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EMERGENCE OF HIV-1 DRUG RESISTANCE MUTATIONS IN PATIENTS AFTER 12 MONTHS OF ANTIRETROVIRAL THERAPY

BY

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DECLARATION

I, Benjamin Asamoah, hereby declare that the data presented in this thesis is the outcome of my research project under the supervision of Dr. Evelyn Yayra Bonney (Virology Department, Noguchi Memorial Institute of medical research), Prof. Osbourne Quaye (Department of Biochemistry, Cell, and Molecular Biology- University of Ghana) and Dr. Peter Quashie (Department of Biochemistry, Cell, and Molecular Biology- University of Ghana). I am aware of and understand the University's policy on plagiarism and categorically state that, except where indicated by referencing, the work presented has not been submitted in support of another degree or qualification from this or any other university or higher institute of learning.

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DEDICATION

My work is dedicated to the memory of My Late father Ch Insp. RTD John Owusu Boadi Mensah whose words have been my guide since infancy to enable me to achieve such success.

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My deepest gratitude to the Almighty, who has been my guide through my educational life.

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LIST OF ABBREVIATIONS AND ACRONYMS

3TC- Lamivudine

AIDS-acquired immunodeficiency syndrome

ART- antiretroviral therapy

ARV-Antiretrovirals

ATV -Atazanavir

AZT- Zidovudine

CCD-catalytic core domain

CCR5- chemokine receptor type 5

CD4-- cluster of differentiation 4

CDC-Centre for disease control

cDNA- complementary DNA

CRIs-co-receptor inhibitors

CTD-C-terminal domain

CXCR4- chemokine receptor type 4

DNA-Deoxyribonucleic acid

DRV-Darunavir

EDDI-estimated date of detectable infection

EFV- Efavirenz

FDA-United States Food and Drug Administration

FIs-fusion inhibitors

FPV- Fosamprenavir

FTC- Emtricitabine

GRID-gay-related immune deficiency

HIV- human immunodeficiency virus

HIVDB-Stanford HIV Drug Resistance Database

HIV-human immunodeficiency virus

INSTIs-integrase strand transfer inhibitors

LPV-Lopinavir

LTR-long terminal repeats

NFAT-nuclear factor of activated T cells

NFkB-Nuclear factor-kappa B

NK-natural killer

NNBS-non-nucleoside binding site

NNRTI – Non-nucleoside reverse transcriptase inhibitors

NRTI - Nucleoside/nucleotide reverse transcriptase inhibitors

NTD-N-terminal domain

NVP- Nevirapine

PHI-pre hairpin intermediate

PI-protease inhibitor

PLHIV-people living with HIV

RNA- Ribonucleic acid

RT-reverse transcriptase

SP 1-specificity protein

TAE-Tris-acetate-EDTA

TDF- Tenofovir

U3-untranslated 3

UNAIDS - Joint United Nations Program on HIV and AIDS

WHO- World Health Organization

ABSTRACT

Background

The United Nations Program on HIV and AIDS (UNAIDS) in 2013 set an ambitious target of 90-90-90, thus 90% of all people living with HIV must have been diagnosed, 90% of those diagnosed put on treatment and 90% of the patients on treatment must achieve viral suppression. The commonest cause of therapeutic failure in HIV patients is the presence of drug resistance mutations. This leads to viral rebound, reduction in CD4 count, and predisposes the patients to opportunistic infections. In addition, chances of transmission increase as a result of increased viral load. Occasionally, viruses carrying drug resistant mutations are transmitted from one person to the other. This study sought to investigate the emergence of HIV drug resistance mutations in protease and reverse transcriptase genes, twelve months after starting antiretroviral treatment.

Method

Ribonucleic acid (RNA) was extracted from 86 (43 at baselines and 43 at follow-up specimen) archived plasma collected from HIV-infected patients before treatment and 12 months after treatment. Complementary DNA was synthesized and a nested PCR was performed targeting the protease and reverse transcriptase genes using gene-specific primers. A total of 80 samples from 20 patients who had their protease and reverse transcriptase genes amplified for baseline and follow-up were sequenced and analyzed using Unipro UGENE, Seqman pro, and Bioedit to generate consensus sequences. The consensus sequences were submitted to the Stanford HIV database to determine the presence of protease and reverse transcriptase mutations. A correlation was drawn between HIV drug resistance and treatment. HIV subtypes were determined using the Rega and Comet databases.

Results

Females accounted for 70% of the patients whiles the remaining 30% were males. Generally, the patient had improved CD4 count after treatment compared to the baseline. Viral loads had also fallen for many of the patients. Out of the 20 patients, 10% (2) were HIV subtype G, 20% (4) were HIV subtype B and 70% (14) were CRF02_AG subtype. All the patients were on First-line drugs, the medications consisted of 34% NNRTI thus 3% were on Nevirapine (NVP) and 31% Efavirenz (EFV), for the NNRTIs, the remaining 66% being NRTIs comprised 3% Emtricitabine (FTC), 13% Zidovudine (AZT), 19% Tenofovir (TDF), and 28% Lamivudine (3TC). With the combination therapy, 45% of patients were on 3TC/TDF/EFV, 9% on FTC/TDF/EFV, 9% on AZT/3TC/NVP, 9% on Comb/EFV, 27% on AZT/3TC/EFV. Transmitted resistant mutations to protease inhibitors include I50N and L90M, K43T, L10F, and 58E. K65R an NRTI resistance mutation and Y181C an NNRTI resistance mutation were observed at baseline. Protease inhibitor mutations at follow-up include K43T, L10F, Q58E, and G73S. The odds ratio of developing drug resistance to protease inhibitors during follow-up was 0.46. Reverse transcriptase resistance mutations observed at baseline were K65R and Y181C. Reverse transcriptase resistance mutations observed at follow-up include M41L, K101Q and V106I, K103E, Y181Q, and D67N. The odds ratio of developing drug resistance to reverse transcriptase during follow-up was 12.67 and a p-value of 0.02.

Conclusion

There is an increase in subtype B viruses in Ghana, subtypes G and CRF02_AG remains the predominant subtype. Drug resistance mutations to protease and reverse transcriptase inhibitors

were observed at baseline and follow-up. Patients on treatment were more likely to develop resistance to reverse transcriptase inhibitors than protease inhibitors. Patients with low CD4 counts were more likely to develop resistance to reverse transcriptase inhibitors.

CHAPTER 1

1.0 INTRODUCTION

1.1 Background

Although HIV was discovered in 1983, treatment was not available until October 1985, when a clinical trial for the first antiretroviral, Zidovudine (3-azido-2,3-dideoxythymidine), was initiated by National Cancer Institute at the Duke University (Reuben *et al.*, 2008). Since then, viral suppression has been mainly by Antiretroviral therapy (ARV) (Nanfack, *et al.*, 2017). This has led to the achievement of a high reduction in the human immunodeficiency virus/ acquired immunodeficiency syndrome (HIV/AIDS) transmission, morbidity, and mortality over the years (Shafer *et al.*, 2017). ARVs target the stages of the viral lifecycle which are composed of entry, reverse transcription, integration, and viral protein synthesis. Essential enzymes such as reverse transcriptase, integrase, and protease encoded by the pol gene are the main targets for most antiretrovirals (McColl *et al.*, 2010).

Antiretrovirals have led to a reduction in HIV-related deaths by 48% between 2005 and 2016 while new infections by 11% between 2010 and 2016. (Chimukangara *et al.*, 2019). As part of the efforts by the United Nations to end the HIV pandemic by the year 2030, United Nations Joint Program on HIV (UNAIDS) and WHO aims to achieve 90, 90, 90 targets by the year 2020. Thus 90% of individuals living with HIV know their status, 90% of those diagnosed are placed on therapy and 90% of those on therapy achieved sustained viral suppression (Beyrer, *et al.*, 2017). This is monitored using the HIV treatment cascade as a care continuum which involves diagnosis, assessments, delivery of treatment, monitoring and support with an overall aim of achieving viral suppression and reducing transmission (Levi *et al.*,2016). For infected people to know their status, efforts are being made to increase the testing rate (Wong *et al.*,2017). In addition, currently there

is a massive global effort to scale up the distribution of antiretrovirals to all HIV patients (Barry *et al*., 2013).

The WHO recommends treatment of all cases once diagnosed as HIV positive, with a combination of two nucleoside reverse-transcriptase inhibitors (NRTIs) and a non-nucleoside reverse-transcriptase inhibitor (NNRTI) being the recommended first-line therapy (Nii-Trebi *et al.*, 2013) and an NRTI and a protease inhibitor (PI) as a second-line drug if the first line fails (Bennett *et al*., 2008; Machnowska *et al*., 2017).

Adverse effects such as drug-to-drug interactions, toxicities, poor tolerability to ART and, the emergence of Resistance are very important issues to deal with. (Zhan *et al*., 2015). The emergence of the resistance strains poses threats to the treatment of HIV patients (Casadellà *et al*., 2016) and limits the available options for treatment (Scherrer *et al*., 2016). Though, with the introduction of combination therapy, resistance development has become less frequent (Bontell *et al.*, 2013) on the Contrary, a Centre for disease control (CDC) report released showed that since the global rollout of ART in 2001the , prevalence of resistance HIV has increase from 11% to 29% (WHO, 2017) Resistance to ART may be acquired by patients on therapy or transmitted to Naïve patients both of which are of public health concern (Shafer *et al.*, 2Drug-resistantistant strains of HIV may be caused by a sub-optimal dose of the ART drug. Chances of developing resistance can be minimized by an uninterrupted supply of optimal dosage (Conradie *et al.*, 2012). Acquired resistant strains can lead to an increase in HIV-related morbidity and mortality due to failed therapy if not properly monitored by the laboratory (DART Trial Team, 2010).

Being one of the most important targets for antiretroviral therapy, antiretrovirals targeting reverse transcriptase do not eliminate but reduce HIV replication. HA high rate of mutation in the pol regions coding for reverse transcriptase (RT) confers acquired HIV RT resistance (Tarasova *et al.*,

2018). These mutations lead to a change in the three-dimensional structure of the HIV reverse transcriptase enzyme (Tarasova *et al.,* 2017). The most relevant and most frequent drug-resistant mutation to reverse transcriptase occurs at the methionine 184 in the active site of the enzyme increasing resistance to as high as a 100-ld (Pouga *et al.,* 2019). Resistant strains evolve due to the high rate of mutation leading to the virus adapting under drug pressure. These mutations may lead to cross-resistance drugs not yet applied as well as the current ones (Riemenschneider *et al.,* 2016). An example of cross-resistance mutations includes K65R/E/N which confers resistance to Abacavir, Emtricitabine, lamivudine, tenofovir didanosine, and stavudine for NRTIs. Cross-resistance mutation for NNRTI includes Y181C/I/V conferring resistance to nevirapine, efavirenz, rilpirvirine, M46I/L mutation confers resistance to ritonavir boosted protease inhibitors such as lopinavir, tipranavir, fosamprenavir, indinavir and Nelfinavir. Integrase strand transfer inhibitors such as bectegravir, cabotegravir, dolutegravir, elvitegravir and raltegravir also have a common strong resistance mutation Q148H/K/R (Wensing *et al.*,2019). Resistance testing aims at personalizing ART and maximizing its efficacy. In low middle-income countries, resistance information provides epidemiological data on resistance pattern to inform meaningful public health action (Noguera-Julian *et al*., 2017). HIV resistance testing may be genotypic or phenotypic or both (Knox *et al.*, 2017). For genotypic resistance testing, sanger sequencing has been the gold standard (Machnowska *et al*., 2017)

1.2 JUSTIFICATION

WHO aims to achieve 90, 90, 90 targets by the year 2020. Thus 90% of individuals living with HIV knowing their status, 90% of those diagnosed placed on therapy and 90% of those on therapy

achieving sustained viral suppression. As at 20th February 2020, Ghana stands at 66 in achieving the third 90 of the 90-90-90 target.

The commonest cause of therapeutic failure for HIV is presence of drug resistant strains (Wensing *et al.*, 2016). Studies have been conducted on HIV-1 and HIV-2 resistance however, there is limited information on the emergence of HIV resistance during therapy in Ghana. This research seeks to provide data on the type of resistance mutations that emerge during therapy. This will help with the better management of HIV.

1.3 HYPOTHESIS

Patient on combination antiretroviral therapy will develop some resistance mutations within the first twelve months on therapy.

1.4 AIM

This research aims to establish the extent of emergence of genotypic resistance to the currently approved ART in the selected HIV clinics during twelve months of anti-retroviral therapy.

1.5 SPECIFIC OJECTIVES

1. To determine drug resistance mutations in the protease and reverse transcriptase genes before therapy and after 12 months on therapy.
2. To determine the association between patients' baseline CD4 count and viral load and the emergence of drug resistance mutations.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 INTRODUCTION TO HIV VIRUS

Human immunodeficiency virus has claimed more than 38million lives since the discovery of the virus (HIV) in 1983 as the causative agent for AIDS (Trivedi *et al.*, 2019). Annually over a million deaths are caused by AIDS (Tarasova *et al*., 2017). The effect of HIV/AIDS caught public health attention in 1981 when it was realized that the causative virus could not be eliminated by the host immune system resulting in the demise of the affected individuals. (Cary *et al*., 2016).

Immune cells such as CD4+ T cells, macrophages and monocytes are the primary targets of the virus leading to the death of bystander and infected cells (Trivedi *et al.*, 2019). Infection involves the binding of gp120, a viral protein, to the CD4, primary receptor and either CCR5 or CXCR4 as co receptors. The virus enters the cell by fusion which is followed by reverse transcription of the viral RNA converting it to a double stranded DNA by reverse transcriptase which gets integrated to the host cell genome. New viral particles are formed when the integrated provirus is transcribed into new viral RNAs that gets packaged (Liu *et al.*, 2017).

HIV-1 consists of four groups M (major), N (non-M, non-O), O (outlier) and P. The reservoirs of groups M and N are traced geographically to distinct chimpanzee communities in southern Cameroon, whiles that of groups O and P remains elusive (D'arc *et al*., 2015). The most dominant in the global pandemic, Group M viruses are subdivided into nine different subtypes (A, B, C, D, F, G, H, J and K). Unlike group M, groups N, O and P are not widely distributed (Bbosa *et al*., 2019). Emerging from Kinshasa and introduced to the Caribbean through Haiti around 1966 and the United States around 1969 via homosexuals, the HIV-1B is the most predominant variant

accounting for approximately 11% of all cases globally (Junqueira *et al.*,2016). Regionally, subtype A predominates in East Africa, former Soviet Union countries and Russia; subtype B being the most prevalent in Americas, Europe and Oceania; Subtype C in India and South Africa and the CRF01_AE and CRF02_AG being ubiquitous strains in Asia and West Africa respectively (Bbosa *et al.*,2019). In Ghana however, 79.2% of the circulating virus is the CRF02_AG, 8.2% unique recombinant for CRF02_AG/A3 mosaic, 8.3% G and 4.2% A3 (Nii-Trebi *et al.*, 2017)

Robust HIV-1 prevention approaches needed to be put in place to reduce the peril of transmission from infected persons to their sexual partners as the virus has been shown to be primarily transmitted by sexual transmission. ART in Serodiscordant patients has however shown a promising result of reducing risk of sexual transmission (Cohen *et al.*, 2016).

2.2 DISCOVERY

HIV/AIDS related death predates to the late 1950s, but the identification of the causative agent to about three decades. The virus was identified in 1983 and confirmed in 1984. HIV a lentivirus has two sets of positive-sense RNAs. Fifteen (15) proteins are produced from 9 genes in the 9000 base pairs of the virus (Becken *et al.*, 2019). The virus sequenced in 1985 had the following nine genes identified, Gag, pol and env coding for the structural proteins, the regulatory proteins Tat and Rev and the accessory proteins Nef, Vif, Vpu (Vpx in HIV-2), and Vpr (Cary *et al.*, 2016). The least prevalent, HIV-2 is found mostly in West Africa while the most prevalent of the two; HIV-1 is found globally (Becken *et al.*, 2019). AIDS as a pathologic entity of HIV was discovered in the 1981 by James Cuman at CDC after a report from New York hospitals on young gay men who are sexually related having opportunistic infections (Montagnier *et al.*, 2010). As a result, the disease was first termed gay-related immune deficiency (GRID). With the emergence of the disease in other groups who were non gay, the name was changed to AIDS (Hepler *et al.*,2019). Similar

disease existed in blood transfused patients and hemophilic patients. Hemophilic patients receiving purified factors 8 and 9 filtered through bacteriological filters gave the clue of the causative agent being a virus (Montagnier *et al*., 2010). Based on previous knowledge and discovered tools such as the biochemical assays in reverse transcriptase by Temin and Baltimore in the 1970s, growth of lentivirus in T lymphocytes cultures, and the discovery of interleukin II previously known as the T-cell growth factor, the search for the retrovirus responsible for AIDS begun in 1980 which became very obvious in 1982 (Gallo & Montagnier, 2003). Subsequently test kit for diagnosing the Virus was available at blood banks in 1985 helping to draw the correlation between HIV and AIDs (Gallo & Montagnier, 2003).

2.3 STRUCTURE

2.3.1 Genomic structure

The HIV has a core which encloses the viral genome made up of two single-stranded viral RNA particles identical to each other which are reverse transcribed to generate the proviral DNA (Blood, G. A. C. 2016). At the two ends of the proviral DNA are the long terminal repeats (LTR) which codes for the promoter and enhancer at the 5' end where transcription is initiated. Over 109 mRNAs are generated from the primary transcript transcribed from the 5' LTR which are translated into all the viral proteins required for the complete virion (Cary *et al*., 2016). The transcription factors such as nuclear factor kapa B (NFkB), nuclear factor of activated T cells (NFAT), specificity protein (SP 1) have binding sites at the cis acting DNA element located on the untranslated 3 (U3) region at the 5 end of the LTR (Cary *et al*., 2016). The HIV-1 has nine viral genes comprising of gag, env, nef, pol, rev, tat, vif, vpr and vpu, essential for all the replicative cycle of the virus which includes receptor binding, membrane fusion, reverse transcription,

integration, viral assembly and proteolytic protein processing (Bobbin *et al.*,2015). Coding for the structural proteins and next to the LTR from the 5' end is the gag reading frame followed by Pol gene, the env reading frame (Blood, G. A. C. 2016).

The four separately folded domains of HIV-1 Gag, a 55kd polyprotein, are the capsid (CA), matrix (MA), nucleocapsid (NC), and p6 (carboxyl terminal). Connecting these domains are the flexible linkers, the Spacer-peptides SP1 and SP2. SP1 connects NC and CA whiles SP2 connects NC and P6 (Lingappa *et al.*, 2014; Schur *et al.*, 2015). Though only CA, MA and NC are required for viral maturation whiles p6 is useful during budding and release, cleavage at five positions by protease leads to structural arrangement resulting in the formation of a conical core by CA to encapsulate the condensed CA-RNA complex while MA oversees the viral membrane interactions (Lingappa *et al.*, 2014; Schur *et al.*, 2015.

HIV-1 env codes for Gp120 and gp41, essential for HIV-1 binding to host cell, results from a precursor glycoprotein cleaved by furin cellular protein (Checkley *et al.*, 2011). Four accessory genes which codes for *nef, vif, vpr* and *vpu* (vpx in HIV-2) and two regulatory genes which codes for *tat* and *rev* (Vicenzi & Poli,2013).

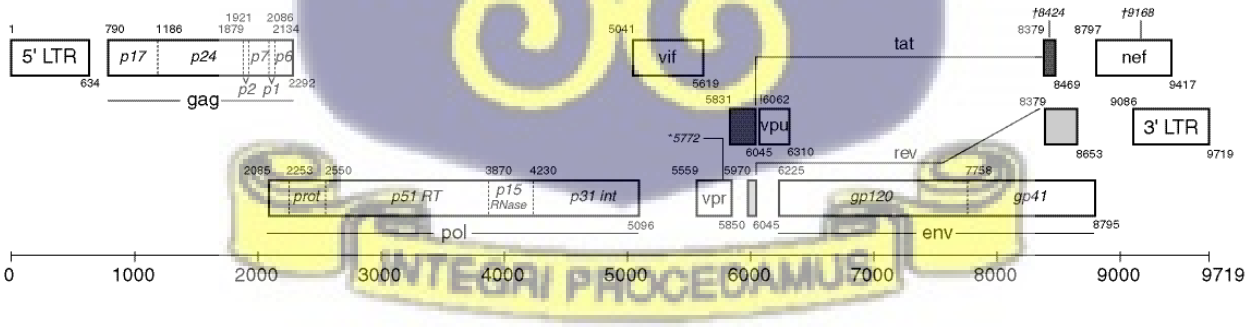


Figure 2. 1: Schematic diagram of HIV-1 gene map showing the segments coding for the structural, accessory and regulatory proteins (https://www.hiv.lanl.gov/content/sequence/HIV/MAP/landmark.html accessed 28/04/2020)

2.4 EPIDEMIOLOGY

In 2018 there were about 37.7 million people living with HIV, of which 36.0 million were adults and 1.7million being children under 15 years. Out of which only 84% knew their status. An estimated 1.5 million people were newly infected people in 2020 and 680,000 people died from AIDS-related illnesses compared to the peak in 2004 with 1.7million deaths a decline of more than 56 percent (UNAIDS 2020).

In Ghana within the same time period, 350,000 people were living with HIV, of which 320,000 were adults with about 29,000 children under 15years. 63% of infected person (220.000) knew their status an estimated 19,000 were newly infected in 2020 and 13,000 deaths as a result of AIDS-related illnesses (UNAIDS 2020).

2.4.1 Molecular epidemiology

The global and regional distribution of HIV-1 is very complex diverse and evolving. It is estimated that between 2010-2015, the global distribution of the HIV subtypes comprised of 46.6% of subtype C. Sub-type B accounted for 12.1% of all infections between 2010 and 2015. Within the same time frame, 10.3% of all infections were sub-type A, CRF02_AG 7.7%, CRF01_AE 5.3%, other CRFs constitute 3.7%, Subtype G4.6%, Subtype D 2.7%, Subtypes F, H, J, and K makes up total of 0.9% and unique recombinant forms makes a total of 6.1% (Hemelaar *et al.*,2019).

A 2017 study in Northern Ghana by Nii Trebi et all showed 79.2% CRF02_AG making it the most predominant followed by 8.3% subtype G, 8.3% unique recombinant form with CRF02_AG/A3 mosaic and 4.2% subtype A3 (Nii-Trebi *et al.*, 2017).

2.5 DISEASE PROGRESSION

Activated T-cells are required for prolific infection by HIV. CD4+ T cells harbours HIV thereby acting as a primary reservoir for the virus during the disease progression to AIDS (Stevenson *et al.*, 2019). Early diagnosis coupled with early treatment lessens transmission by as high as 90% as well as reduce the patients' risk of progression of the disease as a result of the early intervention in the form of antiretrovirals that will be administered. (Drescher *et al*., 2013, Grinsztejn *et al*., 2014). Due to the substantial evolution of testing technologies of HIV, the Fiebig staging of early diagnosis is gradually losing its significance and is likely to be replaced with an improved form, the new estimated date of detectable infection (EDDI) method (Facente *et al.*,2020).

The progression of HIV is classified using the immunological (CD4+ cell counts), clinical stages based on clinical outcomes such as lymphadenopathy and the presence of opportunistic infections such as candida and tuberculosis, as well as viral load (WHO, 2007). The CD4 T-cell count, although is the hall mark of the disease progression, the mechanism of decline is not fully understood. Maintenance of adequate levels is however needed in circulation which depends largely on the ability to renew the depleted lymphocytes through hematopoiesis (Sauce *et al*.,2011). Deficient thymic output may lead to inadequate T-cell count especially naïve CD4 and CD8 compartment. There is also a reduction in the number of natural killer NK and B-cell population which makes it evident that lymphopenia in HIV originates not from weakened Thymus and CD4 t-cell depletion (Sauce *et al*., 2011).

2.6 TREATMENT

Antiretroviral therapy has proven to be effective in significantly plummeting viral loads viral loads thereby helping to minimize transmission between partners. It has also proven to reduce opportunistic infections in patients by revitalizing the immune system via increased CD4 count (Tang et at., 2017).

As at 2017, about half of all people living with HIV globally, 19.7million, were on treatment (Beyer and Pozniak, 2017). Meanwhile in Ghana, 130,000 out of the estimated 310,000 (42% coverage) HIV infected patients are on anti-retroviral therapy with reported 19,000 new infections and 16,000 deaths as at 2017 (Adusei-Poku *et al*., 2019). This is an improvement on the reported 26.6% in 2011 (Nii-Trebi *et al*., 2013).

The Ghana government in 2016 adopted the "treat all" policy a World Health Organization's (WHO) policy on the provision of antiretroviral therapy to all people living with HIV (PLHIV) regardless of their CD4 count. In addition, the nation is highly determined to achieve the UNAIDS target of 90-90-90 (Ali *et al*., 2019).

Currently the approved drugs for treatment of HIV/AIDS fall into one of these five classes: a) receptor antagonist and fusion inhibitors, b) Nucleoside reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), integrase strand transfer inhibitors (INSTI), Protease (PR) inhibitors, which targets the Viral entry, reverse transcription, integration, and viral maturation (Zhan *et al*., 2015).

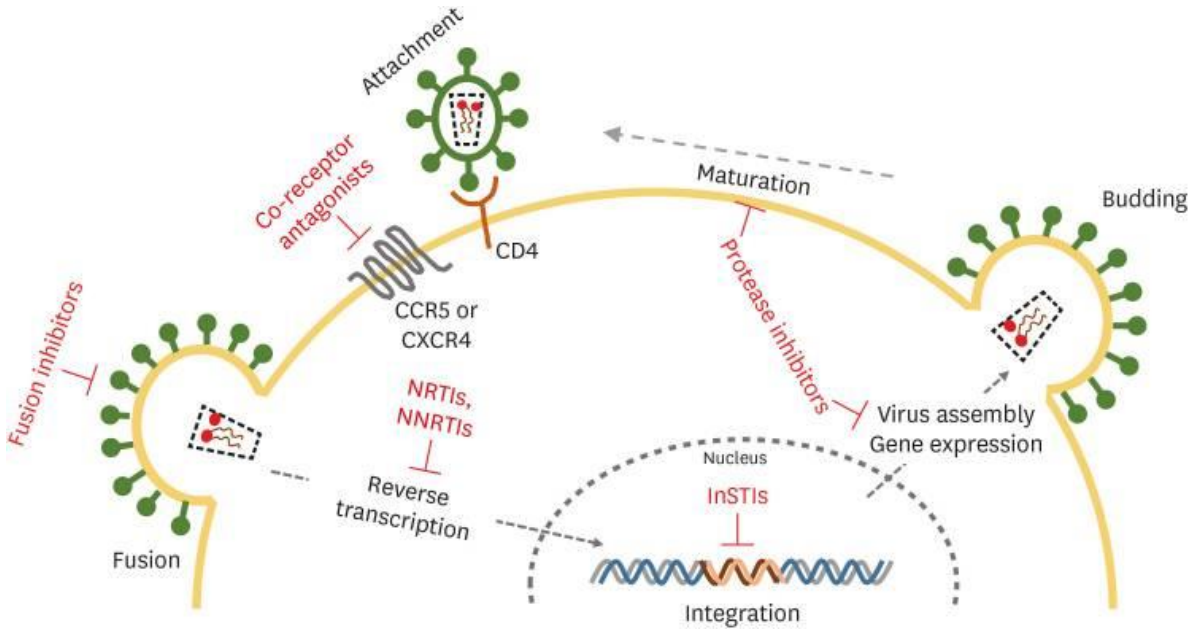


Figure 2. 2 Showing the replication cycle of HIV and the various drug targets (Shin et al., 2021)

Drugs for treatment of HIV targets the viral proteins in the replication cycle yielding exact inhibitory effect resulting in viral suppression (Maeda *et al.*, 2019). Despite the challenges and the inability of the current treatments to eradicate the HIV, as a result of occult reservoirs, the recently developed combination therapies has changed the previously perceived incurable diseases to a manageable chronic infection (Maeda *et al*., 2019). Mortality of HIV positive women have now reduced and mothers are now living longer due to the introduction of the Prevention of Mother-to-child-transmission (PMTCT) (Mkwanazi *et al*., 2015).

In spite of the well-established preventive measures available, the spread of the virus is still eminent as such, pre-exposure prophylaxis, first published in 2010 by iprep and showing reduction in incidence, has been adopted as one of the alternative measures of prevention of transmission (Spinner*et al*., 2016)

Although the WHO has committed to achieving its target of 90-90-90 by the year 2020, that is 90 percent of people with HIV will be diagnosed, 90% of those diagnosed will be on treatment and 90% of those on treatment to achieve viral suppression. However, the emergence of drug resistance strains poses a major treat that is hindering the progress. The WHO reported in 2016 that 70% of People living with HIV (PLHIV) had been diagnosed out of those 77% were on treatment and 82 percent of those on treatment had achieved viral suppression (Fund and WHO, 2017)

2.7 CLASSES OF DRUGS

To prolong the HIV patient's life, antiretrovirals (ARVs) are indispensable in the inhibition of the viral replications as well as slowing the progressions of the disease (Riemenschneider *et al.*, 2016). Antiretroviral therapy in the early 1990s was monotherapy and has evolved through dual therapy and currently triple therapy with the ability to reduce viral load drastically to the barest minimum (Arts & Hazuda2012).

ARVs target the stages of the viral lifecycle which is composed of entry, reverse transcription, integration and viral protein synthesis. Essential enzymes such as reverse transcriptase, integrase and protease encoded by the pol gene are the main targets for most antiretrovirals (McColl *et al.*, 2010). To date, all approved antiretroviral drugs fall into one of the seven categories; fusion inhibitors (FIs), nucleoside reverse transcriptase inhibitors (NRTIs), nucleotide reverse transcriptase inhibitors (NtRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), co-receptor inhibitors (CRIs) and integrase strand transfer inhibitors (INSTIs) (De Clercq, *et al.*, 2010).

From 1987 when the first ART was approved by the FDA till 2019, one fusion inhibitor (enfurvitide) and one CCR5 antagonist (Maraviroc) has been approved in 2003 and 2007 respectively. 8 NRTI, 1 NtRTI, 7 NNRTIs, 9 PI, 3 INSTI. Cobicista (pharmacokinetic enhancer) and Ibalizumab-uiyk (post attachment inhibitors) approved in 2014 and 2018 respectively are the only approved ARTs in these classes. Starting with combivir in 1997, a total of 22 Fixed dose

The WHO recommends one NNRTI and two NRTI as first-line treatment option or two NRTI and a protease inhibitor (PI) as second-line regimen if the first line fails (Bennett *et al*., 2008; Machnowska *et al.*, 2017). Currently there is a massive global effort to scale up the distribution of antiretrovirals to all HIV patients (Barry *et al*., 2013). The WHO recommends treatment of all cases once diagnosed as HIV positive, with a combination of two nucleoside reverse-transcriptase inhibitors (NRTIs) and a non-nucleoside reverse-transcriptase inhibitor (NNRTI) being the recommended first line therapy (Nii-Trebi *et al.*, 2013). Therapies targeting the RT enzyme of HIV-1 are the most prescribed (Pouga *et al*., 2019). The active site of reverse transcriptase protein is target for NRTIs. NRTIs function as chain terminators of the DNA when incorporated into the DNA. Though occasionally leads to mitochondria cytotoxicity as a result of interference with mitochondria DNA polymerase (pol γ) (Bertoletti *et al*., 2019). The usefulness of therapies may be limited due to the high rate of emergence of drug resistance mutants in the most targeted HIV-1 protein, the RT. With M184V and M184I being the commonest drug resistant mutations conferring resistance to these groups of medications (Pouga *et al*., 2019).

2.7.1 Reverse transcriptase inhibitors

Having two binding sites for divalent cations (such as magnesium the most abundant divalent cation in the body) in its active sites, HIV reverse transcriptase (RT) a heterodimer, with an RNAse

H activity has the ability to execute DNA dependent DNA synthesis as well as RNA dependent DNA synthesis (Achuthan *et al*., 2016). There are two groups of reverse transcriptase inhibitors the nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) and the non-nucleoside reverse transcriptase inhibitors (NNRTI) (Rai *et al.*,2018).

2.7.1.1 Nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs)

The HIV reverse transcriptase (RT) is the target enzyme for NRTIs. These are nucleoside and nucleotide analogues which served as chain terminators upon incorporation into the extending DNA chain due to the absence of 3'hydroxyl group (Mislak *et al.*, 2016). The first antiretroviral to be approved for the treatment of HIV Zidovudine (3'-azido-2',3'-dideoxythymidine) belongs to the NRTIs (De Clercq, E. (2010). Being the mainstay of HIV treatment, they are recommended by all treatment guidelines. That notwithstanding NRTIs are associated with inhibiting telomerase activity thereby accelerating age in addition to its ability to cause immune dysfunction with long- or short-term exposure (Gonzalez-Serna *et al.*,2017). In spite of the extent of health advances achieved with the use of NRTI, it is also associated with toxicities especially in the Mitochondria as a result of selectivity of virus and host polymerases. This result in human mitochondria DNA polymerase (mtDNA pol) incorporating NRTIs (Mislak *et al.*, 2016). Though the full risk not well understood, new and promising NRTI such as GS-9131 which is still in development has shown efficacy against resistant mutants (Rai *et al.*,2018).

2.7.1.2 Non-nucleoside reverse transcriptase inhibitors (NNRTI)

Though structurally diverse, NNRTI still show very high specificity for HIV-1 reverse transcriptase RT which is made up of p66 and p51 subunits. NNRTIs do not inhibit HIV-2 RT.

The binding site for NNRTIs, the non-nucleoside binding site (NNBS) is the allosteric site of RT which is a hydrophobic pocket 10A from the active site of DNA polymerase with the following amino acids Leu100, Lys101, Lys103, Val106, Thr107, Val108, Val179, Tyr181, Tyr188, Val189, Gly190, Phe227, Trp229, Leu234, Pro236 and Tyr318 in the p66 palm subdomain, and Glu138 of p51 (Famiglini& Silvestri, 2016). Primer ends are positioned by amino acid residues 227 and 235 in the palm domain which forms part of the NNBS of RT enzyme catalysis is hampered when NNRTI binds to RT due to resulting conformational change at the catalytic site impeding the primer bond and thumb finger assembly (Famiglini & Silvestri, 2016). A component of the cART, NNRTIs are highly selective, with favourable pharmacokinetics and modestly toxic (Kang *et al.*,2016). NNRTIs resistance are more common in naïve patients than PI resistance which contributes to virologic failure on first line therapy in addition to suboptimal adherence. Due to increased risk of resistance related failure, NNRTIs are usually not recommended as part of the second line regimen (Usach *et al.*,2013). Preliminary data of newer NNRTIs such as Doravirine and Elsulfavirine has shown promising signs of favourable side effects such as reduced toxicity than existing NNRTIs in addition to their longer half-life ((Rai *et al.*,2018).

2.7.2 Protease inhibitors (PI)

The Gag-Pol polyproteins are proteolytically processed by the protease enzyme at several cleavage sites to make a mature HIV (Könnyű *et al.*, 2013). Being the essential enzyme required for the maturation of the HIV virion, makes the protease a very important target for most antiretrovirals (Kehinde *et al.*,2019). HIV protease is a noncovalently connected 99 amino acid, C2-symetric active homodimeric aspartyl protease with Asp on position 25 at the active site which cleaves gag and gag-pol polyprotein precursors to produce mature proteins. Upon substrate binding, an

enclosed tunnel is formed from the two monomeric chains assembly which usually opens and closes (Lv *et al*., 2015; Kehinde *et al.*,2019). While Ly et al has reviewed extensively on protease inhibitors and their mechanism of actions, I must emphasize that generally, protease inhibitors interact with the carboxylic group of Asp 25 and 25' via their hydroxyl group to form a hydrogen bond mimicking substrate transition state. Containing Gly 27, Asp 29, Asp 30 and Gly 48 in the conserved active site, the structure of the enzyme is altered with the accumulation of drug resistance mutation ((Lv *et al*., 2015).

Darunavir is known to bind to protease monomers in a one-to-one ratio preventing dimerization which is a crucial step in HIV-1 protease gag-pol auto processing (Hayashi *et al.*,2014). As a result of the shorter half-life and lower systemic exposure, protease inhibitors are boosted with pharmacokinetic enhancers such as ritonavir and cobicister which inhibit cytochrome P450 3A increasing the half-life and enhance plasma exposure. Cobicister is however preferred over ritonavir as ritonavir is known to cause a number of drug-drug interactions via the inhibition of other cytochrome subfamilies such as CYP1A2, CYP2C9, CYP2C19, CYP2D6, drug transporters such as OCT and ABCB1 (P-glycoprotein) and urunosyl-glucuronyl-transferase (Von Hentig, N. 2016).

Patients who fail first-line therapy comprising NRTI and NNRTI, meet the requirement for a second line therapy, a boosted protease inhibitor (bPI) (Sutherland *et al.*, 2015). Relative to NRTIs and NNRTIs, PI are less likely to develop resistance. High toxicities which could be as a result of drug-drug interactions, off target drug effects, as well as overdose coupled with poor bioavailability are the major concern of protease inhibitors (Lv *et al*., 2015)

2.7.3 Integrase Strand transfer inhibitors (INSTI)

Consisting of three separately folded protein domains, the C-terminal domain (CTD), the catalytic core domain (CCD) and the N-terminal domain (NTD), the HIV-1 integrase binds vRNA via the basic residues in the catalytic core domain (Elliott *et al.*,2019)

With no human equivalence, HIV integrase is an indispensable target for HIV antiretrovirals. Integrase undertakes 3'processing of the reverse transcribed cDNA in the cytoplasm cleaving off dinucleotide of GT exposing hydroxy ends of CA-3' for strand transfer (Concerted transesterification). Though random, gene integration occurs in transcribed genes. The 3' hydroxyl at the two ends are ligated into the host chromosome with a five base pair stager across the DNA major groove leaving a two base pair overhang at the 5' end of the viral cDNA which are trimmed and repaired by host DNA repair enzyme (Marchand *et al*., 2006). Integration into heterochromatin is overturned by capsid interaction with cleavage and polyadenylation specificity factor 6 (Engelman *et al.*, 2018). The safety profiles and the efficacy of integrase strand transfer inhibitors puts them ahead of the other classes of antiretrovirals (Walmsley *et al.*, 2013).

Due to the stages and proteins involved in the integration complex there exist several avenues to inhibit the integrase enzyme. Raltegravir, the first HIV integrase inhibitor approved by the FDA impairs the second step of integration, meanwhile 2-(quinolin-3-yl) acetic acid referred to as LEDGINs are known to bind to the catalytic core domain (CCD) dimer at the LEDGF binding pocket inhibiting the activity of integrase (Kessl *et al.*,2012).

All INSTI have two essential components though structurally diverse. Mn++ and Mg++ binding and sequestration motifs and the hydrophobic halobenzolic ring for the displacement of reactive end if the 3' viral DNA from the cavity occupied in integrase (Libre *et al.*,2015).

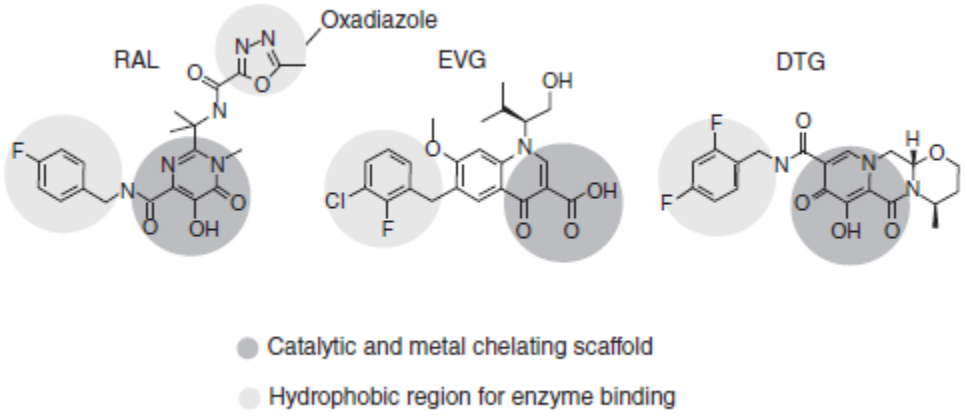


Figure 2. 3:Schematic diagram showing Hydrophobic and metal chelating regions of Protease inhibitors (PIs)

2.7.4 OTHER CLASSES ANTIRETROVIRALS

2.7.4.1 CCR5 inhibitors

Other classes of antiretrovirals include the CCR5 inhibitors which includes vicriviroc, aplaviroc both of which have been discontinued due to adverse effects and lack of clinical efficacy (Roche *et al*., 2011). Maraviroc, the first and still the only CCR5 inhibitor approved by the FDA which is very specific for only CCR5 tropism HIV viruses (Yost *et al*., 2009) It binds to and modifies the CCR5 extra cellular loop (Roche *et al*., 2011). The binding of maraviroc to the extracellular loops of CCR5 leads to a conformational change thereby attenuating the entry of the virus. In Val323 resistance mutation the secondary structure of gp120V3loop and gp120 V3loop-CCR5 N terminus interface is altered. Maraviroc resistant gp120 relies mainly on the charged his88 and his181 on the drug modified extracellular loops 1 and 2 and CCR5 N terminus to enter the host cell as it interacts less efficiently with CCR5 (Roche *et al*., 2013).

2.7.4.2Fusion inhibitors

Approved in 2003 by the FDA, enfurvitide is the only fusion inhibitor ART in use (La Bonte *et al.*,2003). HIV fusses with the host cell via a conformational change in the gp120/41. An exposure of the fusion peptide at the N-terminus of gp41 leads to the formation of a pre hairpin intermediate (PHI) bridge between membranes of the virus and target cell enabling the antiparallel collapse of the C-terminal heptad repeat (CHR) into the N-terminal heptad repeat (NHR)created by hydrophobic grooves bridging the cell and the viral membranes (Ding *et al.*,2017). Enfuvirtide, a 36 amino-acid is a prototype of the C-terminal heptad repeat of gp41 which binds to the N terminal heptad repeat thereby preventing the binding of the endogenous C-terminal repeats and the subsequent trimer of hairpins (six helix bundle) formation required to enhance viral entry (Miller *et al.*,2004). Resistance to enfuvirtide is either direct through mutations at positions 547-556 of the NHR in the enfuvirtide binding site or indirectly through mutations in the allosteric binding sites. In addition, mutations in the CHR and the V3 loop of gp120 and the co receptor binding sites are also known to play compensatory roles in enfuvirtide resistance (Dingens *et al*., 2019).

2.7.4.3 Post attachment inhibitor

Originally TMB-355/TNX355 a monoclonal antibody and now Ibalizumab (Roberts 2018) Approved for the treatment of treatment experienced patients with multidrug resistance failing treatment, Ibalizumab, the first humanized IgG4 monoclonal antibody inhibits HIV-1 entry into CD4 cells post attachment without interfering with its immunological function (Markham, A. 2018; Beccari *et al.*,2019).

Despite its long dosing of 14 days due to its special pharmacokinetic potential, reduced susceptibility is observed with reduced or lost expression of and N-linked glycosylation sites of gp120's V5 loop (Beccari *et al.*,2019).

2.8 CHALLENGES IN TREATMENT

Anti-retroviral therapy fails to fully eradicate HIV in infected individual which is evident when interruption of therapy results in rebound in viral load and loss of CD4 cells. This result from the persistence of non-replicating integrated provirus in resting T-cells. Though majority of which have to carry long stretches of lethal APOBEC3G induced A to G mutation or deletions (Migraine *et al*., 2018). Stimulation of resting T cells and using quantitative virus outgrowth assay (qVOA) showed less than 1% of induced latent cells produced infectious virus (Laird *et al.*, 2013).

The main hurdles in the eradication of HIV are the persistence of latently infected cells and the inability of drugs to reach them, the development of multidrug resistance, the side effects of the drug and issued of dosage which must be very frequent (Dey *et al*., 2013). Reactivation of latent provirus is the currently proposed and widely accepted means of eliminating the virus (Darcis *et al*., 2015). Depending on the cytokine environment, and the local tissue, macrophages can also act as viral reservoir which can make the elimination of the virus from the system more challenging (Bergamaschi *et al.*, 2010).

2.9 RESISTANCE DEVELOPMENT

HIV drug resistance mutations are identified using four main approaches; 1) by site directed mutagenesis for *in vitro* validation, 2) by sequencing the viral genome in patients failing drug therapy,3) phenotypically by susceptibility testing on clinical or laboratory isolates and 4) by

association studies of baseline and exposed viral genotypes in patients on ART (Wensing *et al*., 2016).

Essential proteins necessary for replication of HIV such as reverse transcriptase, protease and integrase are the targets for most antiretrovirals (Lockbaum *et al*., 2018). With majority of the currently prescribed HIV-1 therapy targeting the reverse transcriptase (RT) (Pouga *et al.*, 2019). Being one of the most important targets for antiretroviral therapy, it is worth noting that these reverse transcriptase inhibitors do not totally eliminate but reduces HIV replication. High rate of mutation in the pol regions coding for RT confers acquired HIV RT resistance (Tarasova *et al.*, 2018). These mutations lead to a change in the three-dimensional structure of the HIV reverse transcriptase enzyme (Tarasova *et al.*, 2017). The most relevant and most frequent drug resistant mutation to reverse transcriptase occurs at the methionine 184 in the active site of the enzyme increasing resistance to as high as up to a 100-fold (Pouga *et al.*, 2019).

Analysis of the plethora of structures of drug resistant protease has brought to bear new drug resistance mechanisms leading to the development of tight binding inhibitors for resistant variants. These mechanisms include mutations that alter the interactions between inhibitors/substrates and protease, mutations that alter dimer stability and the transmission of changes to the active sites by distal mutations. These acumens have led to the continuous development of novel antiretroviral drugs for mutant protease (Weber& Agniswamy, 2009).

Resistant strains evolve due to the high rate of mutation leading to the virus adapting under drug pressure. These mutations may lead to cross resistance drugs not yet applied as well as the current ones (Riemenschneider *et al.*, 2016). Finding a resistant mutation in a failed patient on PI drugs is

rare as the determinants remain largely unknown (Sutherland *et al.*, 2015) but is usually attributed to mutations in the gag (Sutherland *et al.*, 2014)

With a high probability of increasing the incidence, treatment and mortality, as a result of increasing frequency of resistance to the commonly used drugs, a joint global action is required to tackle the menace of HIV drug resistance (WHO, 2018). Transmitted drug resistance mutations can persist for a considerable amount of time in the absence of treatment. This generally presents a challenge of fighting infections (Yang *et al*., 2015)

As at 2019, there were 101 drug resistance mutations (DRM) including 93 from WHO list of surveillance drug-resistance mutations (SDRMs) and 8 tenofovir (TDF) resistance-associated mutations (TRAM) (Chimukangara *et al*., 2019). HIV drug resistance is aggravated by factors such as non-compliance by patients and also concentration of drugs at the various body compartments, with low concentrated compartments showing higher resistance as compared to compartments of higher concentration (Fumakia *et al*., 2016). According to Beyrer *et al*,2017, 6 out of 11 countries surveyed had greater than 10% resistance in naïve patients. Although they are not easily implemented by countries, the WHO has a five-point proposed action plan for the monitoring, combating and prevention of drug resistance which includes, prevention, monitoring and surveillance, research, expansion of laboratory capacity, and management and government efforts (Beyrer *et al*., 2017)

Pretreatment NNRTI resistance data for 2016 analyzed from 63 countries with 358 datasets comprising 56,044 adults, revealed a prevalence of 11.0% (7.5-15.9), 10.1% (5.1-19.4), 7.2% (2.9-16.5), 9.4% (6.6-13.2) for Southern Africa, Eastern Africa, western and Eastern Africa and Latin and the Caribbean respectively (Gupta *et al*., 2018). Resistance to Non-nucleoside reverse transcriptase inhibitors NNRTI mutation results from a point mutation near the drug pocket

(K103N) in respect to NRTIs. Three main pathways are involved in the development of drug resistance which includes nucleoside associated mutations (NAMs) which augments the removal of chain terminators from cDNA in formation, mutations which favour the discrimination of physiologic nucleosides over drugs those found in drugs and increased buildup of reverse transcriptase per virion resulting from inserts at the p6 region of the gag increases the chances of viral escape in the presence of NRTI (Soriano *et al.*, 2002).

The commonest in vivo NNRTI resistant mutations are the K103N and Y181C. Other resistant mutations to NNRTI include Y188L single mutant or K103N/Y181C double mutant (Kang *et al.*, 2016). Comparison of nervirapine (an NNRTI) resistance mutation in pregnant women with HIV-1 subtypes A and D showed similar rates of resistance at week one but increased rate of resistance in subtype D than A on week 6 -8 as a result of fading Y181C in women with subtype A and an accumulation of K103N in women with subtype D (Eshleman *et al.*, 2005).

INSTI resistance

The commonest integrase strand transfer inhibitors mutations include Q148H/K/R, 8 N155H, and 6 Y143C/R (Chang *et al.*,2016; Hachiya *et al.*, 2015). Resistance to LEDGIN-6 is an A128T mutation at in the LEDGF binding pocket in the CCD (Kessl *et al.*, 2012)

2.10 AN AMBITIOUS TREATMENT TARGET 90-90-90

HIV-associated morbidity and mortality has significantly declined in the advent of antiretroviral. In 2014 a three-part ambitious goal was set by the UNAIDS/WHO to ensure 90 percent of all persons living with HIV to know their status, 90 percent of those who know their status to be on treatment and those on treatment having 90 percent achieving viral suppression thus is 73% of all infected HIV patients achieving viral suppression (Gisslen *et al.*, 2017). Being the first country to achieve the target, as at 31st December 2015, Sweden had achieved more than the estimated UN

target of 73% viral suppression for all people living with HIV, with an estimated 90% of all people living with HIV knowing their status, 95.1% being on ART and 94.7% achieving a viral suppression of <50 RNA copies/ml (Gisslen *et al*., 2017). Supported with mathematical models and epidemiological data, early diagnosis ("test") and starting of ART in these individuals ("Treat") could lead to significant decrease in the rate of HIV infection (Gardner *et al*., 2011). In Ghana, the "Treat All" national policy and the great virologic suppression and immunological recovery of patients makes the achievement of the 90 90 90 targets attainable (Obiri-Yeboah *et al.*,2018). However, adherence to all six stages of spectrum of care for infected individuals, being diagnosed, linked to health care, retained in health care, being on ART, and having suppressed viral load (Gisslen *et al*., 2017) immensely contribute to achieving better treatment outcomes. Shortfalls in diagnosis ie late detection, suboptimal linkage to care, minimal retention in care, inadequate use of ART, and unsatisfactory adherence to therapy impedes efforts to achieving optimum treatment results (Gardner *et al*., 2011). That notwithstanding, discrimination and stigmatization from health workers may hamper the progress (Nyblade *et al*., 2018)

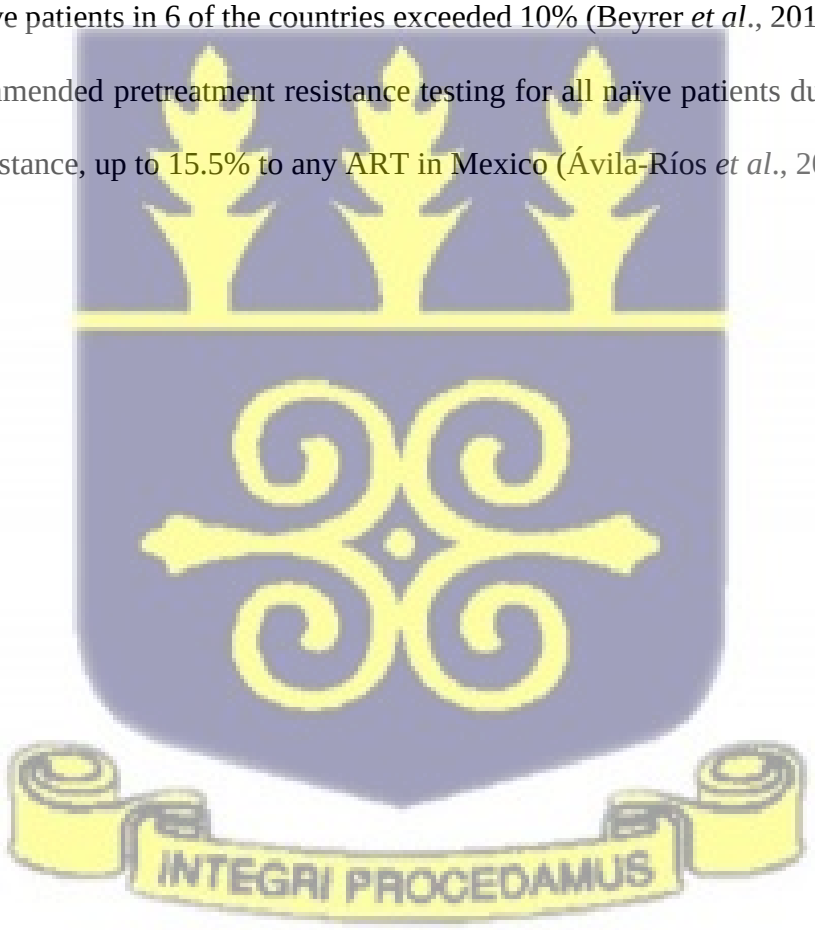
2.11 HIV DRUG RESISTANCE

It has been well established that combination therapy helps reduce the effects of HIV. These hopes may be waned by the emergence of drug resistance strains which is as a result of mutations in the targeted viral proteins by ARVs. thwarting the efforts to control the virus (Clavel *et al.*, 2004). Newly infected patients may acquire drug resistance through the transmission chain or patients during suboptimal antiretroviral treatment (Schmidt et al., 2014)

There are worries as a result of the alarming increase in the number of HIV drug resistance cases since the rollout of ART especially in pretreatment patients. This has been known to be associated

with poor response to treatment with first line therapy and subsequent accumulation of drug resistance mutations (Boerma *et al*., 2017). There is a reduced success to NNRTIs with first line regime that are based on these drugs due to pretreatment resistance (Ávila-Ríos *et al*., 2016).

Though the scale up of ART has had a major influence in achieving the sustainable development goals by reducing new infections, preventing HIV related illness and subsequent AIDS related mortality (WHO, 2017). Reports from 14 countries involving 16 surveys by the CDC, WHO and the global fund to fight AIDS, TB and malaria showed a surge in HIV drug resistance to as high as 29% from 11% since the rollout of anti-retroviral therapy globally in 2001. Meanwhile drug resistance in naive patients in 6 of the countries exceeded 10% (Beyrer *et al*., 2017). Avila-Rios et al in 2016 recommended pretreatment resistance testing for all naïve patients due to high rate of pretreatment resistance, up to 15.5% to any ART in Mexico (Ávila-Ríos *et al*., 2016).



CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 REAGENTS AND CONSUMABLES

All reagents and consumables including ZYMO RNA mini kit for RNA extraction, BIOLAB (New England) cDNA synthesis and PCR kits including the primers and DNTPs were all purchased from INQABA Biotech (South Africa). Agarose powder, TAE (Tri Acetate EDTA buffer), gel red (intercalate), DNA loading dye (cyan orange) is an INVITROGEN (Massachusetts, USA) product and 100kb ladder were all purchased from INQABA Biotech. Sterile nuclease free Eppendorf tubes and PCR tubes were also purchased from INQABA Biotech.

3.2.1 STUDY DESIGN

This study is a retrospective longitudinal study involving eighty-six (86) archived plasma samples made up of forty-three (43) baseline and 43 follow up on the twelfth month. The demographic data, CD4 counts and viral loads of all samples were retrieved and analyzed.

3.2.2 ETHICAL CLEARANCE

Ethical clearance for this study was taken from The Noguchi Memorial Institute For Medical Research. Clearance number CPN 086/14-15.

3.3 STUDY SITE

This study was conducted at the Virology department of the Noguchi Memorial Institute Of Medical Research. All samples were archived plasma from the ART unit (Fevers unit) of the Korle-Bu Teaching Hospital and the ART unit of the Koforidua Regional Hospital. Collected between February 2016 to February 2017.

Korle-Bu Teaching hospital is the premier tertiary health-care facility in Ghana where most cases in the country and other neighbouring West African Countries are referred to.

The Koforidua Regional Hospital also known as the Eastern regional Hospital is the reference hospital for the Eastern region of Ghana.

3.4 INCLUSION CRITERIA

All samples were HIV-1 sero-positive and patients were above the ages of 18years, initially not on ART and follow up samples present on the twelfth (12^{th}) month. Data for the CD4 counts and the viral loads were available for all patients at both baseline and 12^{th} month follow ups.

Protease and reverse transcriptase genes for both baseline and follow up must be amplified by PCR. Patients who had a single gene not amplified by PCR for any of the time points were excluded.

3.5 METHODS

3.5.1 RNA EXTRACTION

3.5.1.1 Reagents reconstitution

Zymo RNA extraction Kits were used for the extraction of all RNAs following an optimized manufactures protocol. RNA concentrations were checked with the nanodrop. Sterile nuclease free Eppendorf tubes were used. The viral RNA extraction reagent was reconstituted by adding beta mercaptoethanol to the viral RNA buffer to make final concentration of 0.5%(v/v) ie 250µl in 50ml or 500µl in 100ml.

The viral wash buffer was also reconstituted by adding 24ml of absolute (100%) ethanol to the 6ml viral RNA wash buffer.

3.5.1.2 Extraction

A 100µl volume of the 2x concentrate DNA/RNA shield was added to 100µl of plasma and pulse vortexed for five (5) seconds followed by 400 µl of RNA buffer and a pulse vortexing five (5) seconds. The mixture was transferred into a Zymo-Spin IC column in a collection tube and centrifuged at 8000rpm for two (2) minutes. The column was transferred into a new collection tube followed by the addition of 500µl of viral wash buffer and centrifuged at 10,000rpm for 30 seconds. The flow through was discarded and the and the washing step repeated. Ensuring the complete removal of the wash buffer, this step was followed by the addition of 500µl absolute (100%) ethanol to the column on a new collection tube and centrifuged at 15000rpm for one minute. The column was transferred onto a labeled nuclease free tube for the final elution. During elution, 15 µl of DNAse/RNAse free water was added to the column and centrifuged at 15000rpm for 30 seconds. the concentration of the Eluted RNA was checked with the nanodrop and recorded. The eluted RNA was stored at -80ºC till use.

3.5.2 COMPLEMENTARY DNA SYNTHESIS

The cDNA master mix contained 3 µl of Viral RNA, 2 µl of DNTPs (d(T)23 or VN(50 µM) primer mix nuclease free waster adding up to make a total volume of 8 µl. All set ups had positive and negative controls. The Random primer mix and the viral RNA were denatured at 64ºC for 5minutes and immediately placed on ice. A total of 10 µl of protoscript II reaction mix (2X) and 2 µl of protocript II enzyme mix (10X) were added. The above mixture was incubated at 42ºC for an hour

for the final annealing and extension. An incubation period of 5 minutes at 20ºC preceded the 1-hour incubation at 42ºC. Finally, enzyme is inactivated at 80ºC for five (5) minutes and the PCR product is either used or stored at -20ºC till use.

3.5.3 PCR

The primers used for protease as reported in Deletsu et al 2020 were AGAGCCAACAGCCCCACCAG and ACTTTTGGGCCATCCATTCC as forward and reverse primers respectively targeting HXB2 positions 2148-2167 and 2611-2592 to yield a product of 463 bases. The reverse transcriptase genes were amplified by PCR using GACCTACACCTGTCAACATAATTGG as the forward primer and TAATCCCTGCATAAATCTGACTTGC as the reverse primer targeting the HXB2 positions 2485-2509 and 3372-3348 respectively for a product of 887 bases.

The Phusion high fidelity PCR kit from Biolabs (New England) was used. Two reactions were set up for both reverse transcriptase and protease genes. The master mix contains 12.5 µl of 2X Phusion master mix, 1.25 µl of 10 µM Forward primer (SK38), 1.25µl of 10 µM Reverse primer (RT 20de), 5.0 µl of cDNA, and 5 µl of nuclease free water for each of the two reactions.

Initial denaturation was at 98ºC for 30seconds, followed by denaturation at 98ºC, annealing at 58ºC and extension at 70ºC for 10seconds, 30 seconds and 90 seconds respectively. The thermocycler was set for thirty (30) cycles. Final extension is at 72ºC for 10 minutes and reaction is held at 4ºC (Biolabs 2020)

3.5.4 TAE DILUTION AND AGAROSE GEL PREPARATION

TAE was prepared by measuring 20ml of 50X Tris-acetate-EDTA (TAE) into a 1litre measuring cylinder and toped up with distilled water up to the 1litre mark. The 1X TAE buffer ready to use.

Agarose gel was prepared by measuring 0.75g of agarose powder into a conical flask and 50ml of 1X TAE added and well mixed. The solution was the microwaved for 4miutes, allowed to cool to 45℃, 5µl of SYBR green was added and gently swirled. Gel was cast in a casting tray with combs already fixed. The gel was allowed to cool at room temperature. The 1.5% agarose gel is ready for use.

3.5.5 AGAROSE GEL AND IMAGING

The 1X TAE buffer prepared is used to prepare a 1.5% agarose gel as well as serve as the buffer for running the electrophoresis. SYBR green/gel red was used as the DNA intercalator whiles cyan orange was used as the DNA loading dye. Precast gels were loaded into tanks ensuring the combed wells were closer to the negative charge of the tank and then flooded with 1X TAE buffer. The first well was loaded with a 100bp marker (ladder) with band sizes ranging from 100bp through to 2kbp, the positive control into the second well, negative control into the third well, subsequent wells were filled with samples. Samples, ladder and controls to be loaded were all mixed with 1 µl of cyan orange (loading dye). The run was set at 100V for 30 minutes. All gels were visualized under the UV using the gel imager and the images taken (Deletsu *et al*., 2020).

3.5.6 SEQUENCING

The DNA Purification and sequencing were done by Inqaba Biotech using the following primers as published in Deletsu et al 2020. Primers for protease were AGCCCCACCAGAAGAGAGCTT and CAACTCCCTCTCAGAAGCAG as forward primers targeting HXB2 positions 2157-2177 and 2198-2217 respectively while the reverse primer targeting HXB2 position 2611-2592 was ACTTTTGGGCCATCCATTCC.

The reverse transcriptase was sequenced using three primers; two forward TTAAAGCCAGGAATGGATG and CAAAAATTGGGCCTGAAAATCCATA targeting HXB2 positions 2692-2716 and 2692-2716 respectively whiles the reverse primer TGGGAAGTTCAATTAGGAATACCACATC targets HXB2 position 2692-2716. (Deletsu *et al*., 2020)

3.5.7 SEQUENCE ANALYSIS

The sequences received were with chromatogram. They were analyzed using three sequence analysis software ie Bioedit version 7.2.5.0, Ugene PRO version 37.0.0 and Seqman ULTRA. The contigs were mapped to a reference sequence, the HXB2, for alignment and also to ensure the right genes were amplified and sequenced. Mixed bases were sorted based on the frequency of occurrence in the three contigs and the height of the peaks. A consensus sequence was generated for resistance determination.

3.5.8 RESISTANCE DETERMINATION

Consensus sequences generated from the Sequence analysis were saved in the fasta format pending analysis in Stanford HIV database. Using the algorithm in HIVDB program, resistance to protease inhibitors and reverse transcriptase inhibitors were generated.

3.5.9 HIV SUBTYPING

Subtyping of HIV was done using three HIV databases, thus Rega, Stanford and Commet. Many of the subtypes were same across different databases. However, when a difference arise a consensus is draw and the subtype with the highest frequencies was adopted. (Struck *et al.*, 2014: Liu *et al* 2006)

3.5.10 STATISTICAL ANALYSIS

Statistical analysis was done using the RStudio version 1.1.463.0 and Excel 2007, whiles the graphs were drawn with GraphPad prism version 9.1.0.221. categorical data were presented using the odds ratio and descriptive data represented as means and standard deviation and proportions.

CHAPTER 4

4.0 RESULTS

4.1 DERMOGRAPHICS

A total of 83 archived samples for 43 patients were retrieved for analysis which comprised of 30 females (70%) and 13 males (30%) (**Error! Reference source not found.**. These samples were collected between February 2016 and February 2017 from diagnosed and confirmed HIV patients who were initially not on ART. However, were put on treatment after the initial samples were taken.

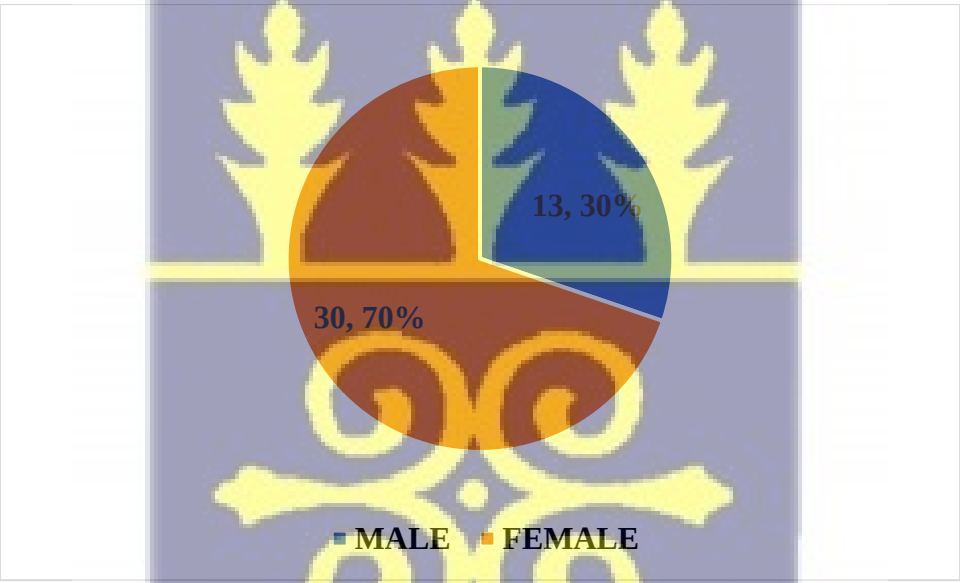


Figure 4. 1 Gender stratification of HIV patients between 2016 and 2017

The majority of the patients were younger than 50 years of age. Nine of them were between nineteen (19) to twenty-eight (28) years of age, eight (8) being between twenty-nine (29) and thirty-eight (38), twelve within the age brackets of thirty-nine (39) to forty-eight (48) years. Eleven (11) patients fell within the ages of forty-nine (49) years and fifty-eight (58). Whiles two (2) and one (1) were within the ages of fifty-nine to sixty-eight and sixty-nine to seventy-eight respectively. The mean age of the participants was (41± 12). The oldest was 72 years youngest being 19 years

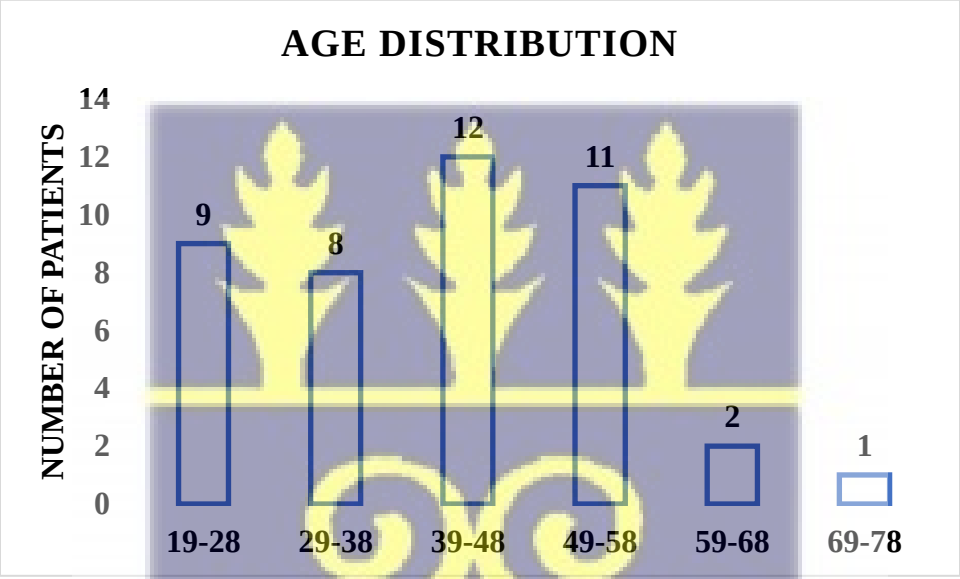


Figure 4. 2 Age distribution of patients

4.2 CD4 COUNT

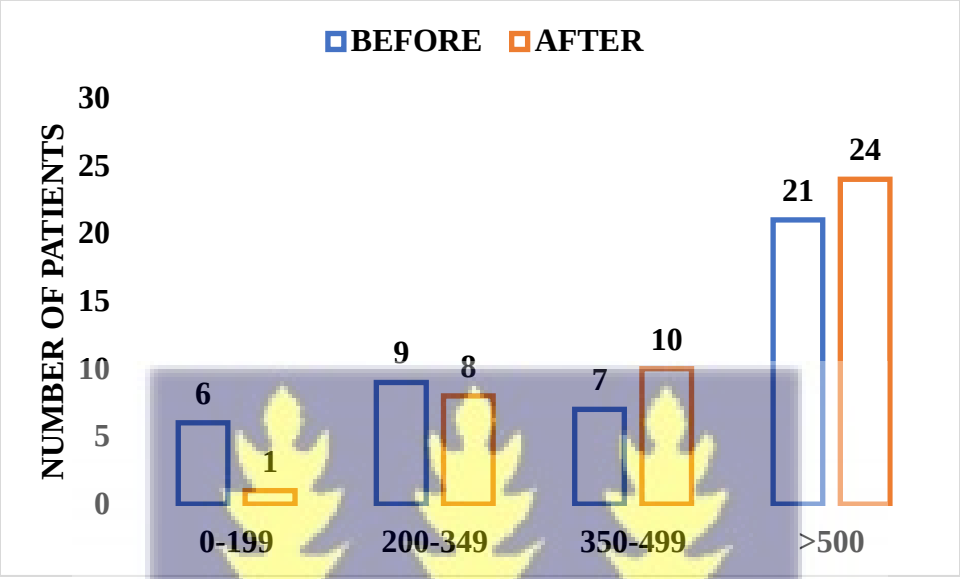


Figure 4. 3 Comparing CD4 counts of patients before and after treatment

Comparing CD4 counts before and after treatment, one patient had CD4 count between 0-99 cell/µl after treatment as against 6 before treatment, 9 patients had CD4 counts between, 200 cell/µl and 349 cell/µl before treatment compare to 8 after treatment in the same range, 10 patients had CD4 counts in the range of 350 cell/µl and 499 cell/µl at follow up three more than the baseline of 7 patients. A total of 24patients had counts greater than 500 cell/µl for the follow up compared to 21 during baseline.

4.3 VIRAL LOAD

With regards to the viral loads of the patients at baseline, 91% (39) of the patients had viral loads greater than 1000copies/ml. while the remaining 9% had viral loads less than 1000copies/ml. one person had a load below the detection limit of assay.

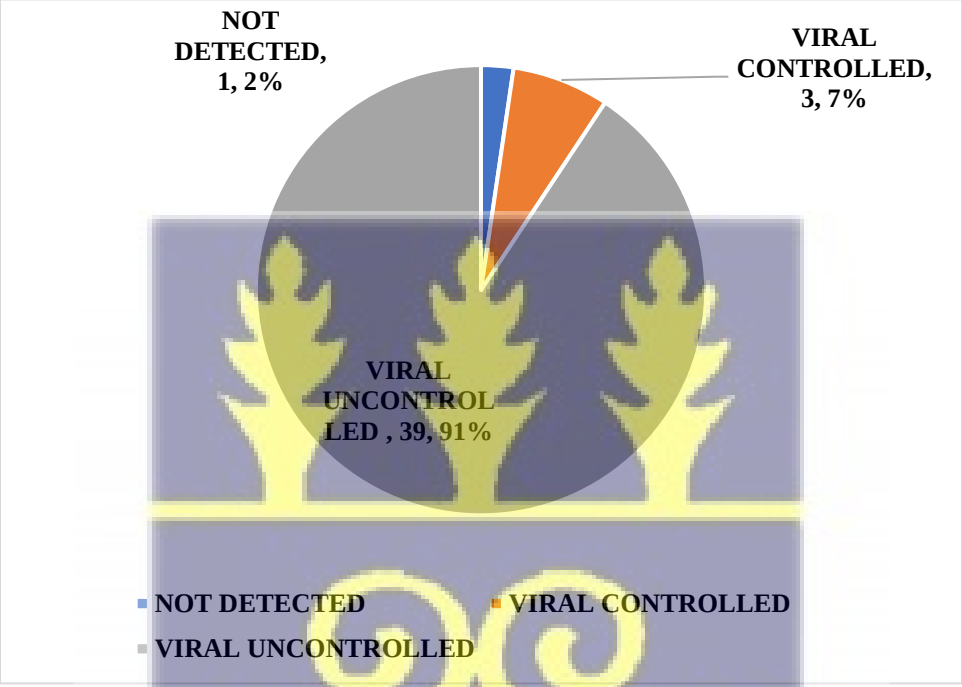


Figure 4. 4 Viral load ranges of patients at baseline

Meanwhile twelve months after treatment, 58% of patients still had viral loads greater than 1000copies/ml while the remaining 42% had achieved viral suppression. Out of the 42%, 19% (8) had undetectable viral nucleic acid and 23% (10) had suppressed viral loads.

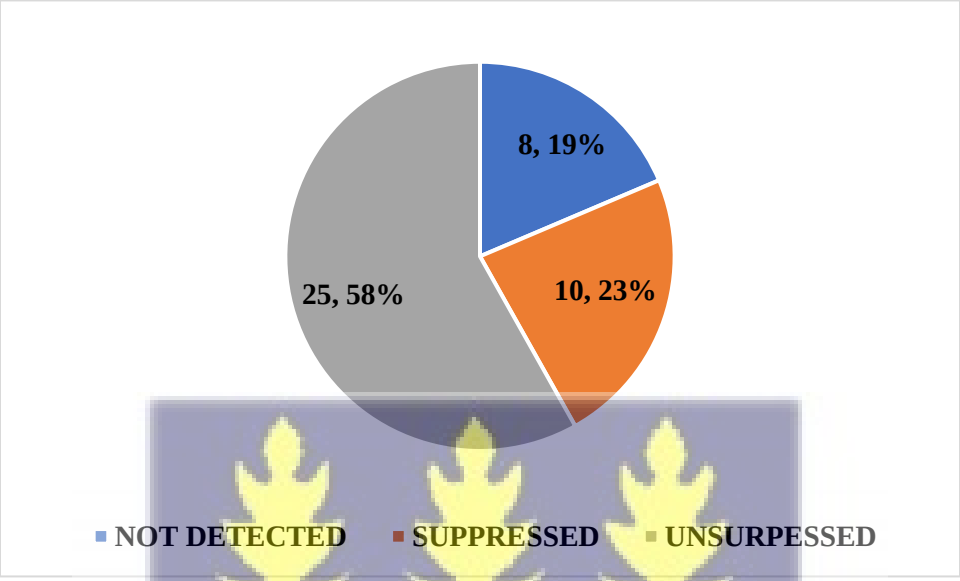


Figure 4. 5 Viral Load distribution after twelve months on therapy

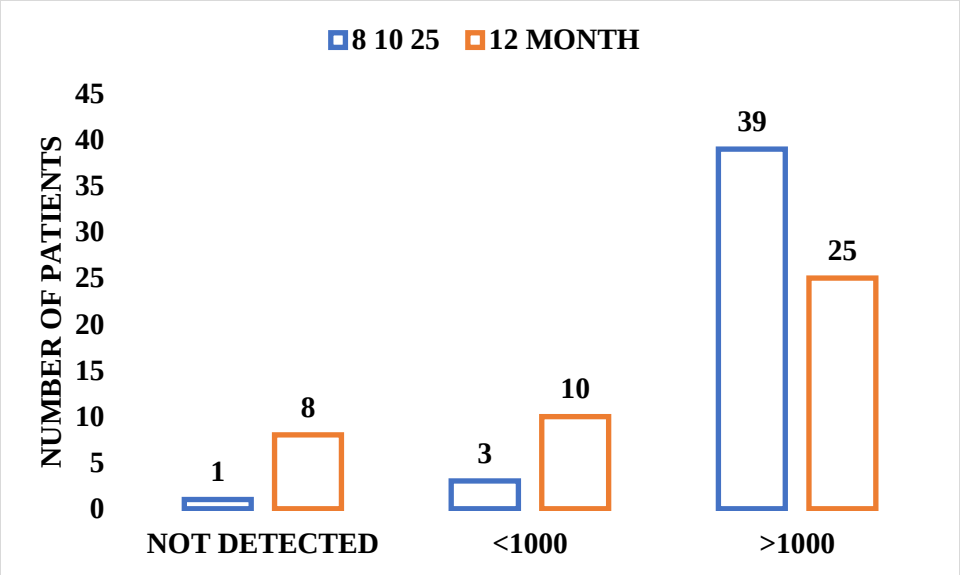


Figure 4. 6 comparing viral loads of patients before and after treatment

Comparing viral loads of patients before and after treatment seven more patients had undetectable viral load during follow ups than in the baseline, thus 8 and 1 respectively. Ten patients had detectable viral loads less than 1000 copies/ml during follow ups whiles 3 had detectable viral load less than 1000 copies /ml at baseline. A total of 25 patients had viral loads greater than 1000 copies/ml during follow ups compared to 39 patients at the baseline.

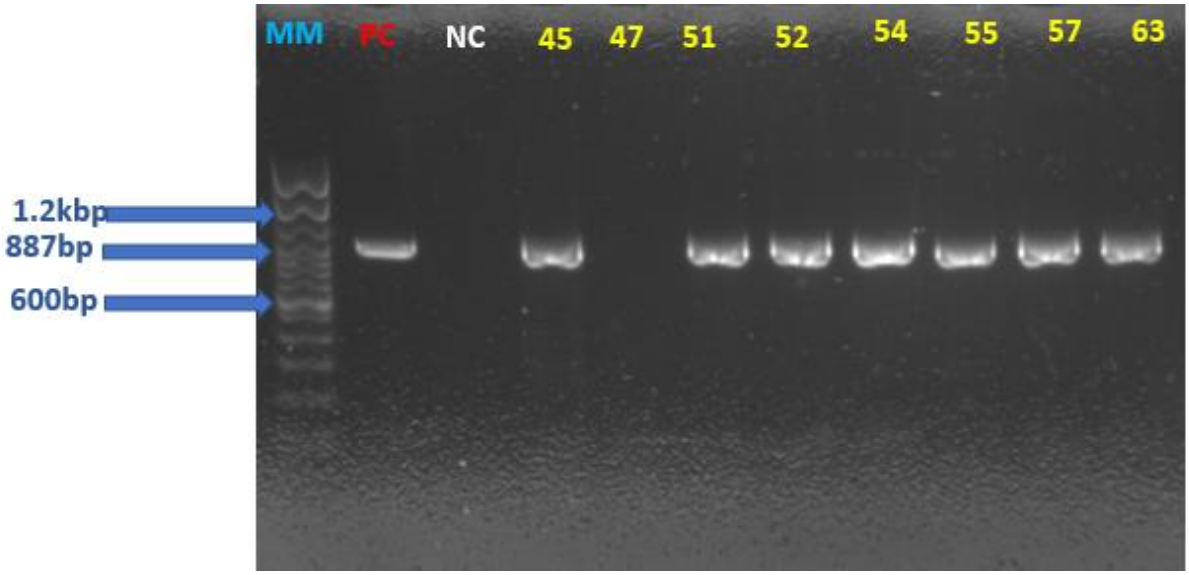


Figure 4. 7:representative gel of PCR amplified Reverse transcriptase gene(887bp)

Figure 4.7 shows an agarose gel electrophoresis of bands on the lane of the predicted size of reverse transcriptase gene. Lane 1 containing MM=molecular marker (ladder) a 100bp from Biolabs, PC positive control in lane 2, NC = negative control in lane 3. Lanes 4 (45) to lanes 11(63) are amplified reverse transcriptase genes lane 5 (47) failed to amplify.

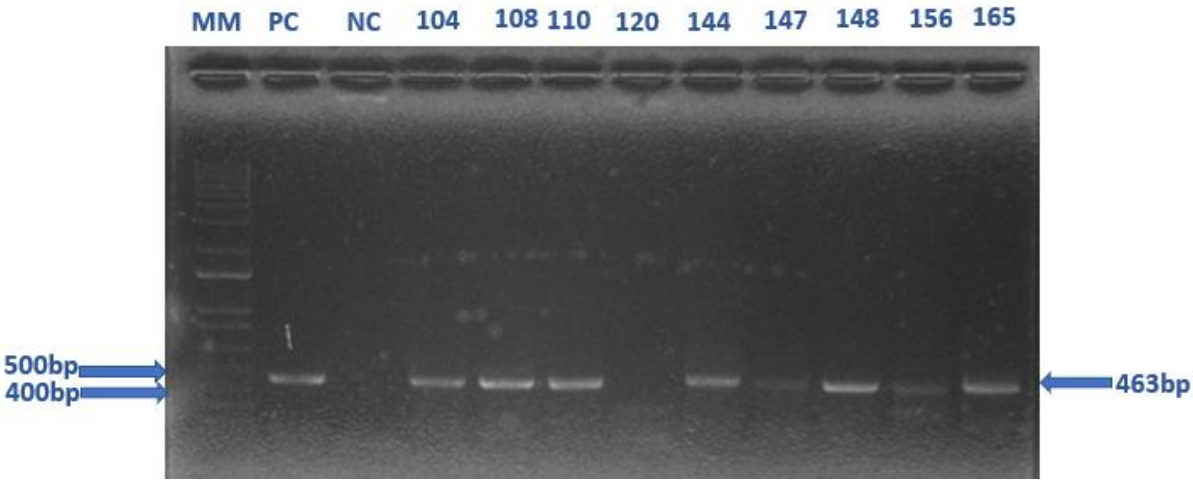


Figure 4. 8:representative gel of PCR amplified Protease gene (463bp)

Figure 4.8 shows an agarose gel electrophoresis of bands on the lane of the predicted size of protease gene. Lane 1 containing MM=molecular marker (ladder) a 100bp from Biolabs (New England) , PC positive control in lane 2, NC = negative control in lane 3. Lanes 4 to lanes 12 are amplified protease (463bp genes for patient. Lanes 7 failed to amplify.

4.4 PCR AMPLICONS

***Table 4. 1: Total* number of achieved PCR amplicons for protease and reverse transcriptase at baseline and follow up**

GENE	PROTEASE	REVERSE TRANSCRIPTASE	TOTAL
BASELINE	37	33	70
FOLLOW UPS	29	35	64
TOTAL	66	68	134

However, 37 protease genes and 33 reverse transcriptase genes were amplified for the baseline.

On the other hand, 29 protease and 35 reverse transcriptase genes were amplified in the follow ups Table 4.1

Table 4. 2: Total number of sequenced PCR amplicons for protease and reverse transcriptase at baseline and follow up

GENE	PROTEASE	REVERSE TRANSCRIPTASE	TOTAL
BASELINE	20	20	40
FOLLOW UPS	20	20	40
TOTAL	40	40	80

Table 4.2 showing samples that were amplified which have their corresponding protease and reverse transcriptase amplifying for both baseline and follow ups.

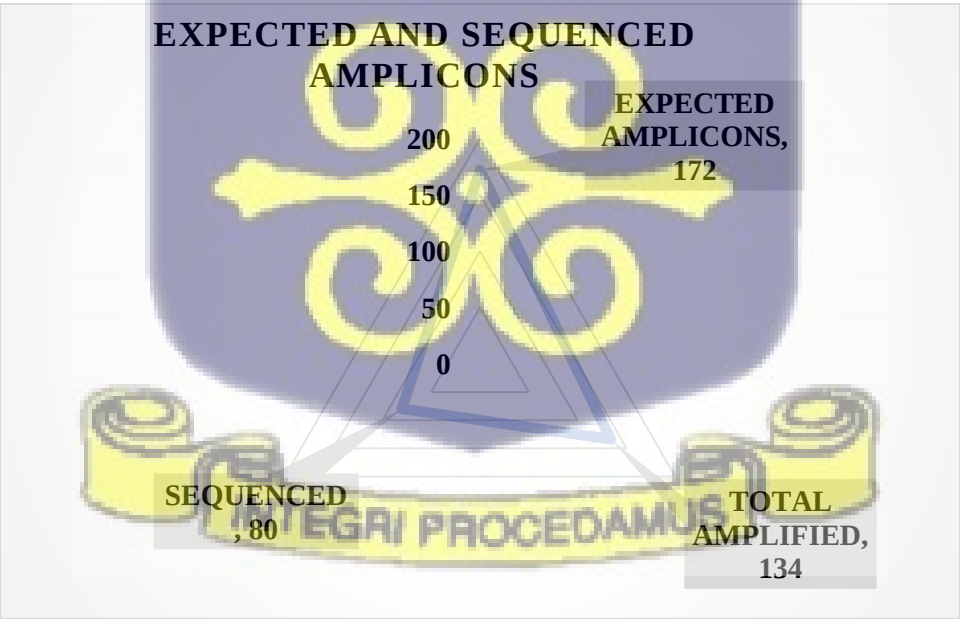


Figure 4. 9: Total number of expected, achieved and sequenced PCR amplicons for protease and reverse transcriptase at baseline and follow up

Figure 4.9 shows the total number of expected amplicons 172 from the 43 patients, thus 43 each of protease and reverse transcriptase at baseline and 43 each of protease and reverse transcriptase genes at follow up, the total number of achieved amplification 134 and the and the number of amplifications that had protease and reverse transcriptase genes amplified for both baseline and follow up that were sequenced, 80, thus 20 each of protease and reverse transcriptase at baseline and 20 each of protease and reverse transcriptase at follow up.

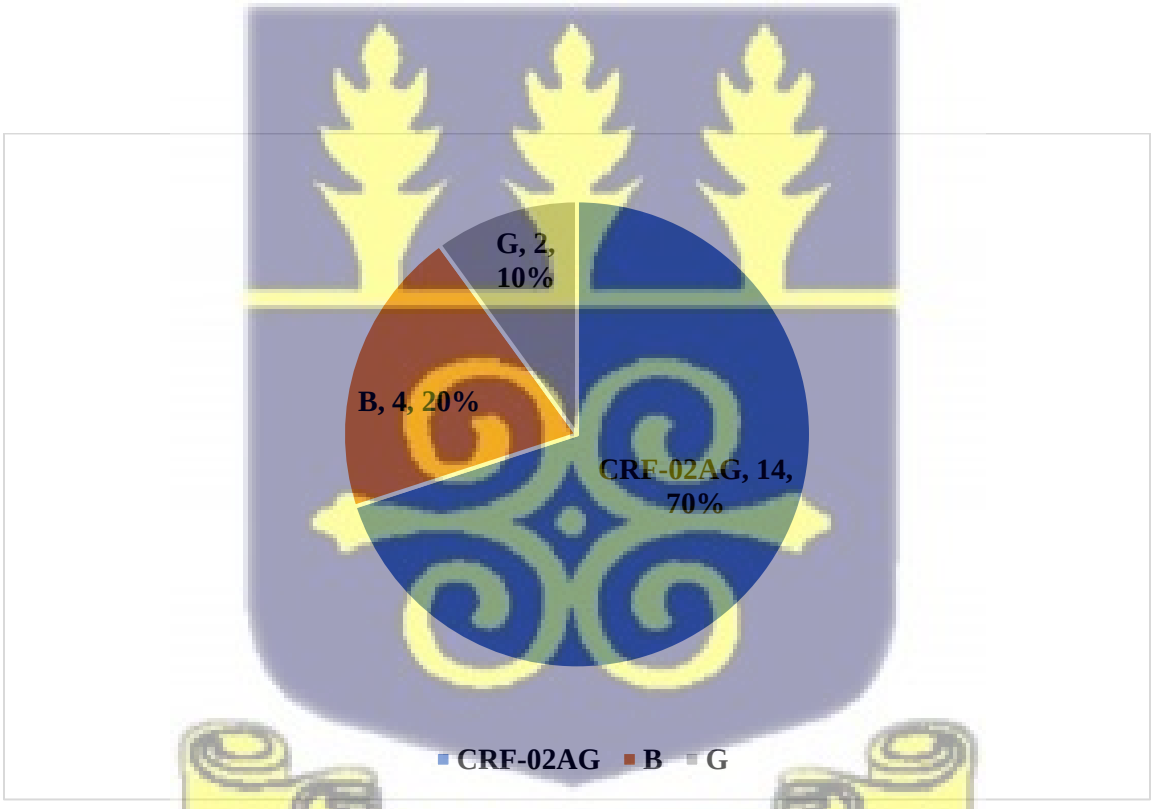


Figure 4. 10:Pie chart showing the percentages of the HIV subtypes

Figure 4.10 shows the subtypes of the sequenced strains. Out of the 20 patients, 10% (2) were subtype G, 20% (4) were subtype B and 70% (14) were CRF-02AG subtype. Subtypes were assigned using the consensus sequences submitted to Rega, Comet and Stanford HIV databases.

4.5 ART COMBINATIONS

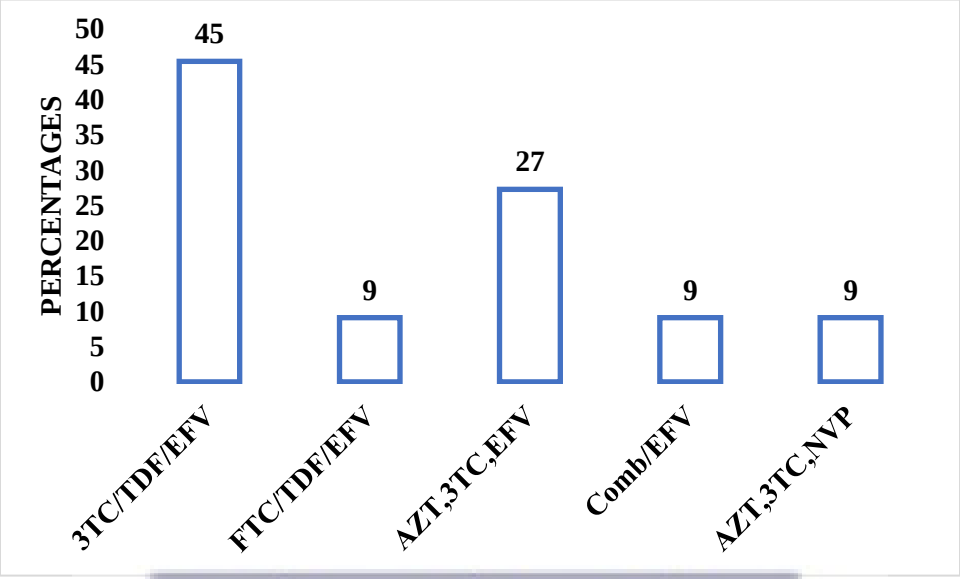


Figure 4. 11:Percentages of combination therapies for the patients with sequenced data

Figures 4.11 shows percentages of combination therapy, 45% of patients were on 3TC/TDF/EFV, 9% on FTC/TDF/EFV, 9% on AZT/3TC/NVP, 9% on Comb/EFV, 27% on AZT/3TC/EFV. Although patients were on Antiretroviral treatment. All the treatment regimen were first line which puts patients at risk of developing resistance strains of the Virus.

4.6 TRANSMITTED RESISTANCE

Table 4. 3: Evidence of transmitted resistance mutations to protease and reverse transcriptase

PROTEASE				REVERSE TRANSCRIPTASE			
MAJOR	FREQ.	ACCESSORY	FREQ.	NRTI	FREQ.	NNRTI	FREQ.
I50N	1	**K43T**	1	**K65R**	1	**Y181C**	1
L90M	1	**L10F**	3				
		Q58E	1				

Table 4.3 shows evidence of transmitted resistant mutations. Two Major resistance mutations to protease inhibitors, I50N and L90M were observed with frequencies of 1 each. K43T occurred once, L10F three times and Q58E once all are accessory protease inhibitor mutations observed at baseline. K65R an NRTI resistance mutation occurred once whiles Y181C an NNRTI resistance mutation was observed once. The major mutations lead to high level resistance while the accessory mutations lead to low level resistance or causing resistance in the presence of other resistant mutations.

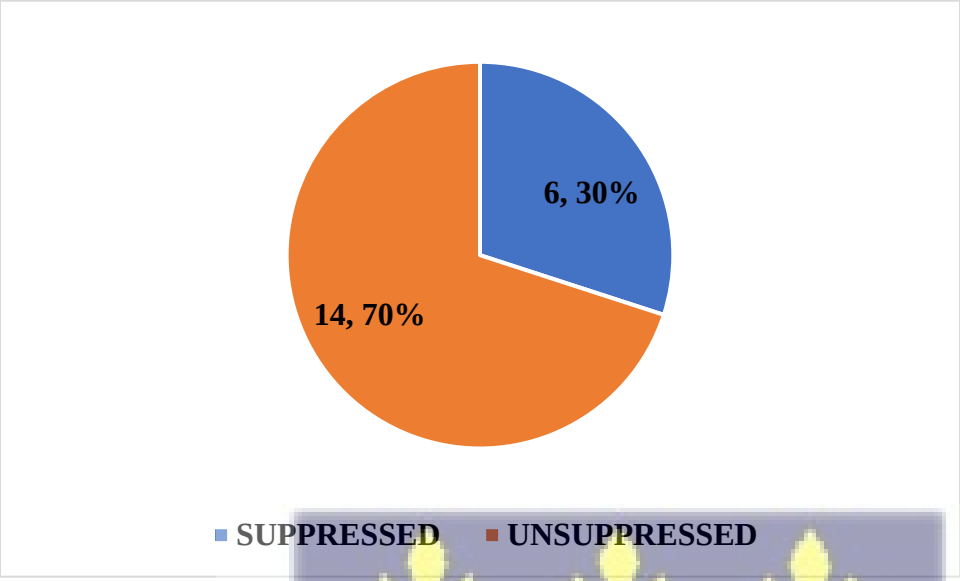


Figure 4. 12:Pie chart showing percentage of patients with suppressed and unsuppressed viral loads after treatment

Defining viral suppression as Viral load <1000 copies/ml, from Figure 4.12, six patients (30%) were virally suppressed after 12 months on antiretroviral, whiles 14 (70%) were still not virally suppressed 12 months after treatment.

Figure 4. 13:Frequencies of reverse transcriptase (RT) resistance and viral loads in follow up

Figure 4.13 shows the comparison of resistance to Reverse transcriptase and Viral load. Low viral load was defined as below 1000 copies/ml and High viral load as >1000copies/ml. Five patients with high viral load had drug resistance mutations to reverse transcriptase at baseline whiles 3 patients with low viral loads developed drug resistance mutation to reverse transcriptase. On the other hand, 9 patients with high viral load developed no drug resistance mutation whiles 3 patients with low viral loads had no drug resistance mutation The Odd ratio of High viral load was 0.455. and p value of 0.6.

Table 4. 4: Reverse transcriptase (RT) resistance and viral loads in follow up

	VIRL LOAD			
RESISTANCE STATUS	**LOW VIRAL LOAD**	**HIGH VIRAL LOAD**	**ODDS RATIO**	**P-VALUE**
RESISTANT	**3**	**5**	**0.45**	**0.6**
NO RESISTANCE	**3**	**9**		

Although per that study findings, it was expected that patients with high viral load were to have higher chances of developing resistance however that was not the case as an Odds ratio of 0.43 showed the was no relationship between having high viral load and developing resistance although the p value of 0.6 was also not significant.

Table 4. 5: Resistance and CD4 counts in follow ups

	CD4 COUNT			
RESISTANCE STATUS	**LOW CD4 COUNT**	**HIGH CD4 COUNT**	**ODDS RATIO**	**P-VALUE**
RESISTANT	**5**	**3**	**3.33**	**0.3**
NO RESISTANCE	**4**	**8**		

Table 4.5 shows the comparison of resistance to Reverse transcriptase and CD4 count. Low CD4 count was defined as below 500 cells/µl and High CD4 count as > 500 cells/µl. Three patients with high CD4 count had drug resistance mutations whiles 5 patients with low CD4 count developed drug resistance mutation. On the other hand, 4 patients with low CD4 count developed no drug resistance mutation whiles 8 patients with high CD4 count had no drug resistance mutation. The Odds ratio of low CD4 count was 3.33.

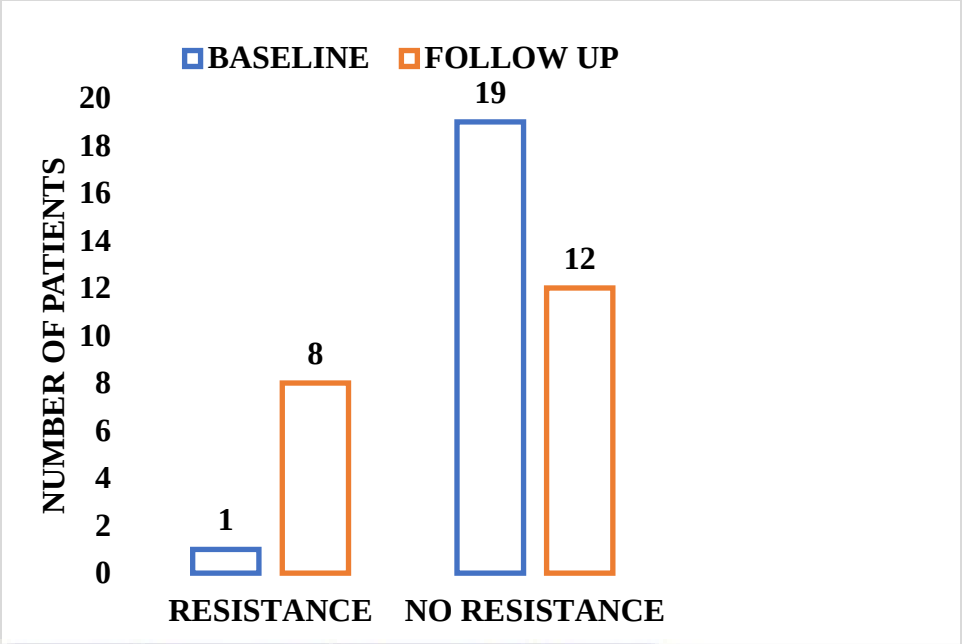


Figure 4. 14: Comparing resistance to reverse transcriptase at baseline and follow up

Figure 4.14 shows the comparison of resistance to Reverse transcriptase at base line and follow up. One patient at baseline had drug resistance mutations to reverse transcriptase whiles 8 patients developed drug resistance mutation to reverse transcriptase at follow up. On the other hand, 19 patients at baseline developed no drug resistance mutation to reverse transcriptase whiles 12 patients in the follow up had no drug resistance mutation to reverse transcriptase. The Odds ratio of developing drug resistance to reverse transcriptase during follow up was 12.67 and p value of 0.02 which is statistically significant that is patients with higher viral loads are more likely to develop resistance to reverse transcriptase as compared to their counterparts with low viral loads.

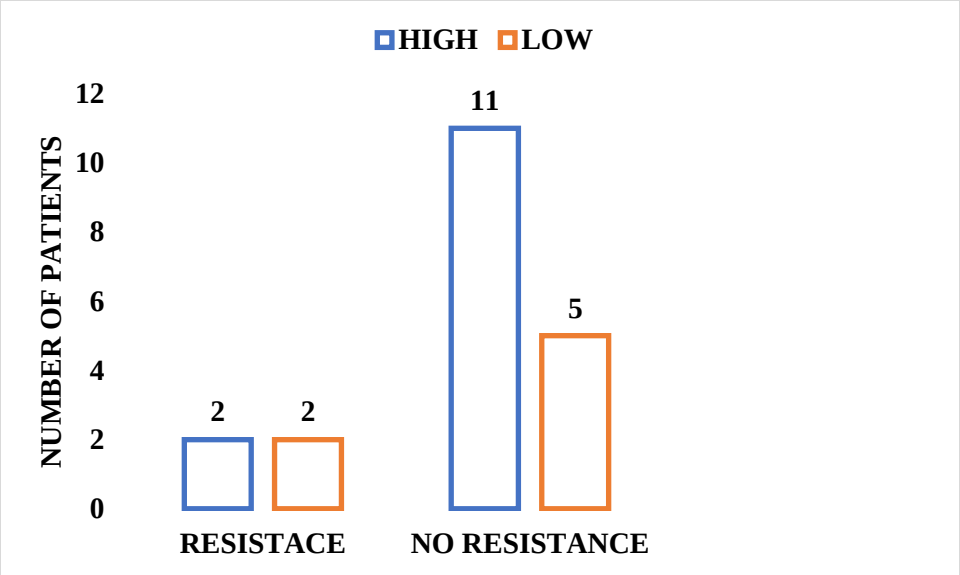


Figure 4. 15: Protease resistance and Viral load during follow ups

Figure 4.15 shows the comparison of resistance to Protease inhibitors and Viral load at follow up. Low viral load was defined as below 1000 copies/ml and High viral load as >1000copies/ml. Two patients with high viral load had drug resistance mutations to protease inhibitors whiles 2 patients with low viral loads developed drug resistance mutation to protease inhibitors. On the other hand, 11 patients with high viral load developed no drug resistance mutation whiles 5 patients with low viral loads had no drug resistance mutation to protease inhibitors. The Odd ratio of High viral load was 0.45.

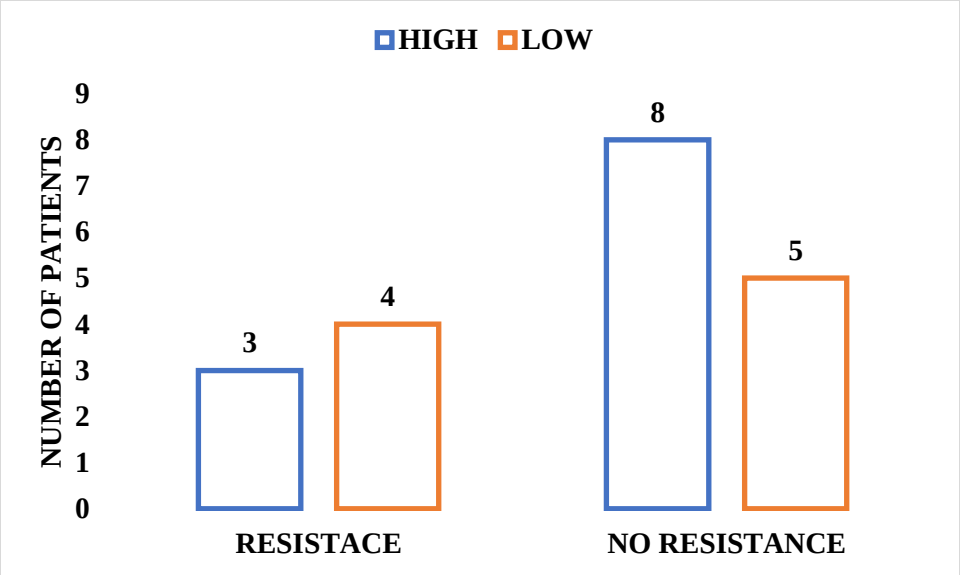


Figure 4. 16: Comparing protease resistance in low and high CD4 counts

Figure 4.16 shows the comparison of resistance to protease inhibitors and CD4 count. Low CD4 count was defined as below 500 cells/µl and High CD4 count as > 500 cells/µl. Three patients with high CD4 count had drug resistance mutations to protease inhibitors whiles 4 patients with low CD4 count developed drug resistance mutation to protease inhibitors. On the other hand, 5 patients with low CD4 count developed no drug resistance mutation to protease inhibitors whiles 8 patients with high CD4 count had no drug resistance mutation to protease inhibitors. The Odds ratio of low CD4 count was 2.133.

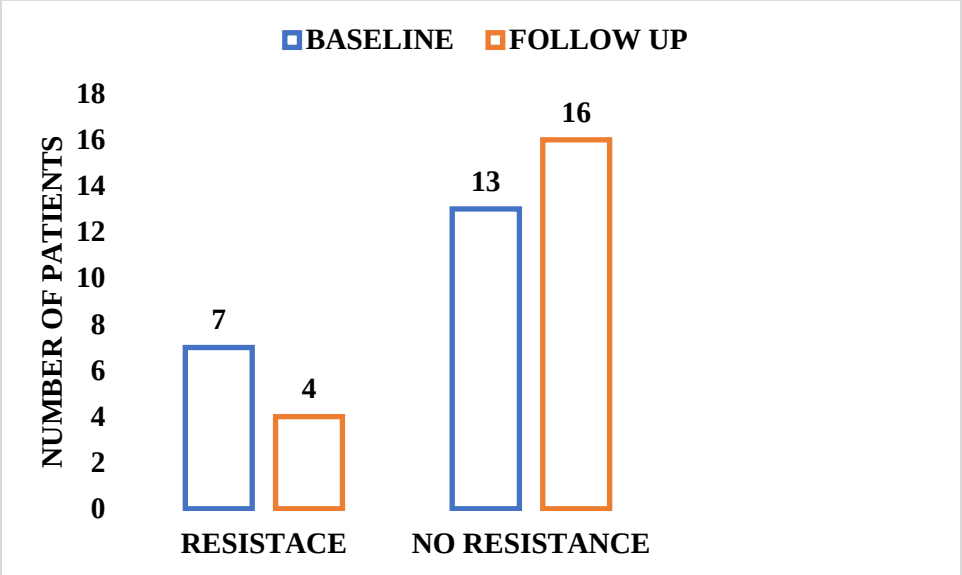


Figure 4. 17:Baseline and follow up protease resistance comparison

Figure 4.17 shows the comparison of resistance to protease inhibitors at base line and follow up. Seven patients at baseline had drug resistance mutations to protease inhibitors whiles 4 patients developed drug resistance mutation to protease inhibitors at follow up. On the other hand, 13 patients at baseline developed no drug resistance mutation to protease inhibitors whiles 16 patients in the follow up had no drug resistance mutation to protease inhibitors. The Odds ratio of developing drug resistance to protease inhibitors during follow up was 0.46 whiles the odds ratio of developing protease inhibitors resistance at baseline was 2.15

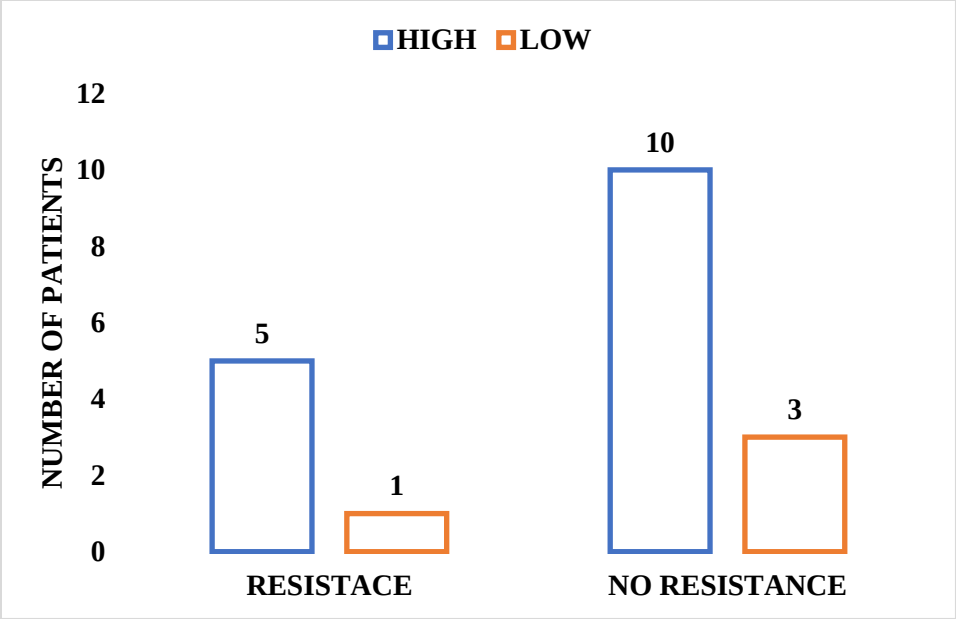


Figure 4. 18: comparing baseline PR resistance in low and high viral loads

Figure 4.18 shows the comparison of resistance to Protease inhibitors and Viral load at baseline. Low viral load was defined as below 1000 copies/ml and High viral load as >1000copies/ml. Five patients with high viral load had drug resistance mutations to protease inhibitors whiles 1 patient with low viral loads developed drug resistance mutation to protease inhibitors. On the other hand, 10 patients with high viral load developed no drug resistance mutation to protease inhibitors whiles 3 patients with low viral loads had no drug resistance mutation to protease inhibitors. The Odd ratio of High viral load was 1.5.

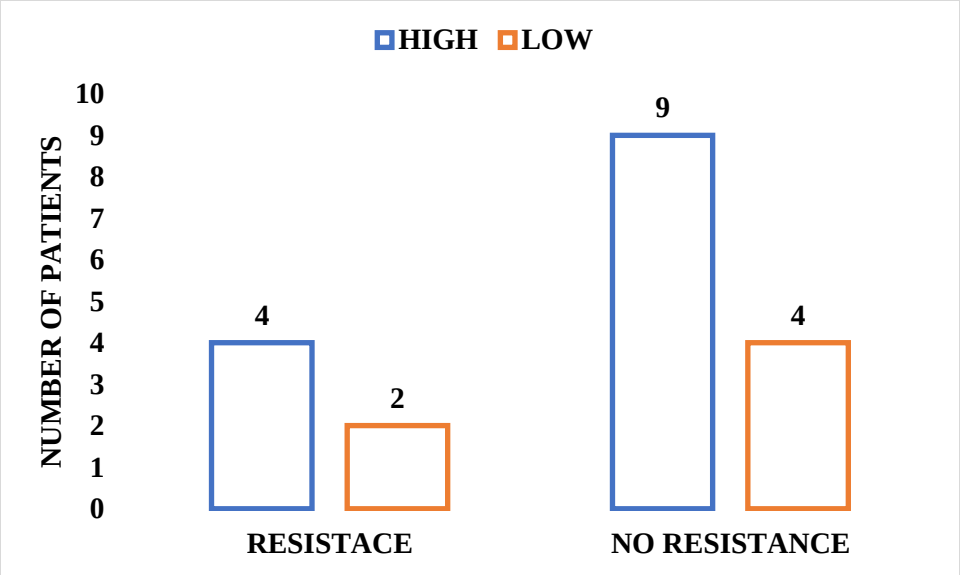


Figure 4. 19: Comparing baseline CD4 count and resistance to PR inhibitors

Figure 4.19 shows the comparison of resistance to protease inhibitors and CD4 count. Low CD4 count was defined as below 500 cells/µl and High CD4 count as > 500 cells/µl. Four patients with high CD4 count had drug resistance mutations to protease inhibitors whiles 2 patients with low CD4 count developed drug resistance mutation to protease inhibitors. On the other hand, 4 patients with low CD4 count developed no drug resistance mutation to protease inhibitors whiles 9 patients with high CD4 count had no drug resistance mutation to protease inhibitors. The Odds ratio of low CD4 count was 1.13.

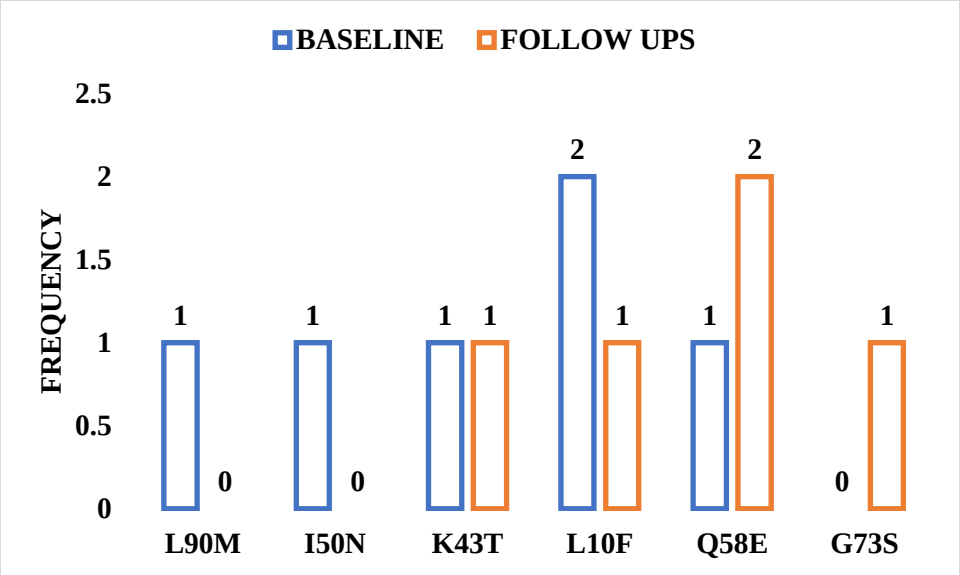


Figure 4. 20: comparing protease mutations at baseline and follow up

Figure 4.20 shows the comparison of protease mutations at baseline and follow up. Each of L90M and I50N appeared once at baseline but not in the follow up. K43T appeared once at baseline and once at follow up. L10F had a frequency of 2 at baseline and 1 at follow up. Q58E appeared once at baseline and twice during follow up. G73S did appear at baseline however did appear once during follow up.

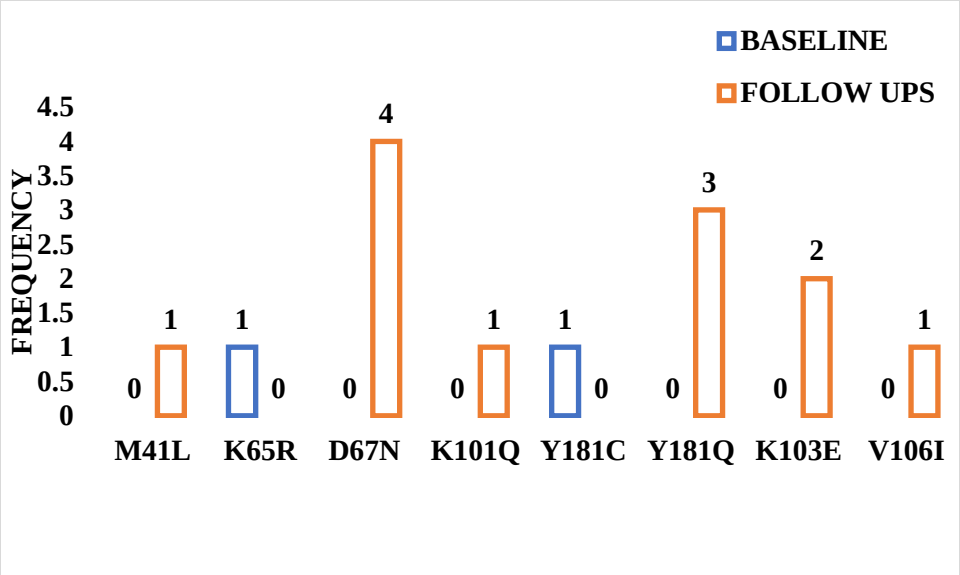


Figure 4. 21: Frequencies of baseline and follow up reverse transcriptase resistance mutations

Figure 4.21 shows the frequencies of reverse transcriptase resistance mutations at baseline and follow up. Each of M41L, K101Q and V106I occurred once in the follow up but not at baseline. K103E, Y181Q, and D67N had frequencies of 2, 3 and 4 respectively at follow up but did not appear in the baseline. Each of K65R and Y181C occurred once in the baseline and not in the follow up.

CHAPTER 5

5.0 DISCUSION

5.1 DEMOGRAPHICS

The 43 patients used in the study comprised of 30 females (70%) and 13 males (30%). These figures are a little higher than figures reported in west central Africa that is 56% women and 44% among men (Girum *et al*., 2018) however an estimated 65% of Ghanaian HIV patients are females whiles 34 percent are males which is in agreement with my reported data (Owusu *et al*., 2020). In other studies, 79% of new infections in sub-Saharan Africa are in adolescent females between fifteen and nineteen years whiles females between the ages of ten and nineteen makes up 75% of new infections in southern and eastern Africa (Owusu *et al*., 2020). This Study's finding is in agreement with UNAIDs report Ghana 2020 which showed that 69% of all HIV patients were women. Some studies attribute this high proportion of women to altered microbiota in the vaginal flora (McClelland *et al*., 2018). Whiles other studies attribute this to immune mediators such as systemic and mucosal cytokines in women (Liebenberg *et al*., 2017). This may also be partially as a result of high number of women compared to men as well as more women having access to HIV screening especially during antenatal

5.2 CD4 COUNT

Though before initiation of treatment, 35%, 65% had CD4 counts <350 cells/µl and > 500 cell/µl respectively, a similar study in China showed 84.5%, 10.0% and 3.7% having CD4 counts within the ranges of <350 cells/µl, 350–499 cells/µl, and ≥500 cells/µl (Tang *et al*., 2017). This means that either the patients I was working with were diagnosed earlier or they generally had better immune status compared to their counterparts in China. This is a very good predictor of better

patients' response when put on treatment (Bordoni *et al*., 2019). Whiles many countries are making the efforts to reduce the transmission rate by adopting the treat all policy, Indonesia only treat patients with CD4 counts <350 cells/µl as such has only 13% of their HIV population on treatment. This means that over 87% of the known HIV population have CD4 counts greater than 350 cells/µl comparable to what was achieved in this study (Tromp *et al.*, 2018)

It is worth noting that 65% of patients had improved CD4 counts after treatment. Comparing CD4 counts before and after treatment, one patient had CD4 count between 0-99 cell/µl after treatment as against 6 before treatment, 9 patients had CD4 counts between, 200 cell/µl and 349 cell/µl before treatment compare to 8 after treatment in the same range, 10 patients had CD4 counts in the range of 350 cell/µl and 499 cell/µl at follow up 3 more than the baseline of 7 patients. A total of 24 patients had counts greater than 500 cell/µl for the follow up compared to 21 during baseline.

Another study conducted in South Africa showed that 81% of patients before treatment had CD4 counts less than 200 cells/µl which drastically reduced to 19% after treatment (Redman *et al*., 2018). The results implies that patients were responding to treatment as such symptoms such as loss of appetite and weight loss were going to be minimized (Wakeham *et al*., 2018).

5.3 TREATMENT REGIMEN

In the combination therapies, 45% of patients were on 3TC/TDF/EFV, 9% on FTC/TDF/EFV, 9% on AZT/3TC/NVP, 9% on Comb/EFV, 27% on AZT/3TC/EFV. This may be as a result of doctors strictly adhering to WHO recommendation of all patient being put on first line drugs (WHO 2018). Another study in Ghana found over 60% of patients of AZT/3TC/EFV combination whiles prescription of stavudine dropped to zero which is in conformity with the findings in this study

(Ankrah *et al*., 2017). In a study a greater percent of patients had switched from first line to second line therapy due to virological failure and immunological failure (Ross *et al.*, 2021). This was not the case in this study as all patients were on first line ARTs. In a similar study in China found, 84.5% of all ART regimen being first line, however 90% of all patients were on first line therapy, whiles the remaining 9.7% were on second line (Tang *et al*., 2017). Although the treatments stated above are the standard first line treatment in Ghana, the WHO in 2020 recommended Dolutegravir (DTG), an integrase strand transfer inhibitor as part of the first line therapy as it was found to be effective in patients with rebound viral loads (Zhao *et al*., 2021). Though most of my patients had efavirenz an NNRTI, as part of their regimen, it was no longer recommended Europe and the USA due to safety concerns and tolerability (Cihla *et al*., 2016). It is known that patients on first line ART for longer periods are less likely to have a viral suppression as the chances of developing resistance to these first line are higher compared to patients of second- and third-line therapies (Ford *et al*., 2019).

5.4 TREATMENT RESPONSES WITH DIFFERENT COMBINATION THERAPIES

5.4.1 Combination therapy and Resistance

On the average, all patients had reduced viral load after treatment whiles the average CD4 increased. At least one patient of 3TC/TDF/EFV, AZT/3TC/EFV and Comb/EFV developed a resistance mutation after treatment while no patient taking FTC/TDF/EFV and AZT/3TC/NVP developed resistance mutation. this study confirms study by Marcelin et al 2012 where resistance mutations were observed in patients on 3TC/TDF/EFV and FTC/TDF/EFV. A similar study by

Bulteel et al 2014 involving 3TC/TDF/EFV and FTC/TDF/EFV also showed the emergence K65R andM184V. Resistance mutations K65R and K103N were observed in a Ugandan study involving AZT/3TC/EFV on HIV 1 patients (Ayitewala *et al.,* 2020) which comparable to observation made in this study.

5.4.2 Combination therapy and CD4 count

This study's finding of increased CD4 count in most of the patients is in lines with Kebede et al's 2017 finding of most patient on first line combination therapy having an improved CD4 count though few experiencing reduced count. This study showed relatively higher CD4 count in TDF based therapies, which is in line with Ayele et al's 2017 finding in which tenofovir based regimen such as 3TC/TDF/EFV and FTC/TDF/EFV were observed to have a better immunological response than other combination therapies though other therapies had improved CD4 counts as well.

5.4.2 Combination therapy and viral load

In this study, 30% of the patients achieved viral suppression while the remaining 70% did not achieve viral suppression however, 70% of the patients had improved viral loads but not all had achieved viral suppression after 12 months on ART. A similar study by in Swaziland Etoori et al 2018 observed a 58% of patients achieving viral suppression.

5.5 VIRAL LOAD

As a result of UNAIDS 90 90 90 target by the year 2020, thus 90% of all HIV patients must have been diagnosed and at least 90% being on therapy whiles 90% of all those on therapy attaining viral suppression. This has made viral loads testing and monitoring very paramount to achieving this target as it will ensure proper management and adherence (Peter *et al*., 2017).

Meanwhile twelve months after treatment, 58% of patients still had viral loads greater than 1000copies/ml while the remaining 42% had achieved viral suppression. Out of the 42%, 19% (8) had undetectable viral nucleic acid.

Ghana AIDs commission reports as at June 2021 indicates that Ghana has so far achieved 58-77-68 of the 90-90-90 (ghanaids., 2021). Meanwhiles UNAIDS report for the same time period for Ghana showed that Ghana has so far achieved 58-45-31 as at June 2021 (UNAIDS, 2021). Although there are differences in the figures for the third of the 90 which is the viral suppression bit. All the values indicate that Ghana has still not only failed in achieving the 90-90-90, we have performed very poorly when it comes to the viral suppression.

Despite using stringent viral suppression of <50 copies /ml as virally suppressed, Sweden by 2015 achieved the UNAIDs target of 90-90-90 (Gisslen et al., 2017).

5.6 HIV SUBTYPING

Out of the 20 patients, 10% (2) were subtype G, 20% (4) were subtype B and 70% (14) were CRF-02AG subtype. Nii-Trebi et al in 2013 reported 66% CRF-02AG as the most predominant strain in Ghana. our data confirms what has been reported in Nii-Trebi with CFR02_AG being the most predominant subtype in Ghana. despite a slightly higher percentage than that report by Nii -Trebi. Epidemiologically about 46.6% of global HIV-1 subtypes are C making it the most predominant globally followed by Subtype B 12.1%, Subtype A makes about 10.36%, 7.7% being CRF02_AG, URF makes 6.1%, CRF01-AE with 5.3%, subtypes G and D makes 4.6% and 2.7% respectively, other CRFs makes 3.7% (Hemelaar *et al*., 2019). In spite of these global distributions, CRF02_AG observed in this study makes up the most predominant subtype in West Africa, subtype B mostly

in Oceania, Americas and Europe (Bbosa *et al*., 2019) whiles subtype G found predominantly in Africa (Murzakova *et al*., 2019).

Sub type B hitherto not reported in Ghana gradually on the rise are likely cases imported (Obeng *et al*., 2020). Globally, there is a surge in the number of subtype B cases hitherto predominantly in Europe and a dip in the number of CRF-02AG (Murzakova *et al*., 2019) which has reflected in this study. The decrease in the number of CRF-02AG the increase in B case in this study is in agreement with the Russian study. Comparing this study to previously reported subtypes in 2017 by Nii- Trebi et al when79.2% of all subtypes were CRF-02AG and no subtype B reported. This study reported a 10% subtype G which is in agreement with the reported 8% in 2017 (Nii-Trebi *et al*., 2017).

5.7 TRANSMITTED RESISTANCE

Thirty percent of the patients in this study had protease resistance mutations as naïve patients. A similar study showed a 21% resistance (Turner *et al*., 2012). Two Major resistance mutations to protease inhibitors, I50N and L90M were observed. K43T, L10F and Q58E all are accessory protease inhibitor mutations observed at baseline. K65R an NRTI resistance mutation occurred once whiles Y181C an NNRTI resistance mutation was observed once. These observations are in line with what was recorded in an Iranian study in naïve patient where resistance mutations to protease inhibitors, NRTIs and NNRTIs were detected (Farrokhi *et al*., 2019). It is worth noting that a cluster of these mutation in a single individual limit therapeutic option (Turner *et al*., 2012).

The mutation I50L is known to increase susceptibility to protease inhibitors except for atazanavir in which it results in resistance (Sista et al., 2008). Energy decomposition increase binding to I50L

due to increase electrostatic energy and Van der Waals. A surge in polar solvation energy cancels out the effect. These leads to increase binding activity to protease inhibitors as a result of the Leu backbone (Meher *et al*., 2012). In the case of I50V, decrease in electrostatic energy is the main driving force for its resistance to inhibitors in addition to the increase in solvation energy between inhibitors and valine 50 residue (Chen *et al*., 2010). Non-polymorphic mutations I50L and I50V are protease inhibitor mutations. Whiles I50L increases susceptibility to all protease inhibitors except ATV, I50V selects resistance to DRV, LPV and FPV however, I50N observed in this study is a highly unusual mutation at this position as such will require further studies to better understand its mechanism of action. Currently no study has reported it. (https://hivdb.stanford.edu/hivdb/by-mutations/report/accessed 07/06/2021)

HIV protease enzymes with L90M mutations instigates structural variations in the enzyme that destabilize binding interactions (Henes *et al*., 2019). The flap region of HIV protease at position 90 is hydrophilic. A mutation in this region renders it hydrophobic thereby physiochemically burying the flap region leading to a reduced susceptibility to protease inhibitors (Su *et al*., 2015). L90M observed in this study is in line with that observed in an Israeli study where clusters of this mutation were detected in treatment naïve individuals though was amongst high-risk sexual behavior individuals, men who have sex with men (MSM) (Turner *et al*., 2012). In a similar study is South Africa, L90M was amongst the protease resistance mutations detected in naïve patient (Digban *et al*., 2019).

For the NRTI resistance mutations, K65R and Y181I/V are amongst the commonest mutations found in treatment naïve patients though no Y181I/V was found in my naïve patients but Y181C (Oliveira e*t al*., 2016). Y181C which usually occurs with K103N as the most predominant NNRTI mutation as well as the most common in patients receiving first generation NNRTIs such as

etravirine, nevirapine and a clue that this may have been transmitted from a patient on one of these medications (Gao *et al.,* 2021). Whiles newer generations of NNRTIs such as rilpivirine are capable of clearing viruses with these mutations, they also come with their own challenges of toxicities, low solubility and hence reduced bioavailability (Sasaki *et al.,* 2019). The homopolymers near the K65 may lead to slippage at this position during DNA synthesis and subsequently in a frame shift which will result in realignment and dislocation of primer and template the outcome is K65R mutation (Coutsinos *et al*., 2011). Despite the reduction in viral fitness as a result of K65R mutation, it is responsible for incorporating natural deoxynucleotide triphosphates and excising NRTIs as such is implicated in varying degrees of phenotypic resistance to a number of NRTIs thymidine analogues excluded. These properties have been attributed to the guanidine plane created between arginine at positions 72 and 65 as responsible for the resistance properties of K65R. (Coutsinos *et al*., 2011). A study in Brazil found a surge in the number of K65R cases in patients on treatment from 2.23 to 12.11% as well as increased viral loads in patients with this mutation (Santos-Pereira *et al.,* 2021). Our finding K65R in naïve patients agrees with Obeng et al (2020) who also found K65R in ART naïve voluntary blood donors.

5.8 RESISTANCE AND VIRAL LOAD

In this study, 5 patients with high viral load had drug resistance mutations to reverse transcriptase at baseline whiles 3 patients with low viral loads developed drug resistance mutation to reverse transcriptase. On the other hand, 9 patients with high viral load developed no drug resistance mutation whiles 3 patients with low viral loads had no drug resistance mutation The Odd ratio of High viral load was 0.455. and p value of 0.6. This result is not in line with what is expected. A study in France showed that resistance to at least one drug rose significantly with increase viral

loads (Assoumou *et al*., 2017). A population-based study in Cameroun evaluating the rate of acquisition of drug resistance mutation and viral suppression in sub-Sahara Africa disclosed that resistance to ART increases with increase viral load (Tchouwa *et al*., 2018). The disparity between this study's outcome and these other two studies may be as result of different samples sizes used, geographical location may play a key role.

5.9 COMPARING NRTI AND NNRTI RESISTANCE MUTATIONS AT BASELINE AND FOLLOW UP

Comparison of resistance to Reverse transcriptase at base line and follow up showed that 1 out of 20 patients at baseline had drug resistance mutations to reverse transcriptase whiles 8 patients out of 20 developed drug resistance mutation to reverse transcriptase at follow up. The Odds ratio of developing drug resistance to reverse transcriptase during follow up was 12.67 and p value of 0.02 which is significant at 95% confidence interval. These findings are in line with a study in Yaoundé, Cameroun that seek to characterize resistant strain in HIV naive patients. Eight percent of patients at baseline had transmitted drug resistance however over 80% of those placed on first line therapy developed resistant mutation confirming this study's outcome. The study suggested educating physicians and patients respectively on the use of genotypic resistance and adherence as a means of curbing the menace (Ceccarelli *et al*., 2012). A similar study conducted by Muwonga et al., 2011 gave a similar outcome where drug resistance mutations were accumulated overtime in patients on ART leading to treatment failure. This finding may mean that either the patients are not adhering to their medication or first line therapies mostly NRTIs and NNRTIs are becoming less effective losing their clinical significance (Rocheleau *et al*., 2018).

5.10 CD4 COUNT AND RESISTANCE

In this study, comparison of resistance to Reverse transcriptase and CD4 count was made. Low CD4 count was defined as below 500 cells/µl and High CD4 count as > 500 cells/µl. Three patients with high CD4 count had drug resistance mutations whiles 5 patients with low CD4 count developed drug resistance mutation. On the other hand, 4 patients with low CD4 count developed no drug resistance mutation whiles 8 patients with high CD4 count had no drug resistance mutation. The Odds ratio of low CD4 count was 3.33. a similar study in Nigeria on the predictors of ART resistance showed that low CD4 count as well as high viral loads and non-adherence were among the factor in predicting virologic failure in patients on ART (Ekong et al., 2020).

With consensus from these studies all efforts must be put in to ensure patients respond to treatment virologically and immunologically by closely monitoring and of switch regimen to a higher generation or higher line of treatment.

5.11 RESISTANCE AT BASELINE AND FOLLOW UP

The reverse transcriptase resistance mutations observed at baseline and follow up include M41L, K101Q, V106I, K103E, Y181Q, and D67N had frequencies of 1,1,1, 2, 3 and 4 respectively at follow up but did not appear in the baseline. Each of K65R and Y181C occurred once in the baseline and not in the follow up. A similar study in India observed, M41L, K65R and D67N, for NRTIs whiles K103N Y181C, and G190A mutation for NNRTIs were also observed. Another study in the United States found the most predominant reverse transcriptase resistance mutations T215Y, M184V, K103N and K65E which was not the case in this study, however, similar

mutations were observed in both outcomes. M41L a member of the thymidine analogue mutation leads to resistance zidovudine and stavudine and cross resistance to Tenofovir. Though stavudine is no longer in use, most of my patients were on zidovudine (Pingen *et al*., 2015). K101Q, V103I, V106I and Y181Q are NNRTI mutations that lead to reduced susceptibility to Nevirapine, Efeverence, Etravirine and rilpavirine (Jiao *et al.*, 2012) The subjects in this study though were not on rilpivirine and Etravirine, they were all on either Nevirapine or Efavirenz.as such presence of these mutations can lead to viral rebound and treatment failure.

CHAPTER 6

6.0 CONCLUSION

This study found 70% of circulating HIV in Ghana were CRF02_AG, 20% subtype B and 10% subtype G making CRF02_AG the most predominant genotype in Ghana. Protease and reverse transcriptase inhibitor mutations were observed at baseline and follow up though some patients with low and high viral loads and CD4 counts had developed resistance mutations. Major resistance mutations to protease inhibitors, I50N and L90M were observed with frequencies of 1 each. K43T occurred once, L10F three times and Q58E once all are accessory protease inhibitor mutations observed at baseline. K65R an NRTI resistance mutation occurred once whiles Y181C an NNRTI resistance mutation was observed. Protease resistance mutations observed during follow up includes K43T, L10F and Q58E. Reverse transcriptase resistance mutations observed during follow ups includes M41L, K101Q, V106I, K103E, Y181Q, D67N. The Odds ratio for developing an RT inhibitor mutation after 12 months on therapy=12.67. The Odds ratio for an RT inhibitor mutation with low CD4 count at follow up= 3.33. The Odds ratio for developing an PR inhibitor mutation after 12 months on therapy=0.46. The Odds ratio for an PR inhibitor mutation with low CD4 count at follow up= 2.1. There seems to be a surge in the number of HIV-1 subtype B infection in Ghana which hitherto only was detected in low frequencies and this could be attributed to subtype importation. Currently only 30 percent of patient on ART achieved viral suppression after one year on treatment

6.1 RECOMMENDATIONS

This study had a short fall of smaller sample size. A scaled up study, be carried out with larger sample size drawn from many sites across the country will provide the national picture of the emergence of drug resistance mutations during antiretroviral therapy.

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