Master’s Thesis

Investigating paediatric HIV-1 drug resistant outcomes in children with maternal exposure to various Prevention of Mother to Child Transmission (PMTCT) interventions at Dr George Mukhari Hospital, South Africa.

Submitted by

Dr Ramokone Maphoto

Student No: 200911680

In partial fulfilment of the requirements for the degree of MMED in Medical Virology

At the

Sefako Makgatho Health Science University

Supervisor

Dr Zinhle Makatini BSc (Hons), MSc, MBChB, FCPATH (Viro)

2016
Declaration

I declare that the Masters dissertation hereby submitted to the Sefako Makgatho Health Sciences University, for degree of MMed Virological Pathology has not previously been submitted by me for a degree at this or any other university; that it is my work in design and in execution, and that all material contained herein has been duly acknowledged.

________________________
Dr R Maphoto

Student Number:

....................day of..................2016
Acknowledgement

This project was made possible by various contributions by certain individual. My outmost gratitude is extended to my supervisor Dr Zinhle Makatini and my colleagues.

To the most precious human beings, the paediatric patients (study patients) and their caregivers, thank you for affording me an opportunity of doing a research.

A very well appreciated gratitude towards my grant awarders, Discovery Foundation Fellowship Awards for granting me the financial resources to proceed with my research.

To my Creator-God thank you, my family and friends thank you for the support and patience
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ABSTRACT

Investigating paediatric HIV-1 drug resistant outcomes in children with maternal exposure to various Prevention of Mother to Child Transmission (PMTCT) interventions at Dr George Mukhari Hospital, South Africa.

Background:

Approximately one-third of children born to mothers living with HIV will acquire HIV infection in the absence of preventive measures. Prevention of mother-to-child transmission programs can reduce MTCT rates to about 1 to 2 percent\(^1\). However, the emergence of HIV drug resistance is a major obstacle to effective antiretroviral (ARV) treatments. The goal of the study was to describe the presence of HIV drug resistance among antiretroviral (ARV) naïve children and to examine resistance in these children in relation to drug exposure in the mother.

Methods:

A total of 29 HIV-1 positive, samples from patients that tested positive with either HIV DNA PCR at 6 weeks/6 months or HIV-1 Elisa positive and had received various PMTCT intervention at any point were collected for the purpose of this study. At enrolment, mean age was 26.1 months. The RNA extraction was done using Qlamp Viral RNA mini kit (Qiagen, Hilden, Germany according to the manufacturer’s instruction. The RT-PCR and Sequencing were performed using the HIV-1 Trugene HIV-1 Genotyping Kit and the Open- Gene DNA sequencing system.

Results:

Of the 29 samples that were sequenced, only 13 (44%) had successful sequence results that could be analysed. HIV-1 subtype C was the predominant circulating subtype with an exception of one (8%) circulating recombinant form B/C in the study patients. HIV-1 sequences from 11 (84.6%) samples were found to exhibit viral genotypic evidence of resistance-conferring mutations to non-nucleoside reverse transcriptase inhibitors (NNRTIs) and nucleoside reverse transcriptase inhibitors (NRTI), with 2/11 [18.2%] having additional minor protease inhibitor (PI) mutations. Wild type virus was detected in 2 of the 13 sequenced samples.

Of the 11 patients with detectable HIV-1 drug resistance mutations, 10 (90.9%) had ≥1 major mutation conferring resistance to antiretroviral drugs. The lamivudine/emtricitabine-related resistance mutation M184V, was detected in 1 individual (9.1%). For NNRTI mutations, K103N (4/11 [36.4%]), Y181C (3/11 [27.3%]), V106M (1/11 [9.1%]), and E138A (1/11 [9.1%]) were observed respectively. The minor PI mutations detected were T74S (2/11 [18.2%]) and L10I (1/11 [9.1%]).
An analysis of 13 sequenced samples showed patients have some polymorphisms; M36I, K20R and V74S. Approximately 6 patients (46%) showed both M36I and K20R polymorphisms associated with reduced drug activity of indinavir and ritonavir; whilst 7 patients (54%) exhibited only M36I. The V74S polymorphism was displayed in one patient (8%).

Conclusion:

From the data, we cannot suggest the occurrence of infant transmitted HIV resistance given the limitations of the current study. The findings, however, do have an important bearing on future HAART options for this population group. While the numbers are too small to be able to draw any conclusions, more such data on HIV drug resistance outcomes in paediatric patients are needed as ARV treatment programs expand around the world.
CHAPTER 1: RESEARCH PROPOSAL

1.1 Study Problem

It is widely known that vertical transmission is one of the routes of HIV transmission. UNAIDS estimates that 90% of transmission of HIV to infants is a result of vertical HIV/AIDS transmission. The HIV vertical transmission rate is around 30% without PMTCT interventions but is reduced substantially with the introduction of PMTCT strategies.[1] There are number of PMTCT strategies employed globally and the impact of such strategies is widely recognized for contributing in the reduction of paediatric HIV morbidity and mortality. The optimum PMTCT intervention in the reduction of vertical transmission is through the use of highly active antiretroviral therapy (HAART). In setting where HAART is used, HIV vertical transmission is estimated at 1% [1]

According to UNAIDS, in Sub-Saharan Africa more women than men are living with HIV/AIDS and therefore vertical transmission becomes an obvious reality. The low socio-economic status of the countries in this region makes the issue of roll-out and access of HAART for HIV-infected pregnant women difficult. In an attempt to address these challenges, WHO has introduced PMTCT guidelines for resource-limited settings which typically advocate the use of monotherapy or dual therapy.

There is no doubt, however, that monotherapy or dual therapy whilst effective in reducing maternal HIV-1 viral load and therefore reducing the probability of HIV-1 transmission presents challenges of HIV drug resistance. The challenge is that of the introduction of monotherapy or dual therapy in a patient with replicating virus- resulting in optimum condition for HIV drug resistant development.

Use of sdNVP in various studies resulted in HIV drug resistant mutations in 35-70% of women who received this PMTCT strategy. However there is paucity of information in the use of sdNVP and AZT as PMTCT intervention in pregnant women. However, it is known that resistance to AZT typically develops only after several months of partially-suppressive ART. The available clinical data document a low prevalence of AZT resistance following short course AZT regimens, hence it is thought not likely that short term administration of AZT for PMTCT will compromise the efficacy of this agent in future HAART regimens for the mother.

Infants born to HIV-infected mothers will also receive a short-course of monotherapy (sdNVP/AZT) as part of the PMTCT intervention which according to WHO 2010 recommendations entails a single dose nevirapine immediately after birth, followed by a seven day course of AZT. South Africa has adopted WHO 2010 option B recommendation of 6 weeks daily nevirapine for infant prophylaxis.
It is therefore generally accepted that the use of just one or two ARVs in pregnancy can induce HIV resistance that could compromise the efficacy of future HAART regimens for the mother as well as for the infant, should the infant become infected despite the PMTCT intervention. The research project in this instance, will address the question of the paediatric HIV drug resistant outcomes from various PMTCT exposures in our setting at DGM, as well as describing the patterns and the extensiveness of HIV drug resistant mutations therefore assisting in anticipation of clinical care for exposed HIV-positive infant.

1.2 Aim
To determine the impact of various PMTCT interventions on paediatric HIV-1 drug resistance outcomes.

1.3 Objectives:
Primary objectives:

- To determine proportion of infants with HIV-1 drug resistant mutations
- To determine the HIV-1 subtype infecting the infants
- To compare the emergence of HIV-1 drug resistant mutations in the Reverse transcriptase region in infants exposed to maternal PMTCT interventions with or without paediatric ARV intervention

Secondary objective:
- To describe the immunological and virological parameters of infants with HIV drug resistance mutations

1.4 Purpose of Study
The purpose of the study is to address the question of what the paediatric HIV drug resistant outcomes are from various PMTCT exposures in our setting at DGM, which will ultimately assist in anticipation of clinical care for exposed HIV-positive infants.
Paediatric HIV infection poses a serious public-health challenge the world over and disproportionately affects children in the poorest parts of the world. [2] World Health Organization (WHO) estimated that 1.4 million pregnant women were living with HIV in the developing world in 2008 and approximately 90% of the HIV infected women reside in Africa. [3]. As a result of the high prevalence of HIV infection in pregnant women, an estimated 260 000 children in sub-Saharan Africa were reported to be infected with HIV in 2012 [4]. The majority of these children transmission of HIV occurred during pregnancy, childbirth or breast-feeding [5]. Globally, 3.2 million children under 15 were living with HIV in 2013, comprising 9.1% of all people living with HIV and 240 000 children worldwide acquired HIV in 2013. In 2013, 1.3 million [1.2 million–1.4 million] women living with HIV gave birth—a figure which is unchanged from 2009 [6]. The rate of mother-to-child transmission also fell—in 2013, 16% [13–18%] of children born to women living with HIV became infected compared to 25.8% in 2009. [6]

The HIV prevalence in South Africa has increased from an estimated 4,09 million in 2002 to 5,51 million by 2014[6] and approximately one-fifth of South African women in their reproductive ages are HIV positive.

The overall risk of mother to child transmission (MTCT) without intervention is 15-30% in Europe and USA but 25-40% in sub-Saharan Africa.[2] In 1994, preventing perinatal transmission of HIV-1 became a reality when the Paediatric AIDS Clinical Trials Group 076 (PACTG076) published data showing that a long complex course of zidovudine prophylaxis given to the HIV-1 infected mother during early gestation, labour and postnatally to the baby, reduced perinatal transmission of HIV-1 by almost two-thirds.[5] Mother to child transmission has fallen to less than 2% in developed countries after the introduction of highly active antiretroviral therapy (HAART) as gold standard PMTCT intervention.[1]

It is a common knowledge that women in the developing world are at higher risk of HIV infection than their male counterparts.

The vulnerability of women is further exploited socio-culturally by the conditions in cultures and communities that remove their control over their own bodies.
Several interventions have been used to reduce the possibility of transmission of HIV from the mothers to the foetuses/infants, with ARV regimen playing crucial part. The role of antiretroviral therapy (ART) in pregnancy is to reduce the maternal plasma viral load and ultimately improve maternal CD4 counts in women who require ART for their own health as well as providing pre and post exposure prophylaxis to the infant.

It has long been established that single dose nevirapine (sdNVP) can also reduce the viral load in pregnant women and prevent MTCT.

According to HIVNET012, sdNVP prophylaxis to the mother, to the mother intrapartum and to the infant showed a reduction of 47%. [3,7] This regimen has been adopted in most of the resource-limited countries because of its efficacy, simplicity and low cost.

A single dose nevirapine given to the mother at onset of labour and a single 2mg/kg oral dose given to the infant at 48 – 72 hours of life was found to be safe and reduced maternal HIV transmission by 47% at 14 weeks to 16 weeks of life and by 41% at 18 months in breastfed infants. [5]

Emergence of viral resistance is a real concern with global use of antiretroviral prophylaxis to prevent perinatal transmission of HIV-1. In the HIVNET012 study, nevirapine induced genotypic resistance was detected in 46% of nevirapine exposed infants who subsequently became infected but mutations faded by 12 months of age. [5]

The predominant NNRTI mutation detected was K103N in 19% of women exposed to single intrapartum nevirapine dose. K103N mutations are known to confer cross resistance to all non-nucleoside reverse transcriptase inhibitors (NNRTIs). Resistance was however, reported to be transient and mutations were undetectable 12 – 24 months after delivery. [5]

What makes nevirapine resistance a topic of major interest is because WHO recommends nevirapine based treatment regimens as first line therapy in resource limited countries. However drugs such as nevirapine have a low genetic barrier. A single gene mutation in HIV-1 reverse transcriptase can confer rapid resistance.

There is evidence that addition of maternal intrapartum/neonatal nevirapine dose to short course maternal AZT (with oral AZT during labour and either no infant prophylaxis or one week of infant AZT
prophylaxis) may provide increased efficacy for reducing perinatal transmission of HIV-1 compared with short course maternal AZT prophylaxis alone. [5]

It has been demonstrated that single dose nevirapine was more effective than AZT alone, however was equivalent to AZT and single dose nevirapine. [8] In terms of HIV drug resistance, nevirapine associated drug resistance emerged rapidly and drug resistance was also reported in women using AZT and single dose nevirapine. [8]

According to the World Health Organization (WHO) 2010 guidelines, the recommended course of drugs for preventing mother to child transmission (PMTCT) in resources-limited settings should be a combination of AZT and single dose NVP.

2.2 HIV Virology

2.2.1 Origin and Taxonomy
The first cases of what is now widely known as Acquired Immunodeficiency Syndrome (AIDS) were first reported in San Francisco in 1981 and 2 years later, the causative agent of AIDS was identified as a lentivirus, later designated HIV-1. In 1986, a morphologically similar but antigenically distinct virus was found to cause AIDS in patients in West Africa. [9] This virus was later designated HIV-2.

Both HIV-1 and HIV-2 are a result of zoonotic transmission of viruses infecting primates in Africa [10] and have been postulated to have evolved from Simian Immunodeficiency Virus (SIV) found in more 35 African primate species and in particular in sooty mangabeys [11] and chimpanzees [12]

The complexity of the HIV increased through constant recombination as such has yielded the four HIV-1 groups (M, N, O and P). This recombination, together with human adaptation accounts for thHIV-1 groups’ genomic, phylogenetic, and virological specificities [13].

HIV-2, is believed to have its origins from the sooty mangabey monkeys (SIVsm) and these are the only primate species naturally infected with viruses related to HIV-2 [14].

2.2.2 HIV structure
The mature HIV virion [Fig1] is an enveloped spherical structure which is approximately 100-120nm in diameter. The envelope consists of a lipid bilayer, made of approximately 72 glycoprotein heterodimers
complexes, arranged as trimers [15]. The trimeric viral glycoproteins (gp) include the external glycoprotein 120 (gp120) and the transmembrane glycoprotein 41 (gp41) bound together.

Below the lipid layer, is the matrix protein (or p17) containing the icosahedral capsid protein (p24) [16]. The viral capsid houses two identical single-stranded RNA genomes with reverse transcriptase.

![Cross sectional structure of HIV-1 showing gp120 & gp41, lipid membrane, matrix, icosahedral capsid and RNA genomes with reverse transcriptase](https://www.aidsfactsheet.com)

2.2.3 Genome Characterization of HIV

The HIV-1 genome consists of two identical 9.2 kb single-stranded RNA molecules, flanked by the 3'-long terminal repeats (LTR) and 5'-LTR on either side.

The HIV-1 genome encodes for both structural and non-structural proteins. The three structural proteins are gag (group antigen), pol (polymerase) and env (envelope). In addition are non-structural proteins regulatory proteins: tat and rev as well as 4 accessory proteins: vif, vpu, vpr and nef. At genome level, HIV-1 and HIV-2 share similarities and only differ in terms of the proteins, with HIV-1 expressing vpu and HIV-2 expressing vpx.
Table 1: The table below illustrates the important HIV-1 genes/proteins

<table>
<thead>
<tr>
<th>Category of genes</th>
<th>Genes</th>
<th>Protein products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structural proteins and enzymes</td>
<td>Gag</td>
<td>P24, p7 and p6</td>
</tr>
<tr>
<td></td>
<td>Pol</td>
<td>Reverse transcriptase, integrase and protease</td>
</tr>
<tr>
<td></td>
<td>Env</td>
<td>Gp160 cleaved into gp120 and gp41</td>
</tr>
<tr>
<td>Essential regulatory genes</td>
<td>Tat</td>
<td>Tat</td>
</tr>
<tr>
<td></td>
<td>Rev</td>
<td>Rev</td>
</tr>
<tr>
<td>Accessory genes</td>
<td>Nef, Vif, Vpu and Vpr</td>
<td>Nef, Vif, Vpu and Vpr</td>
</tr>
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</table>

Figure 2: Genomic organization of HIV (adapted from Sierra et al)
2.3 HIV Genetic Diversity

2.3.1 Molecular Evolution of HIV

One of the unique features of HIV is its exceptional genetic diversity [17]. The virus has several intrinsic mechanisms that ensure rapid viral evolution. The sources of this high genetic diversity are, i) error-prone nature of reverse transcriptase, ii) high replication turnover, iii) rapid generation time of 1 to 2 days and iv) ability to undergo recombination [18].

As a consequence of lack of proofreading activity, the HIV reverse transcriptase enzyme can therefore confer a mutation rate of approximately $3.4 \times 10^{-5}$ mutations per base pair per replication cycle [19]. Since the HIV genome is an estimated $10^4$ base pairs in length and the baseline rate of viral production is approximately $10^{10}$ virions per day, millions of viral variants are produced within any infected person in a single day [20]. This may contribute to the emergence of HIV variants and these may play a role in HIV-1 adaptation to both immune response and antiretroviral therapy.

2.3.2 Genetic variants of HIV

During the course of the HIV epidemics in humans the sequences of the different HIV-1 groups have diversified in the population [21]. Phylogenetic analysis has therefore been used to categorise HIV variants into types, groups, subtypes, circulating recombinants and unique recombinants based on homologies among diverse genomic sequences of HIV viruses. HIV-1 is divided into four groups designated M (major), O (outlier), N (non-M, non-O) and recently reported P designated. HIV-2 has eight distinct groups A-H [21].

Group M is the predominant circulating HIV-1 group and is further categorized into nine subtypes, denoted with letters, and at least 6 sub-subtypes, denoted with numerals. Subtypes A1, A2, A3, A4, B, C, D, F1, F2, G, H, J, and K are currently recognized. HIV-1 subtypes, are phylogenetically linked strains of HIV-1 that are approximately the same genetic distance from one another; in some cases, subtypes are also linked geographically or epidemiologically [19]. Genetic variation within a subtype can be 15 to 20%, whereas variation between subtypes is usually 25 to 35% and is thought to be dependent on the genome region studied [22].

Recombinants between HIV-1 group M subtypes are designated as either circulating recombinant forms (CRFs) if fully sequenced and found in three or more epidemiologically unlinked individuals OR unique
circulating recombinant forms (URFs) if not meeting the CRFs criteria [23]. These are identified by numbers ascending in order of discovery followed by letters of the parental subtype i.e CRF01_AE [24]. More than 70 CRFs have been discovered and linked to a particular geographical location.

Group O was first isolated in 1990 from Cameroonian couple residing in Belgium who presented with generalized lymphadenopathies [25]. Although group O contains very diverse viruses, phylogenetic analysis of gag and env genes do not reveal distinct subtypes as compared to the group M viruses [26].

There is limited data on Group N genetic diversity. However, sequence analysis has shown a high level of HIV-1 Group N intragroup homogeneity which is suggestive of a relatively recent introduction and very slow spread in the human population [27, 28].

Recently, in 2009 HIV-1 P strain was characterized and identified in a Cameroonian patient [29]. Due to paucity of data, evolution of group P cannot be speculated.

2.3.3 HIV-1 Diversity and Epidemiology

Currently Group M is responsible for majority of the HIV infections globally. The HIV-1 non-M variants are mainly restricted to Central Africa and represent less than 5 per cent of infections worldwide [30].

Subtypes are phylogenetically linked strains of HIV-1 that are approximately the same genetic distance from one another [31]. Group M has been classified into nine distinct subtypes denoted with letters, A, B, C, D, F, G, H, J and K.

Some of the pre-existing subtypes, such as A and F have continued to evolve into sub-subtypes i.e A1-4 and F1-2 with lesser degrees of genetic divergence [24].

Some subtypes share a geographic localization, whereas others appear to have a similar ancestry, examples include subtype G viruses from Spain and Portugal, and subtype C viruses from areas as geographically diverse as India, Ethiopia and South Africa.

The epidemics in west and central Africa seem to have stabilised in prevalence but these regions, along with the Congo River basin, continue to be hot spots for HIV diversity [24]. Most if not all the subtypes, sub-subtypes and CRFs have been reported in the DRC, and Cameroon. Although west and central Africa are regarded as the hot spots of the HIV-1 genetic diversity, however prevalence remains surprisingly lower than most regions in the sub-Saharan Africa [24].
The Southern African epidemic is almost entirely attributed to the spread of HIV-1 subtype C contributing 93% - 100% of the HIV-1 infections and is further responsible for more than 51% of HIV-1 infections worldwide [32,33]. Subtype A, B, G and D contribute about 12%, 10%, 6% and 3% respectively whilst subtypes F, H, J and K together account for about 0.94% of all the infections in sub-Saharan Africa[33]. HIV-1 subtype C in the Southern African region it has been linked to sexual transmission and mother-to-child transmission. In Europe, subtype A and B are reported to be the predominant circulating subtypes and have been linked to intravenous drug use and sexual transmission respectively, however the prevalence of non-A and non-B strains has shown an increase as a result of immigrants influx from Africa and Asia [34].

In South America highest diversity and prevalence is clustered in Brazil and Argentina with predominance of subtypes B, C and F, and BF and BC recombinants [24].

In terms of CRFs some developed early in the epidemic in Central Africa [35]. The CRFs are continually been formed in places in the world, particularly in the so-called ‘recombination hotspots’. Highest prevalence of URFs and CRFs are found in West Africa, central Africa, East Africa, South America, Cuba, China and South and South-East Asia [36].

2.4 HIV Replication

The replication cycle of HIV-1 can be successfully completed in approximately 2.5 days. HIV life cycle can be summarized in 6 steps:

i) HIV attachment and entry

ii) Uncoating

iii) Reverse transcription

iv) Provirus integration

v) Virus protein synthesis and assembly

vi) Budding
2.4.1 HIV attachment and Entry

The main target cell for HIV is the activated CD4+ T lymphocyte. Entry of HIV-1 into target cells is a multistep process ultimately involving the fusion of viral and cellular membranes. The process begins with gp120 binding to the CD4 receptor and this results in a conformational change in gp120 [37], allowing for a secondary interaction with CCR5 or CXCR4 coreceptors leading to the release of gp41 from its metastable conformation.

The binding of gp120 to the CCR5 or CXCR4 coreceptor allows for exposure of a fusogenic motif in the amino-terminal ectodomain of gp41. The hydrophobic end terminus of gp41 ectodomain is thereby freed to insert into the target cell membrane. There are two trimeric coiled-coil structures in gp41 comprising of heptad 1 and 2 (HR1 and HR2). Repeat sequences in heptad 1 and 2 interact,
causing the collapse of the extracellular portion of gp41 into a hairpin. It is this hairpin loop structure that brings the virus and cell membranes close together, allowing fusion of the membranes and subsequent entry of the viral capsid into the cytoplasm [38].

2.4.2 Uncoating and reverse transcription

Entry of the viral capsid into the acidic cytoplasm, results in progressive dissociation of the viral capsid, subsequently facilitating the release of the viral single stranded RNA. Although uncoating is thought to be triggered by sudden change in environment, the mechanism of uncoating remains unclear.

Transcription of HIV RNA into cDNA by reverse transcriptase (RT) and tRNA molecule acting as primer occurs in the cytoplasm after viral entry [37]. It is during this transcription of either the minus or plus-strand that strands switching events resulting in genetic recombination occurs [37].

The RNA strand of the RNA:DNA hybrid strand is then removed and digested by the ribonuclease H with subsequent completion of the complementary DNA strand to form a double helix DNA molecule by the polymerase active site of the reverse transcriptase. [39]. This resultant DNA molecule associated with other proteins such as Vpr and Integrase enzyme is referred to as preintegration complex.

2.4.3 Viral Integration and Transcription

The viral integrase cleaves nucleotides on each 3'end of the double helix DNA creating two sticky ends before integration. The preintegration complex then docks to the nuclear membrane directed by HIV-1 Vpr [40] and enters the nucleus through the nuclear pore [41, 42]. The proteins- Nef, Tat and Rev are produced in basal amounts from these DNA forms through activation of the LTR promoter by cellular factors such as NF-κβ [43]. After translocation to the nucleus the preintegration linear double-stranded DNA is integrated into the host chromosome by the virally encoded integrase enzyme (see Fig 3) and integrates the DNA into host cell chromosome with host cell DNA repair enzymes repairing the integrated complex.

Once proviral DNA is integrated, the activation of transcription and gene expression is now dependent on the activity of both the cellular and viral factors [44].

The first rounds of proviral transcription by cellular RNA polymerase II through binding of cellular factors to the viral LTR and translation occurs producing basal amounts of Tat, Rev and Nef [45]. Once sufficient amounts of Tat have been produced, Tat controls further transcription of HIV-1 genes. The Tat protein activates transcription through binding to the TAR element of the LTR and to
other transcriptional activators of cellular origin [46]. The primary transcript is alternatively spliced to
generate numerous viral mRNAs. The early viral transcripts form a group of mRNAs that are 1.8 to
2.0 kb in size and which encode the proteins Tat, Rev and Nef [47]. The incompletely spliced and
unspliced viral mRNAs are exported from the nucleus [48]. These mRNAs are used to encode for
accessory genes, genomic RNA and all enzymes and structural proteins.

2.4.4 Maturation, Assembly and Budding

Enzyme protease, a 99 amino acid aspartyl protein, has a role of cleaving Gag and Gag-Pol
precursor polypeptides generating individual structural and enzymatic proteins [44] Assembly and
maturation of new viral particles is a stepwise process: two viral RNA strands associate together with
replication enzymes, while core proteins assemble over them forming the virus capsid. This immature
particle migrates towards the cell surface. The large precursor molecules are then cleaved by the
HIV-1 protease, resulting in new infectious viral particles, which bud through the host cell membrane,
thus acquiring a new envelope.

2.5 HIV Natural History

The natural history of HIV in the clinical disease context refers to the progression of HIV-1 to AIDS in
the absence of highly active antiretroviral therapy (HAART). This may be divided into three phases:

The initial stage known as the Acute HIV infection is referred to as the time period from the initial
infection with HIV to the time of developing HIV-specific antibodies. During this stage about 40-90% of
individuals may present with transient symptoms, resembling infectious mononucleosis and occurs
particularly during the first 2-3 weeks following exposure [49]. Acute HIV infection is associated with
high HIV replication and a marked decline of CD4+ T cell count which recovers later albeit to a level
below the pre-infection state.

The majority of acute HIV infections are missed due to the inability to detect HIV-specific antibodies
during this stage, hence; the diagnosis is mostly reliant on a high index of suspicion, history of high risk
transmission and p24 antigenemia testing to confirm the diagnosis.
As the immune response to HIV develops, the viral set point is reached and the levels of the set point are usually below $10^{3-5}$ copies/ml [49]. The degree of the viral set point is often correlated with progression of the disease. Individuals with higher plasma HIV-1 RNA viral set point have been postulated to have rapid disease progression and this has stimulated discussions regarding early treatment particularly during the acute infection in order to further reduce the viral set point level. Lastly during this phase there is a rapid depletion of CD4+ T cells from the gut-associated lymphoid tissue which results in the damage of gut mucosa. This allows microbial products translocation to occur, in turn causing immune activation and further deterioration of the CD4+ T cells.

The second phase is the asymptomatic period. In the absence of antiretroviral therapy this stage can last approximately between 8-10 years [50]. The most important pathophysiologic feature of this phase is the gradual loss of CD4+ T cells due to continuous HIV replication. The HIV-1 RNA levels (often referred to as viral load) and the CD4+ T cells both are used to monitor patients at this stage to make a clinical decision of initiating HAART. The plasma HIV-1 RNA level, which is a marker of disease progression, determines how rapidly CD4+ T cells will be lost, and the CD4+ T cells count reflects the degree of impairment of immunologic function and the subsequent risk of opportunistic infections [44].

The observation has been made of person to person variation in the duration of the asymptomatic phase in different persons with some individuals progressing faster to AIDS within two years. The long-
Long-term non-progressors (LTNP) are another person to person variation that has been observed during this asymptomatic phase. This group of cohort, LTNP, achieve good control of HIV viral replication, and maintain high CD4 counts in the absence of antiretroviral medications over many years of infection, albeit some individuals initially identified as long-term nonprogressors (LTNPs) have experienced disease progression over time [51]. Viral and host factors contribute to this exquisite HIV-1 replication control. In many patients during the asymptomatic period, fatigue and generalized lymphadenopathy continue to occur.

Lastly, the AIDS stage may occur after a long period of asymptomatic stage. The occurrence of some of the opportunistic infections such as oral hairy leukoplakia, herpes zoster, oral/vaginal candidiasis may be an indication of early signs of disease progression. In this clinical situation, CD4+ T-cells count of 200 cells/μl is regarded as an important cut-off, below which the risk of AIDS-defining illnesses increases.

It was for this reasons that the earliest treatment guidelines used CD4+ T cell count of <200 cells/μl as a cut-off for initiating HAART. However this cut-off later increased with realisation of new evidence regarding the benefits of early HAART initiation.
2.6 Highly Active Antiretroviral Therapy

Figure 5: Identifying distinct steps in HIV-1 life cycle as potential or current target for antiretroviral drugs [20]

2.6.1 Entry Inhibitors

HIV-1 entry exploits several host proteins for a set of intricate events leading to membrane fusion. The basic steps that are involved in HIV-1 entry to the CD4+ cells are mainly, 1. Binding of HIV to the CD4 receptor, (2) Binding to co-receptors, and (3) Fusion of virus and cell. These steps can be inhibited resulting in distinct classes of entry inhibitors.

2.6.1.1 Fusion inhibitors

The fusion inhibitors were designed based on the discovery that two homologous domains in the viral gp41 protein (HR1 and HR2 peptide) must interact with each other to promote fusion, and that mimicry of one of these domains by a heterologous protein can bind and disrupt the intramolecular interaction of the virus [52].
The first FDA approved fusion inhibitor, Enfurvitide, is a 36 amino acid synthetic peptide homologous to the HR2 region of gp41 (residues 127-162) [53] that has the ability to interfere with the fusion pathway by mimicking the HR2 domain. It is has been approved and licensed for treatment-experienced adults and children over 6 years of age. Enfurvitide is administered subcutaneously making it unfavourable mode of administration particularly in patients with possibility of poor adherence. Currently not used in public sector health in South Africa.

Figure 6: Mechanism of action of fusion inhibitor (as adapted from Arts et al. 2012)

2.6.1.2 Core-receptor antagonists

HIV also binds to core-receptors to enter the target. Currently two core-receptors that play a role in HIV viral entry have been identified i.e CCR5 and CXCR4.

HIV variants use either the CCR5- or the CXCR4-receptors for entry into the target cell, these variants are named according to the receptor tropism i.e those that prefer the CCR5 as a core-receptor are named R5, whereas the ones with preference for CXCR4 are termed X4 and dual-tropic viruses can co-
exist. The R5 variants are thought to be predominant in early infection whereas the X4 seems to be dominant in later stages of the disease.

The CCR5 antagonists such as maraviroc and vicriviroc bind to hydrophobic pockets within the transmembrane helices of CCR5 [54], thereby preventing interaction of the V3 loop of gp120 with the CCR5 core-receptor, thus halting the viral entry process. They are thought to interact with the receptor in an allosteric manner locking the receptor in a conformation that prohibits its co-receptor function [50].

Additional antiviral effects include the inhibition of syncytia formation (fusion between infected and uninfected CD4 cells) and blockade of gp120-induced apoptosis [55].

Currently licensed Maraviroc, is a selective and reversible CCR5 coreceptor antagonist with a broad spectrum activity against clinical HIV isolates, including those with multidrug resistance to other drug classes [56]. Maraviroc was approved for use in treatment-experienced patients by the United States Food and Drug Administration in 2007 at 300 mg orally twice daily. It is not active in patients with non-R5 tropic viruses and those that do not express CCR5 core-receptor; hence tropophile assay needs to be performed to confirm the expression status of CCR5 core-receptor prior to initiating maraviroc. To date it seems maraviroc is well tolerated.

2.6.2 Reverse transcriptase inhibitors

2.6.2.1 Nucleoside/Nucleotide reverse transcriptase inhibitors (NRTIs)

Nucleoside reverse transcriptase inhibitors (NRTIs), also referred to as nucleoside analogs, were the first class of antiretrovirals to be approved by the Food and Drug Agency (FDA). Nucleotide reverse transcriptase inhibitors, N(t)RTI, also belong to this class of drugs.

NRTIs are prodrugs which require entry into the target cell and must be activated to become an intracellular 5'-triphosphate compound. This process is called phosphorylation and is undertaken by cellular kinases and viral kinases prior to the drug achieving its antiviral effects. The activation of nucleotide analogues, however, requires only 2 phosphorylation steps for conversion to its active triphosphate form [57].

The activated intracellular 5'-triphosphate nucleoside acts as a competitive substrate during replication competing with physiological nucleosides for incorporation into the growing DNA. The activated 5'-triphosphate nucleoside differs from the physiological nucleoside by a lack of the 3'-hydroxyl group at the sugar moiety and this prevents the formation of a 3'-5'-phosphodiester bond between the NRTIs
and incoming 5’-nucleoside triphosphate, thereby resulting in premature chain termination of viral DNA [52].

The spectrum of activity of NRTIs/NtRTIs includes HIV-1 and HIV-2, additionally with Tenofovir disoproxil fumarate (TDF), Lamivudine (3TC) and Emtricitabine (FTC) having dual activity against both HIV and Hepatitis B (HBV) viral infection.

Most NRTIs have long intracellular half lives allowing for once-daily dosing and fixed dose combinations. The half life of intracellular NRTI triphosphate ranges from 7 hours for zidovudine (AZT) and stavudine (d4T) to approximately 100 hours for tenofovir disoproxil fumarate (TDF). NRTIs are mainly eliminated renally and thus may require dose adjustment for patients with impaired kidney function and in contrast, abacavir (ABC) and AZT, which are metabolized by the liver, may require dose-adjustment in a case of liver dysfunction.

As a class, NRTIs have few clinically significant drug-drug interactions, because they are not substrates, inhibitors, nor inducers of hepatic cytochrome P450 (CYP) enzymes.

Overall NRTIs have good tolerability. The long-term class side-effects may range from myelotoxicity to mitochondrial toxicity, with latter being regarded as the hallmark toxicity of the NRTI class.

Mitochondrial toxicity may manifest as peripheral neuropathy, lipoatrophy/lipodystrophy, pancreatitis, and hepatic steatosis. The risk of mitochondrial toxicity depends on the affinity of the individual NRTIs for human mitochondrial DNA polymerase gamma, which is the enzyme responsible for the replication of mitochondrial DNA (mtDNA). NRTIs such as stavudine (d4T) and didanosine (ddl) have a 13- to 36-fold binding affinity whereas tenofovir, lamivudine, emtricitabine and abacavir reduced binding affinity and thus associated with minimal risk of mitochondrial toxicity (ddC > ddl > d4T > 3TC ≥ ABC ≥ TDF≥ FTC) [58]. The consequence of mitochondrial toxicity may lead to non adherence in patients ultimately resulting in the emergence of HIV drug resistance mutations.

2.6.2.2. Non-nucleoside reverse transcriptase inhibitors (NNRTIs)

Non nucleoside reverse transcriptase (NNRTIs) is another class of reverse transcriptase inhibitors. The first generation NNRTIs, efavirenz and nevirapine, posed serious toxicity challenges as well low genetic barrier to resistance and this led to the development of second generation NNRTIs – etravarine and rilpivirine. NNRTIs also target enzyme reverse transcriptase enzyme. Unlike NRTIs, NNRTIs require no intracellular activation and instead binds directly and non-competitively to a p66 subunit at a
hydrophobic pocket near the catalytic site of HIV-1 reverse transcriptase. This results in spatial conformation changes of the substrate-binding site which in turn reduces polymerase activity [52].

NNRTIs have antiviral activity against HIV-1, with HIV-2 reported to be intrinsically resistant to NNRTIs. The overall tolerability and long half life are amongst features of the first generation NNRTIs that has played a role in their common selection as part of antiretroviral regimens in many antiretroviral guidelines from developing countries.

Second generation NNRTIs have a broad spectrum of activity against not only wild type HIV-1 but also exhibit activity against isolates with HIV drug resistant mutations such as K103N [50].

Nevirapine and efavirenz are metabolized in the liver by the cytochrome P450 system and as a result, drug to drug interactions of these agents, is inevitable. Adverse events from NNRTIs include Steven-Johnson’s syndrome, grade 4 hepatotoxicity events and a rash.

Central nervous system effects are more commonly seen with patients on an efavirenz based regimen. There are concerns of teratogenicity as a result of efavirenz intake during pregnancy, particularly in the first 28 days. The concerns emanate from preclinical data from teratogenicity studies in animals. However, there is paucity of evidence on the risk of EFV causing neural tube defects in humans. EFV was classified by the US Food and Drug Administration (FDA) as a pregnancy class D drug, resulting in a recommendation against its use during the first trimester of pregnancy. The current data and clinical practise experience has been reassuring, and thus use of EFV during pregnancy has become less of an issue for women initiating on PMTCT programmes. Meta-analysis of birth defects in infants with first-trimester EFV exposure reported no overall increased risk of birth defects associated with EFV exposure during the first trimester of pregnancy [59].

2.6.3 Integrase inhibitors

Enzyme integrase, a 288 amino acid, is responsible for transfer of viral DNA into the host chromosome to allow for viral replication to take place.

The integrase enzyme combines with viral DNA and other cellular cofactors to form the preintegration complex (PIC) [60]. The next step is 3’ processing which involves the removal of a nucleotide from each 3’ DNA terminus, thereby exposing reactive hydroxyl groups. The PIC then enters the host cell nucleus where it binds to host cell DNA with the enzyme integrase nicking each strand of the host cell DNA and exposing the 5’ phosphate groups, thus enabling the covalent bonding of host and viral DNA. After strand transfer is completed, the host cell enzymes repair gaps between the viral and host DNA.
Enzyme integrase is composed of three functional domains – the catalytic core, catalytic triad and viral DNA binding site. Integrase inhibitors, referred to as integrase strand inhibitors (InSTIs) (Raltegravir and Elvitegravir), prevent strand transfer by binding to divalent cations in the catalytic core and preventing covalent bonds from forming between integrase and host DNA [61]. Enzyme integrase cannot incorporate the viral DNA into the host chromosome, ultimately resulting in the prevention of strand transfer and viral replication.

Raltegravir a first generation InSTI has good tolerability, favourable safety profile and absence of significant drug to drug interactions. However, its main limitations, such as the twice daily dosing schedule and modest genetic barrier have prompted the design and development of agents with once-daily dosing, a more robust genetic barrier, and a limited cross-resistance to Raltegravir and other integrase inhibitors.

Dolutegravir is second generation InSTIs with the intention of addressing raltegravir limitations. Dolutegravir is a second generation integrase inhibitor, displaying structural and functional properties superior to the first generation INI raltegravir and elvitegravir. Dolutegravir is able to enter further in the enzyme catalytic site vacated by viral DNA as well as having a slower dissociation from integrase DNA complex.

2.6.4 Protease Inhibitors

These are a very potent class of ARV recommended for the developing country as part of the second-line regimen and/or the salvage therapy. They are favoured for their high genetic barrier to resistance.

HIV-1 protease enzyme is a 99-amino-acid, aspartic acid protein and is responsible for maturation of virus particles late in the viral life cycle [62]. HIV protease systematically cleaves the gag and gag-pol polypeptide precursors into functional proteins for viral capsid formation during or shortly after viral budding from an infected cell [62]. HIV-1 protease inhibitors competitively inhibit the protease enzyme and subsequently preventing cleavage of polypeptide precursor and therefore rendering the virion immature.

HIV-1 protease inhibitors, like NNRTIs, have the first-generation PIs such as ritonavir, indinavir, nelfinavir etc. and the second-generations such as lopinavir/ritonavir, darunavir, and tipranavir etc. The second generation PIs were designed with the goal of inhibiting first generation HIV protease enzyme
resistant isolates as well as to introducing better tolerated drugs with minimized side effects and simple once-daily dosing, which may improve adherence to the treatment.

HIV PIs are metabolized by the cytochrome P450 system, 3A4 and 3A5, except indinavir, therefore drug to drug interactions are likely to occur. HIV-1 protease inhibitors have limited CNS distribution and also exhibit shorter serum half-lives mandating ranging from 1.5-2 hours for indinavir and 7 hours for atazanavir [63].

HIV-1 protease inhibitors are usually boosted with low-dose ritonavir (100-200 mg), with the exception of nelfinavir, to block intestinal and hepatic 3A metabolism improving the pharmacokinetic parameters, thus improves serum concentration and treatment response [64].

The side-effects associated with PIs intake are gastrointestinal such as diarrhoea, metabolic side-effects such as dyslipidemia, insulin resistance, lipodystrophy. Metabolic complications are common in patients receiving protease inhibitor therapy and represent an important consideration in selecting antiretroviral therapy. Dyslipidemia develops in up to 70% of patients receiving protease inhibitors and commonly requires institution of lipid-lowering therapy [62].

2.6.5 Maturation inhibitors

This class of ARV drugs are currently in development and they mainly inhibit maturation of HIV-1 virion. The bevirimat (formerly PA-457), a maturation inhibitor, is a betulinic acid derivative that interferes with the production of the HIV capsid by preventing cleavage of the precursor Gag protein. The drug has had a troubled history with formulation and bioavailability issues, as well as questionable efficacy in treatment-experienced individuals. Newer second generation maturation inhibitors are underway in clinical trials with potential for once-daily dosing and greater potency compared to previous older maturation inhibitors.

2.7 HIV-1 Drug Resistance

2.7.1 HIV-1 resistance and drug resistance development

In response to high burden of HIV/AIDS, the WHO-recommended public health approach to antiretroviral therapy (ART) has been widely implemented. The successes of widely implementing HAART has been hailed and now HIV-1 infection is regarded as manageable chronic infection to those that have access and remain virally suppressed on treatment.
Six classes of ARVs (as discussed in section 5) are currently available and offer effective treatment options for treatment-naïve and treatment-experienced children and adults as well as offering prevention options for PMTCT, PEP and PrEP programmes. However, the use of these agents in clinical practice is largely dictated by their ease or complexity of use, toxicity issues, and drug to drug interactions, efficacy based on clinical evidence, practice guidelines, and clinician preference.

Usage of these drugs also bears some challenges in the form of resistance development, severe toxicity issues warranting drug replacements, co-infections such as TB/HIV, HCV/HBV-HIV.

Multiple factors influence the development of HIV drug resistance, including the virology of HIV, genetic barriers to resistance, regimen potency, and pharmacokinetics of antiretroviral drugs, medication adherence, adverse effects and socio-cultural issues. The reasons implicating HIV-1 virus in contributing to the development of resistance are based on characteristic of the virion and its enzymes.

HIV-1 virion is characterised by high replication rates (>10⁹ produced daily), high infidelity and error-prone reverse transcriptase enzyme. The combination of high rates of replication and frequent introduction of mutations during each round of replication leads to the frequent occurrence of randomly generated mutations, some of which confer drug resistance [65]. This generated mutations results in viral populations that are genetically related yet distinct in a patient often referred to as HIV-1 viral quasispecies.

2.7.2 NRTI resistance

Emergence of NRTI resistance is facilitated by two mechanisms. The first mechanism is the ATP-dependent pyrophosphorolysis, which leads to the removal of incorporated NRTIs from the 3’ end of the nascent chain, and therefore reversing chain termination [66]. The mutations that are collectively referred to as thymidine analogue mutations (TAMs) promote pyrophosphorolysis particularly of thymidine analogues (AZT and d4T) and two distinct pathways have been identified: TAM1 pathway (M41L, L210W, T215Y) and the TAM2 pathway (D67N, K70R, T215F, and 219E/Q) [50].

The second mechanism is the impairment of analogue incorporation into the nascent chain. Mutations associated with this mechanism include the M184V/I, the Q151M complex of mutations and the K65R. The M184V mutation emerges with 3TC or FTC therapy [67], whereas treatment with Tenofovir, ddC, ddl, d4T, and ABC can select K65R [68].

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2.7.3 NNRTI resistance

The mutations that are selected for after the failure of treatment with nonnucleoside reverse transcriptase inhibitors are all located in the pocket targeted by these compounds, and they reduce the affinity of the drug[69]. Various NNRTI have unique subtle differences in how they interact with the targeted pocket on the enzyme, hence the mutations that emerge most frequently are somewhat drug-dependent. NNRTI resistance generally results from amino acid substitutions such as V106, G190, K103, E138, V179, Y181, and Y188 in the NNRTI-binding pocket of RT [70]. However the commonest reported are K103N which confer resistance to the entire first-generation NNRTIs and Y181C particularly conferring resistance to nevirapine. Most NNRTI mutations engender some level of cross resistance among different NNRTIs.

First-generation NNRTIs have a low genetic barrier to resistance, whereby a single mutation in the binding site can decrease the ability of the drug to bind, significantly diminishing activity [71]. The second-generation NNRTIs such as etravirine have a higher genetic barrier to resistance than other currently available first-generation NNRTIs.

2.7.4 Protease Inhibitors resistance

Resistance to protease inhibitors is the consequence of amino acid substitutions that emerge either inside the substrate-binding domain of the enzyme or at distant sites [72]. Directly or indirectly, these amino acid changes modify the number and the nature of the points of contact between the inhibitors and the protease, thereby reducing their affinity for the enzyme [73]. Mutations that confer resistance to PIs can be categorized into major mutations and minor mutations based on phenotype effects. Resistance typical occurs through emergence of one or more major mutations and these mutations are associated with several fold decrease in susceptibility to one or more PIs [50]. The minor mutations also referred to as accessory or silent mutations have less impact on the susceptibility of PIs, however a combination of minor and major mutations has a synergistic resistance effect. Major mutations are usually located within the active site whereas that minor mutations occurs outside the active site.

Major (primary) mutations confer resistance to the first generation PIs are as follows: G48V, L90M (saquinavir) M46I, V82A/L/F, I84V (indinavir), V82A/L/F, I84V (ritonavir), I50L, I84V, N88S (atazanavir). Minor mutations are commonly located at polymorphic sites of non-B subtypes and amino acid changes at positions 20, 36, 63, and 77 are referred to as polymorphisms which have no impact on specific selective drug pressure [74]. HIV-1 protease inhibitors have high genetic-barrier; therefore multiple accumulative mutations are required to cause high-level resistance to ritonavir-boosted PIs.
The second-generation protease inhibitors lopinavir/ritonavir(r), darunavir/r, and tipranavir/r may usually retain activity in the presence of resistance to first-generation agents. Lopinavir/ritonavir requires the accumulation of 7 or more mutations before high-level resistance develops [75], reports have suggested that lopinavir/ritonavir associated resistance may be following two divergent pathways: one consisting of L76V and Q58E mutations and the other the L90M and I54V mutations [76].

Reports suggested that darunavir/r requires accumulation of at least three darunavir-associated mutations together with high number of protease-associated mutation (some report 13 or more mutation) to lose its activity [77]. There about 11 protease mutations that have been described to be associated with a reduced response to boosted darunavir/r (V11I, V32I, L33F, I47V, I50V, I54L/M, G73S, L76V, I84V and L89V) [77].

2.8. Overview of HIV-1 drug resistance assays

HIV-1 drug resistance assays employ different technologies for analysis. The resistance testing assays can be in the form of genotypic and phenotypic analysis, with the former being the commonest method used. Indications for resistance testing are varied in different settings with developing countries typically conducting HIV drug resistance testing in patients with virological failure after 2 failed regimens. In developed countries however, HIV resistance testing forms part of the battery of tests for a patient being worked up for initiation of HAART. Genotype assays provide information about viral mutations that may result in changes in viral susceptibility to particular drugs or classes of drugs. Phenotype
assays directly quantitate the level of susceptibility of a patient's virus in a sample to specific drugs in vitro.

2.8.1 Phenotypic assays

Phenotypic assays use recombinant virus composed of a patient's virus polymerase and reverse transcription genes, which are inserted into a standard reference strain of virus. The recombinant virus is then tested in vitro for the amount of each particular drug needed to inhibit virus replication by 50% (50% inhibitory concentration, IC50), relative to the amount of drug needed to inhibit a reference strain of virus, which is quiet similar to bacteriologic methods[78]. Results are reported as a fold-change in drug susceptibility of the patient sample compared with a laboratory reference strain [78].

2.8.1.1 Advantages

- Measures resistance/susceptibility of the drug directly
- The susceptibility that is measured is the aggregate of the acquired drug mutations in a patient's viral strain, therefore it also measures the unrecognized mutations
- Reports are more familiar to clinicians

2.8.1.2 Disadvantages/limitations

- Unable to detect minority variants when they are present at <20% of the viral population
- Expensive
- Biological cutoffs for clinical resistance have not been developed for all antiretroviral agents
- Longer turnaround times

2.8.2 Genotypic assays

Genotypic resistance assays use state-of-the-art DNA sequencing method to examine the reverse transcriptase (RT) and polymerase (PR) regions of the HIV-1 genome for all possible resistance-associated mutations. Commercially available tests include FDA-approved kits as well as a variety of in-house assays performed by reference laboratories. These assays sequence regions of the HIV protease and reverse transcriptase genes that have been PCR-amplified from the viral quasispecies circulating in a patient's plasma. The sequence data produced from these assays are analysed by an assay-specific computer program that compares the viral mutations present in the clinical strain to a list
of reference drug resistance mutations; and each software mutation algorithm provides an interpretation of the viral genotype, using algorithms established by a panel of experts.

2.8.2.1 Advantages

- Shorter turnaround times as compared to phenotypic assays
- Less expensive

2.8.2.2 Limitations

- Only reports known mutations
- Less is known about the clinical effect of combination of individual mutations
- Difficulty in interpretation when many mutations are present
- Unable to detect minority variants when they are present at <20% of the viral population

Some mutations cause resistance to certain drugs, but increase susceptibility to others; some mutations impact viral fitness; others may be important contributors to major pathways of resistance. Genotypic interpretation is very subjective and so many rule-based genotypic interpretation systems have been proposed.

2.9 Clinical utility of antiretroviral drugs in PMTCT

The goal of administering antiretroviral to any person is to suppress the virus replication, provide better quality of life and prevent transmission to the person at risk of contracting the disease. Antiretroviral drugs can therefore be given in different clinical scenarios with the aim of either treating or preventing HIV-1 infection. WHO and other various guidelines have recommended antiretroviral use for prevention of HIV infection, particularly for pregnant women, young children and key populations exposed to HIV risk. The countries have adapted and implemented the recommendation depending on their epidemiological settings and health needs.

Prevention of mother to child transmission (PMTCT) programme is one of the hallmark prevention programmes that have been implemented with the aim of reducing and eliminating transmission of HIV-1 to children. It was not until 2002 that this programme was implemented in South Africa despite having a recognized high burden of HIV-1 infection and this has eventual evolved through years into a policy that yielded very promising results. The PMTCT programme improved as the ministry of health closely
followed adapted and implemented WHO PMTCT recommendations. Firstly there was a provision of nevirapine for pregnant mothers in 2003, then this was escalated to pregnant women with CD4+ T cell <200 cells/mm³ receiving HAART [79,80]. PMTCT policy was then reviewed and updated in 2008 which included dual prophylaxis with zidovudine and nevirapine from 28 weeks' gestation; nevirapine treatment for pregnant women during labour and for their babies within 72 hours of delivery; and HAART for pregnant women with a CD4+ T-cell count < 200 cells/mm³ [81].

The next revision of PMTCT policy in 2010 included provision of life-long HAART to HIV-positive women with a CD4+ T-cell count ≤ 350 cells/mm³ and dual ART from 14 weeks onwards in the pregnancy for HIV-positive women with a CD4+ T-cell count > 350 cells/mm³, in line with option A of World Health Organization guidelines [82]. Infant prophylaxis was to be given daily for 6 weeks to all infants and continued for all breastfeeding whose mothers were not on HAART.

The final version made a provision of ARV (TDF+FTC/3TC+EFV) to all women irrespective of their CD4+ count, however for the mothers that had CD4+ T cell count of >350 cells/mm³ and are breastfeeding were eligible for these ARVs throughout the pregnancy until 1 week after cessation of breastfeeding [83].

Finally the updated 2014 PMTCT guidelines advocated a lifelong provision of ARVs to all pregnant women and breastfeeding mothers on the same day of diagnosis irrespective of CD4+ T cell count [84].
CHAPTER 3
Study design and Study population

3.1 Study Design

This is a cross sectional study observing for HIV drug resistance outcomes in paediatric patients.

3.2 Population and Clinical Samples

Preselection of patients was done through screening of patient medical files. HIV-positive treatment-naïve patients that had some exposure to maternal PMTCT or to infant prophylaxis were selected. Informed consent was then sort from caregivers/parents.

A total of 29 out of 50 meet the inclusion criteria which is HIV-1 positivity, exposure to PMTCT and samples from patients that tested positive with either HIV DNA PCR at 6 weeks/6 months or HIV-1 Elisa positive at any point were collected for the purpose of this study. Unique sample codes were used for labelling once the samples were aliquoted. Sample were stored at -20°C or processed immediately.

Laboratory Methods

3.3 Sample preparation/Extraction

Ribonucleic acid(RNA) extraction from the plasma was done using the QIamp Viral RNA mini kit (Qiagen, Hilden, Germany according to the manufacturer’s instruction. Samples were lysed with a lysis buffer. This was to expose and stabilise RNA and inactivate RNases and other infectious agents. This step was conducted by pipetting 140ul of sample and 560ul of AVL Buffer containing carrier RNA in an Eppendorf tube (1.5ml tube) and subsequently incubated at room temperature for 10 minutes after vortexing.

Centrifugation was done in between steps to settle the contents down from the sides and the inside of the closing lid. A total volume of 630ul of 96% ethanol was then added to the mixture and vortexed for 5 seconds. This AVL Buffer/Sample/Ethanol mixture was then loaded onto corresponding QIAmp mini spin columns and collecting tubes and centrifuged at 8000 rpm for 1 minute during which the RNA was
forced to bind through a silica gel membrane. This was repeated until the mixture has finished, if larger volume of sample were used.

The contaminants and any remaining impurities were washed away from the membrane by two different wash buffers; 500ul each of AW1 buffer and AW2 buffer.

Finally, the RNA was eluted in 60ul of RNase-free AVE Buffer, loaded onto each the QIAmp mini spin columns and centrifuged again at 8000 rpm for 4 minutes. The RNA extracts were stored at -70°C freezer until further testing can be done.

3.4 TRUGENE HIV-1 GENOTYPING KIT PERFORMANCE

3.4.1 Trugene RT-PCR REACTION

All procedures were performed according to the manufacturer's recommendations. HIV-1 TRUGENE™ kit contains reagents adequate to perform RT-PCR and CLIP sequencing reaction of the protease and the reverse transcriptase regions of the HIV-1 genome on approximately 30 specimens.

This is an integrated system which incorporates reverse transcription-PCR (RT-PCR) from the extracted HIV-1 RNA, a coupled amplification and sequencing step (CLIP), polyacrylamide gel electrophoresis.

An aliquot of 17µl of extracted RNA eluate was mixed with 9µl of Mastermix which is made up of 3.5µl RNase-inhibitor (Rnase-I), 42µl RT-PCR primers, 11µl of dNTP (dATP, dCTP, dGTP, dTTP), 7µl dithiothreitol (DTT), and 70µl RT-PCR buffer1 [90]. The RT-PCR primers used in the kit amplify the entire protease and the first 250 codons of reverse transcriptase as a single amplicon.

The cycling conditions that were used for reverse transcriptase reaction performance were as follows: at 90°C for 2 min, followed by incubation at 50°C for 5 min, at which point 14µl of Mastermix II was added to the reaction mixture. The Master Mix II contains 7µl of reverse transcriptase enzyme (RT-Enzyme), 4µl RNase-I, RT-Enzyme which was incubated for an additional 55 min before the reaction was terminated at 94°C for 2 min. DNA amplification was performed in the same tube using cycling conditions of 94°C for 30 s, 57°C for 30 s, and 68°C for 2
min for 20 cycles; this was followed by cycling conditions of 94°C for 30 s, 60°C for 30 s, and 68°C for 2.5 min for 17 cycles; and the cycling finished with one cycle of 68°C for 7 min and then a 4°C hold.

The RT-PCR product (5µl) from each sample was used in each of 16 sequencing reactions, based on the CLIP principle [85]. The four pairs of primers that sequence the protease reading frame (two pairs) and the beginning and middle of the reverse transcriptase reading frame (one pair each) were used for sequencing reaction [86].

For each segment, four sequencing reactions were used with four dideoxy terminators (ddATP, ddCTP, ddGTP, and ddTTP), Cy5-labeled forward primers, and Cy5.5-labeled reverse primers to generate CLIP sequencing products over 30 reaction cycles of 94°C for 20 s, 56°C for 20 s and 70°C for 1.5 min, followed by a 5-min terminal step at 70°C [86].

Unpurified CLIP sequencing products were analyzed using 6% polyacrylamide–urea–Tris-borate–EDTA gel electrophoresis, which was set to run for 50 min at 2,000 V. This was done with premanufactured Microcel cassettes, which were cast using SureFill 6% sequencing polyacrylamide gel cartridges (Visible Genetics, Inc.). The polyacrylamide gel was polymerized using UV light [86].

3.5 Data Analysis

3.5.1 Sequence data analysis.

The sequence data output and analysis was performed using the Gene Objects software (Visible Genetics, Inc.). The software allows automated and real-time output of sequence data from the DNA sequencers (Long Read Tower Sequencers) [86].

Sequence fragments were assembled automatically and chromatographs patterns were displayed for semiautomated review after alignment with a clade B HIV-1 reference sequence (LAV-1) and a mutated reference sequence containing codon changes typically observed in drug-resistant HIV-1. The aligned sequence of the sample were displayed as, Q Row and the wild-type HIV-1 sequence, LAV-1, displayed as row "W."
The use of both mutant and wild-type reference sequences served to minimize biases toward the wild type during manual editing. Manual editing for this project was limited to bases that were discordant between the two sequencing reactions.

A report was then generated from the edited sequences and displayed in the TRUGENE HIV-1 Resistance Report Generator window and report printed or saved as part of the case to review later.

3.5.2 Stanford University HDRM Database

The edited sequence data was also exported for analysis using the Stanford University HDRM Database. The Stanford HIV Drug resistance database is one of the publicly available algorithms for use in drug resistance interpretation. The online Stanford HIVdb algorithm accepts either a user-submitted sequence or list of mutations and returns inferred levels of resistance to 17 FDA-approved RT and protease inhibitors [87]. The program assigns a drug penalty score to each drug resistance mutation and, adding the scores associated with each mutation, derives the total score for a drug. Applying the total drug score, the program reports one of the following levels of inferred drug resistance: susceptible, potential low-level resistance, low-level resistance, intermediate resistance, and high-level resistance.

3.5.3 Phylogenetic Analysis and HIV subtype Distribution

Aligned sequences were subjected to phylogenetic inference through the neighbour-joining method implemented in the MEGA 5.1 package. One thousand bootstrap replicates were used to assess the phylogenetic robustness of the clusters. Reference subtype sequences were obtained from the Los Alamos HIV Sequence Database and were included in the analysis.

3.5.4 Recombination analysis

In order to analyse recombination two web-based software were used, RIP version 3.0, available HIV sequence database (http://www.hiv.lanl.gov/content/sequence/RIP/RIP.html) using default parameters and Simplot software version 3.5.1 using a 200 bp window, a 20 bp step increment and 1000 bootscan replicates.
3.6 Quality Control

Positive control and a negative control were added to each run to assess quality as well as to assess any contamination should there be any.

3.7 Ethical consideration

Ethics approval was sort from School of medicine research ethics committee (SREC) as well as the Medunsa research ethics committee (MREC). MREC no: MREC/P/18/2012:PG. Specimens were delinked from any personal identifiers to ensure confidentiality
CHAPTER 4: RESULTS

4.1 Study Population

Fifty paediatric samples were eligible for inclusion during the study period. However, 21 (42%) patient samples were excluded due to either insufficient volumes for analytical processing or compromised sample integrity. Of the 29 paediatric plasma samples remaining, amplification was achieved for only 13 (44.8%) using Trugene RT-PCR, resulting in a total of 13 samples for analysis which were all successfully sequenced. (See flow chart 1).

Routine baseline work-up for initiation of HAART in the paediatric population the South African public sector clinics, typically involves baseline HIV RNA PCR (viral load) analysis amongst a number of monitoring and safety tests.

16 of the 29 patient's samples failed to amplify despite displaying intermediate to high baseline viral loads ranging from 3.92 to 6.7 log₁₀. A number of reasons could be postulated for this amplification failure. However, because of resulting insufficient sample volume, repeat testing could not be conducted for trouble shooting purposes.
4.2 PMTCT Exposure History

Prevention of mother to child (PMTCT) interventions including nevirapine (NVP) infant prophylaxis is routinely conducted as part of the antenatal package in the public health hospitals in South Africa. The uptake of PMTCT ARV intervention and nevirapine infant prophylaxis in this study constituted infant prophylaxis with an uptake rate of 100% in the 13 patients reported. Furthermore, two (2 [15.4%]) mothers had documented combination antiretroviral therapy (cART) intervention.

Of the remaining 11 mothers, there was no documentation on PMTCT intervention. Follow up with telephonic interview failed to clarify the type of intervention received although the majority alluded to having received some form of ‘medication’. All in all, there were 2 documented ARV in-utero exposures, with both mothers on combination antiretroviral therapy (cART) containing tenofovir disoproxil fumarate (TDF) with lamivudine NRTI backbone with efavirenz. Maternal viral loads, determinants of both probability of transmission and virological failure, were not available.

4.3 Baseline demographics of Study Population

The median age of the 29 patients was 12 months (interquartile range [6-36] months), with the youngest patient being only 75 days and the oldest, 6 years. Sixteen (16/29 ([55%]) patients were males and (13/29 [44.8%] were females. Of the 24 available CD4 T cell counts, the median baseline CD4 T cell count was 627 cells/mm3 (interquartile range [492 – 918]) and the median HIV-1 RNA level was 253065 copies/mL (interquartile range [30 551 – 2786833 copies/mL]). Maternal variable were not available for analysis.

4.4 Prevalence of HIV resistance mutations

HIV-1 sequences from 11 (84.6%) samples were found to exhibit viral genotypic evidence of resistance-conferring mutations to non-nucleoside reverse transcriptase inhibitors (NNRTIs) and nucleoside reverse transcriptase inhibitors (NRTI), with 2/11 [18.2%] having additional minor protease inhibitor (PI) mutations. Wild type virus was detected in 2 of the13 sequenced samples.

Of the 11 patients with detectable HIV-1 drug resistance mutations, 10 (90.9%) had ≥1 major mutation conferring resistance to antiretroviral drugs. The lamivudine/emtricitabine -related resistance mutation M184V, was detected in 1 individual (9.1%). For NNRTI mutations, K103N (4/11 [36.4%]), Y181C (3/11
[27.3%]), V106M (1/11 [9.1%]), and E138A (1/11 [9.1%]) were observed respectively. The minor PI mutations detected were T74S (2/11 [18.2%]) and L10I (1/11 [9.1%]) See Table 1.

Table 2: Mutational profile with PMTCT history of paediatric patients prior to initiation of HAART

<table>
<thead>
<tr>
<th>Patient</th>
<th>NRTI Mutations</th>
<th>NNRTIs</th>
<th>PI Mutations</th>
<th>PMTCT history</th>
</tr>
</thead>
<tbody>
<tr>
<td>KR</td>
<td></td>
<td>K103N</td>
<td>K20R</td>
<td>Infant prophylaxis given</td>
</tr>
<tr>
<td>MT</td>
<td></td>
<td>K103N</td>
<td>K20R, M36I</td>
<td>Infant prophylaxis given</td>
</tr>
<tr>
<td>DS</td>
<td></td>
<td></td>
<td>T74S, M36I</td>
<td>Infant prophylaxis given</td>
</tr>
<tr>
<td>MR</td>
<td></td>
<td>K103N</td>
<td>M36I</td>
<td>Infant prophylaxis given + Maternal ART</td>
</tr>
<tr>
<td>MG</td>
<td></td>
<td></td>
<td>K20R, M36I</td>
<td>Infant prophylaxis given</td>
</tr>
<tr>
<td>RT</td>
<td></td>
<td>K103N</td>
<td>M36I</td>
<td>Infant prophylaxis given + Maternal ART</td>
</tr>
<tr>
<td>ML</td>
<td></td>
<td></td>
<td>K20R, M36I</td>
<td>Infant prophylaxis given</td>
</tr>
<tr>
<td>MI</td>
<td>Y181C</td>
<td></td>
<td>M36I, L10I</td>
<td>Infant prophylaxis given</td>
</tr>
<tr>
<td>MB</td>
<td>L74V</td>
<td>Y181C</td>
<td>K20R, M36I</td>
<td>Infant prophylaxis given</td>
</tr>
<tr>
<td>TM</td>
<td>Y181C</td>
<td></td>
<td>M36I</td>
<td>Infant prophylaxis given</td>
</tr>
<tr>
<td>CR</td>
<td>M184V</td>
<td></td>
<td>T74S, M36I</td>
<td>Infant prophylaxis given</td>
</tr>
<tr>
<td>BM</td>
<td>V106M</td>
<td></td>
<td>K20R, M36I</td>
<td>Infant prophylaxis given</td>
</tr>
<tr>
<td>KK</td>
<td>E138A</td>
<td></td>
<td>M36I</td>
<td>Infant prophylaxis given</td>
</tr>
</tbody>
</table>
4.5 Prevalence of Polymorphisms

Polymorphisms are defined as mutations that occurred in more than 1% of sequences from untreated persons [88] They are considered secondary mutations that by themselves play a limited, if any, role in resistance. However, their presence might favour a more rapid evolution towards resistance when additional mutations are selected under therapy.

Polymorphisms in our cohort were generally present at varied frequencies, with NRTIs showing the lowest and PIs the highest frequency. All 13 patient’s sequences displayed polymorphisms with the affected codons being 20, 36 and 118 (See Table 2). Two of these sequences were categorised as wild type virus (WT). The most frequent polymorphisms encountered within the protease region were M36I, present in 92.3% followed by K20R present in 46%. The frequency of V118I in the RT region was 15.4%.

A total of seven patients displayed the presence of only one polymorphism with M36I seen in 6 sequences and K20R in one sequence. The remaining 6 sequences displayed more than one polymorphism with the predominance of M36I with K20R (66.6%; [4/6]), followed by the combination of M36I with V118I (16.7%; [1/6] and M36I, K20R together with V118I (16.7%; 1/6)).

There was no significant distinct variation in age observed with patients harbouring K20R polymorphism, whilst patients with M36I polymorphism majority were in the ages of 12-18 months. The majority of PIs polymorphism occurred in combination with major NNRTI mutations or major NRT mutations.
Table 3: Proportion of NRTIs and PIs Accessory mutations/Polymorphism in HIV-1 Subtype C Infected Patients

<table>
<thead>
<tr>
<th>HIV-1 Genome</th>
<th>Codon Position</th>
<th>Subtype Amino acid</th>
<th>Amino Acid Substitution</th>
<th>Frequency of Polymorphisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT</td>
<td>118</td>
<td>V</td>
<td>I</td>
<td>(15.4%; [2/13])</td>
</tr>
<tr>
<td>PR</td>
<td>20</td>
<td>K</td>
<td>R</td>
<td>(46%; [6/13])</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>M</td>
<td>I</td>
<td>(92.3%; [12/13])</td>
</tr>
</tbody>
</table>

RT, Reverse Transcriptase; PR, Reverse Transcriptase; HIV-1, Human Immunodeficiency Virus type 1; NRTIs, Nucleoside/Nucleotide Reverse Transcriptase Inhibitors; PIs, Protease inhibitors

4.6 Age and mutational pattern

Of the 11 patients with detectable HIV mutations, 27% of those with K103N mutation were <6 months of age and 9% were above 12 months of age, those with Y181C majority were between 7-12 months of age, those with V106M, E138A and M184V were in patients above 12 months of age and L74V, T74S and L10I were in patients between the ages of 7-12 months. The K103N mutation noticeably was detected patients below 6 months.
4.6.1 NNRTI-associated HIV-1 resistance mutations

NNRTIs-associated HIV-1 resistance mutations were highest in prevalence, specifically mutations associated with NVP: Y181C, K103N, V106M and E138A. The overall detection rate of NNRTI mutation was 9/11 (81%) in patients. The majority of K103N mutations were seen in patients less than 6 months whilst the majority of Y181C were detected in patients between the ages of 12 months and 18 months. Other major NNRTIs such as V106M conferring high level resistance to both NVP and EFV and E138A mutation weakly selected in patients receiving second-generation NNRTIs, were detected in small proportion of patients over 18 months of age.
Graph 2: Age trend of patients with NNRTI-associated mutations

4.7 Phylogenetic characteristic of HIV-1 variant

All study sequences (Figure 1) clustered under subtype C and in addition study sequence AAZH8847B_1a branched away from other sequences. Further analysis we conducted by constructing a phylogenetic tree with recombinant subtype BC and CD obtained from Locos Alamos and the sequence clustered with recombinant subtypes BC as shown in Figure 2.
Figure 8: Phylogenetic analysis of HIV-1 pol (PR and RT) in comparison with reference subtype strains obtained from Los Alamos HIV database. Study sequences are represented in red and subtype O as an out-group. The tree was constructed using neighbour-joining tree with
a bootstrap value of 1000 replicates. The nucleotide sequence divergent was estimated to be 0.05 bar.

4.8 Recombinant analysis

Study sequence AAZH8847B_1a which clustered with CRF-BC using phylogenetic tree was further analysed using RIP and Simplot software’s for recombination. As shown in Figure 3 and Figure 4, Simplot and RIP showed evidence of recombination between HIV-1 subtype B and C.

![Figure 9: Bootscan analysis of pol (PR and RT) sequence AAZH8847B_1a of HIV-1 B/C recombinant form SA, using 200 bp window, a 10 bp step increment and 1000 bootscan replicates. Y axis represents the number of permuted trees and x axis represents the position of the gene. The straight line indicates the 70% threshold used to denote between subtypes.](image-url)
Figure 10: RIP analysis of AAZH8847B_1a sequence using default settings.
Evidence from many studies has indicated that the use of nevirapine for pMTCT selects for viral mutations such as K103N and Y181C [89]. These mutations are known to be associated with HIV resistance to first generation NNRTIs amongst pMTCT exposed women and those infants who failed nevirapine prophylaxis and seroconverted.

Of the 44.8% successfully sequenced samples in our study, the majority (84.6%) of the samples were found to harbour HIV resistance mutations. Major NNRTI and NRTI mutations were detected amongst 10 (90.9%) of the 11 children.

In keeping with findings from other studies, the majority of clinically relevant HIV mutations were of NNRT origin, with K103N and Y181C reported at 36% and 27% respectively. A number of paediatric studies have reported a detection of Y181C as the most frequently detected mutation compared to K103N. In our study, however, we report the predominance of K103N (36%) and Y181C (27%). Our findings are consistent with K103N predominance reported in adult studies. Although this may be a marginal predominance, the significance finding of K103N predominance in this paediatric population is not understood.

Y181C is a non-polymorphic mutation selected in vitro by nevirapine and has been reported in a number of paediatric HAART naive studies as the most frequently detected NNRTI mutation. Eshleman et al reported Y181C detection rates of 46% in the HIVNET 012 multicenter study [90]. These findings are consistent with the findings of Hunt et al who reported Y181C as the commonest mutation detected with a frequency of 62% ( < 6 months), 39% (6 – 12 months), 22% (18 – 12 months) and 16% (18 – 24%) in a HAART naive paediatric South African cohort using both bulk sequencing and ultrasensitive allele specific PCR resistance platforms [91].

In support of the predominance of Y181C, another South African study reported HIV mutations rates of high rates of 75%.

The findings in our study of K103N as the commonest HIV mutation, is contrary to what has been observed in other paediatric studies. Our study findings are supported by Lehman et al in a cohort of 20 where they reported a detection of K103N by ASPCR in 4 infants at levels ranging from 18.7% to 100% and Y181C in 3 infants at 0.9% – 3.1%. The 3 infants in which K103N was detected in bulk sequencing had K103N levels more than 20% as quantified by ASPCR [92]. Although this may be a
marginal predominance, the significance finding of K103N predominance in this paediatric population is not understood.

It is well established that a proportion of paediatric patients will have NNRTI HIV mutations 6 – 8 weeks after single dose nevirapine. To assess the presence of NNRTI HIV resistance mutations at various time points defined by age, the 13 children were categorised into 4 age groups. The documented age in this cohort corresponded with age at initiation of HAART.

In our study, the majority of K103N mutations were seen in patients less than 6 months, with one patient displaying K103N outside the less than 6 months of age range and within the category of 12 – 18 months. Of note is that Y181C was detected only from the 6 – 12 month age category, with a notable absence at less than 6 months of age. Both K103N and Y181C had declining detectable rates in relation to increasing age. This may be explained by viral DNA archiving in the lymphocyte population.

Other major RT mutations V106M, E138A, M184V and L74V were found in a small proportion of infants particularly in children older than 3 years of age, but were absent in younger children. NRTI M184V was detected in only one of the 13 (10%) children. The detection of HIV mutations conferring resistance to NRTIs was unexpected. Various reasons could account for this occurrence such as inappropriate documentation (patients could be HIV treatment experienced and yet are reported as HAART naive) and possible HIV transmitted drug resistance. HIV transmitted resistance has been reported to occur at low frequencies despite widespread use of antiretroviral drugs in this population [93]

The prevalence of HIV-1 drug resistance among treatment-naïve patients (primary drug resistance) is of paramount importance in informing national policy of appropriate first-line agents.

The 8 different HIV mutation detected (NRTI: M184V and L74V and for NNRTs, K103N, Y181C, V106M and E138A) and for PIs (L10I and T74S) were subjected to the Stanford CPR database for determination of surveillance drug resistance mutations (SDRM).

For NNRTI, we report the presence of surveillance drug resistance mutations for K103N (n=4), Y181C (n=3), V106M (n=1). Reportable NRTIs within the SDRM were M184V (n=1) and L74V (n=1).

Overall none of the mothers to the reportedly HAART naive with evidence of transmitted HIV mutations had documented evidence of antiretroviral exposure during pregnancy. The possibility of these mothers being on a various pMTCT interventions cannot be ruled out. All children had documented
evidence of administration of pMTCT in their medical records, although the duration of the intervention could not be determined.

Although none of our participants were documented to have received HAART, a number of polymorphisms were detected. Different subtypes display a variety of protease polymorphisms related to reduced drug and M36I is the most frequent polymorphism.

In our study, polymorphisms with occurrence at the highest frequency were M36I and K20R. The M36I HIV mutation was detected at a frequency of 92.3% with K20R occurring at a frequency of 46%.

Previous studies have suggested that M36I represents a molecular signature for non-subtype B strains [94] An analysis of 40 sequences from HIV-1 subtype C samples confirmed the predominance of M36I polymorphism, detectable at 90% and nearly half of the sequences reflecting K20R polymorphism [95]

Other evidence in support of this predominance also comes from studies by Handema et al who found high prevalence levels of M36I amongst other polymorphisms in HIV-1 subtype C individuals [96].

A Spanish study by Holguin et al also reported high prevalence of M36I and K20R at 100% and 77.2% respectively from infants and children of immigrants in Madrid. The subjects in this study with viruses harbouring this polymorphism were non subtype B – consistent with our study, where the majority were subtype B [97].

Grossman et al, in a comparative analysis of subtype B and C, in an Israeli cohort, found a significant difference in the prevalence of polymorphisms in the protease region with M36I not detectable in subtype B but identified in subtype C at 21%. K20R in the same cohort was detected at a lower frequency of 7% compared to subtype C [98].

Overall two minor protease mutations (L10I and T74S) were detected in our study, with these detected only 2 patients. Minor mutations may not result in a significant decrease in sensitivity of antiretroviral drugs, but have been reported to be associated with increased viral fitness in viruses with primary mutations. We therefore speculate that the selection of primary mutations under selective pressure could precipitate the development of highly resistant HIV variants during ARV treatment in viruses where one or more secondary mutations are already present.

V118I, an NRTI polymorphic mutation occurring in association with TAMS, was detected in 2 on the 13 samples from our study. This is supported by a study of HAART naive women in South Africa who had the same mutation described [93]
The distribution of HIV-1 sequences in our cohort was subtype C, with one exception with a circulating recombinant form (CRF) B/C, correlating with the known subtype distribution status in South Africa. It is noteworthy that although the predominant circulating subtype in South Africa is C, there has been a reported increase in the number of subtype B cases spread through heterosexual and mother to child transmission route. This is of interest given the fact that traditionally subtype B was frequently observed in South African homosexual transmission cases [99]

In our study, after conducting a recombinant analysis, one of the sequence displayed inter-subtype recombinant B/C which cannot be described as a circulating recombinant form but rather as a unique circulating recombinant form (URF). Inter-subtype recombinant B/C has been described by Jacobs et al in the South African adult female and the researcher found this URF as a new emergence in this population studied [100]. Other than our study, no studies in South Africa have reported URF B/C in HAART naive paediatric population. However, the confidence of reporting this URF is somewhat overshadowed by limitations of failure to identify the exact breakpoint of this sample as well as the fact that the sequence does not classify with any pure subtype. Analysis with a recombinant analysis program (RIP) from Los Alamos, yielded a B/C recombinant. A full length genome or an addition of another HIV gene such as gag p24 as described by Jacobs et al, could confidently identify the trueness of this inter-subtype recombinant [100].
LIMITATIONS

a) The major limitation of this study is that the paediatric patients in the present study were tested for HIV-1 genotypes at pre-treatment rather than at the time of diagnosis. As population sequencing was used for genotyping, the possibility that some HIV resistance overtime were archived, cannot be ruled out. Linked to the failure to detect HIV resistance mutations is the fact that pre-treatment testing was also conducted at one time point, rather than serial sampling at various time points.

b) Paucity of clinical information hindered full understanding of whether the patients were true HAART naive or represented treatment failure patients who were now presenting at another site.

c) The sample size and the fact that the study was not a multi-site study also pose another limitation. Results cannot be generalised to other sites in South Africa.

CONCLUSION

We report on a cohort that is HAART naive, albeit a cohort with a small sample size, who present with evidence of transmitted HIV resistance. The transmissibility of HIV drug resistance mutation constitutes a serious major public health concern. Although available data in South Africa demonstrates a low level of prevalence of primary drug resistance, results generated from our study would support advocating for large surveys of transmitted resistance but as well as the need for testing for HIV resistance in paediatric patients prior to initiation of HAART. The drive to improve reduce transmission of HIV in this population group, should be matched with efforts to improve treatment outcomes.
RECOMMENDATIONS:

a) Use of highly sensitive assays to detect minority variants to assess for the size of transmitted resistance;

b) Baseline HIV genotyping on all patients testing HIV positive and identified for HAART initiation.
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