A STUDY ON THE GENOTYPIC RESISTANCE PATTERNS IN PATIENTS FAILING FIRST LINE ANTI-RETROVIRAL THERAPY REGIMEN IN ZAMBIA

BY

DR. IGNACE GASHONGORE

A dissertation submitted to the University of Zambia in partial fulfillment of the requirements for the degree of Master of Science in HIV Medicine

THE UNIVERSITY OF ZAMBIA

LUSAKA

2012

DECLARATION

I hereby declare that this dissertation herein presented for the degree of Master of Science in HIV medicine is my own work and that it has not been previously submitted either wholly or in part for any other degree at this or any other university nor is it being currently submitted for any other degree.

Signed: _____

Dr. Ignace Gashongore

Signed: _____

Dr Christopher Bositis (supervisor)

Signed: _____

Dr Lottie Hachaambwa (Co-supervisor)

APPROVAL

This dissertation of Dr. Ignace Gashongore is approved as fulfilling the requirements for the award of the degree of Master of Science in HIV medicine by the University of Zambia

Examiner's signature .....Date.....:

Examiner's signature .....Date.....:

Examiner's signature .....Date.....:

DEDICATION

This dissertation is dedicated to my loving and ever encouraging wife Mrs. Diana Gashongore and to our beloved son Travis Gashongore, and to the memory of my late parents Mr. & Mrs. Gashongore (Snr).

ABSTRACT

Background: Few reports have described the drug resistance patterns of patients failing antiretroviral therapy (ART) in areas where HIV subtype C is predominant, and there is little data from Zambia in particular.

Aims: To evaluate the pattern of resistance in patients failing first line regimens in Zambia and determine the impact of first line regimen resistance on second line therapy options. A secondary aim was to evaluate the frequency of non-C HIV subtypes.

Methodology: Charts from patients failing first-line therapy at three urban, outpatient ART clinics (Chreso, Circle of Hope and the Pediatric Center of Excellence at UTH) were reviewed. All available genotypes that were done in patients failing first line ART regimen by December 2010 were included for analysis. The first-line regimen was defined as NNRTI based HAART regimen according to 2004/ 2007 Zambian guidelines. The regimen at failure, and any previous ARV exposure, duration of treatment, the subtype and the viral load were recorded

Results: A total of 126 genotypes were analyzed, 92% of which were from pediatric patients and 8% from adult patients; of these, 19% were found to be wild type while 81% were found to have at least one major mutation. M184V was most common (83.3%), followed by NNRTI mutations (K103 and Y181, 76.4%), and then thymidine analog mutations (TAMs, 59%); 43% of patients had ≥2TAMS. K65R was found in one case of a patient failing on AZT and another one failing on d4T, both in subtype C. 38% of patients were predicted to be resistant to Etravirine (ETR). Subtype C/C was found to be predominant at 95.2%; other subtypes identified were B/C (2.4%), D/C (1.6%) and B/B (0.8%).

Conclusion: The majority of patients failing first line therapy in Zambia have typical mutations found in subtype B populations. Given that ABC/ddI was the preferred NRTI backbone for the second line regimen for pediatric patients in Zambia before the new 2010 guidelines were launched, 43% of patients on this second line regimen would be predicted to have no fully active agents in their NRTI backbone. Although NNRTIs resistance mutations take long to get archived, they may not be seen in situations like here in Zambia where genotyping is not immediately done after

first line failure (i.e. after NNRTI failure).Etravirine (ETR) is therefore likely to be of limited use as a third line agent in this population, and its use should always be guided by a genotype and routine viral load monitoring. A single case of K65R to AZT exposure in subtype C was an unexpected finding.

ACKNOWLEDGEMENTS

1. Dr Christopher Bositis, former Faculty HIV Residency Program for the guidance, encouragement and supervision throughout the study.
2. Dr Lottie Hachaambwa for guidance
3. Dr Robb Sheneberger for the support and encouragement
4. Esnart Chansongo for her support
5. David Rutagwera for his support
6. UTH-PCOE, CoH and Chreso clinics management and staff for the support
7. University of Zambia-Departments of Internal Medicine and DRGS for guidance
8. University of Maryland- Institute of Human Virology for the support

TABLE OFCONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

3TC	:	Lamivudine
69ins	:	Insertion at position 69 of the viral reverse transcriptase
ABC	:	Abacavir
AIDS	:	Acquired Immunodeficiency Syndrome
ART	:	Anti-retroviral therapy
ARVs	:	Anti-retroviral drugs
AZT	:	Zidovudine
CoH	:	Circle of Hope clinic
CRF	:	Circulating recombinant form
d4T	:	Stavudine
DDI	:	Didanosine
DNA	:	Deoxyribonucleic acid
EFV	:	Efavirenz
ETR	:	Etravirine
FTC	:	Emitricitabine
GT	:	Genotype
HAART	:	Highly active anti-retroviral therapy
HIV	:	Human immunodeficiency virus
LPV/r	:	Lopinavir/Ritonavir
NNRTI	:	Non-nucleoside reverse transcriptase inhibitor
NRTI	:	Nucleoside reverse transcriptase inhibitor
NVP	:	Nevirapine
OI	:	Opportunistic Infection
PI	:	Protease inhibitor
RNA	:	Ribonucleic acid
TAM	:	Thymidine analogue mutation
TDF	:	Tenofovir
UTH	:	University Teaching Hospital
WHO	:	World Health Organization

AMINO ACID CODES

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

DEFINITIONS

1. Mutation: A mutation is a permanent change in the DNA or RNA sequence of a gene. Mutations in a gene's DNA or RNA sequence can alter the amino acid sequence of the protein encoded by the gene.[1]

2. Resistance mutation: The term resistance mutation is most commonly used to describe point mutations in virus genes that allow the virus to become resistant to treatment with a particular antiviral drug. Resistance mutations are conventionally listed as a letter, number and letter[1]. For example, the M184V mutation in the reverse transcriptase gene of HIV confers resistance to the drug lamivudine. The letters stand for amino acids and use the conventional one letter abbreviations. *See list of abbreviations.* M stands for methionine, and V stands for valine; 184 is the amino acid position counting from the amino terminus of the protein. M184V means that the 184th amino acid of the protein is normally methionine, but that a mutation in the gene for that protein produces a form of the protein where that amino acid is substituted by valine instead.

3. Major resistance mutations: these are mutations whose presence significantly reduces viral susceptibility to a particular drug.[1]

4. Drug resistance: also known as antimicrobial resistance drug resistance; occurs when microorganisms such as bacteria, viruses, fungi and parasites change in ways that render the medications used to cure the infections they cause ineffective. When the microorganisms become resistant to most antimicrobials they are often referred to as “superbugs”. This is a major concern because a resistant infection may kill, can spread to others, and imposes huge costs to individuals and society[1]. In this study, drug resistance was defined as ≥1 major NRTI or NNRTI resistance mutation and a major mutation defined according to International Antiviral society-USA, 2008 guidelines[1]. Samples with M184V/I were considered to have 3TC and FTC resistance. Some of the NNRTI resistance mutations included: K103N/S, Y181C/I, G190A/S/E, V108I, Y188L, V106M, P225H, and K101E. NRTI mutations included thymidine analogue mutations (TAMs): M41L, L210W, T215Y/F (less forgiving TAM pathway); D67N, K70R, K219Q/E (forgiving TAM pathway); K65R and K70E associated with TDF resistance; L74V associated with ABC and ddI resistance; and

multinucleoside resistance mutations which include 69 insertion and Q151M complex.

5. Genotype: The genotype is the genetic makeup of a cell, an organism, or an individual (i.e. the specific allele makeup of the individual) usually with reference to a specific character under consideration[1].

6. Phenotype: The observable properties of an organism that are produced by the interaction of the genotype and the environment. In case of HIV, the phenotype measures the ability of the virus to grow under different concentration of drugs[1].

7. Wild type HIV virus: This is a virus that has no drug resistance. This virus is stronger and fitter than drug resistant virus and it is the most common form of HIV found in treatment naïve HIV positive individuals. Anything different from it is considered a mutation.[1]

1.0. BACKGROUND

Following the government's efforts and commitment to scale up ART services throughout the country, more people are accessing these services. According to the Ministry of Health, Zambia, approximately 900,000 people were living with HIV as of March 2009. Out of these, approximately 340,000 (37.7%) are in need of life saving drugs (ARVs) but only 231,000 (67.9% of those in need of ART) are so far accessing treatment [2].

This expansion, however, comes with a challenge of having to deal with increasing cases of treatment failure. It is estimated that at least 10% of all patients currently on ART have failed their first line regimen[3] and are in need of second line therapy. In Zambia, like in many other developing countries, ART providers rely on the WHO immunological and clinical criteria for diagnosis and management of treatment failure. This is because viral load monitoring is not widely available in these settings. This presents a challenge in such settings as, in order to maximize the likelihood of durable viral suppression with the 2nd line regimen, the choice of drugs used in this regimen should be based on the resistance patterns that developed with the first line regimen. A good understanding of the likely resistance patterns is therefore vital for one to be able to make a rational choice of the second line regimen. There is a substantial amount of data on the resistance patterns in HIV-1 subtype B, but the little data available to date from countries in sub-Saharan Africa with HIV-1 clade C predominance suggest that there may be some differences in the patterns of resistance between subtype C and subtype B[4,5,6,7,8,9,10,11,12,13,14,15].

1.1. Study justification

Information from various studies on this subject shows that there are differences in the patterns of resistance between HIV subtypes and in particular between subtype C and B:for example, the development of K65R with d4T in subtype C HIV 1, and rapid emergence of K65R with TDF in subtype C compared to subtype B[16,17,18,19].

This serves as evidence that the resistance patterns may be different from region to region and even within the same region from area to area depending on the viral subtype predominant in the area under study. This is particularly of clinical significance because it carries the potential to influence the choice of the first, second line, and other regimens thereafter. Furthermore, while it is widely believed that subtype C is predominant in Zambia, as it is in the rest of the Southern African region, the study cited above by Hamers et al provides evidence that there are other subtypes of HIV1 in Zambia other than subtype C.

2.0. LITERATURE REVIEW

A number of studies looking at genotypic resistance patterns have been carried out in various parts of the world, particularly in the western world where HIV-1 subtype B is predominant. There are a limited number of similar studies in parts of the world where HIV-1 subtype C is predominant, and in the Southern African region in particular. The outcomes of the studies summarized below suggest that there may be some differences in the patterns of resistance between HIV-1 subtype B and HIV-1 subtype C. These differences may particularly be of clinical importance as they may influence future treatment decisions with regards to the choice of drugs to be used either in the first or the subsequent regimens.

A study from Zambia published in 2010 looked at the virologic outcomes in children taking adult fixed dose combination of stavudine, lamivudine and nevirapine (Triomune® 30) [8]. In this study, 103 children were followed up for a period of 6 to 36 months. Viral load monitoring was done every 6months and genotyping was done on those whose viral load was found to be above 1000 copies/ml. It was found that 69% (n=77) achieved viral suppression at 24 months, while 31% (n=26) had viral load greater than 1000 copies/ml. Of those with virologic failure, 21% had extensive NNRTI and Lamivudine resistance; 8% had Q151M, which confers multinucleoside resistance; and, strikingly, 12% of failing patients had either K65R, L74V, or K70E, mutations which are not typically selected for by d4T with subtype B. Extensive

resistance accumulated in spite of the fact that these children were being monitored with routine viral loads. The picture is likely to be much worse where routine viral load testing is not being done.

In a systematic review of evidence [16](1996-2008) which looked at the differences in resistance mutations among HIV-1 non-subtype B infections, it was noted that while most major resistance mutations in subtype B were also found in non-B subtypes, a few novel mutations in non-B subtypes were recognized.
The main differences were as follows:

i. The non-nucleoside reverse transcriptase inhibitor resistance mutation, V106M, has been seen in subtype C and CRF01_AE, but not in subtype B;
ii. The protease inhibitor mutations L89I/V have been reported in C, F and G subtypes, but not in B;
iii. Nelfinavir predominantly selected for a non-D30N containing pathway in CRF01_AE and CRF02_AG, while the emergence of D30N is favoured in subtypes B and D;
iv. Studies on thymidine analogue-treated subtype C infections from South Africa, Botswana and Malawi have reported a higher frequency of the K65R resistance mutation than that typically seen with subtype B.

Additionally, some substitutions that seemed to impact non-B viruses differentially are: reverse transcriptase mutations G196E, A98G/S, and V75M; and protease mutations M89I/V and I93L. (I93L is a secondary resistance mutation in subtype B HIV-1, but causes hyper-susceptibility to PI in subtype C). The authors concluded that these observed differences in resistance pathways may impact cross-resistance and the selection of second-line regimens with protease inhibitors; and that attention to newer drug combinations, as well as baseline genotyping of non-B isolates, in well-designed longitudinal studies with long duration of follow up are needed.

In the South Africa Resistance Cohort Study (SARCS) [17], a cross-sectional observational study, 141 patients failing first-line regimen were recruited and evaluated for resistance and it was found that:

i. Resistance mutations affecting more than one drug class were commonly found in treatment failure
ii. HIV-1 RNA > 300,000 copies/mL was a marker of non-adherence associated with less drug resistance at time of failure
iii. Drug resistance was associated with:
    - Recent opportunistic infection (OI)
    - World Health Organization (WHO) stage IV disease

Lamivudine and efavirenz resistance was most common in a population with few patients on PIs. However, the study was limited by the lack of a genotypic resistance algorithm specific to HIV-1 subtype C and there was no comparison to patients with virologic suppression.

A prospective observational study was carried out in Malawi between December 2005 and June 2007[18]. This study evaluated resistance mutations present in 96 Malawians initiating second-line therapy following failure of first-line regimen. It was noted that Malawians failing first-line therapy according to immunologic/clinical criteria are found to have extensive antiretroviral resistance. Genotype/phenotype testing showed that:

- 17% were predicted to have no active NRTIs
- 22% to 50% were predicted to have no fully active drugs in second-line regimen, depending on NRTI backbone selected
- K65R mutation was seen in some patients failing on d4T-containing regimens

It was also observed in this study that inclusion of zidovudine in the first-line regimen had a protective effect against the emergence of tenofovir and pan-NRTI resistance mutations, but that the risk for thymidine analogue mutations (TAMs) was elevated.

There were similar findings in Botswana where K65R was observed in increased frequency in patients failing didanosine and stavudine containing backbones in HIV1 subtype C compared to subtype B.

The data from the studies cited above suggest that the amount of knowledge in the treatment of HIV infection that we have so far, which is mainly from studies on HIV-1 subtype B, may not be completely generalizable to all subtypes. Given the potential for differences in the selection of ART resistance between subtypes, and the impact that these differences could have on the efficacy of 2nd-line therapy and beyond, additional studies in these area are needed. The table below gives a summary of the common resistance mutations selected for by the drugs commonly used as first line in Zambia.

Table 1-Review of resistance mutations selected for by drugs commonly used as first-line art regimen in zambia.[1, 20, 21, 22, 23, 24, 25]

CLASS	DRUG	Common major mutations
NRTI	3TC	M184V
	FTC	M184V
	D4T	M41L, D67N, K7OR, L210W, T215Y/F, K219Q/E, 69ins, Q151M, K65R
	AZT	M41L, D67N, K7OR, L210W, T215Y/F, K219Q/E, 69ins, Q151M
	TDF	K65R, K70E
	ABC	L74V
	DDI	L74V
NNRTI	NVP	L100I, K101P, K103N/S, V106M, V108I, Y181C/I, Y188C/L/H,G190A
	EFV	L100I, K101P, K103N/S, V106M, V108I, Y181C/I, Y188L, G190S/A, P225H

Comment:

- 69ins confers resistance to all NRTIs
- Q151M complex confers resistance to all NRTIs except TDF
- The presence of 3 TAMs or more inclusive of either M41L or L210W also confers resistance to TDF
- M184V with K65R or M184V with TAMS also confer resistance to ABC and DDI
- The presence of M41L, D67N, L210W, T215Y/F, K219Q/E confers resistance to DDI
- Y181C/I reduces viral susceptibility to ETV. The presence of L100I and K101P with other NNRTI mutations also confers resistance to ETV.

3.0. HYPOTHESIS

The working hypothesis was that the resistance patterns selected by first-line ART regimens in Zambia, where HIV 1 subtype C is predominant, would differ from those commonly observed in HIV 1 subtype B.

4.0. OBJECTIVES

4.1. General objective

The objective of this study was to evaluate the pattern of resistance in patients failing the first line regimen in Zambia.

4.2. Specific objectives

This study was to investigate:

1. Patterns of resistance exhibited under the pressure of the commonly used 1st line regimens in Zambia
2. Prevalence of mutations overall and by class.
3. Risk factors for resistance
4. HIV subtypes present in Zambia and their frequency.

5.0. STUDY DESIGN AND METHODOLOGY

The study was a retrospective, descriptive analysis. It was carried out in Lusaka at the University Teaching Hospital (UTH), Chreso clinic and Circle of hope clinic.

Permission was sought from these health facilities to collect data from their medical records. The principal investigator collected data from the participant's charts and recorded it directly on the data collection sheet which was kept in the password protected computer folder.

5.1. Participants:

All patients with available genotypes within the specified time frame who met the inclusion criteria were considered for inclusion. These patients were drawn from UTH-Pediatrics department, Chreso, and Circle of Hope clinics in Lusaka.

5.2. Sampling:

All available genotypes that were done from 2002, the time ARV's were introduced in public health sector, to 31st December 2010 were screened for inclusion. Purposive sampling method was used and the target sample size of 393 was calculated using the Yamane formula with the level of precision set at 0.05.

5.3. Inclusion criteria

- HIV positive
- On therapy for $\geq$ 24 weeks.
- Has failed the first-line regimen. (Failure being defined as having the viral load of more than 1000 after 6 months on treatment and first-line regimen being defined as NNRTI based HAART regimen according to 2004 and 2007 National guidelines).
- Genotype done prior to switching to second-line regimen.
- There was no age restriction

5.4. Exclusion criteria

- On therapy for ≤24 weeks
- Genotype done on failing 2nd line regimen (Second line regimen being defined according to 2004 and 2007 guidelines for patients whose first regimen was changed due to failure)
- Regimens outside the guidelines
- Genotypes whose corresponding patients records (files) were missing were not included in the main analysis. These genotypes however, were included in the determination of the overall frequency of specific mutations in the population.

5.5. Variables

5.5.1. Independent

- Age
- Sex
- Prior history of ARV exposure
- Failed regimen
- Duration of therapy (time to failure)
- CD4+ cell count (at baseline, peak and at failure)
- WHO disease stage (Pre-HAART and T-staging at failure)
- Viral load
- Viral subtype
- Provider's reason for failure
- OI history

5.5.2. Dependent

- Patient's genotype

5.6. Statistical analysis

- SPSS version 17 was used to analyze data
- Simple descriptive statistics included means, median and range. Chi-square method was applied to determine association as required.
- The prevalence of resistance mutations was analysed overall and by class.

5.7. Ethical considerations

De-identification of patients was achieved by assigning a study number to each patient's file. Other patient's identifiers (e.g.: file number, patient's name) were kept separately in a password-protected computer file. These will be kept for a period of at least five years after which they will be destroyed. This is to allow for all queries that may arise at the end of the study to be answered. Ethical approval was sought from the University of Zambia Biomedical Research Ethics Committee. A waiver for doing the study without patient's consent was requested and granted based on the following:

- This study was only to involve a retrospective chart review
- The research involved no more than minimal risk to the participants; there was no patient interaction, i.e. the data review did not include any direct interaction between the researcher and patients.

6.0. RESULTS

Of 174 genotypes that were done between 2002 and December 2010 in the 3 study sites, 48 were done on failing PI-based regimen and were therefore excluded while 126 met the inclusion criteria for the study and were considered for analysis. 24 were found to be wild type (19%), while 102 (81%) had at least one significant mutation. 92% of 126 genotypes that were analyzed were obtained from pediatric patients while only 8% were from adult patients. 54.8% were from female patients while 45.2% were from male patients (Table 2)

It is important to note that of the 24 wild type genotypes, only 2 of them (8.3%) were from adult while the remaining 22 (91.7%) from children. All the wild type genotypes had viral loads above 100,000 copies per ml. A further analysis of the 102 genotypes that were found to have at least one mutation was done and it was found that the mean treatment duration before genotyping was 39.7 months, the median 39 months while the range was 73 months (11-84months). A closer look at the duration of treatment revealed that 88.2%of patients took medication for 24 to 60 months before a genotype was done; the majority of these (44.1%) took medication for 36 to 47 months before genotyping. Only 5% of patients took medication for more than 60 months before a genotype was done. (Table 2)

Of the few patients that had their WHO staging recorded in their files (45.1% initial and 52.9% at failure), only 37% of them were asymptomatic at failure compared to 57.8% at initiation. P=0.083. 42.6% of those that are symptomatic at failure were in WHO stage 3 or 4 compared to 26.6% in the same stages at initiation P=0.098. (Table 2)

Table 2: Baseline characteristics

Characteristic	Frequency	Percent
Grouped Age (in years)		
Pediatrics (≤15)	116	92
Adults (>15)	10	8
Sex		
Female	69	54.8
Male	57	45.2
Duration of treatment for the 102 who had at least 1 mutation		
>6-<12 months	2	2.
12-23 months	4	3.9
24-35 months	25	24.5
36-47 months	41	40.2
48-60 months	16	15.7
>60 months	5	4.9
Missing	9	8.8
WHO Initial		
WHO stage 1	27	26.5
WHO stage 2	7	6.8
WHO stage 3	6	5.9
WHO stage 4	6	5.9
Missing	56	54.9
WHO at failure		
WHO stage 1	20	19.6
WHO stage 2	11	10.8
WHO stage 3	8	7.8
WHO stage 4	15	14.7
Missing	48	47.1
Genotype		
1TAM	17	16.7

Characteristic	Frequency	Percent
2TAM	19	18.6
≥3TAM	26	25.5
Other mutations	40	39.2

Only 41 patients (40%) had their CD4 initial documented ; of those, the mean was 315.9 cells/mm^3, and the median was 241 cell/mm^3;48 patients (47%) had their CD4 peak documented in the patient's file with a mean of 662.6 cells/ mm^3 and a median of 563 cells/ mm^3; while at failure, 51 patients (50%) had their CD4 documented in the patient's file with the mean of 378.8 cells/ mm^3 and the median of 316 cells/ mm^3.See table 3 below.

Table 3:CD4 values

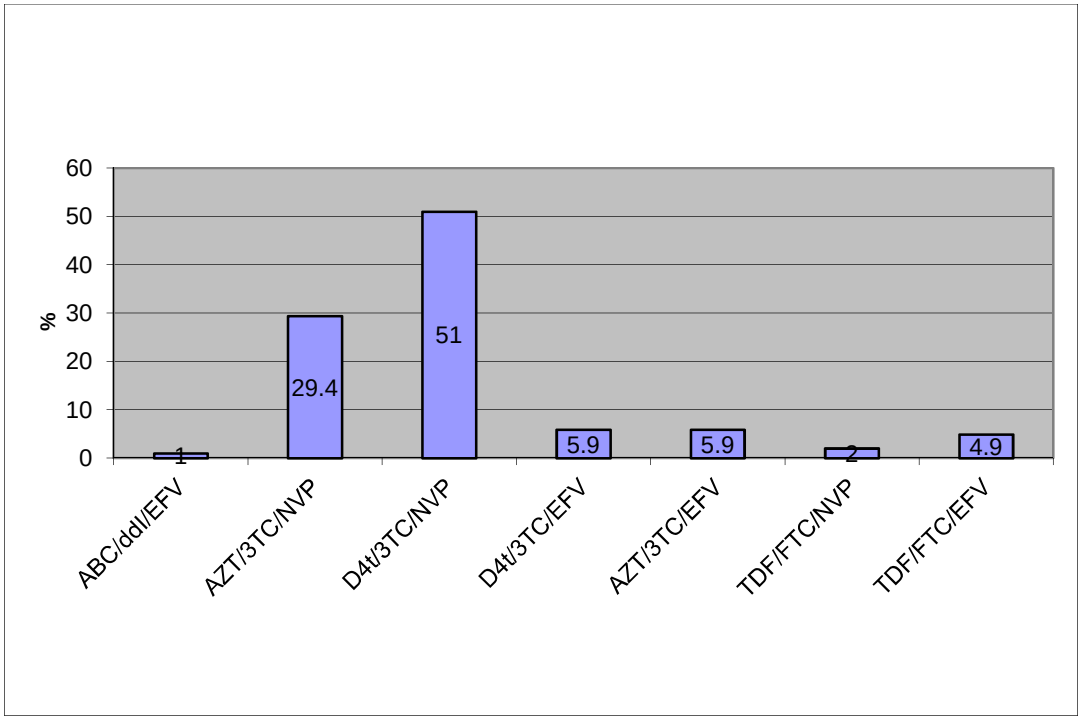
	CD4 Initial	CD4 Peak	CD4 at failure
Mean	315.98	662.58	378.78
Median	241.00	562.50	316.00
Range	1755(4-1759)	1679(49-1728)	1375(6-1381)

According to WHO staging of HIV disease, more patients were found to be symptomatic at failure, that is WHO stage 2 or higher (63%) compared to the time of initiation (42.2%), though this was not statistically significant(P=0.32). See table 2 above

The average viral load at failure was 37,464.2copies/ml, with a median of 20,547copies/ml and a range of 1100-432124 copies/ml. It is important to note that all those that were found to have a wild type genotype had a viral load of above 150, 000 copies/ml.

The majority of patients (92.2%) were on d4T (62%) or AZT (38%) -based regimens; whereas 6.8% were on TDF based and only 1% were on ABC based regimen. See figure 1 below.

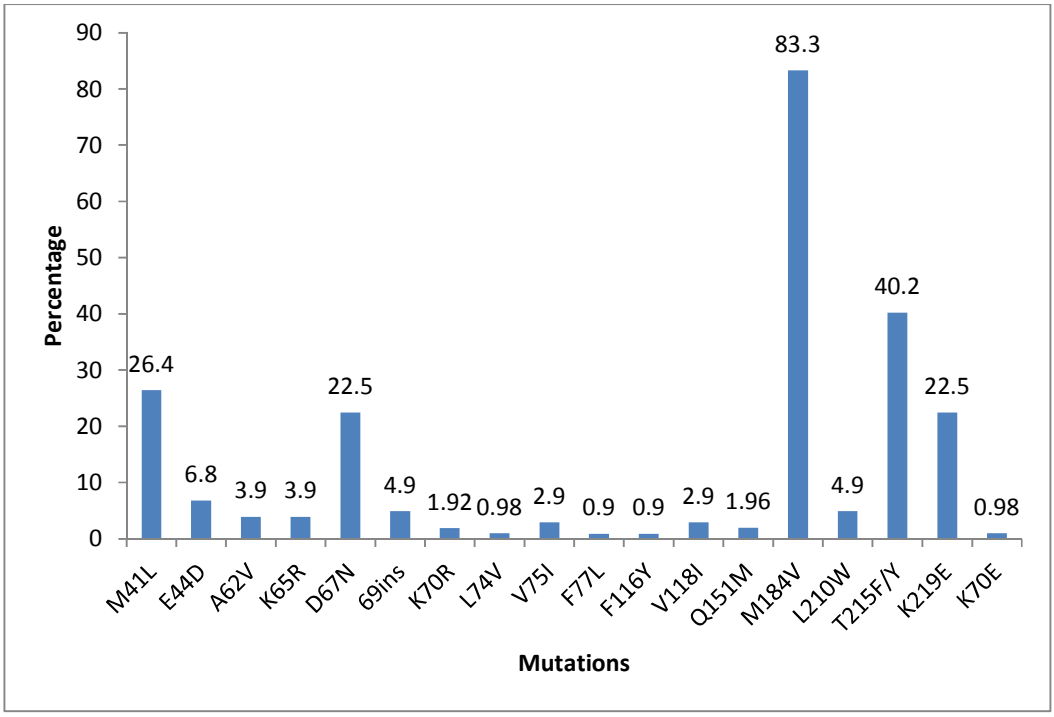
Figure 1: Frequency by regimen



M184I/V mutation was the most common NRTI-associated mutation (83.3%), followed by the thymidine analogue mutations (TAMs) with M41L (26.4%), D67N (22.5%), K70R (20.5%), T215F/Y/C/D/E/V (40.2%), K219E/Q/N/R (22.5%) having the highest frequency. K65R mutation was found in only four patients (3.9%), including one patient failing on AZT and another failing on d4T.

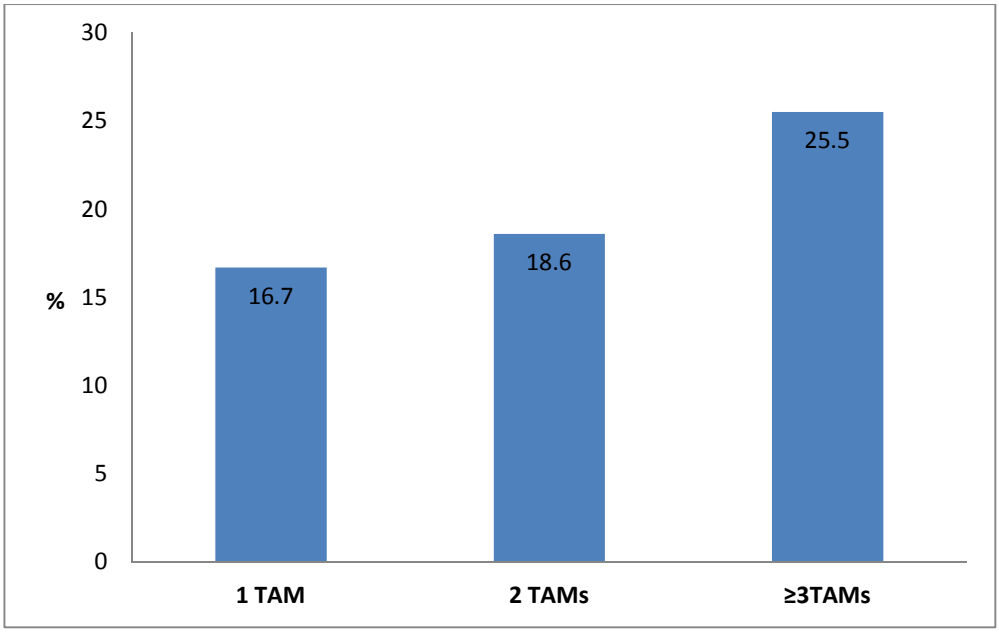
Other important mutations were the pan-NRTI mutations which confer resistance to all the NRTIs, particularly Q151M complex (2.9%) and the 69insertion (1%). The presence of pan-NRTI resistance mutations was associated with a viral load of above 10,000 (*p=0.041)*. Others were A62V (3.9%), V75I (2.9%) and F77L (0.9%). See figure 2 below.

Figure 2: NRTI Resistance Mutations



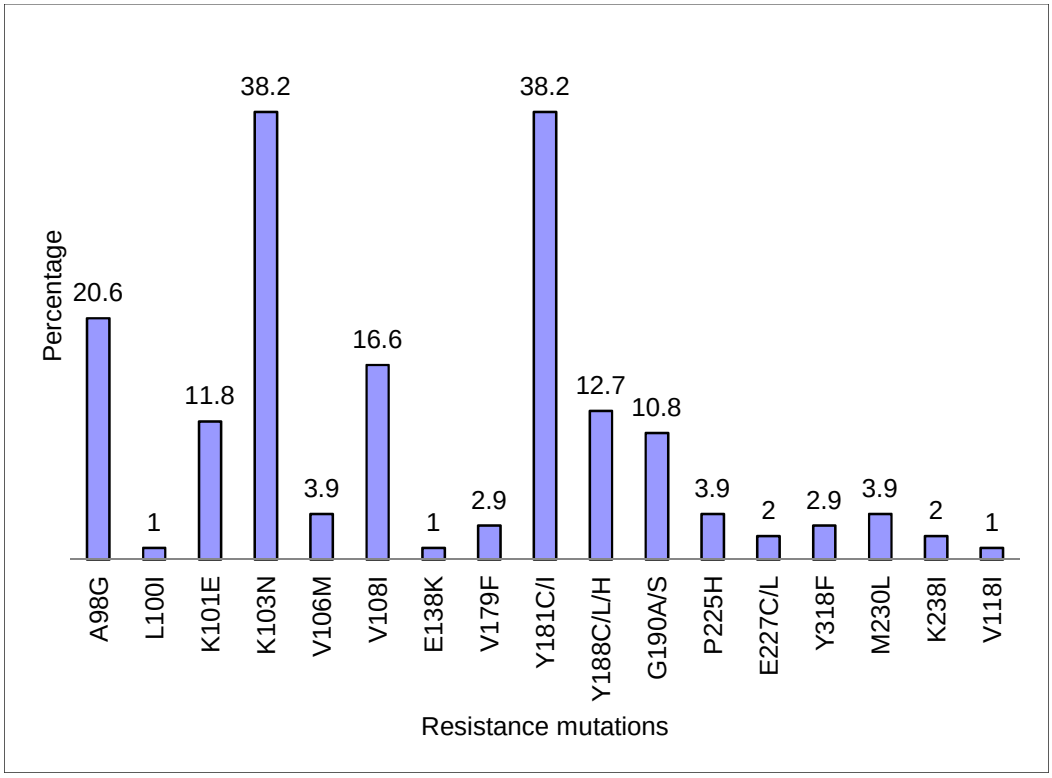
Among patients with TAMs, 16.7% had 1TAM; 18.2% had 2 TAMs and 25.5% had ≥3 TAMs. See figure 3 below.

Figure 3: Frequency of TAMs



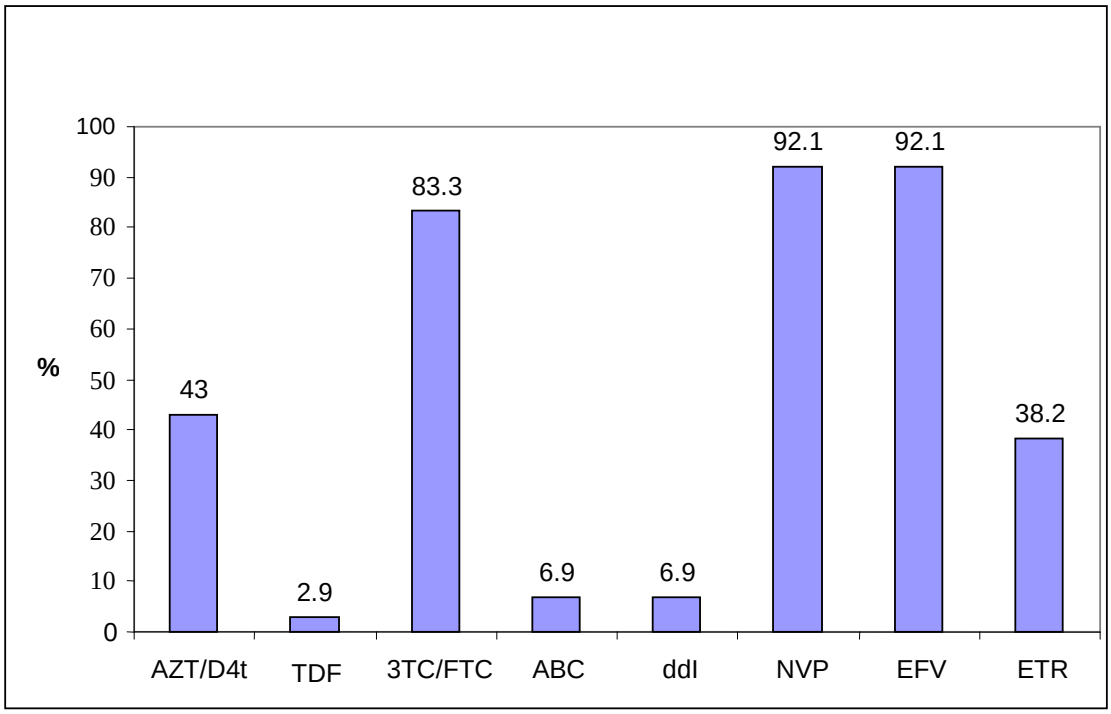
Common NNRTI-associated mutations included K103N/H/S/T (38.2%), Y181C/I/V (38.2%), A98G (20.6%), V108I (16.6%), Y188C/L (12.7%), K101E/P (11.8%), G190S/V (10.8%); others were P225H (3.9%), M230L (3.9%). Of note is the presence of V106M mutation (3.9%) which is relatively uncommon in HIV subtype B[20]. See figure 4 below.

Figure 4: NNRTI Resistance Mutations



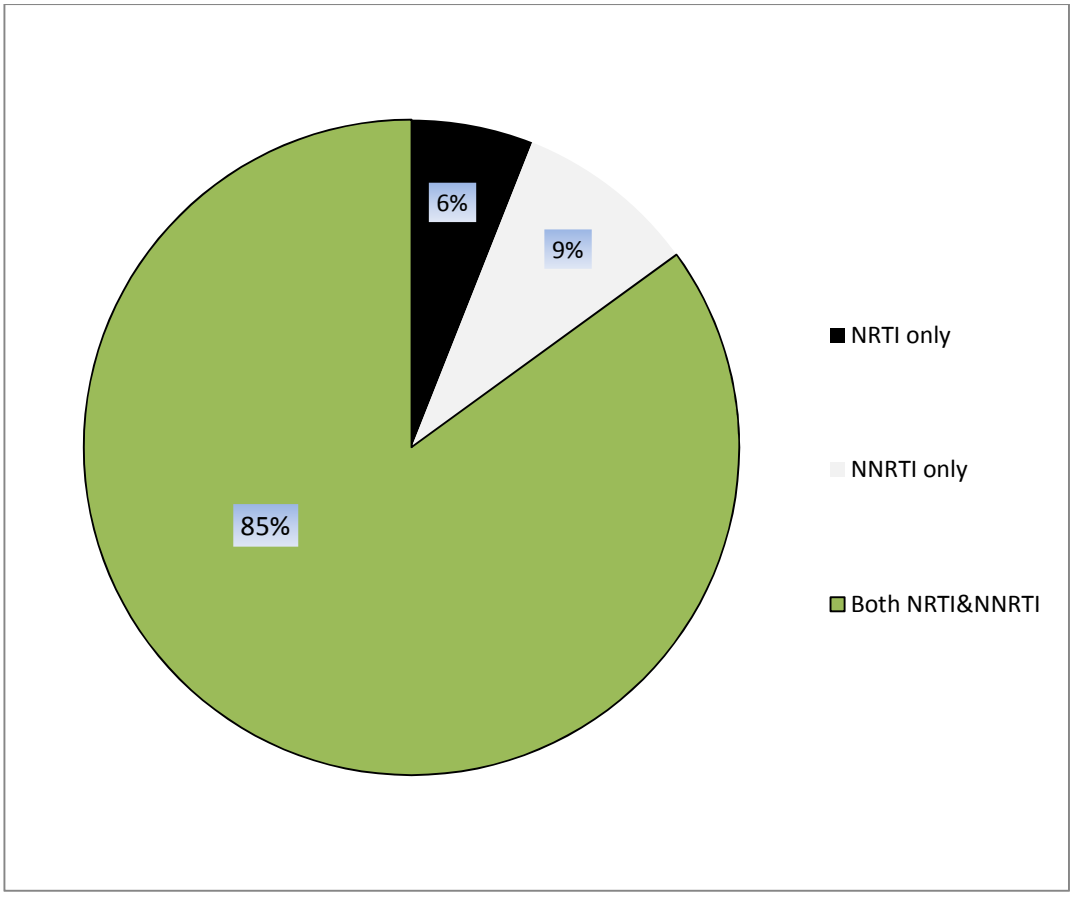
Going by the drugs, viral resistance was highest to NVP and EFV at 92.1%, followed by 3TC/FTC at 83.3, then AZT/D4T 43%, with ETR at 38.2%. See figure 5 below.

Figure 5: Resistance by drug



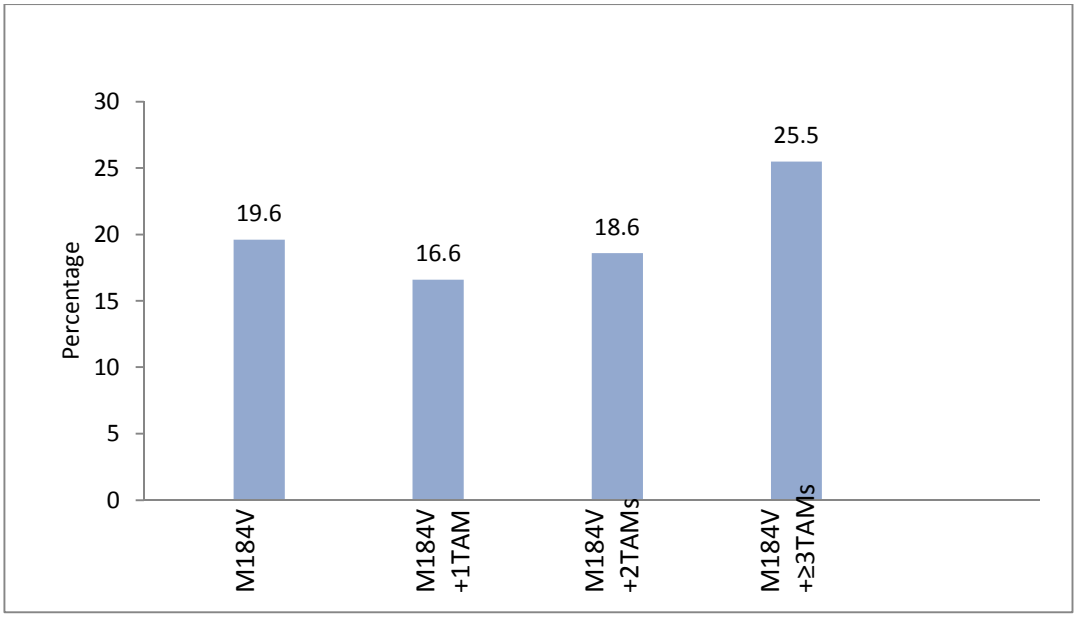
`The majority of patients (85%) had both NRTI and NNRTI–associated resistance mutations at the time of genotyping. See figure 6 below

Figure 6: Frequency of Mutations by Class



More than 60% of patients had a combination of M184V with TAMs. This is a somewhat dangerous combination as it has the potential to affect other NRTIs such as TDF, ABC and ddI. See figure 7 below.

Figure 7: Presence of M184V with TAMs



HIV-1 infection has a global distribution but specific subtypes of HV-1 tend to have specific regional predominance. It is however difficult to predict the subtypes present in a particular area apart from the one expected to be predominant in that particular area and this is largely due to the geo-demographic and socio-economic dynamics of human population. In this study, subtype C was, as expected, found to be the most prevalent at 95.2%. Others were subtype B/C (2.4%), subtype D/C (1.6%), and one patient (0.8%) had subtype B. See table 4 below.

Table 4: Frequency of Subtypes Observed

Subtype	Frequency	Percentage
C/C	120	95.2
B/C	3	2.4
D/C	2	1.6
B/B	1	0.8

There was no difference observed in the pattern of resistance in the different subtypes and recombinant forms of HIV that were found in this study.

The suspected reason for failure was documented only in five patient's files (4%) and all of them cited 'poor adherence'.

Risk factors for resistance: We analyzed multiple variables in attempt to identify correlates of resistance, including WHO stage, CD4 cell count, viral load, OI history, regimen type, duration of regimen, sex and age. Viral loads at failure greater than 150 000 copies/ml were significantly found to be associated with the development of both NRTI and NNRTI resistance mutation (P=0.042). Age less than 15 years and the use of D4T+3TC+NVP (Triomune) also seemed to be statistically significant (P=0.033 and P=0.041 respectively). See summary in table 5 below.

Table 5: Possible Risk Factors to Resistance

Dependent Variable	Independent Variables	Score/strength of a cell	P value
Having both NRTI and NNRTI mutations	Age		
	≤15	4.525	0.033
	>15	0.876	0.759
	Sex		
	Male	0.008	0.929
	Female	0.009	0.876
	CD4 initial		
	<200	1.645	0.649
	200 - 350	0.008	0.929
	351 - 500	1.346	0.246
	>500	0.100	0.752
	CD4 Peak		
	<200	2.904	0.407
	200 - 350	0.100	0.752
	351 - 500	1.346	0.246
	>500	0.623	0.430
	CD4 at failure		
	<200	3.629	0.304
	200 - 350	2.433	0.119
	351 - 500	0.310	0.577
	>500	0.220	0.639
	WHO initial		
	I	2.409	0.492
	II	0.083	0.774
	III	0.310	0.577
	IV	0.310	0.577

Dependent Variable	Independent Variables	Score/strength of a cell	P value
Having both NRTI and NNRTI mutations	WHO at failure		
	I	2.323	0.508
	II	0.075	0.784
	III	1.353	0.245
	IV	0.655	0.418
	Used DDI+ABC+EFV	2.974	0.085
	Used AZT+3TC+NVP	2.143	0.143
	Used D4T+3TC+NVP	4.194	0.041
	Used D4T+3TC+EFV	0.100	0.752
	Used AZT+3TC+EFV	0.001	0.972
	TDF+FTC+EFV	0.655	0.418
	Viral load ≤150 000 copies/ml	0.876	0.387
	Viral load >150 000 copies/ml	4.087	0.0421
	Duration of treatment (months)		
	6-11	0.606	0.354
	12-23	0.411	0.768
	24-35	1.662	0.221
	36-47	0.456	0.260
	48-60	0.178	0.138
	>60	0.156	0.119

7.0. DISCUSSION

In this retrospective descriptive analysis, the vast majority of patients in Zambia with confirmed virologic failure on their first line regimen have evidence of drug resistance. Mutation M184V was found to be the most common. This mutation is commonly selected for by either 3TC or FTC, and one of these two drugs is always part of the NRTI backbone of either the first or the second line regimen. This finding also agrees with a number of studies[6,11,12,17,18] that found that mutation M184V is the first mutation to emerge in a patient on a regimen containing either 3TC or FTC in case of suboptimal adherence.

The thymidine analogue mutations (TAMs) were also found to be very common. These mutations are selected for by d4T and AZT and their high frequency in this study can be attributed to the fact that the majority of patients in this study were on a regimen containing either d4T or AZT. This is because most genotypes that were analyzed were from pediatric patients, and according to the Zambian pediatric ART guidelines that were in use before the year 2010, d4T or AZT based regimens (d4Tor AZT/3TC/NVP or EFV) were the most preferred as first line. The fact that most patients with TAMs had at least 2 TAMs may be explained by the lack of routine viral load monitoring, leading to patients being kept on a failing regimen for longer periods. This probably led to accumulation of TAMs prior to switching to second line regimen. Given that multiple TAMs may lead to cross-resistance to all NRTIs, including TDF and ABC, this has significant implications for the likely efficacy of TDF or ABC-based 2nd line regimens. There was, however, no clear pattern of resistance mutations selected for by those who were either taking AZT or those were on d4T. The pan-NRTI resistance mutations, Q151M complex and 69 ins, usually develop when a patient is kept on a failing regimen containing either d4T or AZT for a long period of time mainly due to non availability of routine viral load monitoring. Their presence is usually associated with increased viral loads; in this study, they were associated with a viral load of above 10,000, and this is consistent with the findings of the study in Malawi[6].

TDF-associated resistance mutations were relatively uncommon, with K65R mutation found in 4 patients; two for these were adults on TDF, and one pediatric patient on d4T, a finding consistent with findings in other studies in HIV-1 subtype C[6, 11, 12].The forth was a pediatric patient who had been taking AZT; this was rather an unexpected finding as it is the first time, to our knowledge, that AZT was found to be associated with K65R mutation. This finding also goes against the knowledge that K65R makes the virus hyper-susceptible to AZT making its selection under AZT pressure less or not beneficial at all to the virus. A possible explanation to this however, is the possibility of transmitted resistance: i.e., a mother who may have had failed on a regimen containing either TDF or D4T could have transmitted the resistant virus with K65R mutation to the child. This unfortunately could not be verified because the health records of the mother to this child could not be traced.

The finding of significant cross-NRTI resistance, due to the presence of M184V plus > 2 TAMs and/or a pan-nucleoside mutation, is concerning. Although mutations selected by ABC, ddI and TDF were relatively uncommon, a number of patients would be predicted to have only partial activity from these drugs due to the frequency of mutations that lead to NRTI-cross resistance.

The presence of high level resistance to NVP, EFV and to some extent ETR implies that ETR will be of limited use in subsequent regimens in a set up where genotyping is not routinely done for patients failing on the NNRTI based first line ART regimen. Its use in the second or third line regimen should always be guided by a genotype done at the time of failure on an NNRTI based regimen. If genotyping is done after the patient has been off NNRTI-based regimen for some time like is the case here in Zambia where genotyping is done after second line failure, (i.e. after failing on PI based regimen), the chances of not finding NNRTI mutations are high as these mutations may be archived with time. If genotyping is not available, or, in situations like here in Zambia where most patients have been exposed to NNRTIs in their first line regimen and PIs in their second line regimen, ETR use as a component in the third line regimen should always be guided by routine viral load monitoring to assess response. The high prevalence of Y181C and K103N mutations however, could also

be attributed to possible vertical transmission as these mutations are known to persist for a long time before they get archived even without NNRTI pressure.[18]

The finding of a significant number of patients with wild type suggest that these patients were not adherent to their treatment i.e. they were not taking their drugs correctly. This could indeed be due to poor adherence as children usually depend on their guardians for their medications; but the other explanation to this could be that these children were possibly taking suboptimal doses of medication due to difficulties associated with upward adjustments of the pediatric doses as children grow up. This may be quite a challenge to many guardians in low socio-economic set up like ours particularly when it comes to handling syrups. These two possible explanations may also justify the observed relatively short stay on the first line regimen by the majority of patients observed. This however contrasts the finding of extensive resistance that was observed which tends to suggest that patients were kept on a failing first line regimen for so long before changing them to second line regimen. Although it was not statistically significant, there was a trend toward higher WHO stage at failure and this trend suggest that clinicians may be relying more on clinical rather than immunologic criteria for detecting failure. One would therefore deduce that a good number of patients may have had taken medications for a much shorter duration before they begun to fail; hence the possibility of transmitted resistance cannot be ruled out.

An attempt to evaluate the risk factors to resistance was made where various independent variables were analyzed for the relationship with having both NRTI and NNRTI resistance mutations. The observed significant relationship between higher viral loads at failure (>15,000 copies/ml) and the development of both NRTI and NNRTI resistance mutations in a client could probably be an indication that patients were kept on failing regimen for so long before changing to second line regimen However, the seemingly significant relationships observed between the age (≤15 years) as well as the use of triomune and the development of both NRTI and NNRTI resistance mutations could be attributed to the fact that the majority of patients in this

study were of pediatric age who took Triomune as their first line regimen as per pediatric ART guidelines that were in place before 2010. There was therefore a bias towards these two variables, hence the observed significance. Other variables were weak in terms of frequency owing primarily to the poor documentation as well as a relatively small sample size.

8.0. STUDY LIMITATIONS

Genotyping is not routinely done in patients failing the first line ART regimen here in Zambia, and the sample size was calculated on assumption that genotyping is done for every patient who is failing on first line before changing to the second line regimen. It was therefore difficult to reach the target sample size in our setting. This reduced the power of the study and some of its objectives could not be conclusively met. Other limitations include:

- This was a retrospective study design.
- This was largely a pediatric study although some studies reported no difference in the patterns of resistance between adults and pediatric patients[16]. The next step is to do a similar study in adults.
- The samples were run from different laboratories with different personnel and instruments.
- Patients getting GTs had access to sites with more resources. This means that these findings may not be generalizable to the entire population. In fact, one could postulate that the problem is likely to be much worse in the general population where access to viral loads and genotyping is extremely limited.
- Phenotyping was not done. This could have helped us know the degree of resistance the virus developed against a specific drug.
- Patients were seen by different providers and therefore the documentation was different

9.0. CONCLUSIONS AND RECOMMENDATIONS

Although the genotypic resistance patterns that were observed in this study under predominantly HIV-1 subtype C were not different from those that were observed in other studies that were carried out with other subtypes, inter-subtype variability may require routine viral load or genotyping if second line options are to be preserved especially if thymidine analogues are used in the first line regimen. This is particularly of significance in HIV-1 subtype C because of its observed ability to unpredictably select for K65Runder pressure of the thymidine analogues (d4T in particular), and an extensive NRTI resistance that was observed in this largely pediatric study. Very few patients were on TDF in this study and even those on d4T did not seem to have many TDF mutations, unlike what was found in the Malawi study.

The findings in this study suggest that TDF or ABC would be a better choice for use as part of first line NRTI backbone as this is likely to preserve future NRTI treatment options. ETR use after NNRTI failure is to be guided by a genotype and routine viral load monitoring. Where routine genotyping is not available, the author would recommend a trial of intensified adherence counseling over 3 months for those with a viral load of over 150,000 copies / ml to see if they suppress before switching to second line regimen. This is because of a significant number of wild type genotypes that were observed in this study.

In summary, while the current study provides valuable information about resistance patterns that are likely to emerge in pediatric patients failing on the most common first-line regimens used in Zambia, additional investigation may be needed to evaluate the generalizability of these findings to adults as well as to children on non-thymidine based first-line ART. this will also help confirm the findings that were different from those seen in published studies to date (e.g., very low frequency of TDF-associated mutations, selection of K65R by AZT, frequency of non-C subtypes, etc.).

10.0. REFERENCES

1. Hirsch MS, Schapiro JM, Brun-Ve´zinet F, Clotet B, Hammer SM, Johnson VA, et al. **Antiretroviral Drug Testing in AdultsHIV1 Infection: 2008 Recommendations of an International AIDS Society-USA Panel**. Clin Infect 2008;47:266-285
2. MoH-Zambia Programmatic data-Personal communication with Dr Peter Mumba, Dr Izukanji Sikazwe and Dr Crispin Moyo
3. AIDSRelief Zambia programmatic data-Personal communication with Dr Chris Bositis
4. Brenner BG, Oliveira M, Doualla-Bell F, Moisi DD, Ntemgwa M, Frankel F, et al**. HIV 1 subtype C viruses rapidly develop K65R resistance to tenofovir in cell culture**, AIDS 2006; F9-13
5. Sung H, Jung YS, Kang MW, Bae IG, Chang H, Woo JH, et al. **High frequency of drug resistance mutations in human immunodeficiency virus type-1 infected Korean patients treated with HAART**. AIDS 2007, 23(10): 1223-1229
6. Kamya MR, Mayanja-Kizza H, Kambugu A, Bakeera-Kitaka S, Semitala F, Mwebaze-Songa P, et al. **Predictors of long-term viral failure among Ugandan children and adults treated with antiretroviral therapy**.JAIDS 2007, 46(2): 187-193
7. Johnson VA, Brun-Vézinet F, Clotet B, Conway B, Kuritzkes BR, Pillay D, et al. **Update on the drug resistance mutations in HIV-1: Fall 2005.** Top HIV Med. 2005;13:125-131
8. Ravindraa KG, Ford D, Mulenga V, Walker AS,Kabamba D,Kalumbi M, et al. **Drug resistance in Human Immunodeficiency virus type-1 infected Zambian children using adult fixed dose combination of Stavudine, Lamivudine and Nevirapine**. Ped Infect J 2010;29: e57-e62
9. Hamers RL, Siwale M, Wallis C, Labib M, van Hasselt R, Stevens W, et al. **HIV 1 Drug Resistance Mutations are present in six percent of persons**

initiating Antiretroviral Therapy in Lusaka, Zambia. J Acquir Immune DeficSyndr 2010; 55:95-101

10. Singh A, Sunpath H, Green TN, Padayachi N, Hiramen K, Lie Y, et al. **Drug resistance and viral tropism in HIV-1 subtype C-infected patients in KwaZulu-Natal, South Africa: implications for future treatment options.** J Acquir Immune DeficSyndr. 2011 Nov 1; 58(3):233-40.
11. Chilton DN, Castro H, Lattimore S, Harrison LJ, Fearnhill E, Delpech V, et al**. Drug resistance in antiretroviral treatment-naive adults infected with non-B subtype virus in the United Kingdom**. AntivirTher. 2010;15(7):985-91
12. Wallis CL, Erasmus L, Varughese S, Ndiweni D, Stevens WS. **Emergence of drug resistance in HIV-1 subtype C infected children failing the South African national antiretroviral roll-out program.** Pediatr Infect Dis J. 2009 Dec; 28(12):1123-5.
13. Sen S, Tripathy SP, Patil AA, Chimanpure VM, Paranjape RS. **High prevalence of human immunodeficiency virus type 1 drug resistance mutations in antiretroviral treatment-experienced patients from Pune, India**. AIDS Res Hum Retroviruses. 2007 Oct;23(10):1303-8
14. Montes B, Vergne L, Peeters M, Reynes J, Delaporte E, Segondy M. **Comparison drug resistance mutations and their interpretation in patients infected with non-B HIV-1 variants and matched patients infected with HIV-1 subtype B**. J Acquir Immune DeficSyndr. 2004 Apr 1; 35(4):329-36.
15. Lwembe R, Ochieng W, Panikulam A, Mongoina CO, Palakudy T, Koizumi Y, et al. **Anti-retroviral drug resistance-associated mutations amongnon-subtype B HIV-1-infected Kenyan children with treatment failure**. J Med Virol.2007 Jul; 79(7):865-72.
16. Martinez-Cajas JL, Pai NP, Klein MB and Wainberg MA. **Differences in resistance mutations among HIV-1 non-subtype B infections: a systematic review of evidence (1996-2008)**. I AIDS 2009, 12:11 doi:10.1186/1758-2652-12-11

17. Marconi V, Sunpath H, Lu Z, Gordon M, Koranteng K, Hampton J, et al. **Prevalence of HIV-1 drug resistance after virologic failure of first HAART regimen in South Africa: initial results of the South Africa resistance cohort study.** Clin. Infect. Dis 2008; 46(10): 1589-97
18. Hosseinipour MC, van Oosterhout JJG, Weigel R, Phiri S, Kamwendo D, Parkin N, et al. **The Public Health approach to identify antiretroviral therapy failure: high-level nucleoside reverse transcriptase inhibitor resistance among Malawians failing first-line antiretroviral therapy**. AIDS 2009, 23 (9): 1127-1134.
19. Doualla-Bell F, Avalos A, Brenner B, Gaolathe T, Mine M, Gaseitsiwe S, et al. **High prevalence of K65R mutation in HIV-1 subtype C isolates from infected patients in Botswana treated with didanosine based regimens**. Antimicrob Agents Chemother 2006; 50:4182-5
20. Liu T F, Shafer R W. **Web Resources for HIV Type 1 Genotypic-Resistance Test Interpretation**. Clin Infect Dis. 2006 June 1; 42(11): 1608–1618.
21. Grossman Z, Istomin V, Averbuch D, Lorber M, Risenberg K, Levi I, et al. **Genetic variation at NNRTI resistance-associated positions in patients infected withHIV-1 subtype C**. AIDS. 2004 Apr 9; 18(6):909-15.
22. Rhee S, Gonzales M J, Kantor R, Betts B J., Ravela J, and. Shafer R W (2003**). Human immunodeficiency virus reverse transcriptase and protease sequence database**. Nucleic Acids Research, 31(1), 298-303.
23. Shafer R W (2006). **Rationale and Uses of a Public HIV Drug-Resistance Database**. J. Infect. Dis. 194 Suppl 1:S51-8
24. Rhee SY, Kantor R, Katzenstein DA, Camacho R, Morris L, Sirivichayakul S. **HIV-1 pol mutation frequency by subtype and treatment experience: extension of the HIVseq program to seven non-B subtypes.** AIDS 20(5):643-651, March, 2006
25. Gifford RJ, Liu TF, Rhee SY, Kiuchi M, Hue S, Pillay D, et al. The calibra**ted population resistance tool: standa**rdi**zed genotypic estimation of transmitted HIV-1 drug resistanc**e. Bioinformatics, Mar, 2009.

11.0. APPENDIX

Data collection sheet

Patients' n^{o} / **Variable**	1	2	…
Age			
Sex			
CD4 initial			
CD4 peak			
CD4 at failure			
WHO Initial			
WHO at failure			
OI History			
Regimen at time of GT			
Duration of treatment(months)			
Other ARV exposure			
Viral load			
VL Date			
Genotype Date			
Subtype			
NRTI mutations			
184V			
TAMs			
TDF mutations			
Pan-NRTI			
Other NRTI mutations			

NNRTI mutations			
103			
181			
Others NNRTI mutations			
PI mutations			
32			
46			
47			
50			
54			
82			
84			
90			
Others PI mutations			
Providers reason for failure			
Comments			