or caregivers who think that the child is doing well and there is no need to continue with medication[94].

5.8 Poverty and HIV

Poverty remains a serious concern globally as well as in South Africa as it impacts on the health and wellbeing of children[95]. Globally HIV is worsened by poverty. Children in South Africa, especially in our study area, rural KZN, are extremely vulnerable since they face both poverty and the plight of HIV. Peadiatric HIV is a multigenerational disease meaning that an HIV positive child may almost always have an HIV positive mother and/ or father[95]. Poverty may impact on the course of disease progression in this vulnerable group. Their families may be unable to access the treatment that is crucial for their wellbeing

and future development. Lack of funds to transport them to clinics that provide treatment or stigmatization by the community may prevent these families from accessing the health care that they require. Many families do not live within close proximity to health care facilities (fig 10) and therefore may not be able to access these facilities on a regular basis. The mean distance travelled by any individual in our study population, to their nearest clinic is 4.72 km (maximum 13.2 km) with a median travel time of 81 min. Travel time to the local hospital is double this (170 min), which is more costly to the patient and a potential barrier to regular clinical follow-up, in addition to overburdening the referral centre[4]. Stigma also plays a vital role in the disclosure and treatment of children in our setting[94]. Parents or care givers are afraid to seek medical assistance or disclose their child's status to other family members and as a result the child does not get tested or does not receive the proper treatment[94].

5.9 Stress of orphans and caregivers

There are a number of children who are orphans and face additional barriers to care. Orphaned children have to deal with economic and sexual exploitation and HIV infection as well as stigmatization and discrimination from their extended families[96] and their community. This may lead to the emergence of child headed households, separation of siblings child abandonment and family breakdown[96]. Disclosure of the child's HIV status, emotional health and reduced conflict with caregivers[97] add to the stress. However we cannot associate non-adherence with the patients that did not have any DRMs as this was not investigated in our study.

There are other added stresses that can negatively impact on HIV affected families. Families may experience losses of members to the disease and this adds to the psychosocial and emotional distress experienced by the family[95].

It has been said that children are the hardest hit by HIV than the adult community[96]. In most cases their parents are ill or die due to the disease and the children do not have the resources to continue socially or economically. The majority of orphans live with their grandparents. The grandparents do not have sufficient resources or the strength to care for themselves and yet they have to care for their grandchildren. There are several challenges that these elderly guardians are faced with. Deteriorating mobility prevents some grandparents from accompanying their grandchildren to the clinics especially with the younger children that need to be carried or if they cannot walk long distances[70]. Some elderly guardians forget the child's clinic dates and as result the child has to be without drugs for a while until the guardian remembers or is reminded. It is also difficult for these guardians to understand the complexity of the child's regimens and this may result in the child not taking their regimen properly at the proper time intervals[70].

These guardians also fear that when they die there will be no one to take care of these children or remind them to take their medication[98]. This is a huge responsibility for these elderly people.

Another reason for the lack or late treatment of children is denial of the parents in finding out the HIV status of the child. When a child is sick it is often ignored or the symptoms are attributed to other causes. Most often the child is tested after the mother has died and there is advanced disease progression in the child[94].

5.10 ART failure

ART failure in this population was high with a wide range of mutations. The number of children failing ART is of great concern. These patients are very young and will have to be on ART for the rest of their lives. The high prevalence of DRMs and TAMS indicate that these patients are on failing regimens for long periods of time without any intervention, severely compromising future drug options. There is therefore an urgent need for early intervention in patients that are found to be failing ART. Failure could mean that these patients are not adhering and counseling can be administered or that these patients have DRMs and appropriate switches can be made. Using our current genotyping system at a fraction of the normal cost and without a pre-genotype viral load shows that genotyping in identifying treatment failure can be implemented in resource limited settings like ours. This would aid in early intervention and management of these patients. Ideally second line drugs should be selected on the basis of resistance testing but the high cost thereof does not warrant this practice for routine patient management. In RLS where second line regimens are limited, children are likely to remain on failing regimens for longer periods of time, promoting the accumulation of drug resistance mutations[83]. We, in our rural cohort, also demonstrated the presence of complex resistance patterns in patients failing therapy and who had been on a failing regimen for a median of 1.75 years (03-3.4). The number of drugs available in South Africa is limited and there is currently no 3rd line option available to children failing therapy. It is therefore important to determine emerging resistance patterns in paediatric patients to ensure that children continue to receive appropriate ART regimens. Children have a higher risk of developing drug resistance and it is therefore clear that interventions within coordinated surveillance strategies are vital to long-term clinical success.

5.11 Cost of genotyping

The current cost of our genotyping reagents are approximately 50US$ at reagent cost and less than 100US$ when staff and transport costs are added. In the public sector, including in South Africa, the cost of genotyping is 250-300US$ and largely prohibitive to the vast majority of patients receiving treatment. In order to perform large-scale genotyping and in the interest of reducing costs, we did not do a pre-genotype viral load yet we successfully genotyped 88% (89/101) of patients in our cohort. Our genotyping system is open source. Any molecular laboratory can perform this assay as it requires routine equipment that can be found in any molecular laboratory. However not all laboratories may have a sequencer. Perhaps the preliminary work can be done and this could be sent to a central laboratory for sequencing. The analysis software is also open access and very user friendly. This system has worked quite well for us as we were able to provide results i.e. sequence the samples, analyze them and produce reports within a specific turnaround time so that the clinician could get feedback and thus manage the patient appropriately. Genotyping is not routinely done to guide clinicians on the management of patients that are failing ART. Clinicians are generally reluctant to switch patient's drugs or regimens and they use clinical markers such as CD4 and viral load before making that decision. However with our cheaper and much more affordable genotyping system, management and guidance of failing patients could become easier for clinicians so that patients will not remain on failing regimens for prolonged periods of time. It could actually be used as part of our national guidelines.

Table 18. Comparison of commercial genotyping assays and the SATuRN in-house genotyping method

Parameter	**ViroSeq**	**TruGene**	**SATuRN In-house method**
Subtypes	Subtype B (FDA approved), non-B	Subtype B (FDA approved), non-B	Major Group M viruses
Diversification possibility	Semi-open system	Closed system	Open system
Cost/kit	$6825 R68 702.06	$ 5150 R5 1841.11	Varies
Cost/test	$144 R1 449.54	$180 R1811.92	$80.49 R810.21
Actual cost/test (incl 10% repeats)	$158.4 R1594.49	$198 R1993.11	**$88.54** **R891.23**

The SATuRN genotyping system is likely to be affordable (Table 18) to upper middle-income countries like South Africa and Botswana but further cost reductions would be required to make drug resistance testing affordable in lower middle-income and low income countries within Africa. This may be possible in the near future given the industry trends of significant reduction in sequencing costs that have been observed over the past 5 years. The added feature of pooling patient samples that is currently possible for next generation sequencing further reduces sequencing costs for individual patients. An additional feature of our study was that genotypes directed subsequent clinical care where a doctor, social worker and other clinic staff managed patients from enrolment to implementing an intervention post-genotyping as was carried out on an adult cohort from the same area[99]. Of the 89 patients that were genotyped, 39 patients managed to suppress their VL to ≤1000cp/ml and of these 23 patients suppressed to ≤25cp/ml. Seventeen patients were switched to 2nd Line regimens and this switch was motivated and guided by the genotyping result. In this way individualized treatment is enabled even in a rural setting such as ours.

5.12 Limitations

Although the number of patients in our cohort was larger than previous studies conducted in South Africa, the number is still fairly small relative to the number of infected children and those on treatment in the country. Future studies should look at larger cohorts including pooling of data and related surveillance from different sites in order to inform national policies. This was a cross sectional study where we identified as many children as possible that were experiencing virologic failure but we are not certain that all the children that were eligible were included in this study. We are however certain that patients who had been failing at the time of the study and who attended any one of the 17 clinics in our surveillance area were recruited. Approximately 1653 children were active in the ART programme. We only identified 101 patients that were failing. However the failure rate in this area was previously described as being approximately 25%. In this study we only managed to recruit 6% of patients that were failing therapy. In this study we did not determine whether the DRMs's detected in the children were acquired or transmitted resistance. Perhaps this can be explored in future analysis of this cohort. We had only one patient that had a PI mutation as we only looked at the protease gene. It is possible that we could have missed mutations at other sites, eg. Gag cleavage sites[100] and env. Although HIV resistance to PIs results from the selection and accumulation of mutations in protease, other genomic regions could be involved in PI resistance.[101] Several studies have shown that gag and gag-pol cleavage sites might influence the virological outcome of a PI-based regimen[101, 102]. As previously mentioned, the impact of pMTCT was not investigated in this study but given the delayed rollout of the ART program as a whole in the country and in this region, we estimate that the majority of children older than 10years of age (n=47) in our study will not have been exposed to combination pMTCT regimens either during gestation or breastfeeding. Their exposure to single-dose nevirapine is highly likely but previous studies have demonstrated the disappearance of any associated mutation within a year[103].

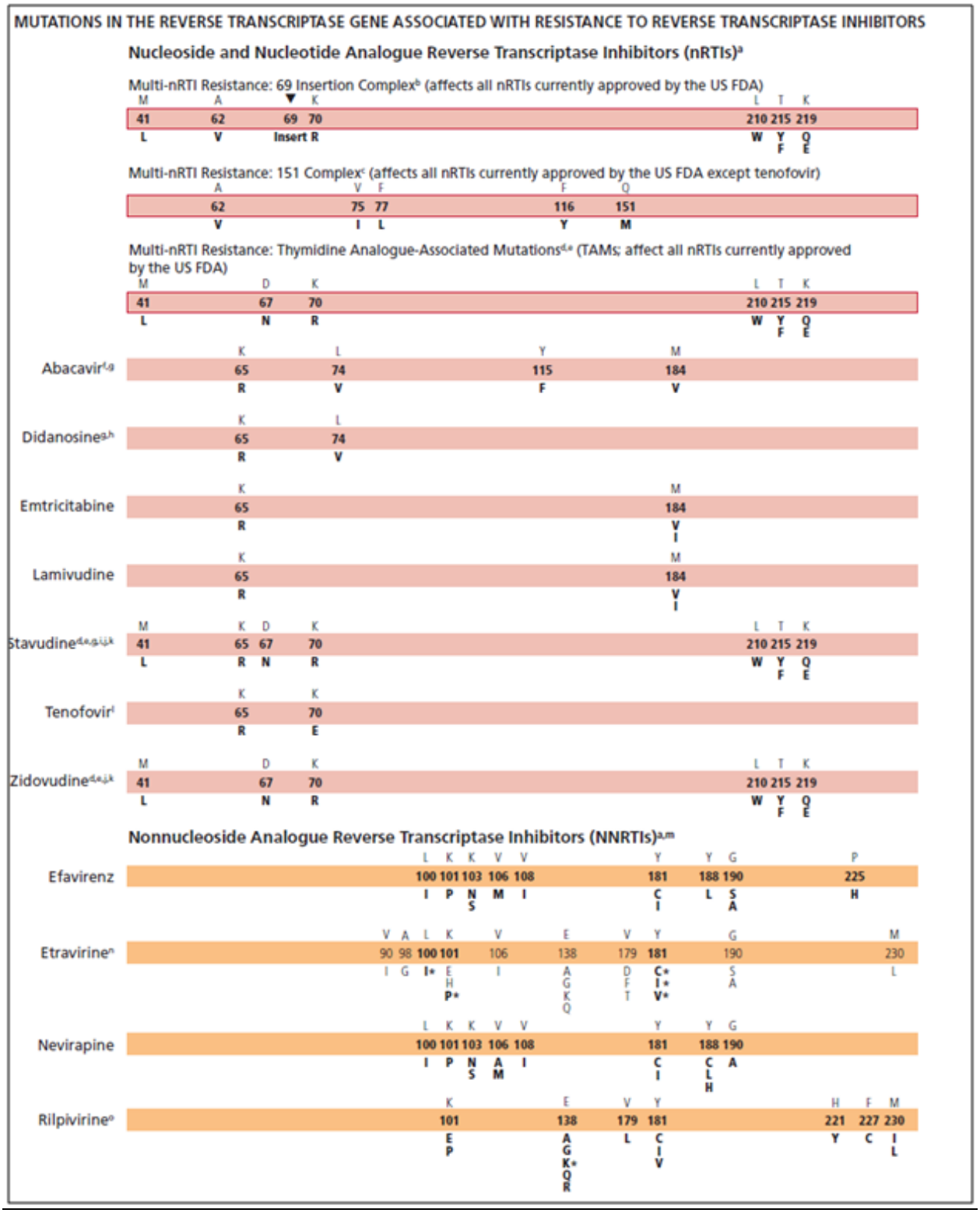
5.13 Conclusion

In conclusion, this study has demonstrated that the spectrum of drug resistance mutations in this rural cohort is complex and varied. The concerning prevalence of high-level resistance mutations particularly the frequency of TAMs amongst this paediatric cohort is an indicator of the time these patients spent on a failing regimen. This highlights the need for timely identification of patients failing ART and the implementation of early interventions, be it drug switches or effective, reinforced, adherence counseling with appropriate follow-up. The results from our study addressed three of the ten goals of the Department of Health 2013 ART Guidelines[22]. We presented a means of achieving the best outcomes for HIV patients receiving ART in a cost-effective manner (Goal B), we used an existing infrastructure for patient management (Goal D), that of a decentralized rural public health clinic facility and we identified DRMs early which ensured patient retention on lifelong ART by early interventions to stop ART failure and prevent mortality and morbidity of our patients (Goal I). In resource limited settings there are few available ART options. This study provides compelling arguments for the recommendation that a genotype be performed on all patients failing ART[91, 104]. This aids in deciding whether a patient's regimen should be switched due to the presence of mutations and prevent unnecessary switching in the absence of mutations. More importantly, genotyping allows for individualized patient care thereby preserving the remaining spectrum of drugs within the program for future use. Genotyping is very expensive and is not routinely performed in resource-limited settings. However we have shown that genotyping can be performed at a fraction of the current cost and can be made freely accessible in settings similar to ours. All laboratory and analysis protocols used in this study are open source, further reducing costs. Thus we propose that there are currently few restrictions to implementing resistance genotyping for patient management at any public health setting including remote rural areas such as in our investigation. Given the spectrum and frequency of complex, high-level resistance mutations we observed, such a management strategy must urgently be considered for successful patient management.

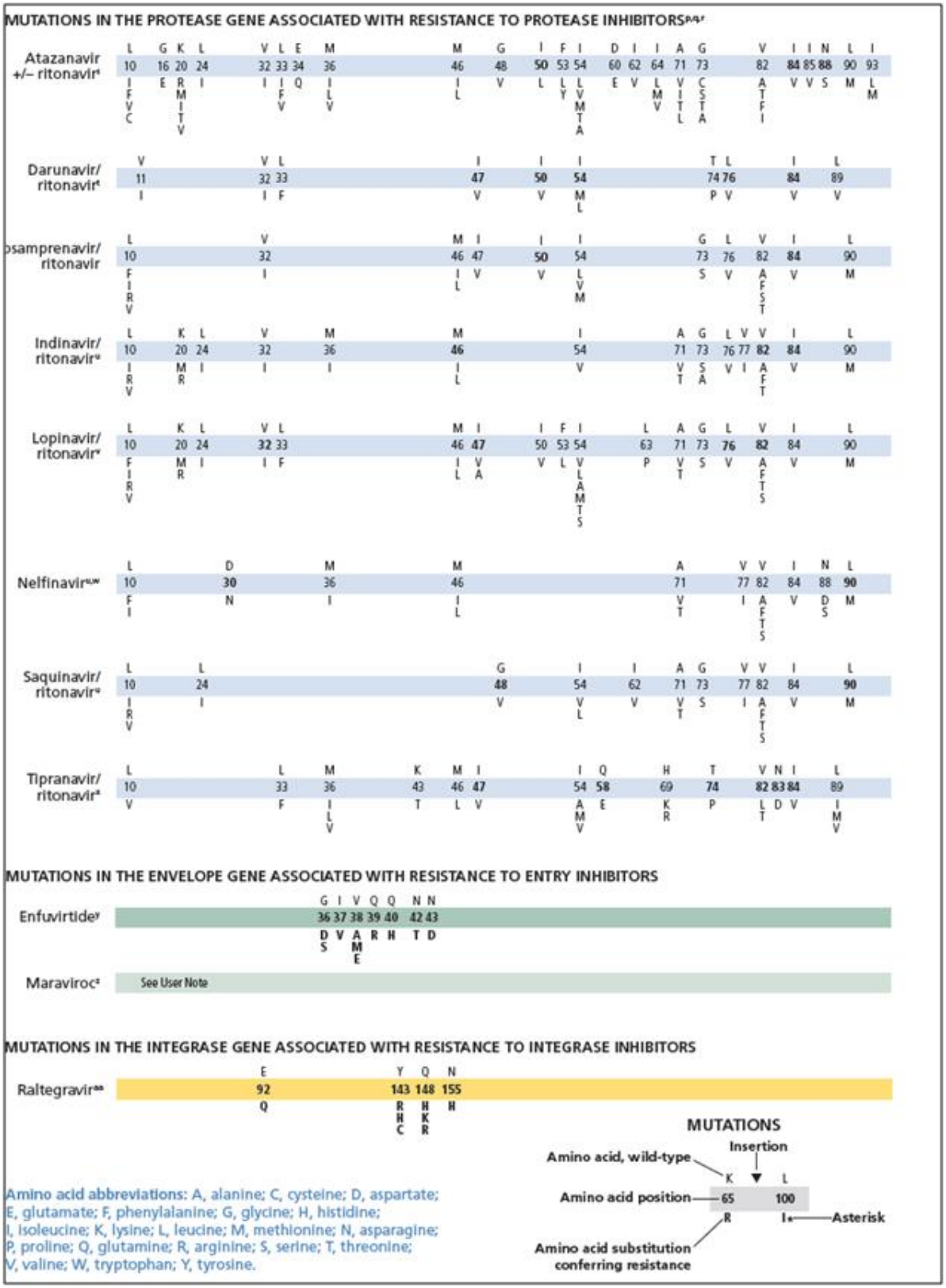
Older treatment programmes in Africa reveal complex resistance patterns[105]. Despite being one of the younger ART programs in the world, we noted complex resistance patterns with DRMs at high frequencies and this may be a reflection of the enormity of the South African ART program and the fact that freely available and widespread distribution of ART often results in the widespread emergence of drug resistance[105]. Although the rate of TR is low (<5%)[99] currently, mathematical modeling suggest that this rate could increase considerably in the next ten years[106]. The impact of TR in children could be greater as the path from drug resistance to virologic failure, immunologic failure and death occurs much faster than in adults[106]. It is therefore imperative that children are managed effectively and that timely interventions be instituted in order to maintain them on long term ART since the possibility of a vaccine or a cure for HIV remains just beyond the horizon.

APPENDICES

Appendix 1



Appendix 2


MUTATIONS IN THE PROTEASE GENE ASSOCIATED WITH RESISTANCE TO PROTEASE INHIBITORS
Atazanavir +/– ritonavir
Darunavir/ ritonavir
Fosamprenavir/ ritonavir
Indinavir/ ritonavir
Lopinavir/ ritonavir
Nelfinavir
Saquinavir/ ritonavir
Tipranavir/ ritonavir
MUTATIONS IN THE ENVELOPE GENE ASSOCIATED WITH RESISTANCE TO ENTRY INHIBITORS
Enfuvirtide
Maraviroc
See User Note
MUTATIONS IN THE INTEGRASE GENE ASSOCIATED WITH RESISTANCE TO INTEGRASE INHIBITORS
Raltegravir
MUTATIONS
Insertion
Amino acid, wild-type
Amino acid position
Amino acid substitution conferring resistance
Asterisk
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 3: Antiretroviral drug classes with modes of action and FDA approved drugs

Antiretroviral drug class	**Abbreviations**	**First approved to treat HIV**	**Mode of action**	**FDA approved drugs**
Nucleoside/Nucleotide Reverse Transcriptase Inhibitors	NRTIs, nucleoside analogues, nukes	1987	NRTIs interfere with the action of an HIV protein called reverse transcriptase, which the virus needs to make new copies of itself.	Lamivudine and zidovudine(Combivir) FTC, emtricitabine (Emtriva) lamivudine, 3TC (Epivir) abacavir/ lamivudine (Epzicom) zalcitabine, ddC, dideoxycytidine (Hivid) zidovudine, AZT, azidothymidine, ZDV (Retrovir) abacavir, zidovudine, and lamivudine (Trizivir) tenofovir disoproxil/emtricitabine (Truvada) enteric coated didanosine (Videx EC) didanosine, ddI, dideoxyinosine (Videx) tenofovir disoproxil fumarate (Viread) stavudine, d4T (Zerit) abacavir, ABC (Ziagen
Non-Nucleoside Reverse Transcriptase Inhibitors	NNRTIs, non-nucleosides, non-nukes	1997	NNRTIs also stop HIV from replicating within cells by inhibiting the reverse transcriptase protein.	Delavirdine DLV, (Rescriptor), efavirenz(Sustiva),nevirapine (Viramune)
Protease Inhibitors	PIs	1995	PIs inhibit protease, which is another protein involved in the HIV replication process.	Amprenavir (Agenerase),Tipranavir (Aptivus) saquinavir mesylate, SQV (Invirase) lopinavir and ritonavir (Kaletra), Fosamprenavir Calcium (Lexiva) ritonavir, ABT-538 (Norvir), darunavir (Prezista), atazanavir sulfate (Reyataz) nelfinavir mesylate, NFV (Viracept)
Fusion or Entry Inhibitors		2003	Fusion or entry inhibitors prevent HIV from binding to or entering human immune cells.	enfuvirtide, T-20 (Fuzeon), maraviroc
Integrase Inhibitors		2007	Integrase inhibitors interfere with the integrase enzyme, which HIV needs to insert its genetic material into human cells.	Raltegravir (Isentress)

http://www.fda.gov/ForConsumers/ByAudience/ForPatientAdvocates/HIVandAIDSActivities/ucm118951.htm

Appendix 4

HIV drug resistance study **Consent form**

Mina……ngiyavuma ukuba umntwana wami abe yingxenye yocwaningo lokuhlola ukungazweli kwemishanguza yesandulela ngculazi. Sengichazeliwe ngocwaningo ngaliqondisisa nephepha lolwazi.

Ngiyayiqonda imithelela yokungenela komntwana wami kulolu cwaningo nokuthi kunokwenzeka kucelwe olunye ulwazi mayelana nempilo kanye nokwelashwa kwakhe ngesikhathi socwaningo.

Ngiyabagunyaza abasebenzi bocwaningo ukuba babheke efayelini kanye nasekhadini lakhe nokuthi ulwazi olutholakala kulolu cwaningo lungahlanganiswa nolwazi oselukhona kwisilondoloza lwazi sase-Africa Centre. Ngiyaqonda nokuthi kuzothathwa elinye isampula legazi kulolucwaningo.

Ngiyaqonda ukuthi ngiyolithola ithuba lokubonisana ngemiphumela yomntwana wami nomhlengikazi noma nodokotela.

Ngiyaqonda ukuthi umntwana wami angashiya noma nini ocwaningweni futhi ngeke abandlululwe ngokwenze njalo. Siyoqhubeka nokusebenzisa imitholampilo ye-ART futhi ngithole ukunakekelwa ngokujwayelekile.

Isishicilelo sobambe iqhaza Usuku……/……/

Isishicilelo sikafakazi Usuku……/……/

Appendix 5

HIV drug resistance study **Consent form**

I..... agree to be part of the **HIV drug resistance study**. The study has been explained to me and I fully understand the information in the study information sheet.

I understand the implications of me / my child/ward joining the study and that I / my child/ward may be asked additional information regarding my / his/her health and my / his/her treatment during the study visit.

I give permission to the research staff to look at my / my child's/ward's clinic file and clinic card and that information from this study may be linked to information already held on the clinical and demographic databases at the Africa Centre. I understand that an extra blood sample will be taken as part of this study

I understand that I will have the opportunity to discuss the results with a nurse or doctor

I understand that I / my child/ward may leave the study at any time and I / he/she will not be discriminated for doing so. I will continue to use the ART clinic and be given appropriate care as usual.

Signature of the study participant:..... date:...../...../.....

Witness signature :.....date:...../...../.....

Appendix 6

HIV drug resistance study **Information sheet (Children)**

Isethulo

Simema umntwana wakho ukuba abambe iqhaza ocwaningeni oluhlola ukungazweli kwemishanguzo yesandulela ngculazi.

Le ncwajana ikunikeza ulwazi ngocwaningo okuzoxoxiswana ngalo nawe. Uma ngabe uluqonda ucwaningo, nokuthi uma uvuma ukuba umntwana wakho abambe iqhaza, uyocelwa ukuba usayine ifomu, noma wenze uphawu efomini ngaphambi kukafakazi.

Incazelo ngalokho esizama ukukwenza

Ukudla amaphilisi okudambisa isandulela ngculazi (ARVs) kuyinto yaphakade. Kunokwenzeka ukuthi ngesikhathi udla ama-ARVs, isandulela ngculazi singafika esigabeni esibizwa ngokuthi 'ngesingezweli'. Lokhu kuchaza ngesimo soshintsho lwegciwane kangangoba ezinye izinhlobo zama ARV's azisakwazi ukumelana naso. Asazi ukuthi lokhu kwande kangakanani ohlelweni lwethu lokwelapha nokuthi imiphi imithelela okungenzeka ihambisane nokwanda kokungezweli kwemithi. Ucwaningo luyithuba lokukhulisa ulwazi lwethu ngalokhu kanye nokusebenzisa ulwazi ekuthuthukiseni uhlelo lwezokwelapha.

Isimemo sokubamba iqhaza

Sicela ukuba umntwana wakho abambe iqhaza kulolu cwaningo ngoba ebedla ama-ARV's isikhathi esingaphezu konyaka kanti imiphumela yakhe iveza ukuthi inani legciwane egazini alehlile ngokwanele.

Kuchaza ukuthini ukuzibandakanya kulolu cwaningo?

Uma evuma ukubamba iqhaza kulolu cwaningo, udokotela noma umhlengikazi wocwaningo uyomubuza imibuzo ngesikhathi elande imithi ngezikhathi ezijwayelekile. Uyomubuza imibuzo ngokwelashwa kwakhe engeke ithathe ngaphezu kwemizuzu engaphezu kwama 20 bese ethatha igazi eliyothunyelwa elabhorethri eThekwini. Inqubo yasemtholampilo iyokwenzeka kuphela kanye nje kuphela. Igazi lakhe liyohlolwa elabhorethri ukuthi alinalo yini igciwane kanye nokungadodobali kwesandulela ngculazi. Ngemvume yakho, ulwazi olutholakale kulolu cwaningo luyongahlanganiswa nolwazi oselukhona kwisilondoloza lwazi sase-Africa Centre. Uyothola ithuba lokuhlangana nomhlengikazi kanye nodokotela

Appendix 7

bioafrica.net/saturn/ Genotypic Resistance Interpretation Algorithm

Durban, 06/09/2011

Dear Clinician,

I enclose the report of the genotyping that you requested

Patient ID on the SATuRN Rega database*: PRES
**Please notice that no patient personal identification information should be stored in this database, please use an sequential study number as patientID.*

Sample ID / Sample Date: PRES - 26/08/2011
Antiretroviral experience: [D4T, 3TC, EFV]
Subtype: HIV-1 Subtype C

Resistance interpretations: HIVDB 6.0.5

HIVDB 6.0.5

Drug	Mutations	Description	Level	GSS
zidovudine	67N 70R 118I 184V 219Q	Intermediate resistance	4	0.5
zalcitabine	N/A	N/A	N/A	N/A
didanosine	67N 118I 184V	Low-level resistance	3	0.5
lamivudine	118I 184V	High-level resistance	5	0.0
stavudine	67N 70R 118I 184V 219Q	Low-level resistance	3	0.5
abacavir	67N 118I 184V	Low-level resistance	3	0.5
emtricitabine	118I 184V	High-level resistance	5	0.0
tenofovir	67N 70R 118I 184V	Susceptible	1	1.0
nevirapine	103N 106M 138A	High-level resistance	5	0.0
delavirdine	103N 106M 138A	High-level resistance	5	0.0
efavirenz	103N 106M 138A	High-level resistance	5	0.0
etravirine	103N 106M 138A	Low-level resistance	3	0.5
saquinavir	N/A	N/A	N/A	N/A
saquinavir/r		Susceptible	1	1.0
ritonavir	N/A	N/A	N/A	N/A
indinavir	N/A	N/A	N/A	N/A
indinavir/r		Susceptible	1	1.0
nelfinavir		Susceptible	1	1.0
fosamprenavir	N/A	N/A	N/A	N/A
fosamprenavir/r		Susceptible	1	1.0
lopinavir/r		Susceptible	1	1.0
atazanavir	N/A	N/A	N/A	N/A
atazanavir/r		Susceptible	1	1.0
tipranavir/r		Susceptible	1	1.0
darunavir/r		Susceptible	1	1.0

Advice:
- Antiretrovirals for which the virus showed a reduced sensitivity, may still be partially active in a combination therapy. Antiretroviral agents against resistant virus are not recommended but may still exhibit a temporary activity when on HAART (> 3 Antiretrovirals).
- The interpretations of enfuvirtide (Envelope entry inhibitor) and tipranavir/r (boosted PI) are based on limited clinical information. These interpretations should be take with care.

List of all amino acid mutations observed in:
Protease: V3I T12S I15V L19I M36I S37N R41K D60E H69K L89M I93L
Reverse transcriptase: E6K K20R V35T T39E S48T V60I D67N K70R K103N V106M V118I D123N E138A T165L K173A Q174K M184V T200A Q207E K219Q V245Q K275R R277K T286A E291D V292I I293V

Appendix 8

PRES-072

Virological Failure Clinical History Sheet: Used by (circle): **DOCTOR/NURSE**

Dates	CD4 Count	Viral Load result	Identified Reason for Detectable VLr (e.g. Non-adherence, Treatment break, Pharmacological...)	(KG)
2008/01/07	69			24 kg
2008/06/30	276	79	Annie worried about adherence.	24 kg
2009/01/26		1200	maybe missing doses in Richard Bay	28 kg
2009/04/15	442	320		28.1 kg
2009/09/14		625		29.4 kg

2009/12/19 1500 2010/02/26 CD4: 349 VL: 2586 2012/04/03 CD4: 290 VL: 15823
2009/09/09 365 2011/05/30 CD4: 368 VL: 24206 2012/04/16 CD4: 760 VL: 14119

2. Antiretroviral Therapy: (Continue overleaf if more regimens than space...)

Dates	Regimen/Drugs			Reasons for changing/Toxicities
FROM: 2009	i. D4T ✓	iv. TDF	vii. LPV/r	
	ii. DDI	v. 3TC ✓	viii. NVP	
TO:	iii. AZT	vi. ABC	ix. EFV ✓	
	x. RTV			
FROM:	i. D4T	iv. TDF	vii. LPV/r	
	ii. DDI	v. 3TC	vii. NVP	
TO:	iii. AZT	vi. ABC	ix. EFV	
	x. RTV			

3) *Perceived Adherence:*

- **Patient estimate of adherence in last 3 months:**
  (**GOOD**) (>90% doses taken) **LESS GOOD** (<90%) **POOR** (<50%) (**NONE**) (since?)
- Details of **any treatment breaks**? Dates: FROM: ______ TO: ______ None reported
  REASON:
- *ASK*: Do you sometimes find it difficult to remember to give your child medicine? (From child) YES/(NO)
- *ASK*: When your child feels better do you sometimes stop giving the medications? YES/(NO)
- *ASK*: Thinking back over the past 4 days, has your child missed any doses? YES/(NO)
- *ASK*: Sometimes if your child feels worse when they take the medicine, do you stop giving it? YES/(NO)
- Do they know names of medications? (YES)/NO
- Do they know about side-effects of the medications? YES/(NO)

- Have you disclosed to anyone at home about your child? (YES)/NO Auntie. (neighbour).
- Do you have a treatment buddy? (YES)/NO mother.
- Have you been to adherence counseling? (YES)/NO At clinic.

4) *Other current medications:* *(Anti-epileptic, Steroids, Warfarin, Statins particularly important)*

1.	2.	3.

5) *TB therapy:* Nil.

Start Date	Regimen/Drugs		
	i. REG 1	ii. REG 2	iii. REG 3 ☐
	vi. MDR		
	i. REG 1	ii. REG 2	iii. REG 3 ☐
	vi. MDR		
	i. REG 1	ii. REG 2	iii. REG 3 ☐
	vi. MDR		

6) *Most Recent Blood Results:*

Dates	HB	ALT
2012/04/03	11.5	20

7) Do you give your child traditional remedies/ immune boosters? YES/(NO)/PREVIOUSLY___ DATES:

8a) Alcohol consumption by caregiver: NONE/AVERAGE/(HEAVY) (>3 drinks most days of week)

8b) Alcohol consumption by child: (NONE)/AVERAGE/HEAVY (>3 drinks most days of week)

9) Hepatitis B Status: POS/(NEG)/NOT TESTED: *(Order test)*

10) Currently Pregnant? YES/NO/(NA)

11) Has there been any exposure to Single Dose NVP at delivery? YES/(NO) If YES; Year:__________

12) Is the caregiver on ARV's? YES/(NO)/Unknown

13) Other Co-morbidities:______________________

Is the patient on or likely to start TB treatment soon? YES/(NO)

Is there significant diarrhea or vomiting causing malabsorption? YES/(NO)

Have you identified any reasons for virological failure? Use the summary below to explain

Poor Adherence ☐	Drug Interactions ☐	Social issues impacting Rx ☐	Transmitted Resistance ☐
Treatment Breaks ☐	Traditional Remedies ☐	Financial ☐	Single Dose NVP ☐
Drug toxicity ☐	Malabsorption ☐	Non-Disclosure ☐	

14) Summary: child aware of HIV status. Child lives in Richard Bay with mum. Comes all way to Mahlabatini for collection of treatment. Aunt worried about adherence.

15) Current action: **HBC/Treatment buddy/Adherence/ Other** ______________

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