THE EFFECTIVENESS OF FIRST LINE ANTIRETROVIRAL THERAPY AND THE IMPACT OF MICRONUTRIENT LEVELS ON PATIENTS' RESPONSE TO TREATMENT IN WINDHOEK, NAMIBIA

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ABSTRACT

Nutritional levels can have profound impact on individuals living with HIV/AIDS and potentially hinder the effectiveness of antiretroviral therapy (ART). But sufficient data is lacking to establish a strong relationship especially in Namibia. In this study, the relationship between CD4 counts, viral load (VL), prescribed first line antiretroviral combinations, micronutrient levels and age was explored. Data spanning from 2006 to 2015 were retrospectively collected from Katutura State Hospital, Windhoek Central Hospital and Katutura Health Centre as well as prospective data on micronutrients. Polymerase chain reaction was used for VL testing, immunoassay for micronutrient analysis and immunophenotyping for CD4 count testing.

ANOVA, Pearson's correlation with cross tabulation determined the relationship between HIV-1 VL, CD4 count, micronutrients, the prescribed combinations and the age of patients. Medical records of 404 HIV positive adults and adolescents (13 – 15 years) and children (1-12 years) under ART took part in this study. In addition correlation between micronutrients levels, VL, CD4 count, at 36 months of treatment was investigated in 30 participants aged 13 – 55 years. HIV-infected individuals with at least 7 records on VL and CD4 count since initiation of treatment qualified for this study.

AZT/3TC/EFV combination was the best treatment across the study population with 83.3% VL decrease in ART naïve adults and adolescents. At the same time, the use of AZT/3TC/EFV and D4T/3TC/LPV-r combinations in children decrease the VL by 50%. Correlation coefficient (*r*) between the first line treatment combinations and

the age of patients in children population was observed (r = 0.38, $P = 0.00$) however, no significant correlation was observed for adult participants (adults and adolescents) and ARV drugs (r = 0.09, $P = 0.11$). A significant correlation between VL and CD4 count in adults and adolescents was observed at initial time (r = 0.24, $P = 0.00$), 6 months (- r = 0.17, $P = 0.00$) and at 18 months (r = 0.19, P = 0.00). In the children population, an inverse correlation between VL and CD4 count was observed throughout 36 months of treatment but was only significant at 18 months (r = 0.03, $P = 0.00$), 30 months (- r = 0.39, $P = 0.00$) and 36 months (r = 0.24, $P = 0.04$) respectively.

Among the 30 participants that took part in micronutrient analysis, normal, low, and high levels of magnesium, iron, folate and vitamin B 12 were observed with no significant correlation to CD4 count and VL. Furthermore, a significant negative correlation was found between ARV combinations and serum folate (- r = 0.4, $P = 0.03$). Micronutrient levels had no role in the treatment failure observed within the population of adults and adolescents in this study. In adults and adolescents, only the combination of AZT/3TC/EFV and D4T/3TC/LPV-r could achieve viral suppression (VL< 1000 copies/mL) at 36 months of treatment and the combination of D4T/3TC/LPV-r was found to be more effective in patients with low VL before ART usage. Treatment failure observed in this study population happened as a result of some combinations losing their potency over the treatment period. The study also showed a greater need in concentrating on the age of patient especially in children younger than 12 years when prescribing anti HIV drugs hence a positive correlation between age and ARV drug combination was found in children.

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ABBREVIATIONS AND ACRONYMS

AIDS	Acquired Immune Deficiency Syndrome
ANC	Antenatal Clinic
ART	Anti-Retroviral Therapy
ARVs	Anti-Retroviral
CD4	Cluster of Differentiation Four
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
KSH	Katutura State Hospital
KHC	Katutura Health Centre
MOHSS	Ministry of Health and Social Services
NRTI	Nucleoside/Nucleotide Reverse Transcriptase Inhibitor
NNRTI	Non-Nucleoside Reverse Transcriptase inhibitor
PI	Protease Inhibitor
RTI	Reverse Transcriptase Inhibitor
SADC	Southern Africa Development Community
STD	Sexual Transmitted Diseases
UNAIDS	Joint United Nations Programme on HIV/AIDS
VL	Viral load
WCH	Windhoek Central Hospital
NGAT	National Guideline for Antiretroviral Therapy

AZT	Azidothymidine also known as Zidovudine
NVP	Nevirapine
EFV	Efavirenz
D4T	Didehydrodeoxythymidine also known as Stavudine
3TC	Dideoxythiacytidine also known as Lamivudine
FTC	Emtricitabine
DTG	Dolutegravir
TDF	Tenofovir
LPV-r	Lopinovir - ritonovir

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DEDICATION

This research is dedicated to my husband and to my children whose presence motivates me to work harder so that I can be their role model. Perhaps in the future they will emulate me to reach greater heights, explore and pursue more in their studies. As the saying goes: “Education is the Power”, so to them I say, let my work inspire you to be the best you can be.

DECLARATION

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CHAPTER ONE: INTRODUCTION

1.1 Orientation of the study

Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome (HIV/AIDS) remains a major health problem in many parts of the world including Namibia. Namibia is recognized as one of the 26 low and middle-income countries in the world, and was ranked 16th in respect to HIV-1 infection rate in developing nations worldwide (UNAIDS, 2013). Furthermore, about 35.5 million people were living with HIV/AIDS (UNAIDS, 2013) which increased to 36.9 million in 2014 (UNAIDS, 2014). UNAIDS reported 1.2 million AIDS related deaths and 2 million new infections at the end of 2014. The report also mentioned that, about 24.7 million people in 2013 and 23.5 million in 2014 (70%) of all people living with HIV and 91% of HIV-positive children live in Sub-Sahara Africa.

The provision of antiretroviral treatment is the only effective way of minimising the spread of HIV. In mid-2014 it was reported that 15.8 million people worldwide were receiving antiretroviral drugs, of which 11.5 million are Africans (UNAIDS, 2014). Namibia is one of many African countries that provide antiretroviral drugs to its citizens living with HIV/AIDS. Furthermore, antiretroviral drugs are also used for the Prevention of Mother To Child Transmission (PMTCT). Highly Active Anti-Retroviral Therapy (HAART) is a combination of three antiretroviral drugs that comprises of either two Nucleoside Reverse Transcriptase Inhibitors (NRTIs) and a Non-Nucleoside Reverse Transcriptase Inhibitor (NNRTI) or one Protease Inhibitor

(PI) (Katende-kyenda *et al.*, 2008; WHO, 2015). However, in many resource-limited countries, HAART consists of two NRTIs and one NNRTI only (Tang *et al.*, 2012). According to the 2015 HIV/AIDS treatment report for the use of antiviral agents issued by the US Department of Health and Human Services (DHHS, 2015) there are new combination therapies that consist of three to four antiretroviral drugs e.g. protease inhibitors (PIs) and Integrase Strand Transfer Inhibitors based regimens (INSTIs) but these are not available in all countries.

Antiretroviral treatment prolongs the life of those living with the virus, protect them from contracting opportunistic infections as well as prevention of mother to child transmission of HIV (*National Guideline for Antiretroviral Therapy* (NGAT), 4^{th} *ed*. 2014). According to this guideline, more than 90% of children born by HIV positive mothers on antiretroviral therapy were HIV free.

1.2 Background and problem statement

Antiretroviral drugs in Namibian state facilities are prescribed to HIV-1 positive patients with a CD4 count of 500 cells per cubic millimetre or lower. This is in contrast with the WHO guideline which recommends initiation of ART for all the people that tested HIV positive regardless of the number of CD4 count. In addition, during the period of this study, only a few HIV patients had records of their viral load before initiation of antiretroviral treatment despite the regulation requiring records on viral load. The prescribed first line therapies contained three drugs which includes two NRTIs and NNRTI or a NRTI, a NNRTI plus a PI. WHO reports of (2013, 2014, 2015), recommends the use of NRTIs Tenofovir Disoproxil Fumarate (TDF) and 3-dideoxythiacytidine (Lamivudine) (3TC) or Emtricitabine (FTC) plus a

NNRTI such as Efavirenz (EFV) as first line for ART naïve adults and adolescents. This is the first recommended combination therapy with 600 mg of EFV a day. The other options of first line treatment combination include:

1. Zidovudine/lamivudine/efavirenz (AZT/3TC/EFV)
2. Zidovudine/lamivudine/nevirapine (AZT/3TC/NVP)
3. Tenofovir/lamivudine/nevirapine (TDF/3TC/ NVP) or FTC and AZT
4. Tenofovir/lamivudine/efavirenz (TDF/3TC/EFV_{400mg}), TDF/3TC/ dolutegravir (DTG)
5. Efavirenz/ dolutegravir (EFV_{400mg} and DTG).

HIV patients in three Windhoek state facilities took part in the study. Patients with the following prescribed combination therapy took part in this study.

<u>Combination of Two NRTIs plus one NNRTI</u>

AZT/3TC/NVP

AZT/3TC/EFV

Stavudine/lamivudine/nevirapine (D4T/3TC/NVP)

TDF/3TC/ NVP

TDF/3TC/EFV

<u>Combination of NRTIs or NNRTIs with PIs included;</u>

Zidovudine/lamivudine/lopinavir-ritonavir (AZT/3TC/LPV-r)

Stavudine/lamivudine/lopinavir-ritonavir D4T/3TC/LPV-r.

According to the third edition of the Namibian Guideline on Antiretroviral therapy (*NGAT, 3rd Ed*. 2010), D4T/3TC/LPV-r is preferred as a combination of choice for infants younger than 24 months. Furthermore, D4T/3TC/NVP is also preferred as a therapy of choice for infants that did not have nevirapine exposure and children that are more than 24 months but weigh less than 14kg. According to the same guideline, children over 24 months that weigh more than 14kg as well as adolescents and adults are prescribed AZT/3TC/NVP and TDF/3TC/NVP. During treatment, patients have to make follow up visits to ART clinics every three months (children) to every six months (adults) to have their CD4 count and viral loads checked. CD4 count and HIV viral load are two parameters mainly used to monitor disease progression and the effectiveness of the drugs used for treatment (Gottlieb *et al.*, 2007). The levels of these two parameters along with the clinical picture of the patient informs if there is any virological or immunological failure (*NGAT, 3rd ed.* 2010).

There are many cases of virological failure in HIV patients under anti-HIV treatment in Namibia. Virological failure may occur because of inactivity of some of the prescribed first line therapies (Tang & Shafer, 2012). Patients that are taking antiretroviral drugs also need enough nutritious food as most of the antiretroviral drugs are taken with a meal or after a meal. Appropriate nutrition is one of the main challenges in HIV treated patients especially in most developing nations around the world. A study by Castleman *et al*. (2009) has shown the effect of micronutrients bioavailability on patient's response to ARV treatment. Like most countries in

Africa, Namibia does not have enough medical doctors or HIV (WHO recommends a doctor to patient ratio of 1:1000). This makes it difficult to set up proper monitoring procedures to determine the outcome of the treatment for each individual. Patients are routinely prescribed antiretroviral drugs on first line as stated in the treatment guideline but, after taking the drugs, some of the patients get viral load suppression (<1000 copies/mL) whilst others fail to reach viral load suppression levels. Prescription of antiretroviral drugs that are not effective increases the chance of developing HIV drug resistance especially in HIV naïve patients (Tang *et al*., 2012). As a result of treatment with antiretroviral drugs that are not effective, many of the treated patients fail to reach undetectable or viral load less than 1000 copies/mL during first 12 - 24 weeks of treatment as per WHO treatment guideline (2010).

1.3 Significance of the study

There is not enough capacity of medical and supporting staff in Namibia to control the whole treatment process and monitor the effectiveness of the first line prescribed therapies for ART naïve patients. Furthermore, in some instances ARVs are prescribed based primarily on their availability and cost which may affect patient treatment and lead to virological failure. To determine the effectiveness of first line therapy, we reviewed and analysed quantitative data (CD4 count and VL) from the initial date of treatment until 36 months of treatment to check how many patients reached the targeted viral suppression. Also worthwhile investigating during this study was the effect of micronutrients bioavailability on patients' response to ART

as vitamins and micronutrients are essential for metabolism and immune function (Carter *et al*., 2014).

Carter *et al.* (2014) stated that micronutrients deficiency especially in HIV patients arise because of inflammatory and immunological effects due to HIV infection in the gut that leads to impaired absorption of the necessary minerals and vitamins. Lack of micronutrients was reported to be one of the factors leading to virological failure as most of the people living with HIV/AIDS in resource limited countries lack access to sufficient quantities of nutritious food (Castleman *et al.*, 2009). Studies have outlined that declining food intake leads to some people living with HIV/AIDS discontinuing their treatment due to the lack of adequate nutritious food (Sanjobo *et al.*, 2008; World Bank & UNAIDS, 2009). In Namibia, micronutrients supplements are not available to all HIV treated patients but offered to patients that showed either virological or immunological failure. A few numbers of patients involved in this study were prescribed multivitamins together with ARV drugs. According to the information obtained from the clinicians, multivitamin to ART patients in Namibia is mainly provided because of lack of food especially nutritious foods to vulnerable patients, without any income.

This is the first study of its kind in Namibia to study the efficacy of first line antiretroviral drug and their association with micronutrients bioavailability in the body of HIV treated patients. It is beneficial for Namibia to know which of the available treatment options may provide the best treatment results. This study investigated whether there was any significant correlation between micronutrient levels in the body (mainly iron, magnesium, folate and vitamin B12) with viral load

and CD4 count. During the period of the study, few HIV adult patients were taking multivitamin supplements alone and some were prescribed multivitamin supplements together with contramoxazole (CTX). Contramoxazole is an antibiotic used as a prophylaxis against possible bacterial infections or any other opportunistic infections for patients that have reached stage 3 of HIV infection. This study was set up to help improve the treatment program in Namibia and also shed more light on the importance of micronutrient intake especially for HIV treated patients as well as possible provision of micronutrient supplements to all HIV treated state patients.

1.4 Research hypothesis

The following four main hypotheses were analysed during the course of the study.

1. There is an inverse correlation between HIV-1 viral load (independent variable) and CD4 count (dependent variable) in patients under anti-HIV-1 drugs.
2. Some of the first line combinations are more effective.
3. There is a relationship between age and ART combinations.
4. Micronutrients levels in the body have an impact on the level of CD4 count and HIV viral load.

1.5 Research questions

The following research questions were formulated and investigated:

a. Which first line therapy is the best based on its efficacy?

b. What impact does micronutrient levels have on patients' response to ART?

c. Is there an association between age and combination therapies?

1.6 Research objectives

The objectives of the study included the following:

1.6.1 General Objectives

To evaluate the treatment response of first line ART combinations based on the changes in CD4 count and HIV-1 viral load trend during treatment as well as to find the impact of micronutrients levels on patients' response to treatment.

1.6.2 Specific Objectives

Specific objectives of this study are as follows:

a. To determine changes in HIV viral load in patients on ARV treatment
b. To determine changes in CD4 count in patients on ARV treatment
c. To determine ART combination that suppresses viral replication the most
d. To assess the impact of micronutrients levels on patients' response to treatment

1.7 Research design and methods

1.7.1 Research Type

This was a descriptive study in which patients' records on (viral load, CD4 count, treatment regimens, age, and gender) were collected retrospectively from patients' hospital files. The information was collected from the outpatient departments in three selected state health facilities. Blood samples for micronutrient analysis were prospectively collected and processed using the facilities in Namibian Institute of Pathology (NIP) laboratories in Windhoek.

1.7.2 Study Sites

Study sites included Windhoek Central Referral Hospital (WCRH), Katutura State Hospital (KSH) and Katutura Health Centre (KHC) in Windhoek were involved in this study. These health facilities were selected because of the comparatively large patient population that receives medical treatment at these state facilities. State health facilities rather than private facilities were selected for the study mainly because most HIV/AIDS patients in Windhoek and the whole country are under the care of the state since most patients cannot afford private health institutions.

1.8 Research methods

This research involved choosing eligible samples for the study and gathering of information from the patients' files as well as blood collection for micronutrient analysis. Some of the data in this study were retrospectively collected (CD4 and VL). A separate study was carried out for micronutrient analysis with consent from patients to give blood samples.

1.9 Data analysis

Data were captured in Microsoft Excel 2010. VL data less than 20 copies were recorded as equivalent to 15 copies per millilitre (15 copies/mL), target not detected (TND) were recorded as 5 copies/millilitre (5 copies/mL). To answer the research questions and objectives, data were analysed using the American IBM Statistical Package for the Social Sciences (SPSS) v.2.2. Analysis of variance (ANOVA) and Pearson's correlations were statistical tools used in the program.

1.10 Study samples

The study used a systematic random selection procedure to select study participants from each visited health facility. After the filtration of patients according to the availability of data, we used the sample size table to calculate the sample size of the study based on the population of ARV treated patients.

1.11 Exclusion and inclusion of the data

Patient's data on VL and CD4 count recorded during the first line of treatment as well as the treatment combination prescribed on first line therapy were used in this study. Patients' data at second and third line of antiretroviral treatment (of those who had) and all those that do not meet the requirements of the study were excluded from this study.

1.12 Ethical consideration

Ethical permission by the Ministry of Health and Social Services (MoHSS) of Namibia was officially granted to conduct this study. Code on patients' confidentiality from the MoHSS as well as NIP laboratory were strongly followed. Consent letter were provided to patients before obtaining blood samples and the standard operating procedure on code of ethics in the laboratory (NIP, STAFF-001) were followed.

1.13 Literature

The literature was reviewed to document known facts about HIV/AIDS and treatment in chapter two. Information on HIV pathophysiology, antiretroviral drugs

and their use in viral suppression is discussed in detail. Information was also compiled on HIV treatment guidelines and the effects on patients' response to treatment when micronutrients and antiretroviral drugs are used concurrently in managing the disease.

2 CHAPTER TWO: LITERATURE REVIEW

2.1 The structure of Human Immunodeficiency Virus

HIV was identified as the causative agent of AIDS in 1983 (BarreA-Sinoussi *et al.,* 1983; Gallo *et al*., 1984; Levy *et al.,* 1984). Human immunodeficiency virus is a virus that causes AIDS *(*Acquired Immune Deficiency Syndrome) (Centre for Disease Control, 2005*).* In 1981, AIDS was discovered in the United States of America by CDC as Pneumocystis carinii pneumonia. According to CDC, AIDS now comprises of many different illnesses that affect people living with the HIV (CDC, 2005). Illnesses of this kind include; wasting syndrome, encephalopathy, tuberculosis, pneumonia, Kaposi sarcoma, multiple lymphoma and candidiasis of bronchi, trachea, oesophagus, or lungs just to mention a few.

In addition, Zack *et al*. (1990) stated that HIV belongs to a special class of viruses called retroviruses. Within this class, HIV is placed in the subgroup of lentivirus or "slow" viruses. Retroviruses genes are composed of RNA (Ribonucleic Acid*).* Other lentivirus include SIV, FIV, Visna and CAEV, which cause diseases in monkeys, cats, sheep and goats. Almost all organisms, including most viruses, store their genetic material on long strands of DNA. Lentiviruses are known for having long waiting periods between initial infection and the beginning of serious symptoms. This is why there are many people who are unaware of their HIV infection and unfortunately can spread the virus to others (Zack *et al*., 1990). HIV has nine genes (compared to more than 500 genes in a bacterium, and around 20,000-25,000 in a human) (Zack *et al.,* 1990). Three of the HIV genes known as *gag, pol* and *env* contain information needed to make structural proteins for new virus particles. The

other six genes are *tat, rev, nef, vif, vpr* and *vpu* which play a major role in coding proteins that control the ability of HIV to infect cells and produce new copies of the virus *(*Zack *et al.,* 1990). *Pol* is the main gene in most retroviruses and encodes four proteins that are used during the process of reverse transcription in which viral RNA is converted into DNA.

According to the study by Van der Kuyl and Berkhout (2012), the *env* helps in encoding the membrane protein 160 (kilodalton) kDa to generate glycoprotein (gp) 41 kDa and 120 kDa. The formation of p160 kDa takes place inside the cell and later transported to the cell surface. At the cell surface carbohydrates are added to p160 kDa which is later broken into gp120 kDa and gp41 kDa (Capon & Ward, 1991). HIV has the long terminal repeat (LTR), which is used in the DNA sequence flanking the genome of integrated proviruses. Capon and Ward (1991) further emphasise that, the LTR contains important regulatory regions, especially those for transcription initiation and poly-adenylation capsid. The HIV core is made up of proteins that are building blocks of nucleotides encoded by the gag gene (Hope & Trono, 2000). Not only does the structure of HIV advocate its replication but it forms the core aspects of treatment regimens. The next section explores the replication and life cycle of HIV as a basis to understand various drug interventions.

2.2 HIV replication and life cycle

HIV replicates rapidly with several billion new viruses made every day in a person infected with HIV (Plantier *et al.,* 2009). Reverse transcriptase, the enzyme that makes DNA copies of HIV's RNA, often makes random mistakes. As a result,

new types and strains of HIV are being developed in a person infected with HIV (Plantier *et al*., 2009). According to Chalmet *et al.* (2010), more virulent and infectious strains of HIV are typically found in people who are in the late stages of infection. More on that Chalmet *et al*. (2009) emphasised that different strains of HIV can also recombine to produce an even wider range of strains. In essence, HIV is constantly changing and trying to evade the immune system. According to the study, the ability of HIV to replicate rapidly is one of the major reasons why HIV is such a deadly virus (Chalmet *et al*., 2010). After entering the cell, the contents within the envelope are released into the host cell cytoplasm where it undergoes replication. As the replicated new cells bud out of the host cell, they acquire a phospholipid envelope to become matured enveloped viral particles (Gottlinger *et al*., 1989).

Bushman *et al*. (1990) outlined the three main enzymes used by HIV to complete its life cycle namely; reverse transcriptase, integrase, and protease, which are found within the nucleocapsid. Protruding from the envelope are peg-like structures that the viral RNA encodes. Each peg consists of three or four gp 41 kDa glycoproteins (the stem), capped with three or four gp 120 kDa. Inside the envelope the nucleocapsid of the virus that is bullet-shaped and composed of protein, surrounds two single strands of RNA. HIV entry into the cell is initiated by the gp 120, the chemokine receptors CCR5 and the receptor on the targeted CD4 positive cells (Bushman *et al*., 1990). HIV provirus can be dormant within a cell for a long time and when the cell becomes activated, it treats HIV genes in much the same way as human genes in the processes of transcription and translation. HIV genes are converted into messenger RNA (mRNA) using host enzymes. The mRNA is then transported outside the nucleus, and it is used as a blueprint for producing new HIV proteins and enzymes (Van der

Kuyl & Berkhout, 2012). HIV has a lot of particles on its surface, of which nine are gp160 spikes. These are the spikes that are used in binding and membrane fusion when the virus attaches itself to host cells (Van der Kuyl & Berkhout, 2012). According to Sundquist and Krausslich (2012) when a CD4+ cell is infected with HIV, the virus goes through multiple steps to reproduce itself and create many more viral particles in a process that can be broken up into several stages (Figure 2.1).

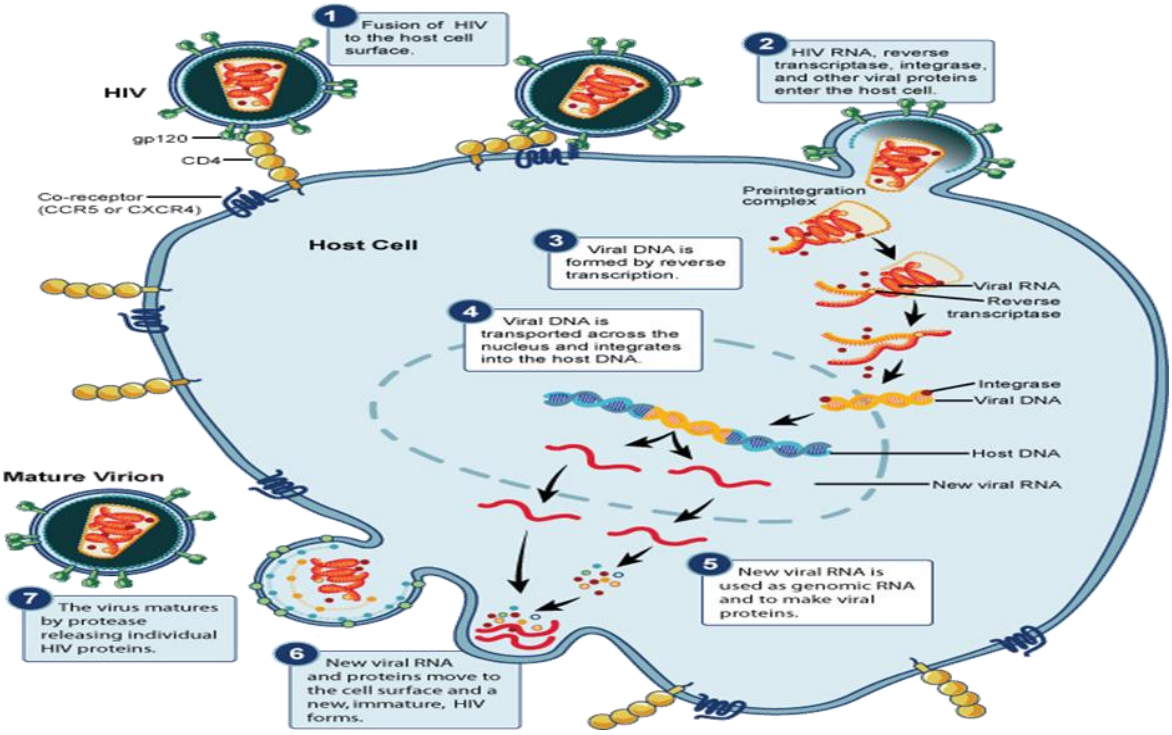

Figure 2.1 HIV replication cycle

http://www.daviddarling.info/encyclopedia/A/acquired_immune_deficiency_syndrome.html

Furthermore, Sundquist and Krausslich (2012) mentioned that the replicative advantages are mediated during physical contact between infected and uninfected cells. These mostly happen in lymphoid tissues where CD4+ T lymphocytes are densely packed and likely to frequently interact. The replicative advantages of HIV from infected to uninfected cells was also reported in the studies by Murooka *et al*.

(2012) as well as Sewald *et al*. (2012); in which they claimed that cell-to-cell virus transfer may be a mechanism by which HIV-1 can also evade ART and thus continue to replicate at low levels in the presence of on-going therapy. The discovery of HIV-1 as the causative agent of AIDS together with increasing understanding of the virus replication cycle have been the most significant advancement in the medical management of HIV infection. The next section deals with the basic principles of antiretroviral drug therapy.

2.3 Anti-retroviral drugs for HIV treatment

The large number of viral particles which are transmitted to uninfected targeted cells during cell-to-cell transfer increases the probability that at least one viral particle escapes inhibition by drugs and proceeds to infect other cells (Sigal *et al*., 2011). This was done by assessing the effects of reverse transcriptase inhibitors (RTIs) on virus spread in an *in vitro* experimental model that showed that cell-to-cell HIV spread was less sensitive to inhibition by RTIs than HIV cell-to-cell free transmission. A similar mechanism of saturation of inhibitors by a large pool of incoming virus particles has also been suggested to explain the resistance of cell-to-cell virus transfer to inhibition by innate antiviral cellular factors (Richardson *et al*., 2008; Jolly, 2011).

However, in a conflicting report, emanating from the results from a study carried out by Permanyer *et al.* (2012); found that RTIs are equally effective at blocking both modes of HIV-1 dissemination. In a study by Martin *et al*. (2010) questions regarding the true impact of antiretroviral therapy on cell-to-cell HIV-1 transmission was raised, where it was confirmed that, virological synapse-mediated HIV-1 spread

is efficient but is not an immune or entry inhibitor evasion mechanism, a result that is encouraging for vaccine and drug design. Antiretroviral drugs are generally classified based on their molecular mechanism and resistance

profiles which includes nucleoside-analog reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, integrase inhibitors, protease inhibitors, fusion inhibitors, and co-receptor antagonists. The next section explores the basic principles of each class.

2.3.1 Nucleoside and non-nucleoside Reverse Transcriptase Inhibitors

NRTIs are also called "nucleoside analogues" or "nukes" NRTIs are DNA chain terminators that compete with endogenous deoxy-nucleotide triphosphates (dNTP) for incorporation into a growing viral DNA chain where they cause chain termination (Tang and Shefar, 2012). NRTIs are analogues of the naturally occurring building blocks of DNA (Hoffman & Landovitz, 2004). NRTIs are the purine nucleosides: adenosine (A), guanosine (G) and the pyrimidines thymidine (T) and cytidine (C). These molecules in their phosphorylated state are recognised by the reverse transcriptase and are incorporated into the viral DNA. The binding of the phosphorylated analogues, prevent an addition of cellular nucleosides that are needed for further viral DNA synthesis (Hoffman *et al.,* 2004). There are six classes of nucleoside-analogue reverse transcriptase inhibitors currently licenced for treatment of HIV-1 (Table 2.2). Nucleotide reverse transcriptase inhibitors are phosphorylated already and they are recognized by the reverse transcriptase (RT) enzymes much easier than nucleosides (Tang & Shafer, 2012). The only approved nucleotide reverse transcriptase inhibitor in the market is Tenofovir.

In addition, NNRTIs were defined as molecules that inhibit the HIV-1 reverse transcriptase (RT) by allosterically binding to a hydrophobic pocket close to the RT active site (Tang & Shafer, 2012). NNRTIs interfere with reverse transcription by directly binding to the catalytic cite of reverse transcriptase enzyme and inhibiting its function (Tang & Shafer, 2012). NRTIs and NNRTIs are available in most countries. Other classes of ARVs on the other hand appeared to be available mainly in resource-rich countries (Tang *et al.*, 2012).

2.3.2 Protease Inhibitors (PIs)

Protease Inhibitors interfere with the HIV enzyme called protease, which normally cuts long chains of HIV proteins into smaller individual proteins. These drugs prevent the HIV protease enzyme from working. Currently there are nine licensed protease inhibitors in the market (Table 2.1). HIV protease act like chemical scissors. They cut the raw material for HIV into specific pieces needed to build a new virus. When protease does not work properly, new virus particles cannot be assembled. Cleavage of polyproteins by the protease enzyme is an essential step in the HIV life cycle. After cleavage, the immature virus proteins are assembled into particles which bud from the cell as mature, infectious viruses (Podzamczer *et al.*, 2002).

Protease inhibitors compete for the active cleavage site on the protease enzyme blocking the cleavage of the polyproteins and thus the maturation of new viral particles. Studies have confirmed that, protease inhibitor (PI)-containing regimens are uncomfortable and are associated with incidence of adverse effects (Podzamczer *et al.*, 2002). The most worrisome situation is the possible association between PIs and the occurrence of lipodystrophy and other metabolic disorders that make

adherence difficult and negatively influence patients' quality-of-life (Ferrer *et al.*, 1999).

2.3.3 Integrase Inhibitors

Raltegravir is the only approved integrase inhibitor for the treatment of HIV. It was approved in August 2012 as part of a fixed-dose antiretroviral combination regimen that incorporates tenofovir and emtricitabine into a single tablet. HIV integrase is one of three enzymes together with reverse transcriptase and protease encoded by HIV, which are essential for its replication.

2.3.4 Co-receptors Inhibitors

The group of co-receptors inhibitors comprises of CCR5 and CXCR4 co-receptor antagonist. According to Moore *et al*. (2004), once the gp120 has bind to the CD4 receptors, gp120 must then bind to a second co-receptor which can be one of the two chemokine co-receptors, CCR5 or CXCR4. Maraviroc which is known by the name Celsentri in Europe and Selzentry in the US) is the only drug in this category approved in 2007 for the treatment of HIV/AIDS. Maraviroc is used in second-line regimens in Europe and the US. Maraviroc was originally approved for use in combination with other antiretroviral, by treatment-experienced in individuals with on-going replication and CCR5-tropic HIV. CCR5 inhibitors only have an antiviral effect in people who are predominantly infected with HIV that is adapted to using the CCR5 receptor, typically patients with higher CD4 count and less advanced HIV disease (https://aidsinfo.nih.gov/education).

2.3.5 Fusion Inhibitors

After HIV's gp120 envelope glycoprotein attaches to a CD4 receptor and a co-receptor on the cell, a different glycoprotein, called gp41, is exposed. The glycoproteins then undergo conformational changes that bring the virus and the cell in a close proximity, allowing them to fuse. There is a single licensed fusion inhibitor: T-20 (enfuvirtide, Fuzeon). T-20 was developed by Roche and Trimeris. Enfuvirtide is a peptide based on gp41 sequence. It blocks the virus from merging with the host CD4+ cells after binding (Eggink *et al*., 2010).

2.3.6 Antiretroviral drug combinations

Various authors argue that the advert of combination therapy, also known as HAART for the treatment of HIV infection was seminal in reducing the morbidity and mortality of associated with HIV infection and AIDS (D'Aquila *et al*. 1996; Staszewski. *et al.* 1996). Currently available drugs according to Kalyan *et al.* (2012) do not cure HIV infection but they do prevent the development of AIDS. Antiretroviral drugs however, do not eliminate HIV from the body (Kalyan *et al.*, 2012). A review by Tang and Shafer (2012) stated that the Unites States Department of Health and Human Sciences (DHHS) guidelines recommends the use of tenofovir/emtricitabine in combination with efavirenz, atazanavir/r, darunavir/r or raltegravir for first-line therapy in ARV-naive patients infected with wild-type. Sax *et al.* (2009) also reported that, Abacavir/lamivudine was downgraded as an alternative NRTI combination. This was done because of recent large clinical trial comparing abacavir/lamivudine with tenofovir/emtricitabine in combination with

efavirenz or atazanavir/r which showed that patients treated with these combinations developed viral load higher than 10^5 copies/mL.

Patients who received abacavir/lamivudine were also at an increased risk of developing virological failure. There are 24 approved ARV drugs for the treatment of HIV as outlined in Table 2.1 (WHO, 2015). The US department of health and human services (2011) recommends the use of Tenofovir/lamivudine or tenofovir/emtricitabine as part of second-line therapy whether or not tenofovir was used in the first line regimen (Table 2.2) (WHO, 2015). Zidovudine/lamivudine is an acceptable alternative in case of Tenofovir/lamivudine or tenofovir/emtricitabine substitution. In the same review, patients receiving regimens with a high genetic barrier to resistance only develop virological failure as result of non-adherence, pharmacokinetic factors or transmissions of drug-resistant HIV strains. It was also observed that in resource-limited settings, additional challenges to ART include intermittent lack of access to ARVs, fewer ARV choices if toxicity or intolerance arises, and incomplete virological suppression in patients with high plasma HIV-1 RNA levels (Tang and Shafer, 2012).

Table 2.1

Classes of ARV drugs approved for HIV/AIDS treatment

ARV DRUGS	HOW THEY TARGET THE VIRUS	EXAMPLES OF BRAND NAMES
1. Nucleoside Reverse Transcriptase Inhibitors (NRTIs)	Inhibit the transcription of viral RNA into DNA, which is necessary for the virus to reproduce.	Zidovudine (AZT), Lamivudine (3TC), Didanosise (ddl), Stavudine (D4T), Abacavir (ABC) and Emtricitabine (FTC).
2. Nucleotide Analogue Reverse Transcriptase Inhibitors	Inhibit the transcription of viral RNA into DNA	Tenofovir (TDF),
3. Non-Nucleoside Analogue Reverse Transcriptase Inhibitors (NNRTIs)	Inhibit the transcription of viral RNA into DNA.	Nevirapine (NVP), Ef avirenz (EFV) and Entravirine (ETV), Delavirdine.
4. Protease Inhibitors (PIs)	Inhibit enzyme that cut long viral proteins into smaller ones for incorporation into new viral particles.	Lopinavir (LPV), Indinavir (IDV), Nelfinavi (NFV), Saquinavir (SQV), ritonavir (RTV), Atazanavir (ATV), Fosamprenavir (FPV), Tipranavir (TPV) and Darunavir (DRV).
5. Fusion inhibitors	Block the virus gp41 hairpin from interacting with the CD4 cell.	Enfuvirtide (ENF).
6. Integrase Inhibitors	Inhibit integrase enzyme from integrating viral DNA into host DNA	Raltegravir (RAL)
7. Co-receptors Inhibitors	Binds to CD4 cells and change their structur	Maraviroc (T-20)

https://aidsinfo.nih.gov/education-materials/fact-sheets/21/58/fda-approved-hiv-medicines

WHO, (2010) report on HIV treatment guidelines recommends zidovudine/lamivudine (AZT/3TC), tenofovir/lamivudine (TDF/3TC), or tenofovir/emtricitabine (TDF/FTC) in combination with nevirapine (NVP) or efavirenz (EFV) as first-line therapy indicated in Table 2.2. Tang and Shafer (2012), in their review, stated that the use of stavudine/lamivudine (D4T/3TC) combination is no longer recommended by the WHO due to increased risk of toxicity, although it is still used in resource-limited regions because of its low cost.

Table 2.2

First and second line ARV combinations approved for the treatment of ART naive patients

First line treatment combination of two NRTIS plus one NNRTIS			
AZT/3TC/ EFV	d4T/3TC/ EFV	ABC/FTC/ EFV	TDF/3TC/EFV
AZT/FTC/ EFV	d4T/FTC/ EFV	ABC/3TC/ NVP	TDF/FTC/ EFV
AZT/3TC/ NVP	d4T/3TC/ NVP	ABC/3TC/ EFV	TDF/3TC/ NVP
AZT/FTC/ NVP	d4T/FTC/ NVP	ABC/FTC/ NVP	TDF/FTC/ NVP
First line regimen/ treatment with 3 NRTIS			
AZT/3TC/ ABC			
AZT/3TC/ TDF			
Second line of ARV treatment due to virological failure			
from TDF	from ABC	from 3 NRTIS	
ddI/ABC/PI ddI/3TC/PI	ddI/3TC/AZT/PI ddI/FTC/AZT/PI TDF/3TC/AZT/PI TDF/FTC/AZT/PI	ddI/EFV/PI ddI/NVP/PI	

https://aidsinfo.nih.gov/guidelines/html/1/adult-and-adolescent-arv-guidelines/11/what-to-start

Tang & Shafer (2012), also acknowledged the important role of boosted PIs in second-line therapy, even in cases where the initial ARV regimen contained a boosted PI. The principles of salvage therapy (combination of more than two drugs) or HAART for patients whom more than one regimen has failed are similar to those for patients whom a single regimen has failed according to Tang and Shafer (2012). They stated that in principle therefore, the salvage regimen should be sufficiently potent to suppress virus levels to below the level of detection, and should have a sufficiently high genetic barrier to resistance to prevent virological rebound. Therefore, the DHHS (2015), panel on antiretroviral guidelines for adults and

adolescence on ART treatment guidelines recommends that salvage regimen to have three optimally active ARV drugs (Table 2.2).

According to Tang *et al.* (2012), in patients that develop virological failure on first-line ART, the extent of drug resistance is roughly proportional to the duration of uncontrolled virus replication in the face of selected drug pressure. Furthermore, it was stated that in resource-limited countries where patients undergo infrequent virological monitoring, samples from patients with virological failure generally contain more drug-resistant mutations and higher levels of cross-resistance than virological samples from patients with virological failure in well-resourced regions (Gupta *et al.*, 2008).

2.4 The role of micronutrients in treatment of HIV patients

Nutritional interventions play a major role in humans and they are more needed in patients living with HIV that are taking HAART (Tiyou *et al.*, 2012). According to the study conducted by Tiyou *et al.* (2012), on nutritional level in HIV treated patients, it was found that 63% of the study population of 319 adult patients had food insecurity or not enough nutritious food. They later found out that the 63% of patients had poor treatment results compared to the other group of patients who had enough nutritious food. Results from a study by Mehta *et al*. (2010) found out that micronutrients in humans are needed for the body's biochemical reactions as well as for the immune system. It was further reported that micronutrient levels may determine susceptibility to HIV infection, transmission and progression, including risk of opportunistic infections (Tiyou *et al.* (2012).

Other studies, concluded that HIV infected patients are vulnerable to malnutrition due to several factors such as inadequate nutrition, nutrient loss due to malabsorption and diarrhoea, metabolic alteration and drug-nutrient interaction (Dudgeon *et al*., 2006; Colecraft, 2008). It is also known that functional status and survival of HIV-infected patients is affected by their nutritional condition (Sanjobo *et al.* (2008). Nutritional support must be considered as an important factor contributing to ART adherence as lack of nutritious food is one of the causes of non-adherence in antiretroviral treatment program (Sanjobo *et al.*, 2008).

2.4.1 Role of Iron

Iron is found in most food products such as liver, meat and cereal. It is an essential mineral that is used in transporting oxygen to all parts of the body. Its deficiency leads to anaemia as well as organ failure due to the lack of oxygen in instances of chronic deficiencies (Nekhai *et al.*, 2013). Too much of iron in the body produces harmful free radicals that may interfere with metabolic processes and cause heart and liver damage (Fuchs *et al.*, 1993; Burtke *et al*., 1994). Iron is known to exist in biological fluids as a component of haemoglobin and myoglobin. It is bound to transferrin which acts as its carrier protein for in both serum and plasma (Jacobs *et al.*, 2001).

Jacobs *et al.* (2001) argued that elevated iron is seen in haemolytic anaemia, hemochromatosis and acute liver disease. Low level of iron is found when there is an iron deficiency or anaemia of chronic disease (Jacobs *et al.*, 2001). Gastrointestinal and menstrual bleeding were recorded to be major causes of iron deficiency,

therefore, for the assessment of the body's iron status, transferrin and ferritin can provide more accurate information (Jacobs *et al.*, 2001). Jones *et al*. (2006) confirmed that in early asymptomatic HIV infection, iron stores decline probably due to impaired absorption, but increase with advancing HIV infection as iron accumulates in macrophages and other cells. Iron may also have adverse effects in HIV metabolism (Doherty, 2007). Mechanism on the involvement of iron in HIV metabolism is yet to be revealed. Metabolism of HIV increases oxidative stress as a result of iron being released from their Apo proteins and this increases iron plasma levels (Award, 2006). The study by Nekhai *et al.* (2013) stated that intracellular iron greatly affects HIV-1 replication since higher intracellular iron stores are associated with faster progression of HIV-1 infection and inversely correlates with the survival of HIV-1 infected patients.

It was demonstrated that serum iron might increase/decrease depending on the stage of the diseases (Frii, 2001). Iron decrease in early asymptomatic stage of HIV infection possibly due to impaired absorption but may also increase with progression of the disease as iron accumulates in microphages and other cells (Drakesmith & Prentice, 2008). A study has shown a negative correlation between serum iron and CD4 count Banjoko *et al.* (2012). In addition, Fuchs *et al.* (1993) and Burtke *et al.* (1994) found out that free radicals and oxidative stress are caused by high levels of serum iron that depleted the number of CD4 cells in HIV patients *in vitro*. Zinc deficiency is also known to cause a decline in the generation of T-lymphocytes resulting in depletion of humoral and cell-mediated immunity (Stambullian *et al.*, 2007).

2.4.2 Role of Magnesium

Magnesium is an essential nutrient found in green vegetables, beans, peas and nuts among others. The element is involved in many biochemical reactions and is observed to strengthen the bones, regulate calcium, zinc, potassium and vitamin D (Burtis & Ashwood, 1994). According to Burtis and Ashwood (1994), magnesium also plays a role in muscles/nerve and heart function. Furthermore, lack of magnesium in the body was associated with vomiting, cramps, nausea, and loss of appetite and heart spasms among others.

Magnesium plays a structural role in nucleic acids and ribosomal particles. It is also required as an activator for many enzymes, and it has a role in energy producing oxidative phosphorylation (Burtis & Ashwood, 1994). The normal body contains 21-28 grams of magnesium, of which more than 50% is bound with calcium and phosphate in bones. Approximately only 1% of the total magnesium is found in the extracellular fluid (Burtis and Ashwood, 1994). Magnesium enters and leaves the cell in the same form and about 35% of magnesium in the plasma is bound to proteins mainly albumin. Therefore, changes in albumin levels also affect the concentration of magnesium in the body (Burtis and Ashwood, 1994). Furthermore, hypomagnesaemia was found to impair neuromuscular function, carbohydrate intolerance and cardiac arrhythmias while, hypomagnesaemia causes hypotension, bradycardia and respiratory depression among others.

The United Nations' National Research Council (UNNRC, 1989), stated that magnesium is an essential micronutrient for humans and that the element plays many important roles including function of over 300 enzymes and in neuromuscular

transmission. Abnormal dietary deficiency of magnesium as well as abnormalities in its metabolism plays important roles in different types of cardiovascular diseases such as ischemic heart disease (Chakraborti *et al.*, 2002).

Specifically, magnesium deficiency is associated with major risk factors for atherosclerotic coronary artery disease, coronary artery spasm, and coronary artery thrombosis and many studies in large population have shown that higher intake of this mineral is associated with lower risk of high blood pressure, stroke, and ischemic heart disease (Saris *et al.*, 2000). Based on this study, magnesium deficiency may usually be reflected in low-magnesium diet, blood loss, and excessive sweating, drug and/or alcohol abuse or due to certain medication use (such as loop diuretics and thiazides, cytotoxic drugs, aminoglycosides, digoxin, steroids). Magnesium deficiency may also manifest in some physiological conditions over utilize magnesium such as pregnancy or growth during infancy (Saris *et al*., 2000). Magnesium also serves as a cofactor of more than 300 enzymes such as MgATP as well as some allosteric activators of many other enzymes used in RNA and cDNA synthesis (Erin, 2012). In contrast, reduction in magnesium concentration in the body leads to high level of neuromuscular excitability as magnesium inhibits calcium from entering into the neurons.

2.4.3 Role of Vitamin B12

Vitamin B12 comprises of a group of substances called cobalamins. These substances are composed of a tetrapyrole structure and a central cobalt atom that differs with respect to the side groups attached to the cobalt atom (Lee & Griffiths, 1985). The predominant form of serum cobalamin is methylcobalamin while the

predominant intracellular form is 5´deoxyadenosylcobalamin. Cynocobalamin is the most stable of the cobalamins and is used as a reference compound for measuring serum cobalamin concentrations (Lee & Griffiths, 1985). Cobalamins are found in food sources such as milk products, poultry, meat and fish. When these products are ingested, they are bound by an-intrinsic factor protein in the gastric juice and are subsequently absorbed in the ileum. Intrinsic factors are essential for their absorption (Chararin, 1987). According to Chararin, (1987) cobalamins are stored in the liver and are released into the plasma and transported by transcobalamin, a B12 binding protein when needed in the body. Vitamin B12 serves as a coenzyme that is involved in two important metabolic functions which are vital to the normal functioning of cell growth and DNA synthesis (Lee & Griffiths, 1985). It is also involved in the synthesis of methionine and the conversion of methylmalonyl CoA into succinyl CoA. Vitamin B12 is also essential for healthy nerve cells, RNA synthesis, red blood cells production and iron function (Lee & Griffiths, 1985).

Lack of vitamin B12 can lead to megaloblastic anaemia, fatigue, constipation, loss of weight, dementia, poor memory and oral soreness (Beuerlein, 1988). Megaloblastic anaemia is characterized by the enlargement and reduction in number of all rapidly proliferating cells of the body, including marrow cells, and is primarily a result of the decreased capacity for DNA synthesis. Vitamin B12 is linked to folic acid during the reaction pathway for methionine synthesis, so deficiency in either will disrupt this metabolic pathway and leads to the same symptoms as mentioned above (Beuerlein, 1988). Vitamin B12 deficiency occurs as a result of several reasons (Taneja *et al.*, 2009). The most common one is when there is a defect in the secretion of an-intrinsic factor, resulting in inadequate vitamin B12 absorption from

foods. Other causes of vitamin B12 deficiency are gastrostomy, malabsorption due to surgical resection, and a variety of bacterial or inflammatory diseases affecting the small intestines. According to Taneja *et al.* (2009) vitamin B12 deficiency due to insufficient dietary intake is very rare and this can only occur after years of abstaining from all animal products. In addition, high vitamin B12 is found during pregnancy, when using oral contraceptives and multivitamins and in myeloproliferative diseases such as chronic granulocytic leukaemia and myelomocytic leukaemia. Elevated B12 itself was shown not to cause any clinical complications (Taneja *et al.*, 2009).

2.4.4 Role of Folate

Folate is vital vitamin for cell growth and DNA/RNA synthesis just like vitamin B12. It is also absorbed in the small intestines and stored in the liver (Chararin, 1987). Sources of folate are fortified grains, tomato juice, green vegetables, black eyed peas, beans and lentils. Folate plays a role in mental health and growth and together with vitamin B12 helps in regulating red blood cells production, iron production and reduces homocysteine. Its deficiency leads to anaemia, poor immune function, insomnia, megaloblastic anaemia, cardiovascular diseases and severe neurological problems (Kones, 1991). Folate deficiency can be caused by insufficient dietary intake, malabsorption or excessive folate utilization. Excessive folate utilization occurs mainly during pregnancy. Other factors such as alcoholism, hepatitis, and other liver damaging diseases can lead to excessive folate utilization. Folate serum level is an indicator of recent folate intake (Steinberg, 1984). The red

blood cell folate is the best indicator of long term folate stores. Vitamin B12 deficiency disrupts the uptake of folate into red blood cells.

Zengin *et al.* (2009) showed that Vitamin B12 and folate deficiency reduce physical activities, cognitive function and megaloblastic anaemia during childhood and infancy (Zengin *et al.*, 2009). Low serum concentrations of vitamin B12 and folate were associated with increased HIV progression with reduction of physical activity megaloblastic anaemia as well as cognitive functions (Zengin *et al*., 2009). It was also confirmed that lack of B group vitamins increases levels of homocysteine which can have detrimental effects on cognition (Elias *et al*., 2006).

2.5 Laboratory methods of micronutrients analysis

There are several methods that directly measure vitamins and minerals and give quantitative measurements (Fazili & Pfeiffer, 2004). The different methods are:

1. High Performance Liquid Chromatography (HPLC)
2. Immunoassays (ELISA, RIA, FIA)
3. Colorimetric and Spectrophotometric assays
4. Fluorometric and Spectrophotometric assays
5. Amperometric assay

There are other methods that indirectly measure the activity of certain enzymes that work under the influence of some vitamins and minerals. Some of this methods semi-quantify or provides a qualitative test of vitamins and minerals. They are:

1. Metabolite loading test
2. Erythrocyte enzyme activation assay
3. Erythrocyte fragility test

4. Prothrombin time
5. Microbiological method
6. Bioassays (*in-vivo* and *in-vitro*)

2.5.1 High Performance Liquid Chromatography

This is a quantitative test of most minerals and vitamins, as it also determines iso-forms of the vitamins. This is one of the direct methods; it is accurate, sensitive, specific and automated with a high through put. Various chromatographic techniques are also used especially for research purposes (Fazili & Pfeiffer, 2004).

2.5.2 Immunoassays

These techniques incorporate the binding reaction of a target substance (antigen) with an antibody. Antibodies are basically immunoglobulin's that bind different natural and synthetic antigens in the body such as carbohydrates, lipids, proteins and nucleic acids. The antibodies have a common structure but have different special components that help them identify and bind to specific antigens (Fazili & Pfeiffer, 2004).

There are several types of immunoassays:

2.5.3 Enzyme Linked Immuno-Sorbant Assay

This assay utilizes antibodies linked to enzymes and it measures the amount of an antibodies present in a solution (Nierenberg & Lester, 1985).

2.5.4 Radio-Immunoassay

This assay uses radio-isotope to determine the number of antibodies needed to bind to known number of radiolabelled antigen. If the antigen is present in the patient sample, then radioactivity will decrease proportionally to the amount of antigen present in the sample.

2.5.5 Fluorescent Immunoassay

Antibodies are labelled with fluorophore. Fluorochrome-labelled anti-immunoglobulin is used to detect binding of the first antibody (Nierenberg & Lester, 1985). The fluorescence produced by the labelled product is then measured on a spectrophotometer.

2.5.6 Colorimetric and Spectrophotometric Assays

These two assays involve a chemical reaction between a chromogen and vitamin that results in a change of colour. For quantifying purpose, this method needs a spectrophotometer to measure the colour intensity (Nierenberg & Lester, 1985).

2.5.7 Fluorometric and Chemiluminescence Assays

Some of the vitamins have an ability to produce fluorescence when reacted with a fluorophore. The fluorescence produced is directly proportional to the concentration of the vitamin. In chemiluminescence assay, a luminal is used to react with the vitamin of interest. The mixture is injected at a controlled rate through capillary where kinetic reaction is measured and a quantitative measurement is given. Radio ligand, enzyme-linked and chemiluminescence that use highly specific intrinsic

factor as vitamin B12-binding protein are used for the analysis of vitamin B12 in serum and in plasma (Jacobs *et al.*, 2001).

2.5.8 Amperometric Assay

This assay is used for vitamins that undergo electro-chemical oxidation (Jacobs *et al.*, 2001). The reaction causes changes in the electrical potential that is directly proportional to the concentration of the vitamin in the sample.

2.6 CD4 count and viral load as monitoring tools for HIV progression

The effectiveness of HIV-1 treatment is monitored by viral load and CD4 count testing (Peterson *et al.*, 2011). According to Peterson *et al*. (2011), HIV targets the most important white cells in the body, the T-helper lymphocytes that have CD4 receptors on their surface. CD4 is a good receptor of HIV. T-lymphocytes are not the only white cells that get attacked by HIV (Peterson *et al*., 2011). Other white cells such as macrophages which hold a lot of virus on their surfaces are also affected but they are hardly killed by the virus. The death of infected CD4 positive cells leads to a weak immune response and high viremia. As a result, the immune system starts to weaken when CD4 + cells get below 500 cells/mm^3 or when they drop by 50% of a previous count (UNAIDS, 2013).

CD4 count in normal infants are considerably higher than in adults, and gradually decline towards adult levels from about 6 years of age (Douek *et al*., 2000). In addition, percentages of lymphocytes that are CD4 positive among normal children is not age related. Thus, CD4 count that identify stages of immune suppression vary with age but the levels of CD4 percentage do not. According to Douek *et al.* (2000),

children generally have greater thymic functions and a greater proportion of circulating CD4 and CD8 cells. But declining CD4 cells in children as well as in adults, leads to progressive HIV infection. HIV infected children loss thymic volume and activity as they grow older. It is highly recommended that CD4 cell count always be monitored along with HIV viral load as together they provide more information on HIV treatment outcome (Vigano *et al.*, 1999; Douek *et al*., 2000).

In a study by Picker (2006), it was shown that' as the number of CD4 cells decrease, the viral load or the viral RNA in plasma increases leading to virological failure. Virological failure was explained as the viral load of >1 000 copies/mL of plasma confirmed by WHO since 2010. CD4 cell count as well as viral load testing, give prognostic information on HIV progression as well as the response to antiviral treatment. CD4 cell count is one of the factors that guide health professionals on when to initiate ARV therapy. Nonetheless, other clinical factors such as viral load, pregnancy, clinical stage of infection and adherence to medications are also taken into consideration. Prophylaxis against opportunistic infections is prescribed based on the number of CD4 cell count. CD4 cell count of < 200 cells/mm^3 or CD4 percentage of < 14% is an indication of prophylaxis against pneumocystis jiroveci pneumonia and CD4 count of < 50 cells/mm^3 is an indicator for mycobacterium avium complex (Gilks *et al*., 2006). Gilks *et al*. (2006), recommend that CD4 count of < 350 cells/mm^3 of whole blood, indicate a need for a patient to start anti-HIV treatment. Viral suppression is said to occur in patients under ART when, CD4 count increase with > 50 cells/mm^3 within few first weeks of treatment and between 50 to 100 cells/mm^3 every year. For ART patients, CD4 count test should be done every

three months and this can be increased in situations such as switching of therapy, rapidly decreasing of CD4 count and ART failure (Kulkarni *et al.*, 2011).

According to the National Guideline for Antiretroviral Therapy (2010), VL testing should be done prior to initiating HAART and then after 6 months followed by every 12 months. Thereafter in adults and adolescents, VL testing should be performed every year (*NTGAT*, 2010). In the case of children under the age of 15 years, the guideline recommends HIV-1 VL testing to be every 6 months. But the *NTGAT*, 2014 guideline recommends viral load testing every 3 months or more often when the viral load is above 1000 copies/mL or when the patient shows poor clinical results. Children in Namibia are started on treatment as soon as they are confirmed to be HIV positive (*NTGAT*, 2014). Furthermore, the 2014 guideline on antiretroviral therapy in Namibia recommends testing of VL before treatment is initiated and every 6 months in all HIV patients.

The HIV VL is a measure of the amount of copies of HIV RNA per millilitre of plasma. VL is the most valuable indicator of virologic failure in ARV treated patients as it indicates how the patient responds to treatment (Gilks & Vitoria, 2006). High plasma viral load is associated with progression of HIV to symptomatic diseases and AIDS as well as higher risk of HIV transmission (Gilks & Vitoria, 2006). A major treatment objective in ART is to get the viral load results decreased by 10 folds or to undetectable levels after 6 – 8 weeks of treatment. This is called viral suppression in ARV treated patients (CDC, 2009). Studies have shown that' despite a broad consensus on CD4 and viral load prognostic patterns, poor correlation between HIV RNA and CD4 count occurs in adult patients. These have led to a renewed debate over the relative merits of HIV RNA and CD4 count being used as prognostic

indicators without treatment (Rodriques *et al.*, 2006 ; Mellors *et al*., 2007) and with HAART treatment (Phillips *et al.*, 2008).

Knowledge of the natural history of HIV infection and the prognostic value of HIV RNA and CD4 count measurements is becoming more important based on; firstly, the population to determine at what threshold antiretroviral treatment is most effective and cost-effective to begin treatment for ART naïve patients, and secondly on individuals to work out management of treatment of each patient (Gilks & Vitoria, 2006). Furthermore, monitoring VL and CD4 count is also important in regulating treatment allocation among patients especially in resource limited settings. This also improves the empirical basis for prognosis and epidemiological models of the treatment impact and the cost-effectiveness of ART (Granich *et al*., 2008). A study by Peterson *et al.* (2011) outlined that most of the current viral load testing platforms are sensitive only to HIV subtype B and this may cause a significant underestimation of viral load if used to process other strains.

Based on the study done by Korenromp *et al.* (2009), HIV prognostic depends on two things: the relative risk of progression per unit difference in RNA or CD4 count and the extent to which viral RNA and CD4 count levels vary within a certain population. In contrast, the interpretation of the published data on HIV RNA and CD4 count in most of the studies done previously are difficult. This is because these two prognostic components (HIV RNA and CD4 count) are likely to vary as infection progresses. Different studies also used variety of measurements as well as analytical methods for their outcome to interpret observed association (Korenromp *et al.*, 2009).

2.6.1 Laboratory methods for HIV-1 viral load testing

Available HIV-1 VL testing assays are different in their sensitivity, target regions, dynamic range and in the methods used in nucleic acid amplification and detection in case of the nucleic based assays. The different methods also differ in quantifying the different HIV subtypes. In a recent study review by Puren *et al.* (2010) viral load assays were divided into three categories namely:

Nucleic Acid based Test (NATs or NAATs)

Non- Nucleic Acid Test (nNAT) which quantifies HIV viral proteins and enzymes

‘’Home brew’’ or in house Nucleic Acid amplification test.

2.6.1.1 Nucleic Acid based Test (NAT)

There are three available NAT technologies used in quantifying the HIV RNA in plasma: 1. Branched Deoxyribonucleic Acid (bDNA), 2. Nucleic Acid Sequence Based Amplification (NASBA) and 3. Reverse Transcription Polymerase Chain Reaction (RT-PCR). These methods start with sample preparation or extraction of the viral RNA followed by target amplification and detection.

a. Branched Deoxyribonucleic Acid

In this method of HIV RNA quantification, a signal is quantified and not the target. The VERSANT HIV-1 Quantiplex v3.0 (Siemens), target probes bind to sequences within the pol gene of HIV-1 RNA. Unlike other methods, the bDNA technique has signal sites that are detected with a series of hybridization reactions involving chemiluminescence technology. The VERSANT HIV-1 Quantiplex v3.0 does not require RNA extraction.

b. Nucleic Acid Sequence Based Amplification

This method of amplification is sensitive, isothermal, transcription-based amplification system specifically designed for the detection of RNA targets. In some NASBA systems, DNA is also amplified though very inefficiently and only in the absence of the corresponding RNA target or in case of an excess (>1,000-fold) of target DNA over RNA (Deiman *et al.*, 2002).

c. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

PCR technologies artificially replicate target nucleic acid sequences which are available for detection. The target sequence that is amplified (amplicons) are captured and visualized by colorimetric enzyme-substrate reaction. Detection of the amplicons happens either during the process of amplification in case of real time PCR or at the end of amplification for endpoint PCR. The RT-PCR is a type of PCR that uses the reverse transcriptase enzyme to convert the viral RNA into complementary DNA (cDNA). It is the cDNA that goes under replication and detection. There are three commercially available RT-PCR based viral load assays: COBAS Ampliprep/COBAS TaqMan (CAPCTM) v2.0 (Roche Molecular System), Real-time HIV-1 (Abbott) and VERSANT HIV-1 RNA 1.0 assay (kPCR) (Siemens).

COBAS Ampliprep/COBAS TaqMan v2.0

The CAPCTM performs an *in vitro* nucleic acid amplification test for the quantification of HIV-1 RNA in human plasma. Ampliprep is for automated specimen processing and the Taqman for automated amplification and detection. The test quantitates the HIV-1 RNA over the range of 20-10,000,000 copies/mL or 33 to

1.67x107 International Units/mL. One copy of the HIV-1 RNA is equivalent to 1.67 international Units (IU) based on WHO 1st International standard for HIV-1 RNA Nucleic Acid-Based Techniques. This test is used for clinical presentation and other laboratory markers for disease progression in clinical management of HIV-1 group M and O patients. The test is used as a prognostic measurement of the HIV-1 RNA baseline as well as for monitoring the effectiveness of ARV therapy (Puren *et al*., 2010).

Abbott Real Time HIV-1

Abbott Real Time is a real time PCR assay that targets the HIV-1 pol integrase region. Quantification of the HIV-1 RNA is automated by using the m2000sp for sample preparation and nucleic acid extraction and the m2000rt for amplification and detection (Puren *et al*., 2010).

VERSANT HIV-1 RNA 1.0 assay (kPCR)

The kPCR assay also targets the integrase section of the HIV-1 pol gene. Sample preparation (SP) Module and amplification with detection (AD) Modules are automated.

2.6.1.2 Non-Nucleic Acid based Test (nNAT)

Non- Nucleic Acid based Tests are technologies that quantify proteins and enzymes that are specific to the HIV to correlate or measure the HIV viral load (Puren *et al.*, 2010). They are:

a. Target amplification Reverse Transcriptase (RT) method of analysis.

There is a functional manual assay ExaVir Load (Cavidi) that extracts and quantifies reverse transcriptase (Malmsten *et al.*, 2005). The first stage involves the separation of viral particles from the plasma which is followed by enzyme linked immunoassay (ELISA) to quantify the RT activity. The RT activity of an unknown sample is compared to the activity of a recombinant RT enzyme standard of a known concentration. Extrapolated results are reported as fgRT/ml of plasma using a conversion factor of HIV-1 RNA equivalents/ml that are provided by a software. This method is able to quantify HIV-1 and HIV-2 as it does not target a specific nucleic acid sequence. The procedure is less expensive and simple but requires a laboratory with standard ELISA and overnight incubation (Greengrass *et al.*, 2005) which takes 2-3 days to complete.

b. The p24 antigen quantification assay

This method involves the use of the ultrasensitive, heat denatured p24 antigen quantification assay (Perkin Elmer Life Science) which is an enzyme immunoassay for quantification of p24 antigen. The method has a high throughput and does not require many rooms of operations. This method is very sensitive and needs external buffer not supplied by the manufacturer.

2.6.2 Laboratory methods of CD4+ lymphocytes cell count

There are different methods that are used in determining the number of absolute CD4+ cell count as well as CD4% (Kovit & Madhuri, 2005). These methods are:

1. Flow cytometric based assays
2. Non-Flow cytometric based assays

3. Manual assays

2.6.2.1 Flow cytometric based assays

They are Single and Dual platforms

Single platforms

The single platform has two technologies. The micro bead based and volumetric technologies.

a. The micro bead technology:

FACSCount^{TM} micro bead-based system, a product of Becton Dickinson Bioscience. This is the only available micro bead-based technology. The technology is designed for enumeration of absolute CD4+, CD8+ and CD3+ T-cell count in a no-lyse, no-wash whole blood. This system is approved by many international organizations as one of the reliable methods. The system uses ready –to-use twin-tube reagent tubes. One tube determines the absolute number of helper/inducer T-cells (CD4+/CD3+) by using a combination of two-colour monoclonal anti-human CD3 antibody conjugated to the tandem dye phycoerythrin + Cy5 (PECy5) and a monoclonal anti-human CD4 antibody conjugated to phycoerythrin (PE). The other tube determines the absolute number of cytotoxic/suppressor T-cells (CD8-+/CD3+). The two tubes give the absolute number of total T-cells (CD3) or CD3+ T-cells, as well as CD4/CD8 ratio (Kovit & Madhuri, 2005).

In addition to the antibody reagents, the reagent tubes also contain a known number of flourochrome-labelled reference beads. The beads in this system function as fluorescence standard for locating the lymphocytes and also as a quantitation standard for enumerating the cells. The control set consist of fluorescent beads at

four different densities: zero (0 beads/µl), low (50 beads/µl), medium (250 beads/µl), and high (1000 beads/µl) (Bergeron *et al*., 2002; Janossy *et al*., 2000). As whole blood is added to the reagents, fluorochrome-labelled antibodies in the reagents bind specifically to lymphocyte surface antigens. After adding the fixative solution, the sample is run on the flow cytometer. During the process, the stained cells come in contact with the green HeNe laser light, which causes the cell to fluoresce. The fluorescent light provides the information that is necessary for the instrument to count the cells. As a result, the calculation of absolute CD3+, CD4+ and CD8+ T-cells is determined automatically by using the built-in Attractors software program

b. Volumetric technology

Available volumetric technologies count CD+ T-cells depending on the volume of the sample. This means that a fixed volume of the sample with known number of beads is used. The Volumetric technologies are as follow:

Guava ®EasyCD4TM System

This is a product of Guava Technologies. The technology is based on a micro-capillary cytometry technology that uses enormous analytical and diagnostic power of conventional flow cytometry in a high miniaturized single platform. This system consists of two-colour, direct immunofluorescence reagents for enumeration of mature CD4+ T-cells in human blood. The instrument has a diode green laser, while the kit consists of two monoclonal antibodies directly conjugated to PECy5 and PE for CD3 and CD4 T-cell antigens. Acquisition of the samples is done by a variable speed fluid pump that does not require sheath fluid and the systems sampling precision depends on the integrity of fluid pathway. The CD3+ and CD4+ cells are identified in the gate.

Partec Cy-Flow volumetric system

This is a volumetric software controlled absolute count system for CD4+ cell count, which is equipped with either a single 532 nm green solid-state laser that is used for one fluorescence parameter or two lasers with a mercury lamp applicable for 2 or 3-colour analysis. Data acquisition and analysis are performed in real time with FlowMax software.

Dual platform

This uses two instruments to generate absolute CD4+ T cells count. They are a flow cytometer for generating a percentage of CD4+ T cells among lymphocyte and a haematological analyser to enumerate the absolute lymphocyte count. An absolute CD4+ T-cell count is then derived by multiplying percentages of CD4+ T cells by absolute lymphocytes count. A whole blood sample that contains a high proportion of lymphocytes, a lymphocyte gate for CD4+ T cell testing is easily derived from a bivariate histogram or homogeneous gate that includes forward scatter (FSC, the size of the cell population) and the right angle side scatter (SSC, granularity of the cell populations) patterns. However, when there is a high proportion of non-lymphocytes, the traditional FSC/SSC lymphocyte gate tends to be unreliable as non-lymphocytes have been shown to contaminate the gate. Due to this the morphological gating is now considered as unacceptable (Kovit & Madhuri, 2005). The only approved dual platform is the PanLeukogating (PLG) technology.

PanLeukogating technology

This is also known as CD45 gating. PLG technology uses two markers which are the CD45 and CD14. The CD45 is used as a marker that is expressed at different intensities on leukocytes, granulocytes as CD45+, monocytes as CD45++, lymphocytes CD45+++ or even bright while the CD14 is selectively expressed by monocytes. The CD45 gating gates all the lymphocytes at a maximum and excludes non-lymphocytes. This flow cytometric method generates three colours (CD3/CD4/CD45) or four colours (CD3/CD4/ CD8/CD45) for immunophenotyping assays of CD4+ T-cells (Niccholson *et al.,* 1993; Pattanapanyasat *et al*., 1994; Bergeron *et al.*, 2002). In the 3 or 4 immunophenotyping techniques, lymphocytes are defined as CD45 bright with a low right angle side scatter which enables a very pure population of lymphocytes. Only after this the lymphocytes gate is established, the percentage of lymphocytes reactive for CD3+/CD4+ T-cells in the 3- colour assay and CD3+/CD4/CD8+/ T-cells in the 4 - colour assay are quantified (Kovit & Madhuri, 2005).

The CD45 gating assay also has a 2-colour panleukogating approach (Bergeron *et al*., 2002; Janossy *et al.*, 2000), which uses the total leukocytes as a common denominator, whereby total leukocytes are identified and gated based on their side scatter and CD45+ characteristics. The CD45 reacts with fluorochrome-conjugated antibody. The 2 regions are formed one with the leukocytes and the other one of lymphocytes. One region will be around all leukocytes and another region will be for all bright CD45+ cells with low SSC. Further only the lymphocytes region is analysed for CD4+ T- cells. By using this method, CD4+T cells are easily distinguished from other cells and the CD4 percentage of total lymphocytes is

obtained. CD4 assisted PLG technique is widely used for its cost effectiveness in resource limiting or poor countries and ease to use. The dual platform can be used as a single platform with addition of micro beads to determine the number of CD4+ and CD8+ T-cells.

The advantages of using PLG assay are that: it is easy to differentiate lymphocytes in the SSC/CD45 bright gate even when there is a big number of debris; the isotope controls (types of negative control designed to measure the level of non-specific background signal caused by primary antibodies, based upon the tissue type of the sample) can be avoided and this reduces the cost of reagents; it is feasible in determining the %lymphocytes among leukocytes from the SSC/CD45 gate.

2.6.2.2 Non-flow cytometry based assay

The Non-flow cytometric technology is mostly used in quantifying the number of CD4+ and CD8+ T-cells. This is a very cost effective method that is suitable for resource poor areas (Bergeron *et al*., 2002). The Beckman-Coulter cytosphere system is currently the one system in the market.

Beckman-Coulter cytosphere system consists of the CD4+ cytospheres reagent, the inert latex spheres coated with monoclonal antibody. Monoclonal antibodies are used to identify and manually enumerate the absolute number of CD4+ T-cells by visible light microscopy in whole blood samples. When using this assay, 10 µL of cytosphere monocyte blocking reagent is added to 100 µL of whole blood. CD4 Cytosphere reagent is added to the mixture after incubation (Janossy *et al*., 2000). 10 µL of blood latex spheres mixture is added and lysed with lysing reagents. After that,

the lysed mixture is loaded into the haemocytometer and this is read under the microscope. The diameter for the CD4 Cytosphere reagents differs from the cytosphere monocyte-blocking reagents, thus CD4+ T-cells rosette with antibody-coated latex beads can easily be distinguished from the monocytes rosette latex beads as they have larger latex sphere size.

2.6.2.3 Manual Methods

Dynabeads T4-T8 quantitative system

This system is based on the use of immunomagnetic cell isolation method that uses Dynobeads magnetic particles coated with antibody to CD4 and CD8 antigens to capture and isolate CD4+ and CD8+ T-cells from whole blood. During the process, whole blood sample is diluted in a buffer solution (Janossy *et al*., 2000). This whole blood sample gets depleted of monocyte using the monocyte depletion magnetic bead reagent that is coated with anti-CD14 antibody. Only after incubation of the sample on the Dynal rotator, magnetic separation of monocytes is done using a magnetic particle concentrator. After that, the supernatant from the monocyte depleted blood is diluted and mixed with magnetic beads coated with anti-CD4 monoclonal antibody. After incubation, the beads are separated from the CD+ T-cells. Isolated CD4+ T-cells are stained with gentian violet and trypan blue. The isolated stained cells are then lysed and the nuclei are identified and counted manually by light microscopy or by an automated cell counter (Janossy *et al*., 2000).

Point Care

This is a closed system for the enumeration of CD4 + T-cells count as well as CD4 %. Lysed whole blood samples are required for this system. It is a simple technology applicable to low volume laboratories as the sample throughput is low.

3 CHAPTER THREE: METHODS

3.1 Research design

Part of this study was carried out retrospectively using data routinely collected at each patient consultation at Katutura State Hospital, Katutura Health Centre and Windhoek Central Hospital between March 2012 and June 2015. Medical records of all HIV-1 positive patients actively taking antiretroviral drugs were reviewed. Patients' information such as personal details (age, gender) and treatment combination were recorded as patients come for their normal hospital visits. CD4 count and viral load records collected from the patients file were verified to see if they match with those in the Namibia Institute of Pathology (NIP) laboratory's Medical Information Technology (MEDITECH) Inc. (USA) database. Patients were than divided into different groups according to their first line treatment combination. Furthermore, 7 records on CD4 count and VL for each individual were recorded. The 7 records on VL and CD4 count were separated in variables recorded before treatment referred to as initial values before treatment, CD4 count and VL recorded at first 6 months of treatment, at 12 months, at 18 months, at 24 months, at 30 months and at 36 months respectively.

The mean, standard deviation, minimum and maximum ranges as well as lower and upper limit of standard deviation and error were calculated for CD4 count and VL values. These variables were also used in calculating the mean difference of CD4 count and VL change over the course of this study. Difference in CD4 count and VL variables were calculated to see the trend as well as the relationship between the two variables from one follow-up to the next and throughout the 36 months treatment.

Furthermore, for the prospective study blood samples collected from consented patients were used for micronutrient analysis. Patients who came for their follow-up were asked to give blood samples for micronutrient analysis. Micronutrients analyses were only done once at 36 months of ARV treatment. The importance of the study on micronutrient analysis was explained to all patients who participated in the study. Patients took different meals before coming to the facilities and the majority had tea and bread at breakfast. Samples were collected in the mornings after the patient's consultation with the doctor. The collected samples were immediately taken to the NIP central laboratory in Windhoek for analysis on surface magnesium (Mg^{2+}), surface iron, folate and vitamin B12.

3.2 Study Population

The Khomas region has 6 clinics, 1 health centre and 2 state hospitals. The region has an approximately 19 155 HIV/AIDS patients actively taking antiretroviral drugs (Mr N. Kamati, personal communication, 25 May, 2015, Katutura Health Centre). Patients on pre-ART (before start of treatment) constitute 11 786 bringing the total number of HIV/AIDS patients on care in the whole Khomas region to 35 645. The study involved children as from the age of 1- 12 years and adults from the age 13-55 years. According to the HIV treatment guideline children are up to 12 years. All patients from 13 years and above are considered adolescents and adults and they are under the same ARV treatment guideline.

3.3 Sampling

A systematic random selection of samples was used during the retrospective study with sampling intervals of 48 samples (19155 (ARV population)) / 392 (sample size according to the table by Israel, 1997). Every 48th person in the ARV list was selected to take part in the study. Most of the patients took part in the study were from Katutura state hospital as it has more patients on ART treatment. Blood samples were collected from 8 am – 10 am in a gel yellow cap tube with heparin. Samples were transported in a cold hamper at 2-8 °C to the NIP laboratory in Windhoek for analysis.

Table 3.1

Number of patients from participated health facilities

Health facility	Number of patients	Total Study Population
Katutura State Hospital	9221	19 155
Katutura Health Centre	5444	
Windhoek Central Hospital	1949	

The sample size of the study was determined using sample size by Israel (1997). According to Israel, (1997) our sample size would be 392 samples based on a population of N = 20 000 (our study population was 19 155 (Table 3.1). We had a sample size of 404 patients for the retrospective study. For the prospective study 30 aged 13 – 55 years of 404 patients gave their consent for micronutrients analysis.

3.4 Data management procedure

Data collection sheets were prepared to capture data for Annexure A (p.120) and B (p.121). The consent letter for reference in Annexure C (p.122). Annexure A was used in recording personal details of patient based on gender and age, ART regimen and the health facility. Annexure B was used to record data on micronutrient levels (iron, magnesium, folate and vitamin B12). Finally, data was entered into Microsoft Excel sheet 2010 based on the data forms.

3.4.1 HIV-1 viral load testing

The COBAS®Ampliprep/COBAS TaqMan® (CAP CTM) (Roche, Germany), was used for HIV-1 quantitative testing. The COBAS®Ampliprep/COBAS TaqMan® HIV-1 Test, v2.0 utilizes real-time PCR technology. It uses a dual-labelled fluorescent probes which allows real-time detection of PCR product accumulation by monitoring the emission intensity of fluorescent reporter dyes released during the amplification process. The probe consists of HIV-1 and HIV-1 Quantitative Standard (QS)-specific oligonucleotide probe with a reporter dye and a quencher dye. The CAP CTM HIV-1 Test v2.0's HIV-1 and HIV-1 QS probes are labelled with different fluorescent reporter dyes (Puren *et al.* (2010).

Reagents used for COBAS®Ampliprep/COBAS TaqMan® HIV-1 Test, v2.0 for viral load testing.

Reagents used were the HIV-1 v2.0 CS1 (HIV-1 Magnetic Glass Particles Reagent Cassette), HIV-1 v2.0 CS2 (HIV-1 Lysis Reagent Cassette), HIV-1 v2.0 CS3 (HIV-1 Multi-Reagent Cassette containing Proteinase solution and the Elution

Buffer), HIV-1 v2.0 CS4(HIV-1 Test-Specific Reagent Cassette containing HIV-1 Quantitation Standard, HIV-1 Master Mix and CAP/CTM Manganese Solution. Controls used were: HIV-1 High Positive Control, HIV-1 Low Positive Control and HIV-1 Negative Control.

The test involves 3 major experimental components:

a) Isolation of HIV-1 RNA

Whole blood samples were collected in BD vacutainer® plasma preparation tubes (PPT) with potassium and etylendiamin tetra acetic acid (K_2EDTA). Potassium and EDTA are preservatives that protect the nucleic acid against degradation. The isolation of HIV-1 RNA was done starting with spinning of whole blood samples at 2000 Round per Minute (RPM) for 10 minutes to separate the plasma. An amount of 1060 µL of plasma was pipetted from each Plasma Preparation Tubes (PPTs) into sample tubes (S-tubes). Sample tubes are special tubes of Roche diagnostics that are loaded in the Ampliprep instrument for nucleic acid extraction. Inside the Ampliprep instrument 850 µL of human plasma was pipetted from each sample and it was then mixed with protease for deproteinisation. The inhibitory proteins and RNases are degraded by protease and nucleases by chaotropic salt reducing agents and detergents. After deproteinasation, 1250 µL of lysis buffer was added to the sample lysing cells to release nucleic acids while stabilizing the cells at the same time. The lysing process was done at 37 ºC for 22 minutes.

After lysing the cells, 100 µL Magnetic Glass Particles (MGP) was added to the mixture to bind total nucleic acids to the silica surface of the MGP in the presence of chaotropic salt at high detergent concentration and low pH at 37 ºC. After that,

unbound substances and impurities like denatured proteins, cellular debris, and potential PCR inhibitors were washed away with the wash buffer. Once the nucleic acids were on the surface of the MGP, Elusion buffer was added to the reaction to release the total nucleic acids at an elevated temperature of 80 ºC with high pH and low salt concentration. After elusion, 35 µL of Master Mix was added to the extracted nucleic acids. The master mix included Mn^{2+}, rTth DNA polymerase, dCTP, dGTP, dUTP, dATP, Biotinylated primers and Amp Erase (Uracil-N-glycosylase) enzyme. Only 50 µL of the prepared nucleic acids was used in the amplification reaction to generate cDNA. This step was followed by the PCR reaction for amplification and detection.

Reverse transcriptase of the target RNA to generate complementary DNA (cDNA)

In reverse transcription, the biotinylated primer anneals to target RNA sequence from 5`→3`. With the presence of rTth DNA polymerase, the primer is extended by incorporating complementary nucleotides. After this a complementary DNA (cDNA) which is complementary to the RNA target sequence is formed. Simultaneous polymerase chain reaction (PCR) and detection of cleaved dual-labelled oligonucleotide detection probe specific to the target. The PCR process involves the following stages:

Denaturation - the cDNA which is attached to the target RNA sequence is being denatured.

Annealing- annealing of primer to the cDNA from 3`→5`.

Extension - the rTth DNA polymerase catalyzes the extension of primer. This forms a double stranded DNA copy (amplicon) of the target sequence. Each new cycle doubles the amount of target resulting in an exponential increase in amplicons.

b) Detection of PCR products in a COBAS TaqMan

The COBAS TaqMan® HIV-1 Test, v2.0 utilizes real-time PCR technology. The use of dual-labelled fluorescent probes allows the real-time detection of PCR product accumulation by monitoring the emission intensity of fluorescent reporter dyes released during the amplification process. The probes consist of HIV-1 and HIV-1 QS-specific oligonucleotide probes with a reporter dye and a quencher dye. In the COBAS TaqMan® the HIV-1 and HIV-1 QS probes are labelled with different fluorescent reporter dyes. Whenever the two probes intact, the fluorescence of the reporter dye is suppressed by the proximity of the quencher dye due to Föster-type energy transfer effects. During the process of polymerase chain reaction (PCR), the probe hybridizes to a target sequence and is cleaved by the 5′ → 3′ nuclease activity of the thermostable Z05 DNA polymerase. After when the quencher and the reporter dyes are released and separated, quenching no longer occurs, and the fluorescent activity of the reporter dye is increased. The amplification of HIV-1 and HIV-1 QS RNA are measured independently at different wavelengths. The process repeats for a designated number of cycles, whereby each cycle effectively increase the emission intensity of the individual reporter dyes, permitting independent identification of HIV-1 and HIV-1 QS RNA. The PCR cycle where growth starts, is equivalent to the amount of starting material at the beginning of the reaction.

Quantitative process starts over a wide dynamic range since the monitoring of amplicon is performed during the exponential phase. The higher the HIV-1 RNA titter of a specimen, the earlier the fluorescence of the reporter dye of the HIV-1 probes rises above the baseline fluorescence level. The amount of the HIV-1 QS RNA in all specimens is the same so for all the samples the fluorescence of the reporter dye of the HIV-1 QS probe should appear at similar cycle. The appearance of the fluorescence signal is reported as a critical threshold (Ct) value. Ct is a fractional cycle number where the reporter dye fluorescence exceeds a predetermined threshold, and starts the exponential growth phase of the signal. The results are interpreted in copies of HIV-1 in copies/mL (Puren *et al.*, 2010).

3.4.2 CD4+ lymphocytes absolute count

CD4+ cells count was performed using the Cell Mek FC 500 an automated flow cytometer. CD4 count and CD4 percentages were measured on whole blood by flow cytometric immunophenotyping. The analyser uses PLG technique for CD4 count Pattanapanyasat & Thakar, 2005). Results were reported in absolute CD4 count as well as in percentages of CD4. CD4% is the percentage of lymphocytes population that are CD4 positive. The absolute CD4 count is calculated from the CD4 cell percentages and the total white blood cell count. The normal CD4 values are between 500-2000 cells/µL (31-55%) for adults and adolescents (Pattanapanyasat & Thakar, 2005).

Reagents used for CD4+ cell PLG technique

The following reagents were used in the analysis of CD4 count: monoclonal antibody CD4-Fluorescein isothiocyanate (FITC)/CD45- Phycoerythrin (PE), which was provided in a phosphate buffered saline with 0.1% sodium azide; Lysing reagents- immunoPrep A that caused a rapid destruction of erythrocytes and left leukocytes intact; Stabilizing reagents- immunoprep B that stabilized the action of lysing reagents; ImmunoPrep C which lyses red cells and fixes cell membrane preserving the form and structure of leukocytes.

Calculation of CD4 count

Samples were loaded on the flow cytometer within the first four hours of finishing the sample preparation. Alternatively, samples were stored at 2- 8 ºC in the dark until processing. Instrument was calibrated according to manufacturer's instruction. The procedure involves two measurements: the white cell count and the percentage of T lymphocytes which are CD4 positive. The resulting CD4% is used to calculate the CD4 cell number (i.e. absolute CD4 count) by the formula:

$$\text{CD4 count} = \frac{white\ blood\ cells}{100}\ \text{X \% CD4 positive lymphocytes}$$

The results are interpreted in number of cells/mm^3 of blood.

Testing Procedure

Whole blood samples were collected in an EDTA tube and processed within 6 hours of collection. Haemolysed samples or samples with suspended cell aggregates did not qualify for this test. An amount of 10µL of CD4-FITC/CD45-PE antibodies were pipetted at the bottom of the labelled tube (special tubes for the flow cytometer) after which samples were mixed on a shaker. After mixing, 100 µL of the mixed whole blood was added into the bottom of the tubes containing the antibodies. The mixture was well mixed to mechanically agitate the sample and the antibody. The tubes were then incubated for 10-20 minutes at a room temperature in a dark place, and then placed into the flow cytometer for processing. Inside the flow cytometer, 100 µL of flow count was added into the samples and samples were allowed to stand for 10 minutes before reading.

3.4.3 Chemistry and Immunochemistry analysis of surface Iron, surface Magnesium, Folate and Vitamin B12

Micronutrients analyses were performed using the Architect *c* 8000 System (Abbott Laboratories, USA). This is a chemistry and immunochemistry analyser, used for the quantification of important minerals and vitamins in human plasma, serum and urine Chemiluminescence, photometry, potentiometric, and turbidimetry immunoassays (Jacobs *et al.*, 2001).

Sample preparation

Specimens were collected by standard venepuncture in heparin containing tubes with silica gel. The tubes were taken to the laboratory and were immediately spun at 1500 rpm for 10 minutes to separate the serum. After spinning, the samples were

loaded into the Architect system for determination of surface iron, magnesium, folate and vitamin B12.

Iron testing

The system accurately quantifies iron by direct colorimetric without deproteinization in human serum or plasma. Results were interpreted in µmol/L and the reportable range for multigent iron is 0.9-179 µmol/L. Reagents used in the analysis of iron were: Reagent 1 that consists of sodium acetate, hydroxylamine HCl and acetic acid and thiourea (animal carcinogen)), Reagent 2 is made up of sodium acetate, acetic acid and ferene. Two levels of controls (normal and abnormal) were run every 24 hours. Calibration is stable for at least 27 days (648 hours) and was required with each change in reagent lot number. Calibration was verified with at least two levels of controls according to the established quality control requirements of the NIP Chemistry and Immunochemistry laboratory. Assay calibrators values were between 0 and 318.4 µmol/L.

Principles of the test

At pH of 4.8, iron is released from transferrin and then quantitatively reduced to a ferrous state. Iron forms with Ferrene-S*, a stable coloured complex and the absorbance is measured at 604 nm of which the colour intensity is proportional to the amount of iron in the sample. Thiourea and detergent agents eliminate the interference from copper. Table 3.2 shows the normal ranges of iron concentrations in adults. Limit of Detection (LOD) for iron is 0.24 µmol/L. Limit of Quantitation (LOQ) for iron is 0.97 µmol/L. Elevated iron is seen in haemolytic anaemia,

hemochromatosis and acute liver disease. Low level of iron is found when there is an iron deficiency or anaemia of chronic disease.

Table 3.2

Iron concentration in adult males and females

	Range (µmol/L)
Adult, Male	5.5 to 25.78
Adult, Female	4.48 to 27.92

Magnesium testing

Reagents used for magnesium testing

Magnesium reagents were supplied as liquid, ready-to-use, single reagent kits which contain, TRIS buffer, arsenazo dye and sodium azide. Calibration was verified with at least two levels of controls according to the established quality control requirements for the laboratory and control results should fall within the acceptable ranges, otherwise recalibration was repeated. Assay calibrators value ranges from 0.063 - 3.90 mmol/L.

Principle of the test

The architect method used for the quantification of magnesium uses an arsenazo dye which binds preferentially with magnesium. The absorbance of the arsenazo-magnesium complex is measured at 572 nm and is proportional to the concentration of magnesium present in the sample. The amount of magnesium is measured in millimoles per liter (mmol/L). Calcium interference is prevented by incorporation of

a calcium chelating agent. Normal levels of magnesium depend on the age of the person as indicated in Table 3.3. On the other hand, in higher levels than those indicated in the table can be seen in adult females (Burtis & Ashwood, (Eds.), 1994). The low detection limit of magnesium in this assay is 0.063 mmol/L. The limit of quantification of magnesium in serum is 0.270 mmol/L (Passey *et al.*, 1986).

Table 3.3

Magnesium values in serum

Age	Mg^{2+} ranges in mmol/L
Newborn, 2- 4 days	0.62 – 0.91 mmol/L
5 months – 6 months	0.70 – 0.95 mmol/L
6 – 12 years	0.70 – 0.86 mmol/L
12 – 20 years	0.70 – 0.91 mmol/L
Adults	0.66 – 1.07 mmol/L

Analysis of Folate

The assay performs a chemiluminescent micro particle Folate Binding Protein (FBP) assay for the quantitative determination of folate in human serum, plasma, and red blood cells on the Architect information system.

Reagents used for folate analysis procedure are: micro particles, conjugate, assay specific diluent, pre-treatment reagent 1 and pre-treatment reagent 2. The Architect folate reagent kits were stored at 2-8°C. Calibrators were stored at -10°C or colder. Calibrators and controls are sensitive to light so they were stored in a carton to protect from light. Un-reconstituted folate lysis reagents were stored at 15-30°C

while reconstituted folate lysis reagents were stored at 2-8°C. Calibrators values ranges from 0.0 - 20.0 ng/mL.

Procedures in folate testing

A two-step assay was allowed for the quantitative determination of folate in human serum using Chemiluminescent Micro particle Immunoassay (CMIA) technology with flexible assay protocols, referred to as Chemiflex. Folate assay diluents and other reagents are well outlined in the folate package insert. Two pre-treatment steps mediated the release of folate from endogenous folate binding proteins. In the pre-treatment step one of the sample and pre-treatment reagent 2 (Dithiothreitol or DTT), was aspirated and dispensed into a reaction vessel (RV). This was followed by pre-treatment step two as follows:

An aliquot of sample/ Pre-Treatment Reagent 2 mixture was aspirated and dispensed into a second RV. Pre-Treatment Reagent 1 (potassium hydroxide (KOH) was then added. An aliquot of the pre-treated sample was transferred into a third RV, followed by the addition of Folate Binding Protein (FBP) coated paramagnetic micro particles and assay specific diluent. Folate present in the sample binds to the FBP coated micro particles. After washing, pteroic acid-acridinium labelled conjugate was added to bind to unoccupied sites on the FBP-coated micro particles. Pre-trigger and trigger solutions was then added to the reaction mixture. As a result, chemiluminescent reaction occurs and is measured as relative light units (RLUs). The amount of folate in the sample is indirectly proportional to the amount of relative light unit detected by the architect's optical system and this was measured in nanograms per millilitre (ng/mL)

Analysis of Vitamin B12

Chemiluminescent Micro -particle Intrinsic Factor (CMIF) assay was used for the quantitative determination of vitamin B12 in human serum on the Architect information system. Reagents used for this procedure are: multi-assay manual diluent, pre-trigger solution, conjugate, micro particles, assay specific diluent, pre-treatment reagent 1, pre-treatment reagent 2, pre-treatment reagent 3, wash buffer and trigger solution. The Architect B12 reagent kits were stored at 2-8°C in an upright position and used immediately. Calibration for B12 assays ranges from 0-2000 pg/mL.

Principles of the test

The B12 assay on Architect was also performed following two-step assay with an automated sample pre-treatment, for determining the presence of B12 in human serum using CMIA technology with flexible assay protocols also called Chemiflex. Pre-treatment reagent 1, pre-treatment reagent 2, and pre -treatment reagent 3 are key reagents for B12 analysis. An aliquot of the pre-treated samples was aspirated and transferred into a new Reaction Vessel (RV). The pre-treated sample, assay diluent, and intrinsic factor coated paramagnetic micro particles were added to the solution.

B12 present in the sample binds to the intrinsic factor coated micro particles. After washing, B12 acridinium labelled conjugate was added in the second step. Pre-trigger and trigger solutions were then added to the reaction mixture; the resulting chemiluminescent reaction was measured as Relative Light Units (RLUs). The inverse relationship between the amount of B12 in the sample and the RLUs detected

by the Architect information optical system was calculated. The results were recorded in picogram/millilitre (pg/mL).

3.5 Data analysis

Data analysis was done with SPSS version 2.2. The values of CD4 count, VL, differences in CD4 count and viral load between follow-ups were calculated 7 times in 36 months (7 times from initial which was zero month of treatment, 6, 12, 18, 24, 30 and 36 months of treatment). Patients taking the same combination were grouped together for each analysis. Children's data (1-12 years) were separated from adult's data (13-55 years) during analysis. ANOVA was used to find the mean in CD4 count and viral load between follow-up as well as the mean in differences of CD4 count and viral load trends between follow-ups among different treatment groups. This was rated in percentages out of 100 depending on the number of times that CD4 count and VL increased/decreased in 6 times (6 is the number of difference in follow-ups from initial to 36 months) i.e. differences between 0 – 6, 6 – 12, 12 – 18, 18 – 24 , 24 – 30 and 30 – 36 months.

Decreases and increases were converted into percentages. The minimum and maximum number of CD4 count, viral load per treatment group, standard deviation, standard of errors as well as upper and lower limit were calculate at a confidence interval of 95%. A two-tailed non-parametric spearman correlation with cross tabulations was used to see the relationship between CD4 count and VL means recorded at different follow-ups. Using the same analysis, the relationship between treatment combination with different micronutrient levels and the relationship between age of patients and the treatment combination was found.

3.6 Determining changes in CD4 count and HIV-1 VL

This was done by finding the VL differences from initial time of antiretroviral treatment and after every six months up to 36 months of treatment. Patients were grouped in seven (7) ART groups according to their first line treatment combinations that were prescribed to the patients involved in this study. The average VL copies as well as the average difference for CD4 count for patients on different combinations were calculated between different months of follow-ups (every six months for 36 months). The average differences with positive values showed VL/CD4 increase between follow-up and a negative average mean shows a decrease between follow-ups. In SPSS differences in VL were calculated by subtracting the previous VL mean for a group of patients taking the same regimen from the current records, which was also done on CD4 count as indicated in Table 3.4. $CD4\text{-}_0$ is CD4 count recorded before treatment and $VL\text{-}_0$ is viral load recorded before treatment. $CD4\text{-}_1$ and $VL\text{-}_1$ are those recorded after six months of treatment than it goes every 6 months until 36 months of treatment.

Table 3.4

Average differences in CD4 count and viral load between follow-ups

Period	Average CD4 differences	Average VL differences
6 months	$CD4_1$- $CD4_0$	$VL_1 – VL_0$
12 months	$CD4_2$- $CD4_1$	$VL_2 – VL_1$
18 months	$CD4_3$- $CD4_2$	$VL_3 – VL_2$
24 months	$CD4_4$- $CD4_3$	$VL_4 – VL_3$
30 months	$CD4_5$- $CD4_4$	$VL_5 – VL_4$
36 months	$CD4_6$- $CD4_5$	$VL_6 – VL_5$

3.6.1 Finding the relationship between CD4 count, HIV-1 viral load

A non-parametric two-tailed coefficient correlation considered significant when $P < 0.05$, was used to identify the relationship between CD4 count and VL for 36 months. At the same time bivariate Pearson's correlation significant at two tailed, $P < 0.05$ and $P < 0.01$ was also used to see the direction of the relationship between CD4 count and VL over the treatment period of 36 months.

3.6.2 Determining ART combination that suppresses viral replication the most in ART naïve patients

This was done by rating the average VL decrease/increase and CD4 count increase/decrease in 7 hospital visits for 36 months. Hence, VL is expected to decrease after treatment, we rated the number of times the viral decreased over the period of 36 months for different combination. Expecting that CD4 count has to increase after the initiation of antiretroviral treatment and viral load has to decrease if treatment is effective.

3.6.3 Assessing the association of micronutrients levels with treatment

Patients were grouped into three (3) categories. Those with normal level of micronutrients; those with low level/deficient and those with high levels. A cross tabulation with Pearson's correlation (two tailed and significant at $P < 0.05$ or $P < 0.01$) was used to analyse the relationship between micronutrients level with ART combination.

4 CHAPTER FOUR: RESULTS

4.1 Demographic characteristics of patients per combination

Table 4.1 indicates the age category of participant on each treatment combination. Furthermore, in this study the data on adults represents both adults and adolescents population. According to the Table 4.1, 404 participants took part in the study of which 326 (80.7%) were adults and adolescents whilst 78 (19.3%) were children. The most prescribed combination was AZT/3TC/NVP for both children, adults and adolescents whilst combinations with protease inhibitors were least prescribed. More significantly, Table 4.1 shows that among the study group, no child was treated with the combination of TDF/3TC/EFV and TDF/3TC/NVP whilst the treatment combinations with protease inhibitors (AZT/3TC/LPV-r and D4T/3TC/LPV-r) and TDF/3TC/EFV were lowly prescribed.

Table 4.1

Age categories of participants on each combination

ARV Therapy	Children	Adults and adolescents	Count
AZT/3TC/NVP	16(16.3)	161(39.9)	227
AZT/3TC/EFV	3(0.7)	33(8.2)	36
TDF/3TC/EFV	0(0.0)	15(3.7)	15
TDF/3TC/NVP	0(0.0)	51(12.6)	51
AZT/3TC/LPV-r	3(0.7)	7(1.7)	10
D4T/3TC/NVP	4(1.0)	56(13.9)	60
D4T/3TC/LPV-r	2(0.5)	3(0.7)	5
Total (%)	**78(19.3)**	**326(80.7)**	**404**

Table 4.2, indicates gender segregated relative frequencies of participants. There was slightly higher male participation of 211 (52.2%) individuals compared to female participation at 193 (47.8%). There were more males on treatment

combination of with AZT/3TC/NVP 136 (33.7%), (Table 4.2). AZT/3TC/NVP treatment combination was the most prescribed first line treatment of which the majority of participants were males. Although marginal, some combinations were prescribed more often to females compared to males, despite the majority of participants being males (Table 4.2). The treatment combinations prescribed often to females were: AZT/3TC/EFV at 4.7%, TDF/3TC/NVP at 6.7%, D4T/3TC/NVP at 10.9%, D4T/3TC/LPV-r at 0.7% and AZT/3TC/LPV-r at 1.7%.

Table 4.2

Relative frequency of gender categories of participants under different first line combination

ARV Therapy	**Female patients No: (%)**	**Male patients No: (%)**
AZT/3TC/NVP	91 (22.5)	136 (33.7)
AZT/3TC/EFV	19 (4.7)	17 (4.2)
TDF/3TC/EFV	2 (0.5)	13 (3.2)
TDF/3TC/NVP	27 (6.7)	24 (5.9)
AZT/3TC/LPV-r	7 (1.7)	3 (0.7)
D4T/3TC/NVP	44 (10.9)	16 (4.0)
D4T/3TC/LPV-r	3 (0.7)	2 (0.5)
Total	**193 (47.8)**	**211 (52.2)**

Table 4.3 shows the distribution of participants on different combinations of treatment at a confidence interval (CI) of 95% across the study population. D4T/3TC/LPV-r combination was prescribed to female participants' age between 29 and 40 with the mean age of 34 years (standard deviation (SD ± 0.5). Whilst the age mean for male participants was 2 years (SD ± 0.7) for the same treatment combination. Female patients on AZT/3TC/NVP combination had a mean age of 33.6 years (SD ± 14.0) (Table 4.3). Male patients on the same therapy

(AZT/3TC/NVP), their mean age was at 25 years (SD ± 16.5). The mean age per gender under treatment combination AZT/3TC/EFV was 34 years (SD ± 11.9) and 35 years (SD ± 13.4) for female and male participants respectively. Moreover, for TDF/3TC/NVP combination the mean age was 41 years (SD ± 9.2) and 44 years (SD ± 3) for female and male participants respectively (Table 4.3).

Table 4.3

Age distribution for females and males on different combinations

ARV Therapy	Female			Male		
	Mean ± SD	95 % C.I		Mean ± SD	95 % C.I	
		Lower limit	Upper limit		Lower limit	Upper limit
D4T/3TC/LPV-r	34.3 ± 5.0	28.6	40	1.5 ± 0.7	0.5	2.5
AZT/3TC/NVP	33.6 ± 14.0	30.7	36.5	25.3 ± 16.5	22.6	28
AZT/3TC/EFV	34 ± 11.9	28.7	39.3	35.1 ± 13.4	28.8	41.4
TDF/3TC/NVP	40.5 ± 9.2	27.8	53.2	44 ± 3	42.4	45.6
AZT/3TC/LPV-r	38 ± 7.8	22.8	53.2	36.1 ± 4.5	27.2	45
TDF/3TC/EFV	30.3 ± 0.8	29.7	30.9	4.0 ± 3.0	36.3	43.1
D4T/3TC/NVP	37.7 ± 11.2	30.8	44.4	39.7± 13.4	33	46.3

The mean age of female participants for D4T/3TC/NVP treatment was 38 years (SD ± 11.2) and for male participants it was 40 years (SD ± 13.4). The mean age of female participants that were under treatment combination AZT/3TC/LPV-r was 38 years (SD ± 7.8) (Table 4.3). In contrast, mean age of male participants was 36 years (SD ± 4.1) for the same treatment combination. This data suggests that AZT/3TC/LPV-r was prescribed to similar age profiles, ages of thirties, irrespective of the gender. The mean age of female participants treated with combination

TDF/3TC/EFV was 30 (SD ± 0.8). At the same time, the mean age of male participants for the same treatment combination was 4 years (SD ± 3.0). The data suggests that TDF/3TC/EFV was mostly prescribed to women in their thirties and for children.

4.2 Descriptive statistics on VL and CD4 count in adults and adolescents

4.2.1 Descriptive statistics on CD4 count of adults and adolescents

The population of adults and adolescents in this study compromised participants from ages 13 – 55 years. In this section, means, minimum and maximum number of CD4 count of each treatment combination as well as the standard deviation and the lower and upper boundaries of CD4 count at the confidence interval of 95% were calculated for all the participants from 13 years and older. This information is in different Annexure at the end of this document. In the current section, trends of CD4 count was observed for over a period of 36 months for patients/participants taking different first line ARV combinations. Average CD4 count trends in adults and adolescents are indicated on Figure 4.1. As clearly seen on Figure 4.1, there was no one single treatment combination that gave a consistent rise in CD4 count throughout all 36 months. Data suggest that there was some form of fluctuation in the CD4 count for any given combination over the study period.

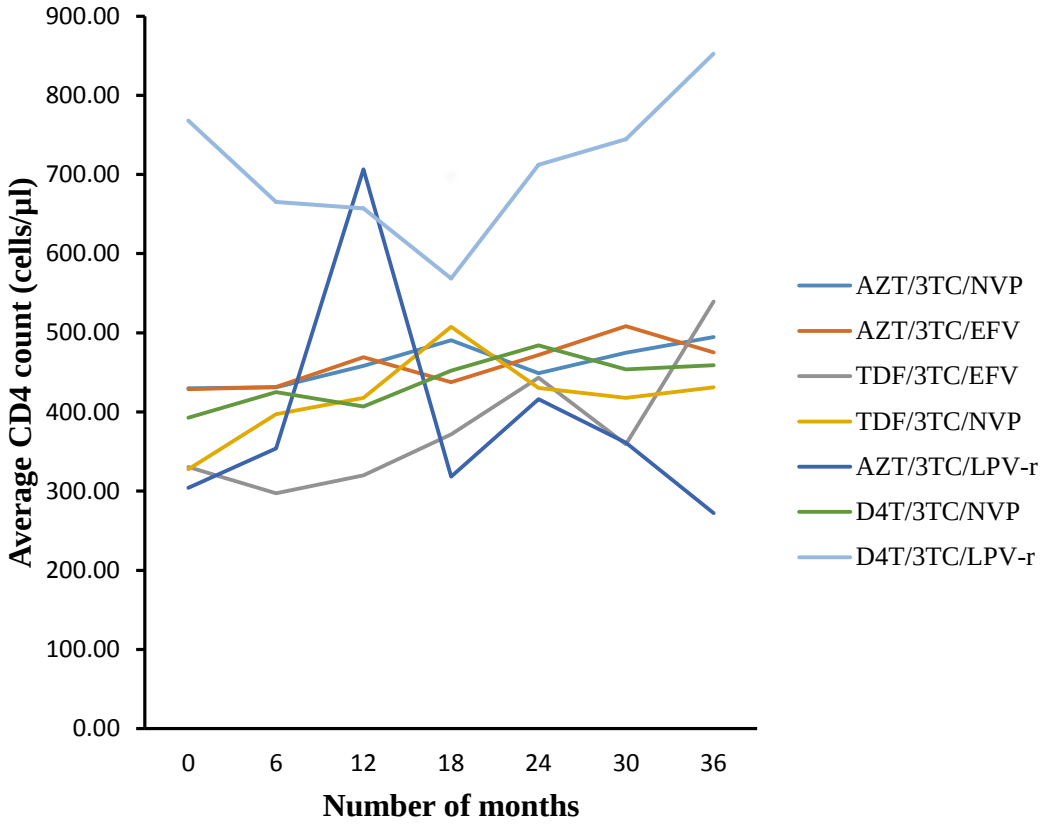

Figure 4.1. Average CD4 count trends in 36 months of treatment in adults and adolescents

Participants falling under the mostly prescribed combination (AZT/3TC/NVP) showed the highest increase in CD4 count at 18 and 36 months of treatment with (490.45 cells/µl) and 494.81cells/µl respectively. All treatment combination showed an increase in CD4 count as early as 6 months except for TDF/3TC/EFV and D4T/3TC/LPV-r (Figure 4.1). Interestingly, combination D4T/3TC/LPV-r recorded high CD4 count consistently over the study period than any other treatment combination despite that it was the least prescribed treatment combination. More information on minimum and maximum ranges on CD4 count trends can be found in Annexure D on page 123. These results provide a partial insight in line with the

objectives of this study. The results suggest that the most effective treatment indicative of increase CD4 is D4T/3TC/LPV-r.

4.2.2 Descriptive statistics on HIV-1 VL within the population of adults and adolescents

VL trend of adult participants over a period of 36 months is shown on Figure 4.2. Different first line ARV combinations showed an increase in VL at 6 months of treatment except for TDF/3TC/EFV combination (Figure 4.2).

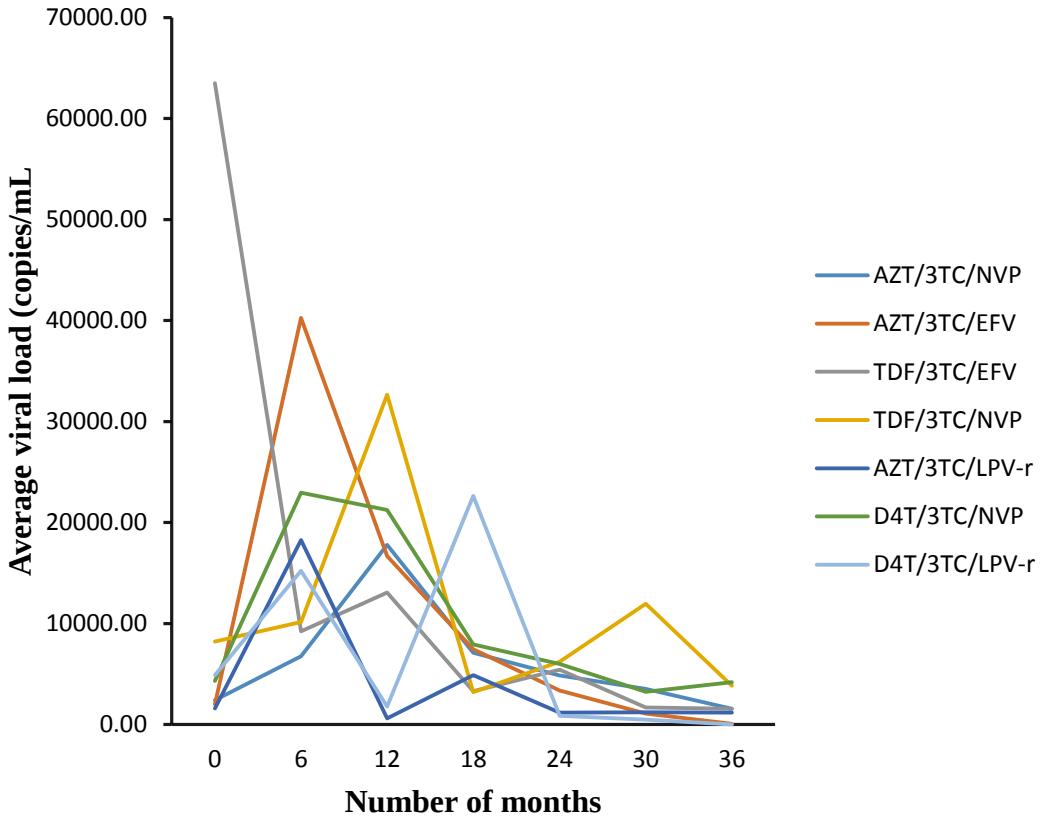

Figure 4.2. Average viral load trends in 36 months of treatment in adults and adolescents

Figure 4.2 show that AZT/3TC/NVP combination gave a rise in VL from initial treatment until 12 months (Figure 4.2). The VL only started to decrease at 18 months of treatment for most of the combinations and this was observed until 36 months of treatment (Figure 4.2). Furthermore, VL dropped consequently for AZT/3TC/EFV treatment from 18 months (7453.92 copies/mL) to 24 months (3354.62 copies/mL) and the decrease was still observed at 36 months (Figure 4.2). Moreover, TDF/3TC/EFV and TDF/3TC/NVP showed greater VL decrease at 18 months with 3294.87 and 3217.41 copies/mL and only some slight increase could be observed from 24 months and at 30 months for TDF/3TC/NVP.

AZT/3TC/LPV-r showed some viral load increase at 18 months with some slight decrease at 30 and 36 months (Figure 4.2). D4T/3TC/NVP combination recorded high viral load with the only decrease at 30 months (3248.91 copies/mL) followed by a slight increase at 36 months (4176.58 copies/mL). Evidently, Figure 4.2 shows VL decrease at 36 months across all treatment combinations compared to VL recorded at initial months of treatment. The most significant reduction in VL from 24 months was achieved with D4T/3TC/LPV-r although this treatment was availed to fewer patients. Annexure E page 124 provides more data on VL trends. These results are somewhat consistent with the results observed with CD4 count in terms of the efficacy of the treatment combination. Once again D4T/3TC/LPV-r appears to be the most effective in suppressing viral replication; however, it is the least prescribed and availed mostly to women. Subsequent statistical analysis such as analysis of variance was required to evaluate this observation.

4.3 Analysis of variance on average differences in VL and CD4 count among adults and adolescents

4.3.1 Analysis of variance on average differences in VL among adults and adolescents

In this section VL differences were calculated between follow-ups to see if there was an increase or decrease in viral load. A more detailed table on VL differences between follow-ups is shown in Annexure F on page 125.

Figure 4.3 shows average differences between VL recorded after every 6 months of treatment within the population of adults and adolescents. According to the Figure 4.3, VL records for all 7 ART combinations showed an increase after treatment but it is visible that VL recorded after 6 months (0 and 6) of treatment was less compared to an increase observed between 6 and 12 months. This was evident as VL values for the 6 combination were less than a 100 copies and 102.67 copies/ mL for D4T/3TC/LPV-r average VL between initial and 6 months.

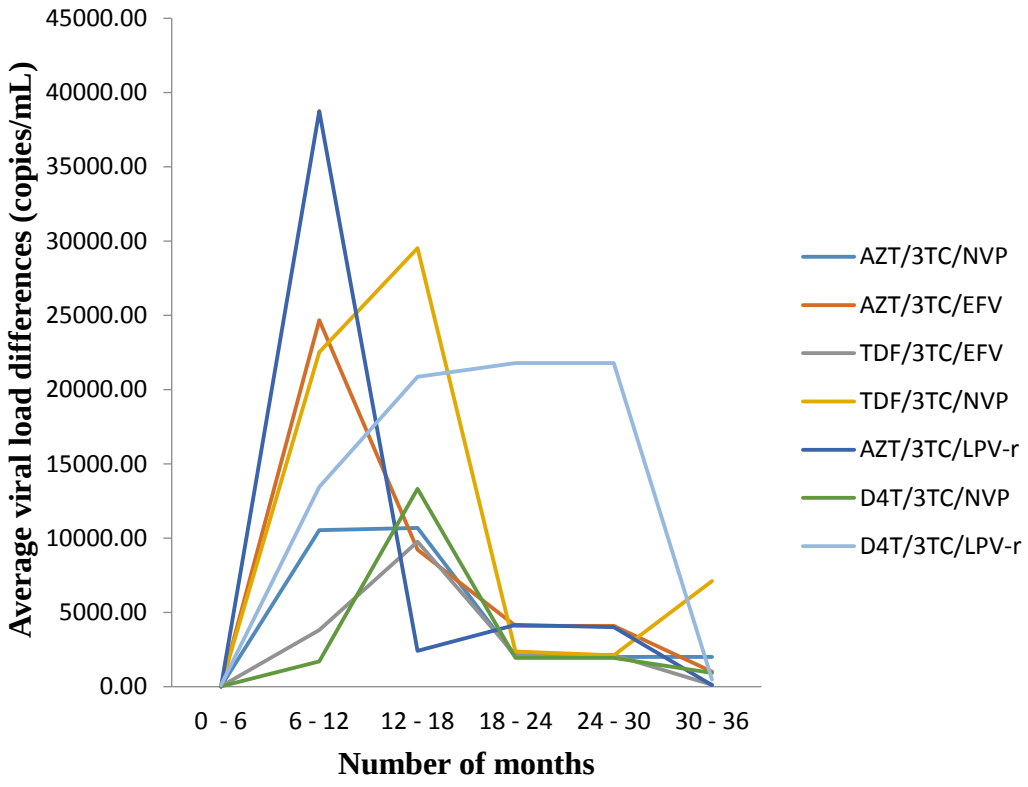

Figure 4.3. Trends on differences in viral load of adults and adolescents

VL differences trends shown on Figure 4.3. AZT/3TC/LPV-r combination recorded the highest VL between 6 and 12 months. VL recorded from initial to 6 months was less than 200 copies/mL and VL recorded between 18-24 and 24-30 where closer to each other. VL increased at a very high rate from 6 months to 18 months (6 and 12 and 12- 18 months) for all 7 combinations. AZT/3TC/LPV-r combination had the highest average plasma VL of 38 738.20 copies/mL while the lowest VL of 1 715.09 copies/mL was recorded for D4T/3TC/NVP (Figure 4.3). Additionally, between 18 and 36 months VLs were below 10 000 copies/mL and decreased with most combinations; with exception of a recorded VL of 21 777.05 copies/mL for D4T/3TC/LPV-r until 30 months of treatment. Most combinations

suppressed VL from 24 months of treatment. Specifically, average VL decreased is more between 30 and 36 months compared to decrease in VLs recoded between 24 and 30 months (Figure 4.3). The results suggest that a longer consistent treatment has the most desirable reduction in VL and also suggest that some treatment combinations are most effective than others.

Table 4.4

Total increase and decrease in viral load in adults and adolescents

ARV	VL difference between 0 and 6 months	VL difference between 6 and 12 months	VL difference between 12 and 18 months	VL difference between 18 and 24 months	VL difference between 24 and 30 months	VL difference between 30 and 36 months	Total decrease in VL N (%)
AZT/3TC/NVP	**decrease**	increase	increase	**decrease**	increase	**decrease**	3 (50)
AZT/3TC/EFV	**decrease**	increase	**decrease**	**decrease**	**decrease**	**decrease**	5 (83.3)
TDF/3TC/EFV	increase	**decrease**	increase	**decrease**	**decrease**	**decrease**	4 (66.7)
TDF/3TC/NVP	**decrease**	increase	increase	**decrease**	**decrease**	increase	3 (50)
AZT/3TC/LPV-r	**decrease**	increase	**decrease**	increase	**decrease**	**decrease**	4 (66.7)
D4T/3TC/NVP	increase	**decrease**	increase	**decrease**	**decrease**	**decrease**	4 (66.7)
D4T/3TC/LPV-r	**decrease**	increase	increase	increase	increase	**decrease**	2 (33.3)

Table 4.4 also shows 5 VL increases between 6 and 12 months compared to 2 decreases for all 7 combinations. The table also shows that AZT/3TC/EFV recorded more decreases with 83.3% from initial treatment. This combination was followed by TDF/3TC/EFV, AZT/3TC/LPV-r and D4T/3TC/NVP with recorded VL decrease of 66.7% for 36 months. AZT/3TC/NVP and TDF/3TC/NVP combinations recorded 50% decrease in VL.

4.3.2 Analysis of variance on average differences in CD4 count among adults and adolescents

CD4 count means recorded every 6 months for 36 months in adult participants' showed increments on CD4 count (Figure 4.4). For most combinations, CD4 count increased at a low rate less than 100 cells/µL for most of the combinations. AZT/3TC/EFV showed a low rate of CD4 count growth over the period of 36 months especially from 0 to 6 months (6.69 cells/µL). The same situation was observed in 12 to 18 months (4 cells/µL) and 30 to 36 months (6.39 cells/µL). More Information on differences in CD4 counts in adults is provided in Annexure G on page 127.

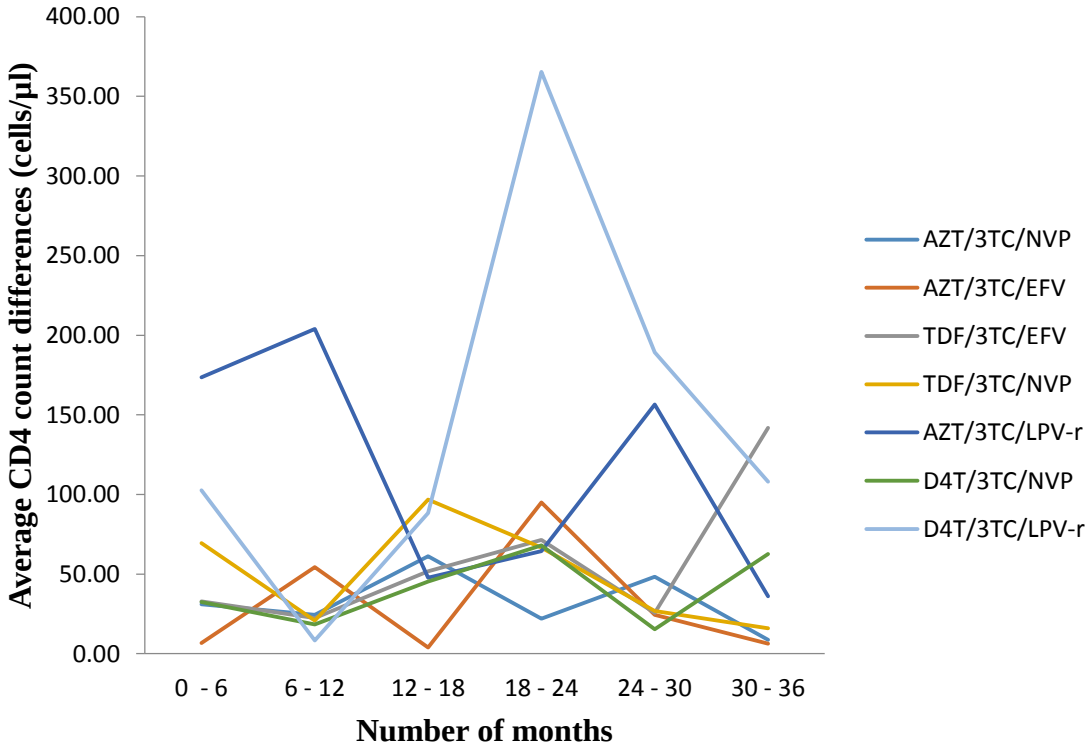

Figure 4.4. Trends on differences in CD4 count in adults and adolescents

Table 4.5 indicate that AZT/3TC/EFV combination also had only 2 CD4 increases (33.3%) among all. TDF/3TC/NVP combination also had the lowest CD4 count with only 2 increases between 0 to 6 months (69.41 cells/µL) and 12 to 18 months (96.84 cells/µL). Furthermore, 2 combinations showed increments in CD4 counts in most follow- ups (Table 4.5). These combinations were TDF/3TC/EFV with an increase in CD4 counts between 0 to 6 months (32.87 cells/µL), 12 to 18 months (51.80 cells/µL), 18 to 24 months (71.53 cells/µL) and 30 to 36 months (141.80 cells/µL) indicated on Figure 4.4. D4T/3TC/NVP showed an increase between 0 to 6 months (32.46 cells/µL), 12 to 18 months (45.23 cells/µL), 18 to 24 months (67.96 cells/µL) and 30 to 36 months (62.54 cells/µL) (Figure 4.4). The highest CD4 increase can be seen between 18 to 24 months for the combination of D4T/3TC/LPV-r (Figure 4.4).

Table 4.5.

Total increase and decrease in CD4 count in adults and adolescents

ARV	CD4 difference between 0 and 6 months	CD4 difference between 6 and 12 months	CD4 difference between 12 and 18 months	CD4 difference between 18 and 24 months	CD4 difference between 24 and 30 months	CD4 difference between 30 and 36 months	Total increase in CD4 count N (%)
AZT/3TC/NVP	**increase**	decrease	**increase**	decrease	**increase**	decrease	3 (50)
AZT/3TC/EFV	decrease	**increase**	decrease	**increase**	decrease	decrease	2 (33.3)
TDF/3TC/EFV	**increase**	decrease	**increase**	**increase**	decrease	**increase**	4 (66.7)
TDF/3TC/NVP	**increase**	decrease	**increase**	decrease	decrease	decrease	2 (33.3)
AZT/3TC/LPV-r	decrease	**increase**	decrease	**increase**	**increase**	decrease	3 (50)
D4T/3TC/NVP	**increase**	decrease	**increase**	**increase**	decrease	**increase**	4 (66.7)
D4T/3TC/LPV-r	**increase**	decrease	**increase**	**increase**	decrease	decrease	3 (50)

Table 4.5 provides further information showing that TDF/3TC/EFV and D4T/3TC/NVP recorded 66.7% CD4 count increase. All other combinations

achieved 50% and below in CD4 count: AZT/3TC/NVP (50%), AZT/3TC/EFV (33.3%), AZT/3TC/LPV-r (50%) and D4T/3TC/LPV-r (50%) (Table 4.5).

4.4 Correlation analysis in adults and adolescents

The correlation analysis was done with the notion that treatment should result in increased CD4 count whilst the VL should be decreasing. The correlation between VL and CD4 count recorded at different follow-ups is indicated in Table 4.6. An inverse correlation was observed between VL and CD4 count recorded after 6 months of treatment r = - 0.17, *P = 0.00* (Table 4.6).

Table 4.6

Correlation between CD4 count and viral load in adults and adolescents

	CD 4 count		Viral load		Coefficient of correlation	
	Mean	± SD	Mean	± SD	r	*P-value*
CD4 count and viral load at initial time	403.22	157.58	6417.01	36742.29	0.24	***0.00***
CD4 count and viral load after 6 Months of treatment	420.47	229.25	13907.88	47269.23	-0.17	***0.00***
CD4 count and viral load after 12 Months of treatment	443.01	224.01	19859.79	106433.49	-0.05	*0.41*
CD4 count and viral load after 18 Months of treatment	472.74	210.50	6587.10	15045.54	0.19	***0.00***
CD4 count and viral load after 24 Months of treatment	456.37	214.84	5022.06	24404.72	-0.04	*0.50*
CD4 count and viral load after 30 Months of treatment	459.80	207.15	4393.81	15816.95	-0.12	*0.35*
CD4 count and viral load after 36 Months of treatment	476.52	230.63	2186.49	8642.01	-0.14	*0.12*

Negative correlations but not significant were found between CD4 counts and VL recorded after 12 months of treatment r = - 0.05, *P = 0.41*, after 30 months of treatment (r = - 0.12, *P = 0.35*) and after 36 months treatment (r = - 0.14, *P = 0.12*). In addition, a positive significant correlation was observed between VL and CD4

counts recorded at initial time of treatment (r = 0.24, *P = 0.00*) and at 18 months of treatment (r = 0.19, *P = 0.00*) (Table 4.6). All correlations were calculated at a correlation significant at $P \leq 0.05$.

There was no correlation between ARV therapy and age of adult participants (r = 0.09, *P = 0.11*; Table 4. 7), at which correlation was significant when $P \leq 0.05$.

There was no correlation between ARV therapy and age of adult participants (r = 0.09, *P = 0.11*; Table 4. 7), at which correlation was significant when $P \leq 0.05$.

Table 4.7

Relationship between age and ART in adults and adolescents

				r	*P-value*
Age and ARV therapy correlation				0.09	*0.105*

Correlation was significant at 0.05 (two- tailed)

4.5 Descriptive statistics on VL and CD4 count among children population

4.5.1 Descriptive statistics on HIV-1 VL for children population

Children population consisted of participants of 1 – 12 years old. This group was also referred to as the population below 13 years of age or just children. Detailed information on children data on VL is available in Annexure H on page 127.

On Figure 4.5 averages of VL recorded at different follow-ups for four different first line combinations prescribed to 78 children between 1 and 12 years were recorded. AZT/3TC/NVP combination showed poor results in VL suppression as VL kept on increasing from 18 months (6687.54 copies/mL), 24 months (11 729.70

copies/mL) and 30 months (21 146.71 copies/mL) and only decreased at 36 months (1968.86 copies/mL) (Figure 4.5). AZT/3TC/EFV combination also had a VL increase recorded at 6 months (18375.40), 18 months (5799.65 copies/mL) and a decrease from 30 months (149.38 copies/mL) and 36 months (8.33 copies/mL). Figure 4.5 also shows that AZT/3TC/LPV-r combination recorded VL increase at 12 months (313.00 copies/mL) and 24 months (2118.94 copies/mL) while D4T/3TC/LPV-r combination recorded VL decreasing from date of treatment until 36 months of treatment which was either less than 20 copies or target not detected. The most significant viral suppression was observed with D4T/3TC/LPV-r but remains the least prescribed for children. The highest VLs were recorded at 6, 24 and 30 months in patients under AZT/3TC/EFV and AZT/3TC/NVP treatment combination. The other two combinations recorded VL copies below 5000 copies/mL (Figure 4.5).

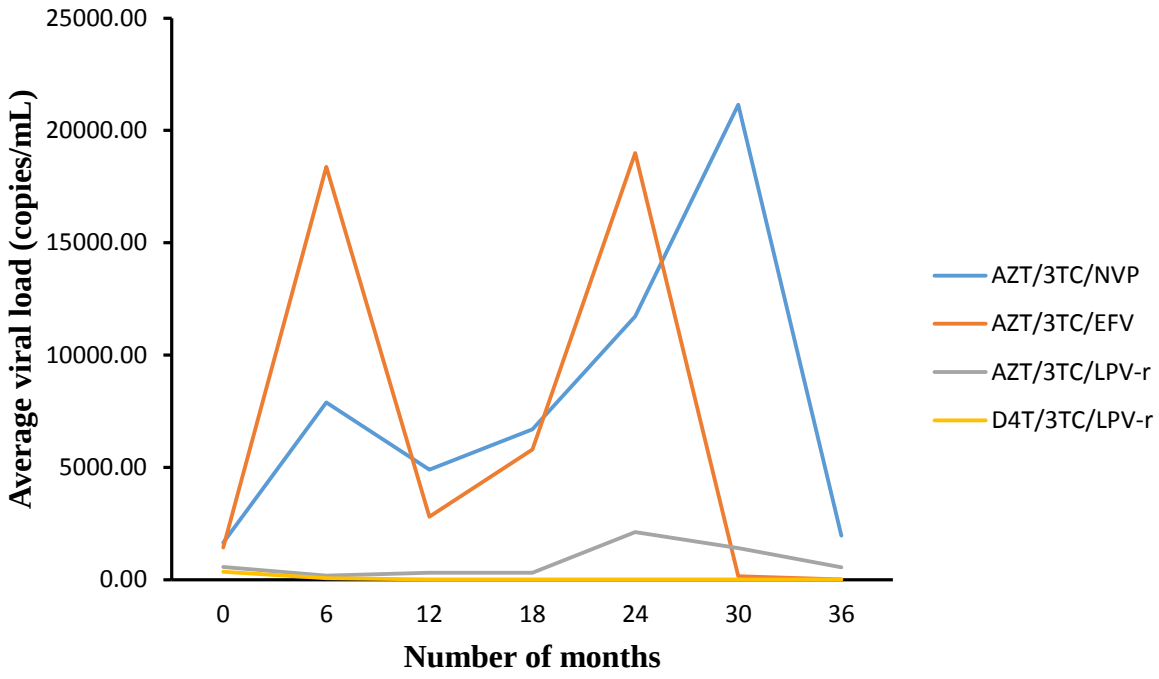

Figure 4.5. Average viral load trends in 36 months of treatment in children

Consistent with the viral suppression observed for children, D4T/3TC/LPV-r appears to be the most effective for children over 36 months in relative comparison to other treatments. However, such observation needs to be firm up with analysis of variance and correlation analysis.

4.5.2 Descriptive statistics on CD4 count for children population

CD4 count trend in children participants is shown on Figure 4.6. Detailed information on CD4 count on Figure 4.6 is provided in Annexure I on page 128. In 6 months of treatment the number of CD4 count increased but decreased at 12 and 18 months of treatment for AZT/3TC/LPV-r combination (1186.67 cells/µL and 1122.33 cells/µL) as well for D4T/3TC/LPV-r combination at 12 months (992.00 cells/µL) and 18 months (985.00 cells/µL). Figure 4.6 also indicates that, 4

combinations prescribed to children showed a slight decrease in the number of CD4 count at 24 months.

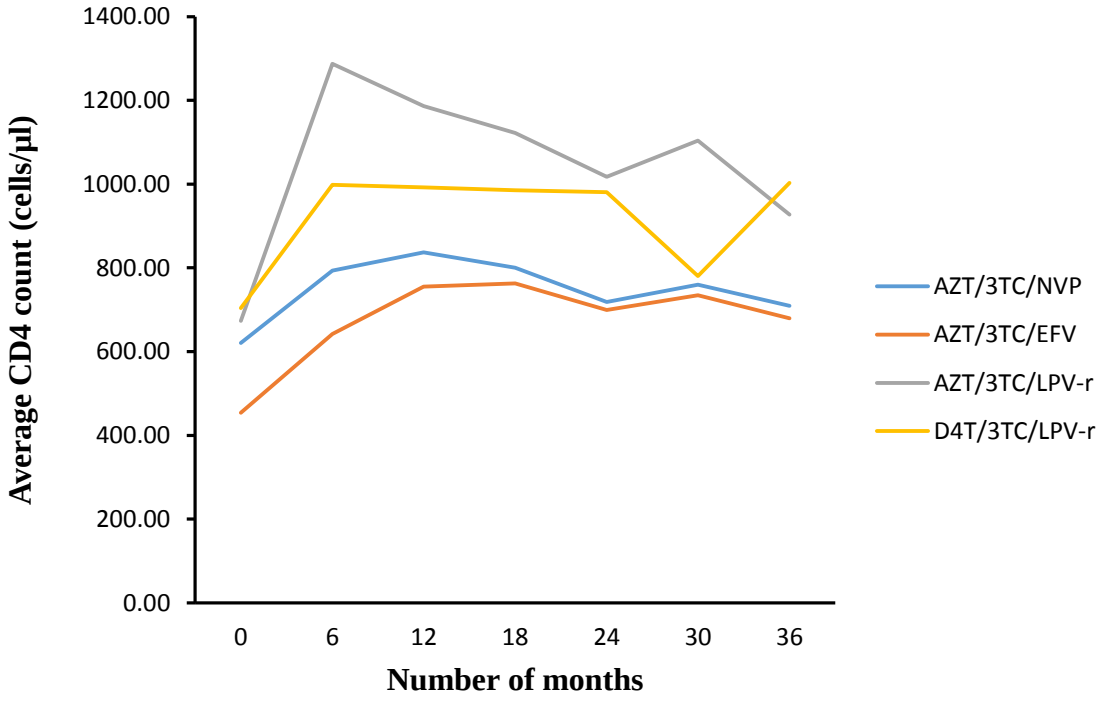

Figure 4.6. Average CD4 count trends in 36 months of treatment in children

Overall number of average CD4 counts across the 4 combinations showed a decrease but with slight differences (Figure 4.6).

4.6 Analysis of variance on average differences in CD4 count and VL among children

4.6.1 Analysis of variance on average differences in CD4 count among children

This section describes the differences in CD4 count recorded between follow-ups for a period of each 6 months while participants were on ART. Trend differences in

averages of CD count for participants below 13 years of age at different follow-ups are provided on Figure 4.7 and more details can be found in Annexure J on page 129.

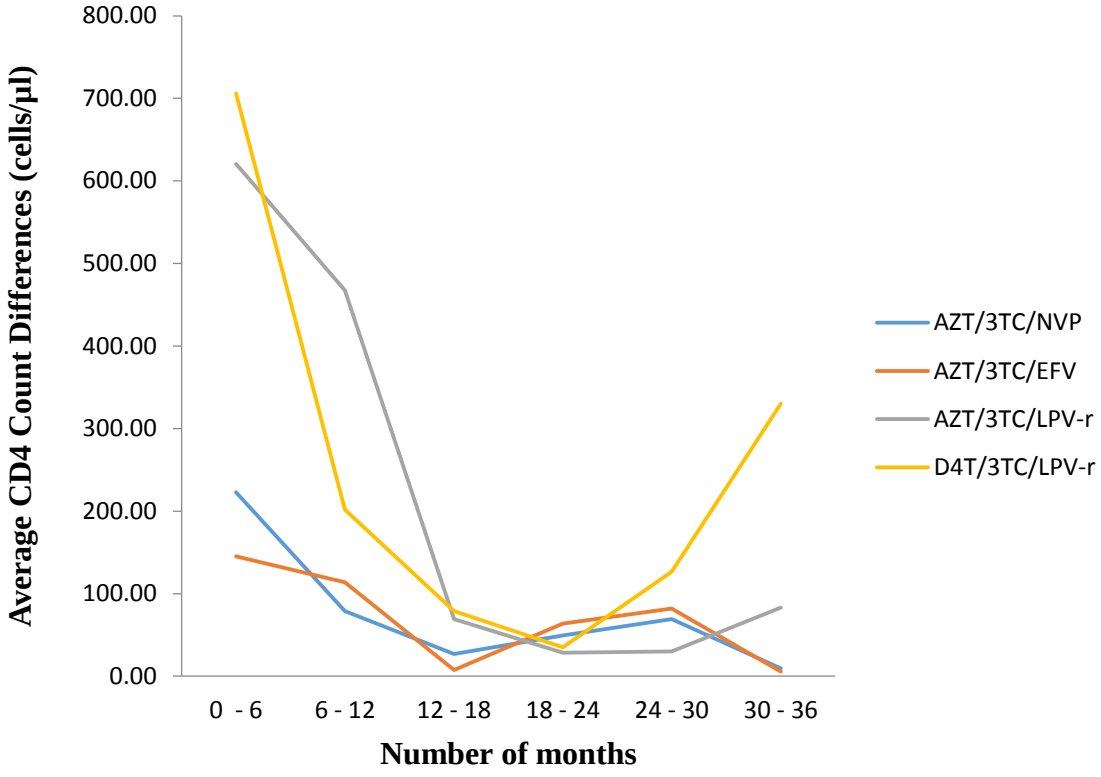

Figure 4.7. Trends on differences in CD4 count between months of treatment in children

A high number of CD4 count increases were recorded between initial and 6 months of treatment for all four combinations (Figure 4.7). Furthermore, between 6 and 12 months of treatment and 12 and 18 months of treatment a decrease in CD4 count was observed across 4 combinations prescribed. Similar information is also observed for AZT/3TC/NVP and AZT/3TC/EFV combinations recorded some rise in CD4 count between 24 and 30 months and a decrease between 30 and 36 months of treatment.

AZT/3TC/LPV-r and D4T/3TC/LPV-r recorded a decrease in CD4 count between 18 and 24 months (28.33 cells/µL and 35 cells/µL) and an increase between 24 and 30 months (30 cells/µL and 126.5 cells/µL) as well as between 30 and 36 months (83.33 cells/µL and 330.33 cells/µL) (Figure 4.7). Average CD4 count increase and decrease is indicated in Table 4.8. AZT/3TC/EFV and D4T/3TC/LPV-r combinations obtained 50% CD4 count increase. AZT/3TC/NVP and AZT/3TC/LPV-r combinations obtained a 33.3% CD4 increase over 36 months of treatment (Table 4.8).

Table 4.8

Total increase and decrease in CD4 count in children

ARV	CD4 difference between 0 and 6 months	CD4 difference between 6 and 12 months	CD4 difference between 12 and 18 months	CD4 difference between 18 and 24 months	CD4 difference between 24 and 30 months	CD4 difference between 30 and 36 months	Total increase in CD4 count N (%)
AZT/3TC/NVP	**increase**	decrease	decrease	**increase**	**increase**	decrease	2 (33.3)
AZT/3TC/EFV	**increase**	decrease	decrease	**increase**	**increase**	decrease	3 (50)
AZT/3TC/LPV-r	**increase**	decrease	decrease	decrease	**increase**	**increase**	2 (33.3)
D4T/3TC/LPV-r	**increase**	decrease	decrease	decrease	**increase**	**increase**	3 (50)

4.6.2 Analysis of variance on average differences in VL among children

Figure 4.8 shows data on VL average difference between follow-ups in participants below 13 years of age. More data on VL differences in children are available in Annexure K on page 130.

AZT/3TC/NVP and AZT/3TC/EFV combinations had an average increase in VL from initial months of treatment between 6 and 12 months, 12 and 18 months and 18 and 24 months (Figure 4.8). AZT/3TC/NVP combination recorded an average

difference of 735.82 copies/mL between 6 – 12 months, 12 – 18 months (969.71 copies/mL) and 18 – 24 months (3483.08 copies/mL). AZT/3TC/EFV combination had average differences of 169.33 copies/mL, 1857.1 2 copies/mL, 15186.98 copies/mL (18 – 24 months) and this was the highest across all 4 combinations (Figure 4.8).

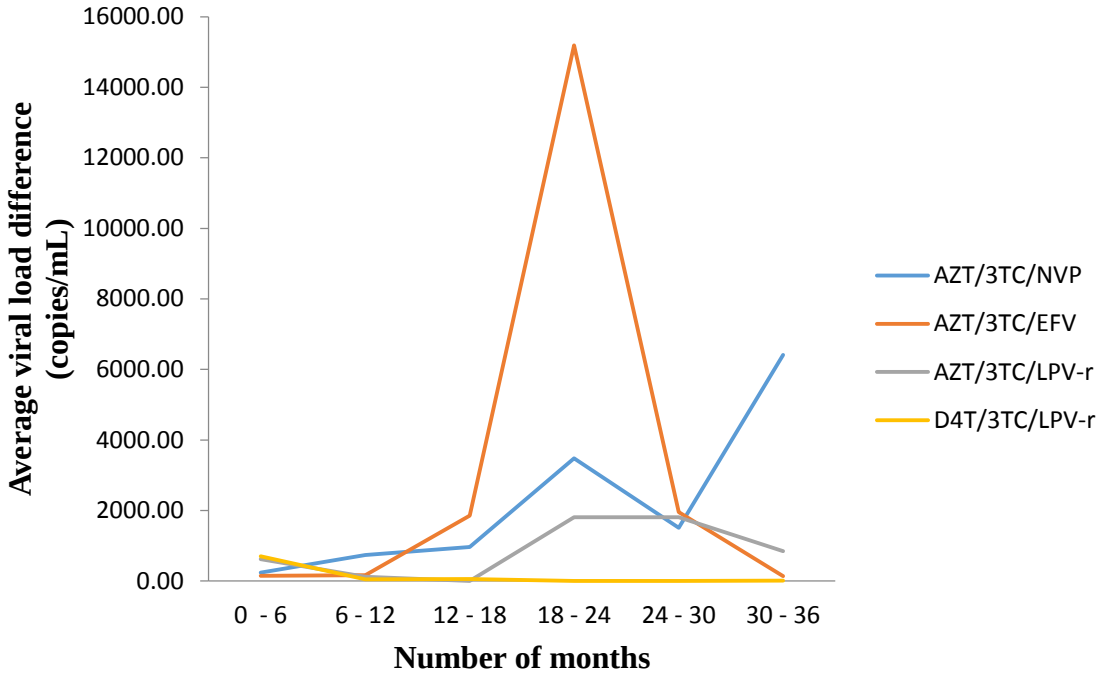

Figure 4.8. Trends on differences in viral load between months of treatment in children

AZT/3TC/LPV-r recorded an increase in VL between 0 and 6 months (620.67 copies/mL), 18 and 24 months (1812.27 copies/mL) and 24 and 30 months (1812.27 copies/mL) and a decrease in VL between 6 - 12 months (126 copies/mL, 12 - 18 months (6.33 copies/mL), 30 - 36 months (852.2 copies/mL). On the other hand, D4T/3TC/LPV-r combination only showed VL increases between 0 - 6 months, 12 - 30 months and at 6 - 12 months and 30 - 36 months VL decreased. Also shown on

the same Figure 4.8, VL was suppressed below 2000 copies/mL in most cases. These decrease and increase in VL are indicated in Table 4.9 where three combinations obtained 50% VL decrease and only one combination with 33.3% increase.

Table 4.9.

Total increase and decrease of viral load in children

ARV	VL difference between 0 and 6 months	VL difference between 6 and 12 months	VL difference between 12 and 18 months	VL difference between 18 and 24 months	VL difference between 24 and 30 months	VL difference between 30 and 36 months	Total decrease in VL N (%)
AZT/3TC/NVP	**decrease**	increase	increase	increase	**decrease**	increase	3 (50)
AZT/3TC/EFV	**decrease**	increase	increase	increase	**decrease**	**decrease**	3 (50)
AZT/3TC/LPV-r	increase	**decrease**	**decreas**	increase	increase	**decrease**	3 (50)
D4T/3TC/LPV-r	increase	**decrease**	increase	increase	increase	**decrease**	2 (33.3)

4.7 The relationship between VL and CD4 count in ART naïve children

Table 4.10 indicates the relationship between VL and CD4 count for participants less than 13 years of age. An inverse significant correlation was observed between VL and CD4 count after 18 months of treatment r = - 0.03, *P = 0.00,* at 30 months of treatment r = - 0.39 *P = 0.00* and at 36 months of treatment r = - 0.24, *P = 0.04)* all significant at $P \leq .05$ (Table 4.10).

Table 4.10.

Correlation between CD4 count and viral load in children

	CD 4 count		Viral load		Coefficient of correlation	
	Mean	± SD	Mean	± SD	r	*P-value*
CD4 count and viral load at initial time	620.23	273.31	1550.56	3654.99	-0.02	*0.84*
CD4 count and viral load after 6 Months of treatment	814.56	352.15	7704.27	14103.42	-0.06	*0.59*
CD4 count and viral load after 12 Months of treatment	851.32	426.85	4450.64	9423.33	-0.16	*0.17*
CD4 count and viral load after 18 Months of treatment	818.83	298.58	6151.21	15428.25	-0.03	***0.00***
CD4 count and viral load after 24 Months of treatment	739.17	312.26	11188.89	46259.90	-0.22	*0.06*
CD4 count and viral load after 30 Months of treatment	775.62	299.07	18768.43	48843.52	-0.39	***0.00***
CD4 count and viral load after 36 Months of treatment	727.65	307.53	1763.48	6339.42	-0.24	***0.04***

Correlation was significant at 0.05 (two- tailed).

Non- significant negative correlations were recorded between CD4 counts and VL at initial month of treatment (r = - 0.02, *P = 0.84)*, after 6 months of treatment (r = - 0.06, *P = 0.59*), after 12 months (r = - 0.16, *P = 0.17),* and after 24 months treatment (r = - 0.22, *P = 0.06*). All correlations were calculated at correlation significant when $P \leq 0.05$. Positive correlation between VL and CD4 count was not observed within this population (Table 4.10). These results suggest that all treatment regiments varied in the level of their efficacy for children patients.

4.8 The relationship between age and ART in ART naïve children

There was a positive significant relationship between ARV therapy and age of children participants (r = 0.38, *P = 0.00;* Table 4.11*)* at which correlation was significant when $P \leq 0.05$.

Table 4.11.

Relationship between age and ART in children

	r	*P-value*
Age of the patient and treatment combination	0.38	*0.00*

Correlation was significant at 0.01 (two- tailed)

4.9 Effects of micronutrients levels on treatment response in adults and adolescents

A total of 30 patients participated in this study for micronutrient analysis including 13 females and 17 males (Figure 4.9). Participants' ages ranged between 13 – 55 years.

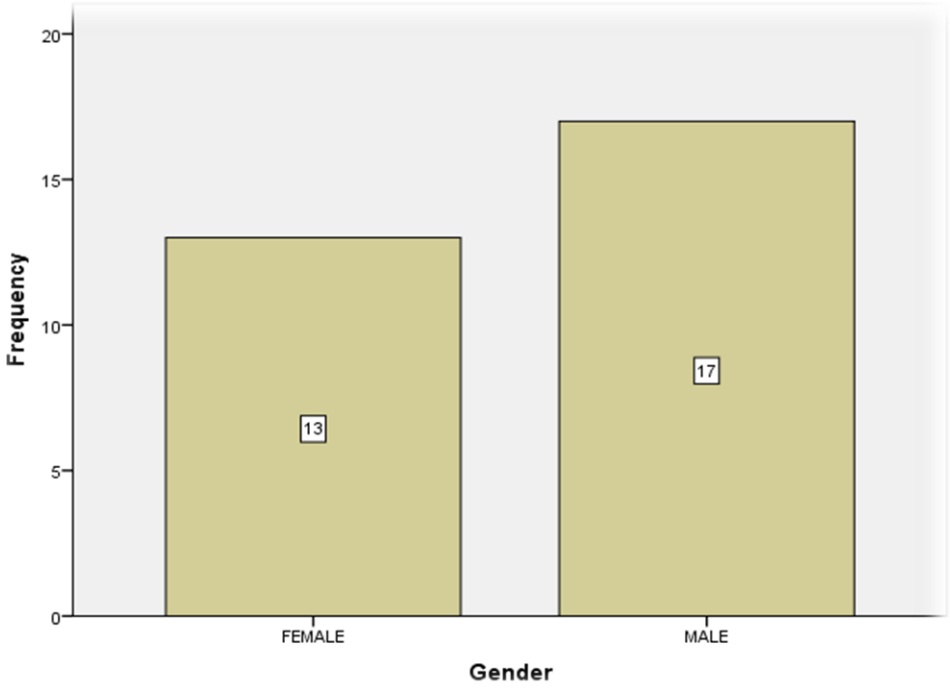

Figure 4.9. Gender of participants in micronutrient study

Table 4.12 shows the average molar concentrations of micronutrients for participants under the same ARV treatment. The micronutrient levels are indicated with their corresponding mean values as well as minimum and maximum concentration.

Table 4.12.

Levels of micronutrients recorded per combination in adults and adolescents

Descriptives						95% Confidence Interval for Mean			
		N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Min	Max
Vitamin B12	AZT/3TC/NVP	16	565.54	172.78	43.20	473.48	657.61	254.00	928.20
	AZT/3TC/EFV	3	665.20	483.36	279.07	-535.54	1865.94	335.00	1220.00
	TDF/3TC/EFV	1	343.50	.	.	.	.	343.50	343.50
	TDF/3TC/NVP	6	604.28	299.51	122.27	289.97	918.60	286.00	998.70
	D4T/3TC/NVP	4	710.10	416.16	208.08	47.89	1372.31	434.20	1321.00
	Total	30	595.13	264.49	48.29	496.37	693.89	254.00	1321.00
Folate	AZT/3TC/NVP	16	8.42	4.77	1.19	5.88	10.96	2.50	20.19
	AZT/3TC/EFV	3	8.94	4.50	2.60	-2.24	20.12	4.62	13.60
	TDF/3TC/EFV	1	4.88	.	.	.	.	4.88	4.88
	TDF/3TC/NVP	6	3.49	1.98	0.81	1.41	5.56	1.20	7.12
	D4T/3TC/NVP	4	5.79	3.29	1.64	0.56	11.02	1.68	8.54
	Total	30	7.02	4.40	0.80	5.37	8.66	1.20	20.19
Magnesium	AZT/3TC/NVP	16	0.84	0.13	0.03	0.78	0.91	0.71	1.24
	AZT/3TC/EFV	3	0.87	0.08	0.05	0.66	1.07	0.80	0.96
	TDF/3TC/EFV	1	0.87	.	.	.	.	0.87	0.87
	TDF/3TC/NVP	6	0.86	0.05	0.02	0.80	0.91	0.78	0.93
	D4T/3TC/NVP	4	1.02	0.19	0.09	0.73	1.32	0.86	1.23
	Total	30	0.87	0.13	0.02	0.83	0.92	0.71	1.24
Iron	AZT/3TC/NVP	16	14.51	10.75	2.69	8.78	20.23	2.30	38.00
	AZT/3TC/EFV	3	12.08	10.13	5.85	-13.08	37.24	0.84	20.50
	TDF/3TC/EFV	1	11.30	.	.	.	.	11.30	11.30
	TDF/3TC/NVP	6	17.39	8.84	3.61	8.12	26.67	1.26	24.50
	D4T/3TC/NVP	4	19.73	10.20	5.10	3.50	35.95	10.60	28.90
	Total	30	15.43	9.83	1.79	11.76	19.10	0.84	38.00

Table 4.12 indicates mean levels for different micronutrients, their minimum and maximum concentrations for adult participants. Vitamin B12 levels among patients taking different ART combination were above normal maximum serum concentrations in patients prescribed AZT/3TC/NVP, AZT/3TC/EFV,

TDF/3TC/NVP and D4T/3TC/NVP combinations. This implies that vitamin B12 is not a limiting factor toward the efficacy of the AZT/3TC/NVP, AZT/3TC/EFV, TDF/3TC/NVP and D4T/3TC/NVP. For magnesium, only patients under AZT/3TC/NVP and D4T/3TC/NVP treatment combinations recorded higher levels above the maximum level. In contrast to vitamin B12, folate and iron concentrations fluctuated above and below the expected normal concentrations in serum. Furthermore, patients under AZT/3TC/NVP, TDF/3TC/NVP, D4T/3TC/NVP combinations had folate levels that were below 3.56 ng/mL which is known to be the minimum level of folate in serum (Burtis & Ashwood, 1994).

D4T/3TC/NVP AZT/3TC/EFV, TDF/3TC/EFV and TDF/3TC/NVP combinations recorded folate levels above 20 ng/mL which is the maximum normal level (Burtis & Ashwood, 1994). Some patients also recorded low iron levels which were below 5 µmol/L known as the minimum normal level of iron concentration in serum. This situation was observed within patients under AZT/3TC/NVP, AZT/3TC/EFV and TDF/3TC/NVP and only patients under AZT/3TC/NVP showed iron levels above normal level of 32 µmol/L (Burtis & Ashwood, 1994).

4.9.1 Categorisation of patients based on their relative molar micronutrient concentration values

In this section, patients were categorised based on their expressed micronutrient concentration levels. The first category included patients with ‘normal micronutrient’ levels. For each micronutrient it was considered normal if a patient has the following values: vitamin B12 ranges from 133 – 675 pmol/mL, folate ranges between 3.56 –

20 ng/mL, magnesium 0.70 – 1.15 mmol/L and iron 5 – 32 µmol/L (Burtis & Ashwood, 1994). The second category defined as low micronutrient value included patients with concentration values that are relatively lower than the corresponding normal values. The third category defined as high micronutrient value included patients with concentration values that are relatively higher than the corresponding normal values.

The levels of vitamin B12, folate, magnesium and iron in participants under different first line combination are indicated in different figures below (figure 4.10 – 4.13). Figure 4.10 illustrates the concentration of vitamin B12 in pmol/L. The figure indicates that of 30 participants in this study, 21 of them recorded normal vitamin B12 levels and 9 participants were found to have high levels of vitamin B12.

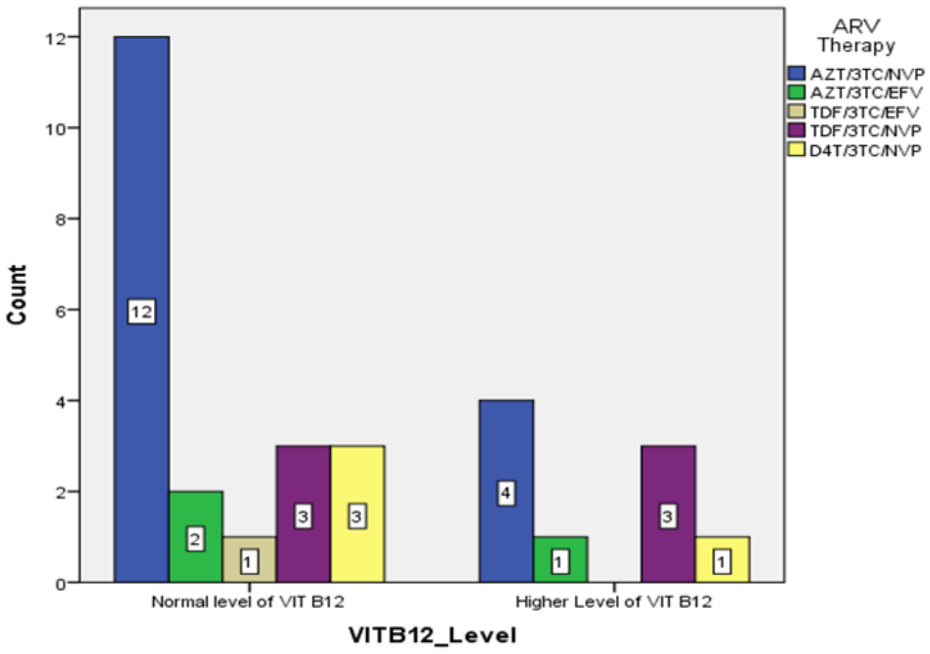

Figure 4.10. Recorded vitamin B12 levels in pmol/L

In Figure 4.11 the concentration of magnesium is expressed in mmol/L. Data reflects that 28 participants had normal levels of magnesium and only 2 participants had high magnesium level.

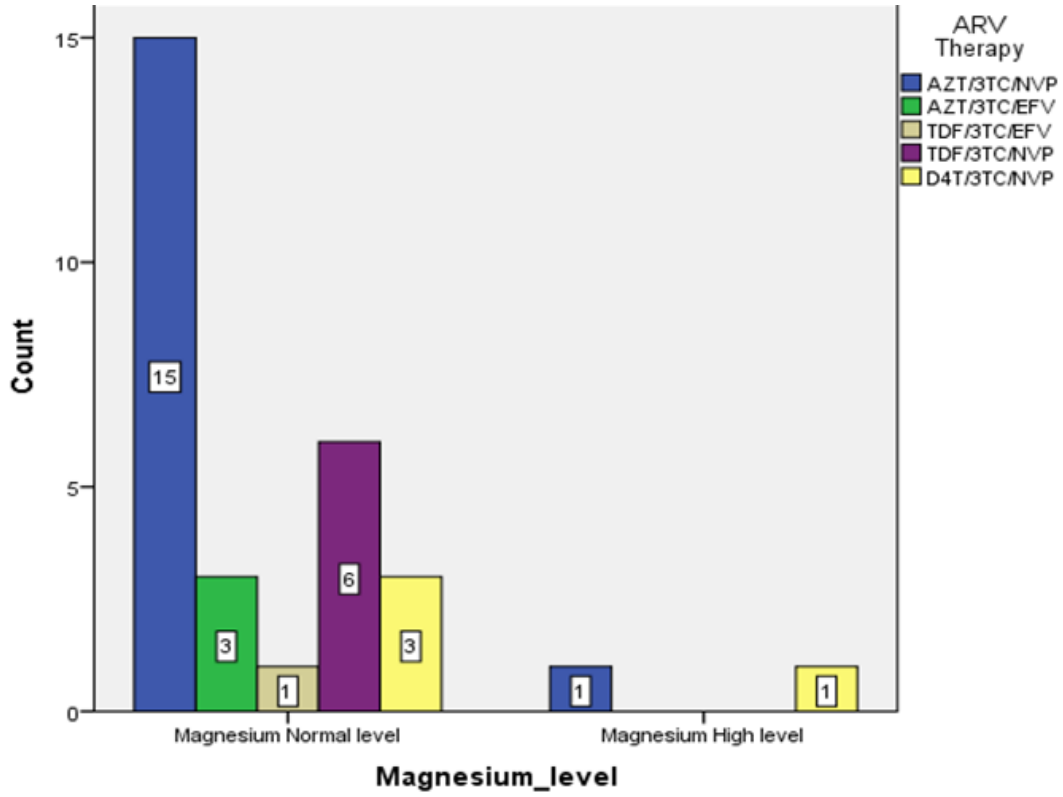

Figure 4.11. Recorded magnesium levels in mmol/L

The molar concentration of folate was expressed in ng/mL and values are illustrated in Figure 4.12. Results show that 6 participants recorded low levels of folate, 21 had normal levels and only one participant had high levels of folate.

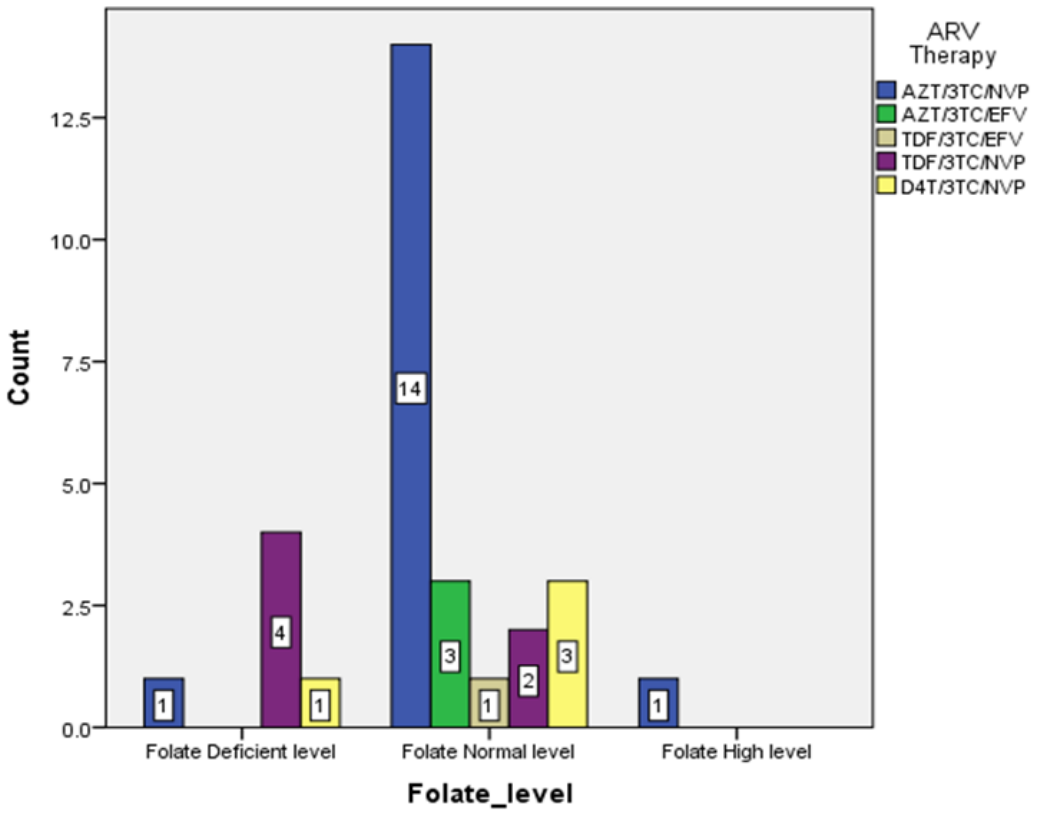

Figure 4.12. Recorded folate levels in ng/mL

The data suggest that folate could be a putative limiting factor contributing to the effectiveness of the ARV treatment amongst the six patients relative to others. However, further analysis would be required to confirm if folate deficiency could be ruled out as a limiting factor. Concentration of iron was expressed in µmol/L and recorded values are shown in Figure 4.13. Results show that 22 participants had normal iron level, 6 had low iron levels and 2 participants recorded high levels of iron (Figure 4.13).

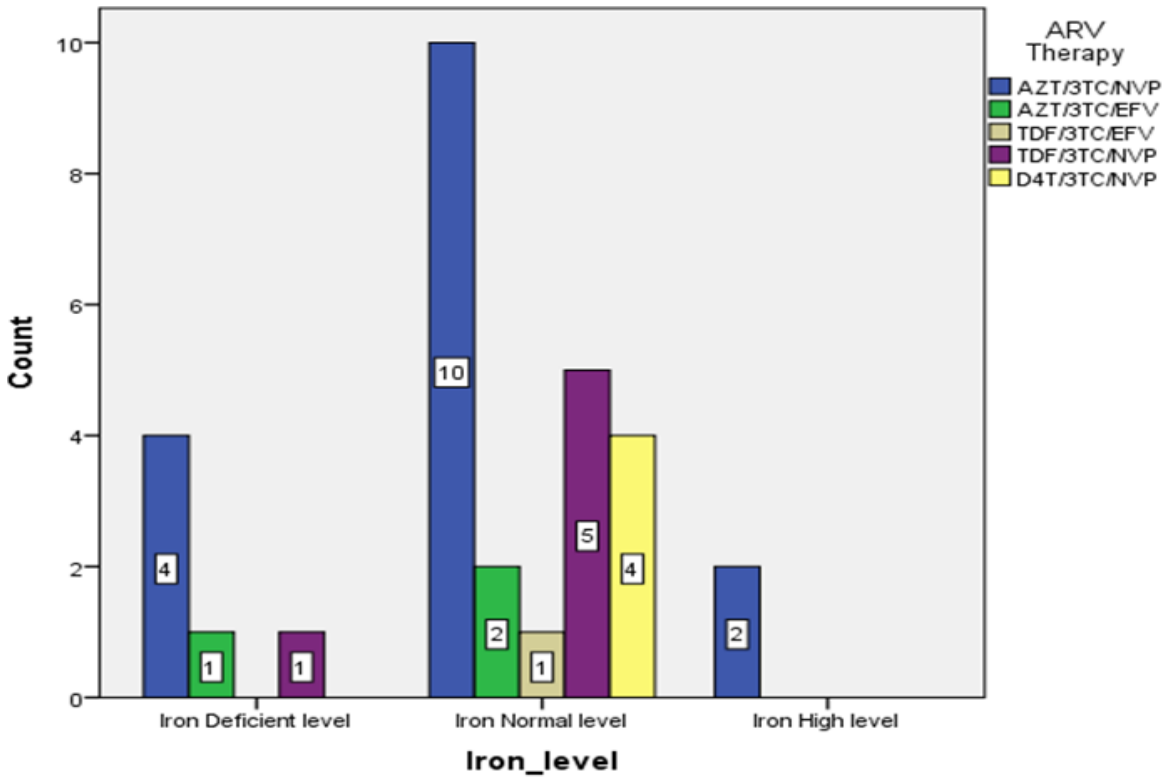

Figure 4.13. Recorded iron levels in µmol/L

4.10 Analysis of the impact of micronutrient availability on CD4 count and VL in adults and adolescents

4.10.1 Correlation between micronutrient levels, CD4 count, VL and ARV combinations

This section illustrates results comparing the relationship between micronutrient levels, CD4 count and VL. Data in Table 4.13 represents correlations between micronutrients, CD4 counts and VL in adults and adolescents. The association between micronutrient levels, CD4 counts and VL was present but not significant.

Table 4.13.

Correlation between micronutrients, CD4 count and VL in adults and adolescents

Correlations			
		CD4 36 months	Viral load 36 months
Vit B12 Level	Pearson Correlation	-0.151	-0.058
	Sig. (2-tailed)	0.425	0.76
Magnesium level	Pearson Correlation	0.327	-0.12
	Sig. (2-tailed)	0.078	0.529
Folate level	Pearson Correlation	0.239	-0.06
	Sig. (2-tailed)	0.203	0.754
Iron level	Pearson Correlation	0.159	-0.005
	Sig. (2-tailed)	0.401	0.979

In Table 4.14, the association between micronutrient levels and antiretroviral therapy was analysed and it was found that there is indeed a correlation but not significant for magnesium, iron and vitamin B12 levels in this study.

Table 4.14

Correlation between micronutrients and ARV therapy in ART naive adults and adolescents

		B12 Level	Magnesium level	Folate level	Iron level	ARV Therapy
ARV Therapy	Pearson Correlation	0.084	0.157	**-.397***	0.064	1
	Sig. (2-tailed)	0.659	0.408	***0.03***	0.738	
	N	30	30	30	30	30

Significant negative correlation was observed between folate levels and ARV therapy (r = - 0.397, *P = 0.03*) (Table 4.14).

5 CHAPTER FIVE: DISCUSSION

A specific objective of this study was "To determine changes in HIV VL in patients on ARV treatment". In this regard, the effectiveness of different first line ART combinations was done by correlating HIV-1 VL with CD4 count every 6 months of treatment for a period of 36 months. Based on our results viral suppression (viral load < 1000 copies/mL) could not be achieved in 36 months of treatment in most adults and adolescents as VL recorded between follow-up were above 1000 copies/mL (Figure 4.2).

These findings demonstrate no remarkable improvements on virological suppression time lines at 48 weeks of treatment reported by Gallant *et al.* (2004). Gallant *et al.* (2004) observed virological suppression in 48 weeks of treatment with the use of Tenofovir and Efavirenz combinations. The difference in virological suppression timelines reported by this study and those reported by Gallant *et al.* (2004) are perhaps not comparable as there are many variables that are different. Nonetheless, despite the improvement in virological suppression reported by this study, the suppression is not linear with time but rather the data of this study supports the argument that there is inconsistency in the efficacy of various combinations.

High levels of VL that were recorded at various intervals can be attributed to treatment failure by some prescribed combinations. Findings of this study suggest that the efficacy of particular combinations depends on accurate determination of the initial VL (VL0). This is a clear indication that patients with viral load more than 1000 copies/ mL initially need different interventions especially in designing different treatment to avoid virological failure. Virological failure might also occur

as a result of unmonitored VL for longer periods as some patients that visit hospitals when they are very sick and the VL has increased in their system. This finding is supporting the results from a study reviewed by Tang and Shafer (2012), where they found out that virological failure in most cases is found in unmonitored VL for a longer period of time especially in resource limited countries where there are no proper facilities for VL testing as well as genotyping.

This study set out as one of its specific objectives "to determine ART combinations that suppress viral replication the most". The only first line combination that achieved significant and consistent viral suppression in children throughout the 36 months was D4T/3TC/LPV-r. This combination achieved viral suppression in adults of < 1000 copies/mL from 24 months of treatment amongst all prescribed combinations. However, D4T/3TC/LPV-r combination had the lowest VL and highest CD4 count at initial treatment in children (Figure 4.5 and 4.6). But comparing the decrease in VL, children maintained VL below 1000 copies with 33% VL decrease and 50 % CD4 increase for the combination of D4T/3TC/LPV-r (Table 4.8 and 4.9). D4T/3TC/LPV-r is the only combination that was prescribed to patients with low VL especially in children which was maintained for the rest of 36 months of treatment in children.

Based on the results on D4T/3TC/LPV-r in children it seems like this combination is good for patients with low VL to keep the VL suppressed. In adults D4T/3TC/LPV-r could not give sufficient viral suppression as adults that were prescribed this combination had high copies of VL. In adults AZT/3TC/EFV combination gave 83.3% VL decrease (Table 4.4) but recorded poor CD4 increase of

only 33.3% (Table 4.5). These findings are consistent with those reported by (Gallant *et al*., 2004). Despite the high efficacy observed for D4T/3TC/LPV-r, it remains the least prescribed combination.

Interestingly, findings of this study shows that demographically D4T/3TC/LPV-r is mostly prescribed to women and children. Our findings are consistent with the report by the Namibian Guideline on Antiretroviral therapy (NGAT, 2010), that D4T/3TC/LPV-r is preferred as a combination of choice for infants younger than 24 months. It is reasonable to assume that D4T/3TC/LPV-r is the least prescribe given that studies have confirmed that, protease inhibitor (PI)-containing regimens are uncomfortable and are associated with incidence of adverse effects (Podzamczer *et al.*, 2002) as well as high cost. It was stated that, the most worrisome condition is the possible association between PIs and the occurrence of lipodystrophy and other metabolic disorders that make adherence difficult and negatively influence patients' quality-of-life (Ferrer *et al.*, 1999).

This study also set out to "determine changes in CD4 count in patients on ARV treatment" as one its specific objectives. The average number of CD4 count in all adult participants showed normal levels in CD4 count over the treatment period of 36 months. CD4 count below 250 cells/µL is regarded as an immunological failure. The results in Figure 4.1 and 4.6 exclude the situation of immunological failure in adult and children populations as averages in CD4 count recorded were above 250 cells/µL. According to these data there were virological failure in most of the adult participants and no signs of immunological failure observed.

Furthermore, D4T/3TC/LPV-r maintained VL suppression for 36 months in children with 350 copies/mL (Figure 4.5) at initial and 4896.07 copies/mL in adults at initial (Figure 4.2). This situation agrees with the study by Phillipus *et al.* (2001) where it was found out that if high VL (VL > 100 000 copies/mL) is recorded before treatment, it takes much time to obtain viral suppression in patients with high VL copies than in patients with low copies of VL before treatment.

Our study also found that different combinations were behaving differently in both adult and children participants. This was confirmed when only AZT/3TC/EFV combination in adult participants achieved 83.3% of VL decrease and TDF/3TC/EFV, AZT/3TC/LPV-r and D4T/3TC/NVP achieved 66.7% VL decrease each. Looking at the CD4 count increase, the TDF/3TC/EFV and D4T/3TC/NVP combinations achieved 66.7% and AZT/3TC/EFV combination obtained 33.3% (Table 4.5). Based on this finding the three combinations gave better treatment outcome as they decreased the VL from 12 and 24 months in adult participants. Contradicting our finding on the efficacy of D4T/3TC/NVP combination in ART naïve adult patients' was a systematic review of all available publications on efficacy of first line combinations by Tang *et al*, (2012). In their study, they found out that D4T/3TC/NVP combination gave poor treatment results associated with higher rate of virological failure and drug resistance. They also noted that TDF/3TC/NVP combination was one of the least studied regimens among the first line combinations recommended by the world health organisation in the treatment of HIV (Tang *et al*., 2012). Nonetheless, this study found TDF/3TC/NVP with 50% VL decrease and 33.3% CD4 increase which shows better treatment outcome compared to other

combinations in this study. AZT/3TC/NVP combination gave poor treatment results and it was the most prescribed in both study populations.

In all study participants, AZT/3TC/EFV and D4T/3TC/LPV-r combinations showed a better treatment results which make the two combinations of efavirenz and nevirapine and stavudine with lopinovir-ritonovir the combination of choice for ART naïve patients. These findings supported our second hypothesis that ARV drug's efficacy is not the same especially in ART naïve patients. In addition, poor efficacy of some combinations was observed as some of these combinations are the one that were shown by some studies that they give poor treatment results. Tang and Shafer (2012) in their study review outlined the use of unreliable ART combinations in developing nations because of their low costs. This is one of the statements that cannot be ruled out when it comes to ART treatment in Namibia as a developing nation.

5.1.1 Impact of ART treatment on VL, CD4 count in relation to the age of patients

In line with our specific objectives the question of a likely association between age of patient and the impact of the treatment combinations was investigated. This study found a significant correlation between age and treatment combination only in children participants coefficient of correlation (r) and *P value* (r = 0.38, $P = 0.00$, significant at $P \leq 0.05$). A correlation that was not significant was found in adults (patients age 13 – 55 years) and ARV combination equivalent to r = 0.09, $P = 0.11$. The study also found out that there is an inverse correlation between CD4 count and VL. However, such an inverse correlation is only observable at 6, 12, 24, 30 and 36 months in adults but throughout the 36 months of treatment in children. The study

outcome suggests that the efficacy of treatment was somehow related to the age of patients especially in children. According to these findings treatment is more effective in young ART naïve patients than adult patients. It is likely that is not age of the patient but rather the early detection and treatment of HIV infection. Such notion is supported by the observation in HIV-1 treatment initiated in children as soon as they are tested positive and this makes treatment to be effective once initiated immediately. Based on these results, VL was uncontrollable at some points mostly in adults where VL was increasing even with the use of antiretroviral drugs.

5.1.2 Relationship between micronutrient levels, CD4 count, VL and treatment combinations

This study was particularly interested to find out what impact does micronutrient levels have on patients' response to ART. Several studies reviewed this question from many functional areas. Tiyou *et al.*, 2012 argued that patients responded poorly if they had weak nutrient status and that micronutrient levels may be determinants of susceptibility to HIV infection, transmission and progression, including risk of opportunistic infections. Other studies, concluded that HIV infected patients are vulnerable to malnutrition due to several factors such as inadequate nutrition, nutrient loss due to malabsorption and diarrhoea, metabolic alteration and drug-nutrient interaction (Dudgeon *et al.*, 2006; Colecraft, 2008).

Sanjobo *et al.* (2008) stated that nutritional support must be considered as an important factor contributing to ART adherence as lack of nutritious food is one of the causes of non-adherence in antiretroviral treatment program. Given the above

narrative this study analysed the micronutrient availability (specifically iron, folate, magnesium and vitamin B12) and patient response to ARV treatment.

Our study did observe a significant correlation between folate levels in adult and adolescents and ARV combinations (r = - 0.397, *P = 0.03*). However, correlations were not significant with other micronutrients in this study. Nonetheless, our finding suggests that iron, Mg^{2+}, vitamin B12 levels could not be a limiting or impact factor on patients' response to ARV treatment in this study population. Participants in this study had normal to above normal iron, Mg^{2+} and vitamin B12 levels therefore a comparative analysis with a below normal level ARV response was not possible.

Micronutrients levels also showed no significant association with CD4 count and VL. Our findings also suggest that micronutrient levels recorded within the study group had no impact on the level of neither CD4 count nor VL. It is therefore likely that in the cohort of adult participants in this study, micronutrients availability could be ruled out as one of the causes of virological failure. Time was limited as patients cannot be interviewed on food availability in their households to be able to give more evidence on our findings. In addition, the missing data on height and weight of patients could also have contributed a lot to our findings on the relationship of micronutrient levels and treatment response in ART naïve patients.

CONCLUSION

The study successfully addressed the specific objectives set out in the scope of this study and the scientific questions that guided its mission. One of the key findings of this study is that AZT/3TC/EFV combination in adults can be the first choice of antiretroviral treatment on ART naïve patients in Khomas region followed by TDF/3TC/EFV, AZT/3TC/LPV-r and D4T/3TC/NVP. Moreover, D4T/3TC/LPV-r combination can be regarded as first line best choice for patients with low copies of VL. The significant positive correlation observed between the first line treatment combinations and the age of patients shows a great need in concentrating on the age of the patient especially in children younger than 12 years when prescribing anti HIV drugs.

Furthermore, the observed non-significant correlation between the prescribed first line antiretroviral drugs and the serum levels of iron, Mg^{2+}, vitamin B12, indicates that these micronutrients levels had no intervention in the treatment failure observed within adult participants. With regard to this outcome treatment failure was possibly a result of some of the combinations that lost their efficacy over the treatment period and patients that had very higher VL at initial time of treatment.

Based on the results it is likely that iron deficiency could be a contributing factor to the effective biochemical functioning of the ARV drugs amongst the 6 patients with low values. However, further analysis would be required to confirm or rule out this observation. Further studies will be required to observe how patients with low micronutrient levels respond to each treatment as well as folate levels on ART combinations. Such a study will also have to look at how patients under particular

age group (children, adolescent and adult) respond to ARV treatment given their micronutrient status.

RECOMMENDATIONS

Given the findings from this study, there is a big concern on finding the main causes of virological failure in adults. There is a need to re-visit the treatment guidelines on ART naïve patients within the region. This group of patients did not show good treatment outcomes evident from the very high VLs recorded throughout 36 months of treatment. Initiating treatment for patients as soon they are confirmed to be HIV positive is likely the best way to treat ART naïve patients. Early treatment was found to be one of the effective ways of viral suppression in ART naïve patients (Grinsztejn *et al*., 2014). Our study gave clear evidence of the difficulties to suppress VL in patients with very high VL copies at the time of ART initiation thus early treatment is best way to suppress the virus.

STUDY LIMITATIONS

1. There were a lot of missing data on weight and height of patients as this was a retrospective study. This is the reason why we did not include this data in our study on micronutrients analysis that could be used to describe nutritional levels of the ART naïve patients in the region.

2. Funding was not enough to sample from a large number of patients, especially for micronutrients analysis, limiting the study to only 30 participants.

3. Difficulties in getting patients back to the hospital and convince them to give blood samples for micronutrients analysis.

4. Children below 13 years were excluded from the micronutrient study due to the low population size to represent a good clinical picture.

5. Insufficient time to interview patients about the availability of food in their households.

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ANNEXURES

ANNEXURE A: Primary data collection form

Patient Code	Patients name	Gender	Age	ART Combination	Health facility
Hospital follow-up	CD4 count (cells/µL)	Viral load (copies/mL)	Comment:		
Treatment initiation date					
After 6 months					
After 12 months					
After 18 months					
After 24 months					
After 30 months					
After 36 months					

ANNEXURE B: Data collection form for micronutrients analysis

Patient Name/ Code	Gender	Age	ART combination
Micronutrients Analysis	Levels	Comment:	
Iron (µmol/L)			
Magnesium (mmol/L)			
Folate (ng/mL)			
Vitamin B12 (pg/mL)			

ANNEXURE C: Consent letter

University of Namibia
Private Bag 13301
Windhoek - Namibia
Telephone: +264 206 3111

Consent letter

RESEARCH TITLE: THE EFFECTIVENESS OF FIRST LINE ANTIRETROVIRAL THERAPY AND THE IMPACT OF MICRONUTRIENT LEVELS ON PATIENTS' RESPONSE IN WINDHOE, NAMIBIA.

The study was described in the language that I understand and I freely and voluntarily agree to participate. I understand that my identity will not be disclosed and that I may withdraw from the study without giving a reason at any time and this will not affect me in any way.

Name of the participant**:**
Signature of the participant or guardian**:**
Date**:**
Regarding this study, please feel free to contact me.

Ndahafa Frans
Molecular Diagnostic Unit
Namibia Institute of Pathology
P.O.Box 277
Windhoek - Namibia
Tel: 061-295 4032, Cell: 0814507159

ANNEXURE D: Adults and adolescents descriptive statistics on viral load

		N	Mean	Std. Deviation	Std. Error	Mean		Min	Max
						Lower Bound	Upper Bound		
Viral Load at initial time -0 Month	AZT/3TC/NVP	161	2404.17	2015.10	158.81	2090.54	2717.81	5.00	14521.30
	AZT/3TC/EFV	33	1992.33	872.57	151.90	1682.93	2301.73	23.60	4231.30
	TDF/3TC/EFV	15	63483.76	162533.73	41966.03	2524.42	153491.94	890.00	463811.00
	TDF/3TC/NVP	51	8210.20	15227.95	2132.34	3927.27	12493.13	5.00	45622.00
	AZT/3TC/LPV-r	7	1584.46	761.26	287.73	880.40	2288.51	541.60	2341.60
	D4T/3TC/NVP	56	4328.05	8452.82	1129.56	2064.37	6591.73	21.60	46811.00
	D4T/3TC/LPV-r	3	4896.07	4424.47	2554.47	949.92	15887.05	2341.60	10005.00
	Total	326	6417.01	36742.29	2034.97	2413.64	10420.38	5.00	463811.00
Viral Load after 6 Months of treatment	AZT/3TC/NVP	161	6771.14	24094.71	1898.93	3020.95	10521.34	5.00	241000.02
	AZT/3TC/EFV	33	40250.11	99759.04	17365.82	4877.09	75623.13	5.00	362500.01
	TDF/3TC/EFV	15	9236.73	16239.26	4192.96	243.73	18229.74	5.00	48655.00
	TDF/3TC/NVP	51	10155.72	19219.17	2691.22	4750.24	15561.20	5.00	80009.00
	AZT/3TC/LPV-r	7	18276.74	37445.98	14153.25	355.02	52908.50	1008.20	102608.20
	D4T/3TC/NVP	56	22954.10	66879.27	8937.12	5043.71	40864.48	5.00	345556.00
	D4T/3TC/LPV-r	3	15234.34	26255.98	15158.89	989.12	80457.80	5.00	45552.02
	Total	326	13907.88	47269.23	2618.00	8757.52	19058.25	5.00	362500.01
Viral Load after 12 Months of treatment	AZT/3TC/NVP	161	17780.23	126859.87	9997.96	964.76	37525.21	5.00	1.40E+06
	AZT/3TC/EFV	33	16689.19	58483.90	10180.74	4048.29	37426.68	5.00	242899.80
	TDF/3TC/EFV	15	13055.93	27288.35	7045.82	2055.85	28167.72	5.00	80009.00
	TDF/3TC/NVP	51	32666.42	112467.57	15748.61	1034.41	64298.44	5.00	575635.00
	AZT/3TC/LPV-r	7	621.43	147.16	55.62	485.32	757.53	298.57	698.57
	D4T/3TC/NVP	56	21239.01	78691.55	10515.60	165.28	42312.75	5.00	575635.00
	D4T/3TC/LPV-r	3	1790.04	2435.01	1405.85	4258.86	7838.94	15.00	4566.00
	Total	326	19859.79	106433.49	5894.81	8263.00	31456.58	5.00	1.40E+06
Viral Load after 18 Months of treatment	AZT/3TC/NVP	161	7092.99	16425.49	1294.51	4536.46	9649.52	5.00	111246.13
	AZT/3TC/EFV	33	7453.92	15929.69	2773.00	1805.49	13102.34	5.00	63392.85
	TDF/3TC/EFV	15	3294.87	2985.55	770.87	1641.53	4948.22	15.00	8642.00
	TDF/3TC/NVP	51	3217.41	9265.36	1297.41	611.48	5823.34	5.00	49075.24
	AZT/3TC/LPV-r	7	4882.14	6841.41	2585.81	1445.10	11209.39	145.00	14855.00
	D4T/3TC/NVP	56	7925.31	15598.02	2084.37	3748.13	12102.48	5.00	74930.47
	D4T/3TC/LPV-r	3	22646.95	35359.22	20414.65	65190.22	110484.12	15.00	63392.85
	Total	326	6587.10	15045.54	833.30	4947.77	8226.43	5.00	111246.13
Viral Load after 24 Months of treatment	AZT/3TC/NVP	161	4856.56	32359.41	2550.28	179.99	9893.11	5.00	403036.40
	AZT/3TC/EFV	33	3354.62	12987.83	2260.89	1250.67	7959.90	5.00	74930.47
	TDF/3TC/EFV	15	5442.26	4047.47	1045.05	3200.84	7683.67	5.00	9860.00
	TDF/3TC/NVP	51	6210.98	11628.08	1628.26	2940.53	9481.43	5.00	74930.47
	AZT/3TC/LPV-r	7	1176.29	495.44	187.26	718.08	1634.49	452.00	1522.00
	D4T/3TC/NVP	56	5988.30	15525.28	2074.65	1830.60	10145.99	5.00	63392.85
	D4T/3TC/LPV-r	3	869.90	756.93	437.01	1010.41	2750.21	63.06	1564.34
	Total	326	5022.06	24404.72	1351.65	2362.97	7681.15	5.00	403036.40
Viral Load after 30 Months of treatment	AZT/3TC/NVP	161	3533.70	18109.44	1427.22	715.07	6352.33	5.00	215610.60
	AZT/3TC/EFV	33	1085.42	4270.37	743.38	428.78	2599.63	5.00	24446.67
	TDF/3TC/EFV	15	1677.59	1408.79	363.75	897.43	2457.76	5.00	4856.00
	TDF/3TC/NVP	51	11970.68	19409.27	2717.84	6511.74	17429.63	5.00	74930.47
	AZT/3TC/LPV-r	7	1221.43	39.34	14.87	1185.05	1257.81	1200.00	1300.00
	D4T/3TC/NVP	56	3248.91	10160.92	1357.81	527.80	5970.03	5.00	74930.47
	D4T/3TC/LPV-r	3	492.83	627.12	362.07	1065.01	2050.67	5.00	1200.20
	Total	326	4393.81	15816.95	876.02	2670.42	6117.19	5.00	215610.60
Viral Load after 36 Months of treatment	AZT/3TC/NVP	160	1555.47	6382.39	504.57	558.94	2552.00	5.00	73435.00
	AZT/3TC/EFV	33	72.78	260.59	45.36	19.62	165.18	5.00	1456.13
	TDF/3TC/EFV	15	1552.56	1461.79	377.43	743.04	2362.07	5.00	5047.41
	TDF/3TC/NVP	51	3842.26	11223.64	1571.62	685.56	6998.96	5.00	73435.00
	AZT/3TC/LPV-r	7	1173.29	698.45	263.99	527.32	1819.25	100.00	2500.00
	D4T/3TC/NVP	56	4176.58	14050.37	1877.56	413.87	7939.30	5.00	73435.00
	D4T/3TC/LPV-r	3	11.67	5.77	3.33	-2.68	26.01	5.00	15.00
	Total	325	2192.79	8654.59	480.07	1248.34	3137.24	5.00	73435.00

ANNEXURE E: Adults and adolescents descriptive statistics on CD4 count

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min	Max
						Lower Bound	Upper Bound		
CD4 at Initial time -0 Month	AZT/3TC/NVP	161	429.89	130.74	10.30	409.54	450.24	46.00	1011.00
	AZT/3TC/EFV	33	428.67	219.68	38.24	350.77	506.56	45.00	1016.00
	TDF/3TC/EFV	15	330.33	139.92	36.13	252.85	407.82	49.00	446.00
	TDF/3TC/NVP	51	327.59	148.85	20.84	285.72	369.45	132.00	742.00
	AZT/3TC/LPV-r	7	304.29	177.21	66.98	140.40	468.17	46.00	456.00
	D4T/3TC/NVP	56	392.75	147.64	19.73	353.21	432.29	42.00	742.00
	D4T/3TC/LPV-r	3	768.00	142.67	82.37	413.58	1122.42	608.00	882.00
	Total	326	403.22	157.58	8.73	386.05	420.39	42.00	1016.00
CD4 after 6 Months of treatment	AZT/3TC/NVP	161	431.13	245.56	19.35	392.91	469.35	10.00	1114.00
	AZT/3TC/EFV	33	431.39	273.52	47.61	334.41	528.38	60.00	1045.00
	TDF/3TC/EFV	15	297.47	164.62	42.51	206.30	388.63	197.00	851.00
	TDF/3TC/NVP	51	397.00	192.43	26.95	342.88	451.12	98.00	895.00
	AZT/3TC/LPV-r	7	353.86	202.54	76.55	166.54	541.18	81.00	581.00
	D4T/3TC/NVP	56	425.21	180.02	24.06	377.00	473.42	119.00	904.00
	D4T/3TC/LPV-r	3	665.33	414.11	239.08	-363.36	1694.03	193.00	966.00
	Total	326	419.15	229.60	12.72	394.13	444.16	10.00	1114.00
CD4 after 12 Months of treatment	AZT/3TC/NVP	161	458.10	230.21	18.14	422.27	493.93	15.00	1100.00
	AZT/3TC/EFV	33	469.09	280.79	48.88	369.53	568.65	80.00	1152.00
	TDF/3TC/EFV	15	319.80	137.29	35.45	243.77	395.83	232.00	724.00
	TDF/3TC/NVP	51	417.88	199.77	27.97	361.70	474.07	90.00	809.00
	AZT/3TC/LPV-r	7	706.43	75.59	28.57	636.52	776.34	535.00	735.00
	D4T/3TC/NVP	56	406.84	191.67	25.61	355.51	458.17	108.00	978.00
	D4T/3TC/LPV-r	3	657.00	206.76	119.37	143.37	1170.63	503.00	892.00
	Total	326	444.91	224.75	12.45	420.43	469.40	15.00	1152.00
CD4 after 18 Months of treatment	AZT/3TC/NVP	161	490.45	207.46	16.35	458.16	522.74	100.00	1092.00
	AZT/3TC/EFV	33	437.82	270.71	47.12	341.83	533.81	26.00	994.00
	TDF/3TC/EFV	15	371.60	156.54	40.42	284.91	458.29	241.00	823.00
	TDF/3TC/NVP	51	507.43	190.09	26.62	453.97	560.89	145.00	823.00
	AZT/3TC/LPV-r	7	318.43	241.48	91.27	95.10	541.76	65.00	644.00
	D4T/3TC/NVP	56	452.07	189.49	25.32	401.33	502.82	116.00	855.00
	D4T/3TC/LPV-r	3	568.67	292.26	168.74	-157.35	1294.69	262.00	844.00
	Total	326	472.74	210.50	11.66	449.81	495.68	26.00	1092.00
CD4 after 24 Months of treatment	AZT/3TC/NVP	161	448.85	214.83	16.93	415.41	482.29	15.00	985.00
	AZT/3TC/EFV	33	472.15	251.36	43.76	383.02	561.28	78.00	978.00
	TDF/3TC/EFV	15	443.13	188.08	48.56	338.98	547.29	255.00	855.00
	TDF/3TC/NVP	51	430.49	227.41	31.84	366.53	494.45	150.00	976.00
	AZT/3TC/LPV-r	7	416.29	93.43	35.31	329.88	502.69	222.00	532.00
	D4T/3TC/NVP	56	484.21	189.29	25.30	433.52	534.91	156.00	952.00
	D4T/3TC/LPV-r	3	712.00	168.65	97.37	293.04	1130.96	580.00	902.00
	Total	326	455.87	213.92	11.85	432.56	479.18	15.00	985.00
CD4 after 30 Months of treatment	AZT/3TC/NVP	161	474.81	200.78	15.82	443.56	506.06	100.00	927.00
	AZT/3TC/EFV	33	508.27	279.34	48.63	409.22	607.32	56.00	989.00
	TDF/3TC/EFV	15	359.07	174.30	45.00	262.54	455.59	75.00	581.00
	TDF/3TC/NVP	51	417.63	183.32	25.67	366.07	469.19	102.00	862.00
	AZT/3TC/LPV-r	7	361.29	119.28	45.08	250.97	471.60	127.00	427.00
	D4T/3TC/NVP	56	453.68	187.64	25.07	403.43	503.93	75.00	860.00
	D4T/3TC/LPV-r	3	744.67	273.00	157.62	66.49	1422.84	450.00	989.00
	Total	326	460.34	206.76	11.45	437.82	482.87	56.00	989.00
CD4 after 36 Months of treatment	AZT/3TC/NVP	161	494.81	213.76	16.85	461.54	528.08	105.00	963.00
	AZT/3TC/EFV	33	475.39	278.81	48.53	376.53	574.25	99.00	978.00
	TDF/3TC/EFV	15	539.33	191.47	49.44	433.30	645.36	296.00	820.00
	TDF/3TC/NVP	51	431.22	210.75	29.51	371.94	490.49	68.00	832.00
	AZT/3TC/LPV-r	7	272.43	87.88	33.21	191.15	353.70	156.00	356.00
	D4T/3TC/NVP	56	459.21	256.11	34.22	390.63	527.80	105.00	860.00
	D4T/3TC/LPV-r	3	852.67	151.02	87.19	477.52	1227.82	685.00	978.00
	Total	326	477.35	229.93	12.73	452.30	502.40	68.00	978.00

ANNEXURE F*:* ANOVA adults and adolescents differences in VL between follow-ups

		N	Mean	Std. Deviation	Std. Error	Mean		Min	Max
						Lower Bound	Upper Bound		
Difference Viral Load between initial time and 6 Months of treatment	AZT/3TC/NVP	161	30.99	271.28	21.38	73.21	11.24	1185.00	533.00
	AZT/3TC/EFV	32	6.69	170.82	30.20	54.90	68.27	454.00	357.00
	TDF/3TC/EFV	15	32.87	228.66	59.04	93.76	159.49	405.00	249.00
	TDF/3TC/NVP	51	60.47	198.97	27.86	116.43	4.51	449.00	456.00
	AZT/3TC/LPV-r	7	6.86	89.87	33.97	89.98	76.26	135.00	65.00
	D4T/3TC/NVP	56	32.46	181.81	24.29	81.15	16.22	458.00	400.00
	D4T/3TC/LPV-r	3	102.67	287.87	166.20	612.43	817.76	152.00	415.00
Difference Viral Load between 6 Months and 12 Months of treatment	AZT/3TC/NVP	161	10534.55	118671.27	9352.61	29005.03	7935.93	1248319.81	226998.02
	AZT/3TC/EFV	33	24664.25	65600.43	11419.57	1403.35	47925.15	4547.00	358850.00
	TDF/3TC/EFV	15	3819.20	11185.12	2887.98	10013.31	2374.91	31354.00	819.00
	TDF/3TC/NVP	51	22510.70	112871.80	15805.21	54256.40	9235.01	575477.00	71367.00
	AZT/3TC/LPV-r	7	38738.20	45164.32	17070.51	3031.83	80508.23	1009.63	101909.63
	D4T/3TC/NVP	56	1715.09	102292.86	13669.46	25679.12	29109.29	574791.00	322112.00
	D4T/3TC/LPV-r	3	13444.30	23853.93	13772.07	45812.15	72700.75	643.12	40986.02
Difference Viral Load between 12 Months and 18 Months of treatment	AZT/3TC/NVP	161	10688.01	127946.90	10083.63	9226.16	30602.19	98566.81	1.40E+06
	AZT/3TC/EFV	33	9235.28	50058.05	8713.99	8514.53	26985.09	58826.85	197232.24
	TDF/3TC/EFV	15	9761.06	25033.25	6463.56	4101.89	23624.01	3214.12	71367.00
	TDF/3TC/NVP	51	29527.64	112277.25	15721.96	2050.85	61106.12	44519.34	575614.81
	AZT/3TC/LPV-r	7	2420.43	5230.37	1976.89	7257.71	2416.85	14156.43	14.43
	D4T/3TC/NVP	56	13313.70	80914.82	10812.70	8355.43	34982.84	73687.47	575374.60
	D4T/3TC/LPV-r	3	20856.91	32936.17	19015.71	102674.90	60961.08	58826.85	2354.20
Difference Viral Load between 12 Months and 24 Months of treatment	AZT/3TC/NVP	161	2008.32	36805.87	2900.71	3720.29	7736.93	402360.40	111241.13
	AZT/3TC/EFV	33	4099.30	21409.18	3726.86	3492.06	11690.66	74830.47	62410.55
	TDF/3TC/EFV	15	2147.38	3993.96	1031.24	4359.16	64.40	5354.00	8637.00
	TDF/3TC/NVP	51	2369.07	14518.38	2032.98	6452.43	1714.29	73687.47	41528.90
	AZT/3TC/LPV-r	7	4154.29	6333.53	2393.85	1703.25	10011.82	132.00	13403.00
	D4T/3TC/NVP	56	1937.01	21117.60	2821.96	3718.32	7592.34	63367.83	74662.23
	D4T/3TC/LPV-r	3	21777.05	35221.96	20335.41	65719.14	109273.24	48.06	62410.55
Difference of Viral Load between 24 Months and 30 Months of treatment	AZT/3TC/NVP	161	2008.91	36802.28	2900.43	7736.97	3719.14	111241.13	402360.40
	AZT/3TC/EFV	33	4099.30	21409.18	3726.86	11690.66	3492.06	62410.55	74830.47
	TDF/3TC/EFV	15	2147.38	3993.96	1031.24	64.40	4359.16	8637.00	5354.00
	TDF/3TC/NVP	51	2127.19	14326.17	2006.07	1902.11	6156.49	41528.90	73687.47
	AZT/3TC/LPV-r	7	4016.43	6435.86	2432.53	9968.60	1935.75	13403.00	403.00
	D4T/3TC/NVP	56	1937.01	21117.60	2821.96	7592.34	3718.32	74662.23	63367.83
	D4T/3TC/LPV-r	3	21777.05	35221.96	20335.41	109273.24	65719.14	62410.55	48.06
Difference of Viral Load between 30 Months and 36 Months of treatment	AZT/3TC/NVP	160	2000.22	17402.42	1375.78	716.94	4717.39	24814.34	215605.60
	AZT/3TC/EFV	33	1012.65	4287.64	746.38	507.68	2532.98	1036.13	24431.67
	TDF/3TC/EFV	15	125.04	1131.41	292.13	501.51	751.59	892.52	2435.00
	TDF/3TC/NVP	51	7113.25	17503.92	2451.04	2190.19	12036.31	26134.46	64631.48
	AZT/3TC/LPV-r	7	108.57	146.56	55.40	26.98	244.12	125.00	400.00
	D4T/3TC/NVP	56	-927.67	9376.32	1252.96	3438.66	1583.32	61216.89	10326.98
	D4T/3TC/LPV-r	3	481.16	625.39	361.07	1072.39	2034.72	10.00	1185.20

ANNEXURE G: ANOVA adults and adolescents differences in CD4 count between follow-ups

		N	Mean	Std. Deviation	Std. Error	Mean		Min	Max
						Lower Bound	Upper Bound		
Difference CD4 between initial time and 6 Months of treatment	AZT/3TC/NVP	161	30.99	271.28	21.38	11.24	73.21	533.00	1185.00
	AZT/3TC/EFV	32	6.69	170.82	30.20	68.27	54.90	357.00	454.00
	TDF/3TC/EFV	15	32.87	228.66	59.04	159.49	93.76	249.00	405.00
	TDF/3TC/NVP	51	69.41	185.06	25.91	17.36	121.46	400.00	449.00
	AZT/3TC/LPV-r	7	173.57	138.50	52.35	45.48	301.66	85.00	485.00
	D4T/3TC/NVP	56	32.46	181.81	24.29	16.22	81.15	400.00	458.00
	D4T/3TC/LPV-r	3	102.67	287.87	166.20	817.76	612.43	415.00	152.00
Difference CD4 between 6 Months and 12 Months of treatment	AZT/3TC/NVP	161	24.52	344.99	27.19	29.18	78.21	1040.00	1093.00
	AZT/3TC/EFV	32	54.38	136.78	24.18	5.06	103.69	261.00	456.00
	TDF/3TC/EFV	15	22.33	61.00	15.75	11.45	56.12	127.00	123.00
	TDF/3TC/NVP	51	20.88	190.11	26.62	32.59	74.35	504.00	504.00
	AZT/3TC/LPV-r	7	204.00	81.45	30.78	128.68	279.32	124.00	354.00
	D4T/3TC/NVP	56	18.38	170.91	22.84	64.14	27.39	469.00	446.00
	D4T/3TC/LPV-r	3	8.33	291.11	168.07	731.49	714.82	261.00	310.00
Difference CD4 between 12 Months and 18 Months of treatment	AZT/3TC/NVP	161	61.27	233.31	18.39	24.96	97.59	1146.00	889.00
	AZT/3TC/EFV	33	4.00	187.65	32.67	70.54	62.54	630.00	386.00
	TDF/3TC/EFV	15	51.80	61.93	15.99	17.51	86.09	24.00	140.00
	TDF/3TC/NVP	51	96.84	138.55	19.40	57.87	135.81	272.00	301.00
	AZT/3TC/LPV-r	7	47.71	305.54	115.48	234.86	330.29	421.00	456.00
	D4T/3TC/NVP	56	45.23	165.08	22.06	1.02	89.44	331.00	641.00
	D4T/3TC/LPV-r	3	88.33	476.83	275.30	1272.83	1096.17	630.00	268.00
Difference CD4 between 12 Months and 24 Months of treatment	AZT/3TC/NVP	161	21.96	233.19	18.38	58.26	14.33	1050.00	754.00
	AZT/3TC/EFV	33	94.94	252.70	43.99	5.34	184.54	335.00	900.00
	TDF/3TC/EFV	15	71.53	90.45	23.35	21.44	121.62	13.00	200.00
	TDF/3TC/NVP	51	66.69	240.40	33.66	134.30	0.93	430.00	641.00
	AZT/3TC/LPV-r	7	64.43	336.24	127.09	246.54	375.40	242.00	512.00
	D4T/3TC/NVP	55	67.96	303.51	40.93	14.09	150.01	499.00	1004.00
	D4T/3TC/LPV-r	3	365.33	385.13	222.35	591.37	1322.04	54.00	796.00
Difference of CD4 between 24 Months and 30 Months of treatment	AZT/3TC/NVP	161	48.24	182.21	14.36	19.88	76.60	407.00	790.00
	AZT/3TC/EFV	33	24.48	251.93	43.86	113.82	64.85	649.00	444.00
	TDF/3TC/EFV	15	25.60	167.52	43.25	118.37	67.17	412.00	147.00
	TDF/3TC/NVP	51	26.82	199.28	27.91	29.23	82.87	520.00	365.00
	AZT/3TC/LPV-r	7	156.57	150.87	57.02	17.04	296.11	51.00	368.00
	D4T/3TC/NVP	55	15.33	247.56	33.38	82.25	51.60	1098.00	522.00
	D4T/3TC/LPV-r	3	189.33	113.71	65.65	471.81	93.14	295.00	69.00
Difference of CD4 between 30 Months and 36 Months of treatment	AZT/3TC/NVP	161	8.73	204.05	16.08	23.03	40.49	1183.00	689.00
	AZT/3TC/EFV	33	6.39	228.06	39.70	74.47	87.26	700.00	585.00
	TDF/3TC/EFV	15	141.80	343.42	88.67	48.38	331.98	285.00	745.00
	TDF/3TC/NVP	51	15.90	129.57	18.14	20.54	52.34	376.00	346.00
	AZT/3TC/LPV-r	7	36.14	316.75	119.72	256.80	329.09	451.00	426.00
	D4T/3TC/NVP	56	62.54	258.45	34.54	6.68	131.75	345.00	745.00
	D4T/3TC/LPV-r	3	108.00	123.19	71.13	198.03	414.03	11.00	235.00

ANNEXURE H: Children descriptive statistics on VL

		N	Mean	Std. Deviation	Std. Error	Mean		Min	Max
						Lower Bound	Upper Bound		
Viral Load at initial time -0 Month	AZT/3TC/NVP	69	1652.04	3869.61	465.85	722.46	2581.62	64.00	32341.60
	AZT/3TC/EFV	3	1441.61	953.92	550.74	928.05	3811.28	441.64	2341.60
	AZT/3TC/LPV-r	3	559.47	389.04	224.61	406.96	1525.89	235.60	991.00
	D4T/3TC/LPV-r	3	350.00	149.91	106.00	996.86	1696.86	244.00	456.00
	Total	78	1550.56	3654.99	413.85	726.49	2374.64	64.00	32341.60
Viral Load after 6 Months of treatment	AZT/3TC/NVP	69	7896.82	14401.58	1733.75	4437.18	11356.46	15.00	60605.00
	AZT/3TC/EFV	3	18375.40	16216.49	9362.60	21908.60	58659.40	230.00	31452.21
	AZT/3TC/LPV-r	3	187.00	159.14	91.88	208.34	582.34	5.00	300.00
	D4T/3TC/LPV-r	3	62.75	0.00	0.00	62.75	62.75	62.75	62.75
	Total	78	7704.27	14103.42	1596.90	4524.44	10884.10	5.00	60605.00
Viral Load after 12 Months of treatment	AZT/3TC/NVP	69	4895.17	9919.59	1194.18	2512.22	7278.11	5.00	54662.98
	AZT/3TC/EFV	3	2796.13	2638.77	1523.49	3758.94	9351.20	630.00	5735.00
	AZT/3TC/LPV-r	3	313.00	437.67	252.69	774.24	1400.24	5.00	814.00
	D4T/3TC/LPV-r	3	10.00	7.07	5.00	53.53	73.53	5.00	15.00
	Total	78	4450.64	9423.33	1066.98	2326.01	6575.27	5.00	54662.98
Viral Load after 18 Months of treatment	AZT/3TC/NVP	69	6687.54	16304.96	1962.89	2770.67	10604.42	5.00	111246.13
	AZT/3TC/EFV	3	5799.65	2706.71	1562.72	924.18	12523.48	3456.00	8762.21
	AZT/3TC/LPV-r	3	306.67	150.44	86.86	67.06	680.39	150.00	450.00
	D4T/3TC/LPV-r	3	10.00	7.07	5.00	53.53	73.53	5.00	15.00
	Total	78	6151.21	15428.25	1746.91	2672.68	9629.74	5.00	111246.13
Viral Load after 24 Months of treatment	AZT/3TC/NVP	69	11729.70	48891.76	5885.88	15.38	23474.78	5.00	403036.40
	AZT/3TC/EFV	3	18997.34	26364.56	15221.59	46495.87	84490.55	1564.34	49327.68
	AZT/3TC/LPV-r	3	2118.94	2831.84	1634.97	4915.75	9153.63	134.00	5361.82
	D4T/3TC/LPV-r	3	10.00	7.07	5.00	53.53	73.53	5.00	15.00
	Total	78	11188.89	46259.90	5237.90	758.89	21618.88	5.00	403036.40
Viral Load after 30 Months of treatment	AZT/3TC/NVP	69	21146.71	51493.57	6199.10	8776.60	33516.81	5.00	215610.60
	AZT/3TC/EFV	3	149.38	164.79	95.14	259.98	558.74	15.00	333.24
	AZT/3TC/LPV-r	3	1402.33	1464.30	845.41	2235.19	5039.86	234.00	3045.00
	D4T/3TC/LPV-r	3	5.00	0.00	0.00	5.00	5.00	5.00	5.00
	Total	78	18768.43	48843.52	5530.44	7755.92	29780.95	5.00	215610.60
Viral Load after 36 Months of treatment	AZT/3TC/NVP	69	1968.86	6717.23	808.66	355.21	3582.51	5.00	54222.98
	AZT/3TC/EFV	3	8.33	5.77	3.33	6.01	22.68	5.00	15.00
	AZT/3TC/LPV-r	3	550.14	472.16	272.60	622.76	1723.04	15.00	908.00
	D4T/3TC/LPV-r	3	5.00	0.00	0.00	5.00	5.00	5.00	5.00
	Total	78	1763.48	6339.42	717.80	334.17	3192.80	5.00	54222.98

ANNEXURE I: Children's descriptive statistics on CD4 count

		N	Mean	Std. Deviation	Std. Error	Mean		Min	Max
						Lower Bound	Upper Bound		
CD4 at Initial time -0 Month	AZT/3TC/NVP	69	620.81	278.55	33.53	553.90	687.73	124.00	1106.00
	AZT/3TC/EFV	3	453.67	13.28	7.67	420.68	486.65	446.00	469.00
	AZT/3TC/LPV-r	3	673.00	308.67	178.21	93.77	1039.77	423.00	1018.00
	D4T/3TC/LPV-r	3	704.00	401.64	284.00	904.56	1212.56	420.00	988.00
	Total	78	620.23	273.31	30.95	558.61	681.85	124.00	1106.00
CD4 after 6 Months of treatment	AZT/3TC/NVP	69	793.74	339.05	40.82	712.29	875.19	200.00	1057.00
	AZT/3TC/EFV	3	641.67	398.27	229.94	347.69	1031.02	263.00	1057.00
	AZT/3TC/LPV-r	3	1287.33	513.37	296.40	12.04	2562.62	797.00	1821.00
	D4T/3TC/LPV-r	3	998.00	33.94	24.00	693.05	1302.95	974.00	1022.00
	Total	78	814.56	352.15	39.87	735.17	893.96	200.00	1057.00
CD4 after 12 Months of treatment	AZT/3TC/NVP	69	836.68	440.52	53.03	730.86	942.51	270.00	1050.00
	AZT/3TC/EFV	3	755.33	448.42	258.90	358.61	1069.28	345.00	1234.00
	AZT/3TC/LPV-r	3	1186.67	75.06	43.33	1000.22	1373.11	1100.00	1230.00
	D4T/3TC/LPV-r	3	992.00	16.97	12.00	839.53	1114.47	980.00	1004.00
	Total	78	851.32	426.85	48.33	755.08	947.56	270.00	1057.00
CD4 after 18 Months of treatment	AZT/3TC/NVP	69	800.57	299.50	36.06	728.62	872.52	257.00	1058.00
	AZT/3TC/EFV	3	763.00	333.16	192.35	64.62	1190.62	424.00	1090.00
	AZT/3TC/LPV-r	3	1122.33	85.94	49.62	908.84	1235.83	1030.00	1200.00
	D4T/3TC/LPV-r	2	985.00	360.62	255.00	1225.08	1245.08	730.00	1240.00
	Total	78	818.83	298.58	33.81	751.51	886.14	257.00	1240.00
CD4 after 24 Months of treatment	AZT/3TC/NVP	69	718.28	314.27	37.83	642.78	793.77	188.00	1230.00
	AZT/3TC/EFV	3	699.33	334.05	192.87	130.50	1029.17	439.00	1076.00
	AZT/3TC/LPV-r	3	1017.33	250.94	144.88	393.97	1040.70	741.00	1231.00
	D4T/3TC/LPV-r	3	981.00	1.41	1.00	968.29	993.71	980.00	982.00
	Total	78	739.17	312.26	35.36	668.76	809.57	188.00	1231.00
CD4 after 30 Months of treatment	AZT/3TC/NVP	69	759.71	300.75	36.21	687.46	831.96	284.00	1321.00
	AZT/3TC/EFV	3	734.67	400.75	231.38	260.86	1230.20	274.00	1003.00
	AZT/3TC/LPV-r	3	1104.00	95.02	54.86	867.97	1240.03	1010.00	1200.00
	D4T/3TC/LPV-r	2	780.00	0.00	0.00	780.00	780.00	780.00	780.00
	Total	78	775.62	299.07	33.86	708.19	843.04	274.00	1321.00
CD4 after 36 Months of treatment	AZT/3TC/NVP	69	709.09	308.06	37.09	635.08	783.09	213.00	1093.00
	AZT/3TC/EFV	3	679.67	297.13	171.55	58.45	1217.78	344.00	909.00
	AZT/3TC/LPV-r	3	927.33	370.58	213.95	6.76	1047.90	518.00	1240.00
	D4T/3TC/LPV-r	3	1002.50	0.71	0.50	996.15	1008.85	1002.00	1003.00
	Total	78	727.65	307.53	34.82	658.32	796.99	213.00	1240.00

ANNEXURE J: ANOVA CD4 differences between follow-ups in children

		N	Mean	Std. Deviation	Std. Error	Interval for Mean		Min	Max
						Lower Bound	Upper Bound		
Difference CD4 between initial time and 6	AZT/3TC/NVP	69	222.59	315.35	37.96	146.84	298.35	533.00	1185.00
	AZT/3TC/EFV	3	145.33	287.36	165.91	859.17	568.50	412.00	159.00
	AZT/3TC/LPV-r	3	620.67	825.74	476.74	1430.58	2671.92	281.00	1340.00
	D4T/3TC/LPV-r	3	706.00	155.56	110.00	691.68	2103.68	596.00	816.00
Difference CD4 between 6 Months and 12 Months	AZT/3TC/NVP	69	78.86	237.10	28.54	21.90	135.81	677.00	577.00
	AZT/3TC/EFV	3	113.67	54.85	31.67	22.58	249.92	82.00	177.00
	AZT/3TC/LPV-r	3	467.33	315.53	182.17	1251.17	316.50	721.00	114.00
	D4T/3TC/LPV-r	3	202.00	319.61	226.00	3073.60	2669.60	428.00	24.00
Difference CD4 between 12 Months and 18 Months	AZT/3TC/NVP	69	27.02	200.48	24.13	21.14	75.18	600.00	659.00
	AZT/3TC/EFV	3	7.67	131.42	75.88	318.81	334.14	144.00	88.00
	AZT/3TC/LPV-r	3	69.00	148.94	85.99	300.99	438.99	93.00	200.00
	D4T/3TC/LPV-r	3	79.00	479.42	339.00	4386.40	4228.40	418.00	260.00
Difference CD4 between 12 Months and 24 Months	AZT/3TC/NVP	69	49.59	172.05	20.71	90.92	8.26	404.00	467.00
	AZT/3TC/EFV	3	63.67	112.08	64.71	342.09	214.76	192.00	15.00
	AZT/3TC/LPV-r	3	28.33	84.03	48.52	180.41	237.08	57.00	111.00
	D4T/3TC/LPV-r	3	35.00	35.36	25.00	282.66	352.66	10.00	60.00
Difference of CD4 between 24 Months and 30 Months	AZT/3TC/NVP	69	69.01	170.94	20.58	27.95	110.08	334.00	554.00
	AZT/3TC/EFV	3	82.00	254.83	147.13	551.04	715.04	165.00	344.00
	AZT/3TC/LPV-r	3	30.00	262.34	151.46	681.68	621.68	331.00	150.00
	D4T/3TC/LPV-r	3	126.50	156.27	110.50	1530.54	1277.54	237.00	16.00
Difference of CD4 between 30 Months and 36 Months	AZT/3TC/NVP	69	9.28	163.77	19.72	30.07	48.62	457.00	278.00
	AZT/3TC/EFV	3	5.67	117.47	67.82	297.49	286.16	141.00	70.00
	AZT/3TC/LPV-r	3	83.33	12.06	6.96	113.28	-53.39	96.00	72.00
	D4T/3TC/LPV-r	3	330.00	90.51	64.00	483.20	1143.20	266.00	394.00

ANNEXURE K: ANOVA viral load differences between follow-ups

		N	Mean	Std. Deviation	Std. Error	Mean		Min	Max
						Lower Bound	Upper Bound		
Difference Viral Load between initial time and 6	AZT/3TC/NVP	69	236.33	350.98	42.25	320.65	152.02	1185.00	533.00
	AZT/3TC/EFV	3	145.33	287.36	165.91	568.50	859.17	159.00	412.00
	AZT/3TC/LPV-r	3	620.67	825.74	476.74	2671.92	1430.58	1340.00	281.00
	D4T/3TC/LPV-r	3	706.00	155.56	110.00	2103.68	691.68	816.00	596.00
Difference Viral Load between 6 Months and 12 Months	AZT/3TC/NVP	69	735.82	4604.04	554.26	370.19	1841.83	3594.30	34439.90
	AZT/3TC/EFV	3	169.33	493.06	284.67	1055.50	1394.17	400.00	456.00
	AZT/3TC/LPV-r	3	126.00	342.83	197.93	977.64	725.64	514.00	136.00
	D4T/3TC/LPV-r	3	52.75	7.07	5.00	10.78	116.28	47.75	57.75
Difference Viral Load between 12 Months and 18 Months	AZT/3TC/NVP	69	969.71	7405.36	891.50	2748.67	809.25	53772.44	4311.53
	AZT/3TC/EFV	3	1857.12	1972.08	1138.58	6756.05	3041.81	3157.35	412.00
	AZT/3TC/LPV-r	3	6.33	340.96	196.85	840.65	853.31	315.00	364.00
	D4T/3TC/LPV-r	3	60.00	84.85	60.00	702.37	822.37	0.00	120.00
Difference Viral Load between 12 Months and 24 Months	AZT/3TC/NVP	69	3483.08	18175.05	2188.02	883.04	7849.21	24210.98	98195.23
	AZT/3TC/EFV	3	15186.98	26595.45	15354.89	81253.74	50879.78	45871.68	1230.00
	AZT/3TC/LPV-r	3	1812.27	2698.69	1558.09	8516.20	4891.66	4911.82	16.00
	D4T/3TC/LPV-r	3	0.00	14.14	10.00	127.06	127.06	10.00	10.00
Difference of Viral Load between 24 Months and	AZT/3TC/NVP	69	1506.30	13745.92	1654.81	4808.43	1795.83	98195.23	13552.40
	AZT/3TC/EFV	3	1952.54	4549.42	2626.61	13253.92	9348.84	7197.87	919.26
	AZT/3TC/LPV-r	3	1812.27	2698.69	1558.09	4891.66	8516.20	16.00	4911.82
	D4T/3TC/LPV-r	3	0.00	14.14	10.00	127.06	127.06	10.00	10.00
Difference of Viral Load between 30 Months and	AZT/3TC/NVP	69	6408.05	20480.80	2465.60	1488.03	11328.08	6059.77	111241.10
	AZT/3TC/EFV	3	141.05	166.38	96.06	272.27	554.37	10.00	328.24
	AZT/3TC/LPV-r	3	852.20	1903.42	1098.94	3876.17	5580.56	493.41	3030.00
	D4T/3TC/LPV-r	3	9.50	6.36	4.50	47.68	66.68	5.00	14.00