THE EFFECT OF LIPID-BASED NUTRIENT SUPPLEMENTS CONTAINING VITAMINS AND MINERALS ON RENAL EXCRETION OF ELECTROLYTES AND TENOFOVIR CONCENTRATIONS IN BLOOD AMONG ZAMBIAN HIV/AIDS PATIENTS

By

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PHILOSOPHICAL QUOTES

“AIDS is indeed brought about by the collapse of the immune system - but not because of a virus. The cause is poverty, bad nourishment and general ill-health. The solution is not expensive western medicine but the alleviation of poverty in Africa”

~Thabo Mbeki (2000)

“Let food be thy medicine and medicine be thy food”

~Hippocrates (460 BC-370 BC)

“It is self-explanatory, in right dosages food and exercise together are as good as medicine”

~Derick Munkombwe (2012)

DEDICATION

It is with deepest gratitude that I dedicate this thesis to;

My wife Mutinta Eustekia Hadunka our sons Derick Jr. and Habwami, my father Isaac Munsaka Munkombwe, my late mother Saliya Munsaka whose affection I will always cherish.

To you all, I say, God bless.

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ABSTRACT

The Effect of Lipid-Based Nutrient Supplements Containing Vitamins and Minerals on Renal Excretion of Electrolytes and Tenofovir Concentrations in Blood among Zambian HIV/AIDS Patients by Derick Munkombwe

Background: Advanced HIV-infection combined with under-nutrition and anti-retroviral therapy (ART) places patients at high risk of electrolyte abnormalities and/or losses as well as increased mortality. **Aims:** This study evaluated if nutritional supplements would help curtail renal electrolyte loss and determined tenofovir blood concentrations in HIV/AIDS patients. **Methods:** 130 malnourished HIV-positive patients were enrolled in a sub-study embedded in the 'Nutritional Support for African Adults Starting ART (NUSTRAT) trial. Inclusion criteria were, ART-naive, body mass index (BMI) <18.5 kg/m^2 and CD4 count <350 cells/μL. Patients received lipid-based nutrient supplements alone (LNS, n=63) or together with vitamins and minerals (LNS-VM, n=67). Blood and spot urine samples were collected and assayed for creatinine, potassium, magnesium and phosphate concentrations using spectrophotometric methods. Tenofovir in serum and dried blood spot (DBS) was assayed by chromatographic methods. **Results:** Eighteen (28.6%) patients from the LNS and 16 (23.9%) from LNS-VM groups died. Phosphate excretion at baseline, was high in both LNS (mean ± SD: 1.2 ± 0.6 mg/mg creatinine) and LNS-VM (1.1 ± 0.8 mg/mg creatinine) groups relative to normal physiological ranges. Phosphate excretion remained high in the LNS group (1.1 ± 0.41 mg/mg creatinine) but significantly decreased in the LNS-VM group (0.6 ± 0.28 mg/mg creatinine; $p < 0.001$) after 12 weeks of ART. This difference is probably explained by increased renal tubular reabsorption of phosphate in the LNS-VM group (88.3 ± 5.7%) compared to the LNS group (76.6 ± 8.9%). The fractional excretion of potassium (FEK) was not significantly different at baseline (p=0.69) between the two groups although the values were above normal physiological ranges (i.e. >6.4%); FEK was significantly lowered in the LNS-VM group (6.2 ± 3.4%); $p < 0.001$) but not in the LNS group (12.8 ± 4.7%) after 12 weeks of ART. Patient blood tenofovir trough concentrations ranged from 67.0 to 421.1 ng/mL (means±SEM: males, 213±12.7, n=53; females, 223.5±14.2, n=35). Multivariable linear regression of tenofovir concentrations with age, sex, BMI, and CD4 cell count revealed BMI as an independent predictor of blood tenofovir (coefficient -34.3; 95% CI -41.4, -27.1; p<0.001). Stratification of BMI into <18.5 kg/m^2 (n=47) or >18.5 kg/m^2 (n=41) categories at 12 weeks of ART showed that patients with lower BMI had significantly elevated blood tenofovir concentrations than those with higher BMI (253.7±95.7 ng/mL; 95% CI 224.1-282.3 versus 166.2±85.9 ng/mL; 95% CI 136.7-195.7, p<0.001). **Conclusions**: The LNS-VM regimen appeared to offer protection against phosphate and potassium loss. This offers potential opportunities to improve care and support of poorly nourished HIV-infected patients. Malnourished HIV/AIDS patients attained a relatively higher blood tenofovir concentration that was inversely correlated with their BMI. High drug concentrations may predispose patients to drug-related adverse events.

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ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
ARI	Acute Renal Injury
ART	Antiretroviral Therapy
ARV	Antiretroviral Drug
BMI	Body Mass Index
CASR	Ca^{++}/Mg^{++}-sensing receptor
Cr	Creatinine
EDCTP	European and Developing Countries Clinical Trials Partnership
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
K^+	Potassium
LNS	Lipid-Based Nutrient Supplementation
LSHTM	London School of Hygiene and Tropical Medicine
MDGs	Millennium Development Goals
Mg^{++}	Magnesium
NUSTART	Nutritional Support for Africans Starting Antiretroviral Therapy
PO_4^-	Phosphate
ROS	Reactive Oxygen Species
TDF	Tenofovir Disoproxil Fumarate
UNZABREC	University of Zambia Biomedical Research Ethics Committee
UTH	University Teaching Hospital

CHAPTER 1

INTRODUCTION

Since the time antiretroviral therapy (ART) was introduced, management of patients infected with human immunodeficiency virus (HIV) has been a great success worldwide in as far as viral suppression is concerned (Hull and Montaner, 2010, Rockstroh et al., 2013). However, in HIV endemic regions and sub-Saharan Africa in particular, high rates of early mortality within the first 3 months of ART initiation remain a formidable challenge in malnourished patients (Stringer et al., 2006, Chisenga et al., 2015, Filteau et al., 2015). Advanced HIV-infection combined with under-nutrition and tenofovir-based ART places HIV/AIDS patients at high risk of electrolyte abnormalities leading to increased morbidity and consequent mortality (Maggi et al., 2012b).

In sub-Saharan Africa and Zambia in particular, a high number of HIV/AIDS patients starting ART die but the causes remain unclear (Heimburger et al., 2010). However, HIV infection, malnutrition and tenofovir-based ART are known to contribute to renal tubular injury resulting in life-threatening electrolyte loss (Maggi et al., 2012a). For instance, malnutrition is linked to depletion or altered metabolism of vitamins and minerals (Musoke and Fergusson, 2011). Similarly, tenofovir-based ART can exacerbate bone loss leading to electrolyte wasting (Cotter and Mallon, 2011). Renal injury and subsequent electrolyte wasting can occur with HIV infection per se (Kalyesubula et al., 2014). Thus, the pathophysiological overlaps of malnutrition, HIV infection and/or tenofovir-based ART affect renal function that could drive clinically significant electrolyte losses.

For instance, studies have shown that tenofovir-based antiretroviral therapy induces proximal tubular injury and Fanconi syndrome (Hall et al., 2009a, Baxi et al., 2014, Kalyesubula and Perazella, 2011). The hallmark of this renal injury is renal phosphate wasting.

Despite the reports of association with renal toxicity, tenofovir remains a component of first line treatment regimen in resource limited countries such as Zambia due to its excellent efficacy and cost effectiveness (Jimenez-Nacher et al., 2008b, Jimenez-Nacher et al., 2008a, Wandeler et al., 2012). However, there is increasing acknowledgement of the potential for tenofovir nephrotoxicity especially in undernourished patients (Herlitz et al., 2010). Therefore, recent guidelines recommend monitoring of renal function in HIV patients receiving tenofovir-containing regimen (Nishijima et al., 2011). Unfortunately, frequent laboratory monitoring for toxicity in resource limited countries is a huge challenge due to paucity of cheap monitoring tools, trained health personnel and laboratory facilities among other things (Lamorde et al., 2014). Therefore, management of HIV-infected patients in poor settings, if appropriate interventions, cheaper, effective and sustainable monitoring methods are not found, patients at higher risk of renal dysfunction will forever be faced with undiagnosed drug toxicities. Further, at the time this study was carried out there was insufficient data on renal handling of electrolytes. Furthermore, despite the fact that many studies demonstrated low serum levels of electrolytes in HIV positive patients, there was little known regarding electrolyte-drug interactions. This wide knowledge gap warranted the need for the present study to be undertaken.

The current study investigated the effects of a nutrition supplement with added minerals and vitamins on the renal excretion of electrolytes and tenofovir concentrations in blood among malnourished HIV/AIDS patients initiating tenofovir-based treatment. The study hypothesis is that a lipid-based nutrient supplement containing vitamins and minerals (LNS-VM) during tenofovir based therapy in HIV/AIDS is associated with improved renal excretion of electrolytes compared to a lipid-based nutrient (LNS) alone. To achieve this, several blood and spot urine experimental assays were conducted to determine creatinine, potassium, magnesium and phosphate concentrations using spectrophotometric methods. These were investigated because they are essentially dependent on the kidney for their excretion from the human body. Since tenofovir-based antiretroviral therapy is known to (a) contribute to proximal renal tubular dysfunction resulting in excessive loss of electrolytes via the kidney, and (b) be affected by fat (high lipid) meals that lead to increased tenofovir bioavailability, blood concentrations of tenofovir were also determined. Tenofovir in serum and dried blood spot (DBS) was assayed by chromatographic methods. Specifically, HemaSpot dried blood spot using LC-MS/MS was used with a view of providing evidence for its cost effectiveness in terms of requiring only 3 drops of blood and storage without refrigeration for low and middle income countries. This study was nested under the Nutritional Support for Africans Adults Starting Antiretroviral Therapy (NUSTART) trial. Briefly, the NUSTART trial was a phase III randomized controlled trial that was initiated in 2011 in Zambia and Tanzania. The NUSTART trial was registered as PACTR201106000300631.

CHAPTER 2

HIV INFECTION AND NUTRITION

2.1 HIV Infection and Metabolic Disorders

The Human Immunodeficiency Virus (HIV) is an RNA blood-borne retrovirus that attacks the immune system of the human body (Cunningham et al., 2010). HIV uses a range of viral enzymes to incorporate itself into human DNA in certain types of immune cells (Bushman et al., 1990, Cervio, 2009, Maplanka, 2007, Ji et al., 2006, Engelman et al., 1991). Once present in the DNA strand, HIV can use the cell's own mechanisms to produce more HIV viral particles, to infect more human immune cells. HIV binds to receptors such as CD4, CCR5 and CXCR4 on the surface of human immune cells, primarily CD4+ T-helper immune cells also known as CD4+ cells that fight infections in the body (Clapham and McKnight, 2001). The HIV virus then gains entry into the cell cytoplasm, where an enzyme called viral reverse transcriptase creates viral DNA from HIV RNA. This DNA moves into the cell nucleus, where it is incorporated into the human DNA strand by way of viral integration catalyzed by the enzyme called integrase. As normal cellular DNA transcription takes place, the HIV DNA within the human strand is also transcribed, producing HIV-derived mRNA. This mRNA is then translated into the proteins required to generate more HIV viral particles by viral protease enzyme (Cohen, 2007, Engelman et al., 1991). If this process is left unimpeded, the HIV infection will rapidly spread amongst all CD4+ immune cells, eventually exhausting their ability to defend the body and destroying them at the same time (Weiss, 1993, Douek et al., 2009, Zhang et al.,

2015). These cells are critical to the human immune defences against a range of other pathogens. The loss of CD4 cells renders the human body vulnerable to a number of opportunistic infections, cancers and other diseases (WHO, 2011, Masur, 2015, Pollock et al., 2015, Galisteu et al., 2015, Mitiku et al., 2015). ARV drugs work by inhibiting the various viral enzymes critical to the HIV replication cycle, specifically reverse transcriptase, integrase and protease (Figure 2.1).

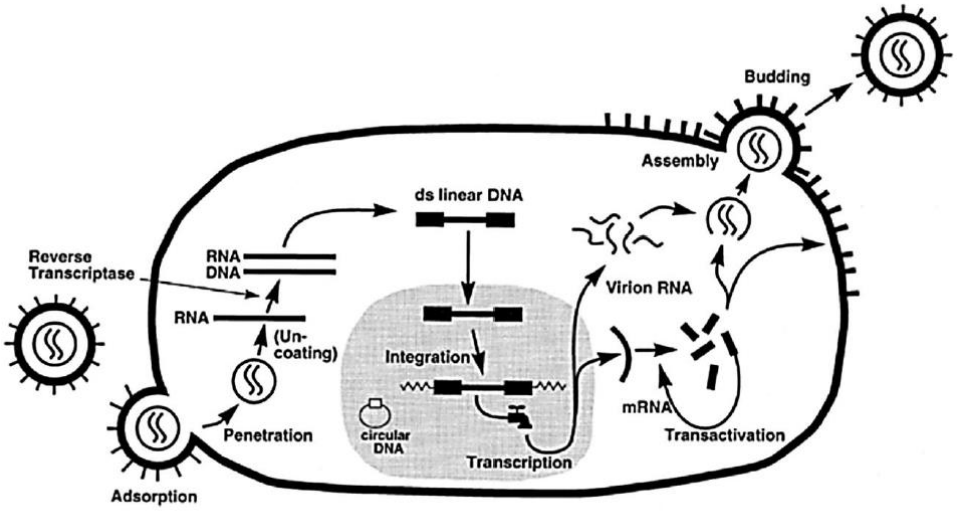

Figure 2.1: Replication cycle of HIV. Main steps in the HIV-1 replication cycle involve, HIV binding to a specific type of CD4 receptor and co-receptor on the surface of the CD4 cell; a special enzyme called reverse transcriptase changes the genetic material of the virus, so it can be integrated into the host cell DNA; the virus' new genetic material enters the nucleus of the CD4 cell and uses an enzyme called integrase to integrate itself into the host cell genetic material; the virus uses host enzymes to create more of its genetic material; a special enzyme called protease cuts the longer HIV proteins into individual proteins, when these come together with the virus' genetic material, a new virus assembles; the new viruses bud off and are released to infect more immune cells (Source: Adapted from Sakuma et al., 2012).

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When HIV severely damages the immune system, it leads to Acquired Immune Deficiency Syndrome (AIDS) (Weiss, 1993, Douek et al., 2009). HIV infection can be acute or chronic. Acute HIV infection is the earliest stage that develops generally within 2 to 4 weeks after infection has taken place (James and Kligman, 2006, Kahn and Walker, 1998). At this stage of infection the virus multiplies rapidly throughout the body and symptoms include but not limited to fever, upset stomach, esophageal sores, headache, and rash. The second stage is the chronic infection of the virus also known as clinical latency stage or seroconversion stage (Burton et al., 2002, Clapham and McKnight, 2001). During this stage HIV continues to multiply but a very slow rate. The stage 2 can present with or without symptoms and if not treated it advances to AIDS in 10 to 12 years. AIDS is the final stage of HIV infection in which HIV destroys the immune system to the extent that the body can longer fight off opportunist infections and cancer. The symptoms during this time can range from weight loss, chronic diarrhea, nausea and vomiting, night sweats, fever, mouth and skin problems and serious illnesses or diseases (Guss, 1994, Holmes et al., 2003). Without treatment the infected person in this stage can typically survive about 3 year (Holmes et al., 2003). The staging system for HIV infection and disease in adults and adolescents was first produced in 1990 and later updated in 2005 by the World Health Organization. Clinically, WHO classifies HIV infection into 4 stages; Stage 1 is when the CD4+ cell count is at least 500 cells/µL, stage 2 when the CD4+ cell count is 350 to 499, stage 3 when the CD4+ cell count is 200 to 349 and stage 4 is when the CD4+ cell count is less than 200 cells/µL or the percent

of CD4+ cells is less than 15% of all lymphocytes (WHO, 2011, Morgan et al., 2002).

In HIV-infected patients the prevalence of nutritional abnormalities has been well documented. HIV infection has been shown to alter nutrition in infected persons through various modes especially changes in food intake. In addition to impaired swallowing from oral and esophageal opportunistic infections, diarrhea from parasitic infections and HIV enteropathy in advanced disease can lead to malabsorption of fat and carbohydrates (Babameto and Kotler, 1997, Stack et al., 1996, Pencharz, 1997, Paton et al., 2003, Arenas-Pinto et al., 2015). In a study to characterize enteropathy in Zambian AIDS patients, Kelly and co-workers found that patients with HIV related diarrhea had reduced villous height and increased crypt depth compared with controls. He concluded that nutritional disturbances were associated with enteropathy, accounting for over one third of the variation in mucosal morphometric parameters (Kelly et al., 1997). In a related study among Zambian children Amadi reported that although intestinal and systemic infections did not differ for HIV-seropositive and HIV-seronegative children, HIV influenced nutritional status of all children (Amadi et al., 2001) and mortality was attributable to diarrhea. In fact, in the era before HIV treatment was readily available persistent diarrhea occurred in up to 75% of patients with HIV/AIDS in Western populations (Dworkin et al., 1985, Antony et al., 1988, Smith et al., 1988, Rene, 1989, Rolston et al., 1989) and in up to 100% of those infected in some developing countries (Colebunders et al., 1987). This is because the patients with HIV are at high risk of gastrointestinal tract infections

resulting in either loss of micronutrients through diarrhea or poor absorption of nutrients due to impaired intestinal mucosa. Studies have long shown that gut immune cells are potential targets for direct HIV infection (Griffin et al., 1996). Moreover, HIV infection is principally a mucosal disease and the gastrointestinal tract is the major site of HIV replication, which results in massive depletion of lamina propria CD4+ T cells (Brenchley et al., 2004, Mehandru et al., 2004, Mehandru et al., 2007, Cavarelli et al., 2013, Rossetti et al., 2013). As such immunocompromised patients, even when asymptomatic, need an increased food intake of up to 400 kcal/day (10-15% increased energy intake), with a minimum consumption of 2,500 kcal/day (Macallan et al., 1995, Grunfeld et al., 1992, Bell et al., 1993, Stack et al., 1996, Schwenk et al., 1996).

A study that assessed the effect of persistent HIV infection on host metabolism found that HIV-infected patients had 8% ($P < 0.05$) higher rates of resting energy expenditure than did control subjects without HIV infection. Fat-oxidation rates were significantly higher in the HIV patients (means +/- SE: 2.90 +/- 0.08 vs 2.19 +/- 0.17 g.kg FFM-1.d-1, patients vs. control subjects, $P < 0.01$). The results indicate that HIV infection affects host metabolism in the early asymptomatic stage, before CD4+ T cell numbers start to decline (Hommes et al., 1991). There is evidence that a critical interplay exists between HIV infection and adipose tissue storage and protein metabolism (Mody et al., 2014). HIV infection has also been associated with a significant down-regulation of whole-body protein flux (Paton et al., 2003).

Micronutrients play important roles in maintaining immune function and neutralizing the reactive oxygen intermediates produced by activated macrophages and neutrophils in their response to microorganisms (Semba and Tang, 1999). Serum or plasma measurements of vitamins and trace elements have shown that deficiencies are common among HIV-infected persons, especially women in developing countries. Many of the data on micronutrients and HIV/AIDS have come from observational studies suggesting that the B vitamins, vitamin C, and vitamin E, all potent antioxidants, are associated with reduced risk of HIV complications and progression (Abrams et al., 1993, Tang et al., 1993).

2.2 Nutrient Deficiency in HIV Infection

In Africa, where more than 25 million people are living with HIV/AIDS, nutrition deficiency is endemic as well. Sub-Saharan Africa is the region hardest hit by the HIV epidemic and has 67% of the estimated 33 million people living with HIV/AIDS worldwide (Ahoua et al., 2011, Hajizadeh et al., 2014, Nathan et al., 2003, Sharma et al., 2015, Stabinski et al., 2015). Malnutrition in general has also been shown to hasten HIV disease progression and mortality (Koethe and Heimburger, 2010).

In 2010 there were 925 million undernourished people in the world. Malnutrition contributed 1.4% to all disability adjusted life years (Murray et al., 2012). One of the most affected regions is sub-Saharan Africa. In this region, a significant proportion of patients who require antiretroviral therapy are malnourished because of a combination of HIV-associated wasting and

inadequate nutrient intake (Chapman et al., 2003). For that reason, adjuvant micronutrients therapy has been encouraged in the management of HIV/AIDS patients to avert or ameliorate the complications of AIDS related infections and slow the progression of the disease (Olayemi et al., 2012). A study that examined the availability of nutritional support services in HIV care and treatment sites across sub-Saharan African countries found that availability of nutritional support services was high but efforts to determine uptake, quality and effectiveness of services and their impact on patient and programme outcomes required further research (Anema et al., 2012, Anema et al., 2011).

The prevalence of biochemical abnormalities in HIV-infected patients has been well documented. One study found that in HIV-infected patients with CD4 count of less than 200cell/mm^3, 9.2% had low serum calcium (versus 0.5% if CD4 count > 200cell/mm^3, $p<0.002$), 11.4% had increased creatinine plasma level (versus 2.3% if CD4 count > 200cell/mm^3, $p<0.0001$) and 24.5% had low serum bicarbonate (versus 13.7% if CD4 count > 200cell/mm^3, $p<0.0001$) (Bagnis et al., 2009). Moreover, low body mass index in HIV-infection has been associated with complications that include electrolyte disorders, micronutrient deficiencies, and severe infections, which contribute to the high mortality (Mwagomba et al., 2010, Heimburger et al., 2010, Musoke and Fergusson, 2011). This is because it is assumed that malnutrition contributes to depletion or altered metabolism of vitamins and minerals (Musoke and Fergusson, 2011). Similarly, antiretroviral therapy has been implicated in bone mineral loss leading to mineral wasting in HIV patients (Cotter and Powderly, 2011).One study

conducted in Ivory Coast, reported a significantly lower level of Selenium but not vitamins A and E in HIV–infected patients. This study involved 30 asymptomatic HIV1-infected patients, aged from 18 to 50 years old. Selenium, vitamin E and vitamin A concentrations were evaluated but results showed that all patients were deficient in selenium only (0.58 +/- 0.12 micromol/L vs 1.80 +/- 0.31 micromol/L, $p < 0.0001$) (Djinhi et al., 2009). In the study conducted by Mehta et al., significantly lower levels of lipid soluble vitamins were reported in HIV-positive patients. This was an observational analysis of 1078 HIV-infected pregnant women enrolled in a trial of vitamin supplementation in Tanzania. Approximately 35, 39 and 51% of the women had low levels of vitamins A, D and E respectively (Mehta et al., 2010). In a study conducted by Baum et al., a significantly lower level of plasma antioxidant concentrations were observed in HIV/hepatitis C virus co-infected patients when compared with HIV-monoinfected controls. Significant differences were found between HIV/HCV-coinfected and HIV-monoinfected participants in vitamin A, (39.5 +/- 14.1 vs. 52.4+/-16.2 mug/dL, P=0.0004); vitamin E, (8.29+/-2.1 vs. 9.89+/-4.5 mug/mL, P=0.043); and zinc, (0.61+/-0.14 vs. 0.67+/-0.15 mg/L, P=0.0160 (Baum et al., 2011).These studies are consistent with the findings of Akiibinu and Olusegun. as shown in table 2.1 and 2.2. The following tables show trace element deficiency and oxidative stress in HIV/AIDS patients. Attempts to improve nutrition status in HIV/AIDS patients have not been completely successful. A study in France conducted by Ahoua et al found that 14% of the patients on nutritional rehabilitation did not recover from malnutrition (Ahoua et al., 2011).

Table 2.1: Levels of trace metals in symptomatic HIV/AIDS patients and controls

	N	Co (µg/dL)	Fe (µg/dL)	Zn (µg/dL)	Mn (µg/dL)	Cu (µg/dL)	Se (µg/dL)
Controls	65	63.5±6.8	75.8±8.2	122.1±18.3	65.4±13.1	71.5±16.0	57.8±11.7
HIV-patients	70	38.0±6.5	67.0±11.6	97.4±22.0	62.8±9.5	59.4±12.5	42.3±8.2
p values		<0.01*	<0.05*	<0.05*	>0.05	<0.05*	<0.01*

* = significantly different from the controls. N = number of subjects used in the study (Source: Akiibinu and Olusegun, 2012).

Table 2.2: Levels of antioxidants and markers of oxidative stress in HIV/AIDS patients and controls

	N	TAP (µMol Trolox equiv./ L)	TPP (µMol H_2O_2/L) (%)	OSI (nM/ml)	MDA (mg/L)	Vitamin C (mg/L)	Vitamin E (mg/L)
Controls	65	1652±380	11.8±4.3	0.71±0.46	8.2±3.2	22.6±8.7	12.7±5.3
HIV-patients	70	930±370	33.9±12.0	3.70±1.50	17.5±5.8	12.5±4.1	8.3±4.0
p values		<0.01*	<0.01*	<0.01*	<0.01*	<0.01*	<0.05*

* = significantly different from the controls. N = number of subjects used in the study Abbreviations: Total Antioxidant Potential (TAP), Total Plasma Peroxide (TPP), Oxidative Stress Index (OSI) and Malondialdehyde (MDA) (Source: Akiibinu and Olusegun, 2012).

A study conducted in the South of India that involved a total of 464 HIV-infected ART experienced and ART naïve patients analyzed for abnormalities in potassium, sodium, chloride and bicarbonate levels. Of these 278 (60%), 312 (67.2%), 319 (68.7%) and 176 (38%) had abnormal potassium, sodium, chloride

and carbonate levels respectively. Interestingly, patients who had abnormal potassium levels were significantly younger than those that reported normal levels (p=0.03). Patients with abnormal sodium levels had relatively lower BMI as compared with normal group (p=0.05) (Sundaram et al., 2010). In the abnormal chloride group, 102 (32%) individuals had some AIDS-defining illness as compared to 32 (26.9%) normal individuals. In the bicarbonate group 88 (30.6%) individuals had AIDS-defining illness compared to 53 (30.1%) normal individuals (p=0.03). In the multivariable analysis low absolute CD4 cell count and male gender were identified as significant co-factors of abnormal electrolyte levels in HIV disease (Sundaram et al., 2010). A two-by-two factorial randomized, controlled trial done in Zambia found low serum iron among children aged between 7 and 15 years (Nchito et al., 2004). Micronutrient deficiencies are well documented among infants in Zambia (Hautvast et al., 2000, Gitau et al., 2005, van Rheenen et al., 2008, Kafwembe et al., 2009) more than in adult populations. The prevalence of stunting among children in Zambia is considered severe at 25% among 6-to-8 month-old children nationally (Zambia Demographic and Health Survey, 2014). This might be the proxy for adult micronutrient deficiencies in Zambia. A study from Tanzania, reported that approximately 51% of the women had low levels of vitamins A, D and E respectively (Mehta et al., 2010). This study demonstrated that low concentrations of lipid-soluble vitamins were widely prevalent among HIV-infected women in Tanzania.

In one study, food enriched with animal protein was shown to enhance the content of absorbed iron in a household dietary study whose objective was to

enhance the content and bioavailability of iron of selected rice-and maize-based Philippine complementary foods (Perlas and Gibson, 2005). By contrast, a study carried out in Malawian children showed that the community-based intervention reduced the predicted prevalence of inadequate intakes of protein, calcium, zinc and vitamin B12, but not iron. In this study intervention children had diets that were significantly more diverse and of a higher quality than those of controls. Median daily intakes of protein, calcium, zinc, haem iron, vitamin B12 and animal foods were higher ($P < 0.05$) in the intervention group (Yeudall et al., 2005).

HIV has been implicated in lowering the total antioxidant potential in HIV-infected lactating mothers. The study found that breast milk of HIV-infected lactating mothers had low antioxidant capacity (Rahamon et al., 2013). Much has been learnt about the relationship between ART and nutrition, but much remains to be accomplished to ensure the effective integration of nutritional considerations into the effective and safe use of ART in HIV-infected patients (Raiten, 2011). It is now known that in as much as ARVs are essential to prolong lives, sound nutrition is essential to efficacious implementation of ARVs. On the other hand key multilateral, US agencies, public and private organizations reached consensus on role of food and nutrition in achieving global health promotion and disease prevention goals (Raiten et al., 2011). Another study demonstrated that provision of high levels of vitamins and minerals to patients referred to ART, delivered with substantial macronutrients, increased nutritional recovery in patients with body mass index of < 18.5 kg/m^2 (Rehman et al., 2015b). Studies have demonstrated increased mortality in HIV-infected patients

with hyponatremia. A study that was done in 1990s showed that 30 of the 96 HIV-infected patients had hyponatremia, for an incidence rate of 31.3%. Of the 30 hyponatremic patients, 20 had hyponatremia as outpatients and 10 had hyponatremia while in hospital. None of the patients had a serum sodium concentration higher than 150mmol/L. the CD4 cell count at the time of hyponatremia in 20 patients was 103.5±17.5 cells/mm^3 of blood. Approximately 70% of the patients with hyponatremia died as compared to a significantly lower rate of 36.4% of the patients without hyponatremia over a comparable period of follow up, $p<0.005$ (Cusano et al., 1990). A study that was conducted in Zambia and Kenya among 661 HIV-infected women showed that hyponatremia coupled with hypochloremia predicted death in HIV-infected patients. Women were more likely to die if they were both hyponatremic (sodium <135 mmol/L) and hypochloremic (chloride < 95 mmol/L). It was further observed that mortality was higher among women with both hyponatremia and hypochloremia compared with women who had only one electrolyte abnormality. This is because serum chloride and sodium levels are closely regulated together and conditions that result in hyponatremia can also cause hypochloremia. Therefore, monitoring of serum sodium and chloride was recommended to identify HIV-infected patients at highest risk for mortality during the first year of antiretroviral therapy (Dao et al., 2011).

By 2009, more than 800 million people worldwide were chronically undernourished, and 133 million were living with HIV infection. In an effort to improve the prognosis of HIV-1 infection in sub-Saharan Africa, a significant

proportion of malnourished HIV-positive patients eligible for therapy were recommended to concurrently start antiretroviral therapy and nutrient supplementation (Rehman et al., 2015b, Ndekha et al., 2009). Some studies however, have been inconsistent regarding benefits of micronutrients supplementation in HIV/AIDS patients, with some even showing worsened outcomes for certain supplements. Some studies reported that it remained unknown if pharmacologic doses of micronutrients were safe, favorable or contra-indicated in HIV/AIDS patients (Olsen et al., 2004, Tang et al., 2005b). Olsen argued that low doses (≤60 mg) of elemental iron are more beneficial than higher doses in that higher doses may promote HIV replication resulting in increased viral load and subsequent increase in the rate of progression to HIV/AIDS (Olsen et al., 2004). By contrast, a study on the impact of nutritional supplementation in HIV-positive patients starting ART provided evidence that at 6 months of antiretroviral therapy, patients in the nutritional supplement arm demonstrated a greater increase in weight (12.7% vs. 4.9%; p=0.047), BMI (7.8% vs. 5.5%; p=0.007), CD4+ count (83.0% vs. 46.4%; p=0.002) and hemoglobin (9.5% vs. 1.0%; p=0.026) when compared to controls on ART alone. Patients in the nutritional supplement arm also showed an increase in the number of red blood cells (7.0% vs. -4.5%; p=0.043) and white blood cells (28.6% vs. -2.8%; p=0.035) when compared to the controls. These results suggest that nutritional supplements if taken concomitantly with antiretroviral therapy could promote weight gain and improved immune response in HIV-positive patients that present at antiretroviral therapy initiation with weight loss (Evans et al., 2013). In people

living with HIV/AIDS, nutritional support is now increasingly recognized as a critical part of the essential package of care, especially for patients in sub-Saharan Africa. Concomitant administration of antiretroviral therapy and ready-to-use therapeutic food has been found to increase the chances of nutritional recovery in these high-risk patients (Ahoua et al., 2011). Nevertheless, potential renal function benefits have not been explored in undernourished HIV/AIDS patients taking nutrient supplementations together with antiretroviral drugs. Moreover, mortality rates in underweight patients have not been curtailed.

2.3 Renal Function in HIV Infection

In one of the early kidney research works, the renal function of 32 malnourished Jamaican children was investigated by serial tests from admission until recovery. It was established that in the malnourished as compared to the recovered children, there were several functional renal lesions. The etiology of these lesions was associated with deficiencies of magnesium and potassium and three of the patients presented with renal phosphaturia (Alleyne, 1967).

It is well documented that renal abnormalities are common in a large percentage of patients with HIV-infection. Studies done in Sub-Saharan Africa have reported increasing prevalence of renal dysfunction ranging from 6% in South Africa, 33.5% in Zambia and 48.5% in Uganda (Brennan et al., 2011, Mulenga et al., 2014, Izzedine et al., 2009a). The HIV-associated renal disease has been linked with progression to AIDS and death (Maggi et al., 2012a). A study that evaluated the incidence and risk factors for acute kidney injury in HIV-infected patients found that Black race and low body mass index (BMI) were

among factors that had significant but weak acute kidney injury associations. The study argues that although acute kidney injury incidence has decreased during the ART era, it remains common in HIV-infected patients and seems attributable to HIV-related factors (Li et al., 2012) Nevertheless, the pathogenesis of kidney tubular dysfunction is complex and may differ according to the etiological trigger (Ishikawa et al., 2010). Renal disease can be relatively complicated by other infections such as hepatitis C virus co-infected with HIV (Izzedine et al., 2009c). Other factors including opportunist infections and non-communicable diseases such as hypertension, diabetes mellitus and cardiovascular disease, have been implicated in renal dysfunction (Kalyesubula et al., 2014).

2.4 Renal Handling of Nutrients and Electrolytes

The kidneys play an extremely important function of clearing the blood of toxins and regulating the amounts of other substances dissolved in blood (Ganong, 2003). The actual excretion occurs in tiny functional units inside the kidney called nephrons (Ugwuja and Eze, 2006). Filtration of substances from the blood that enters the kidney is the first process in each nephron. The resultant filtrate flows along the length of the tubular structure of the nephron lined with a layer of specialized cells surrounded by blood capillaries. The main functions of the cells in the renal tubules are the reabsorption of water and small molecules from the filtrate into blood and the secretion of waste substances from the blood into the urine (Miller, 2013). The balances of electrolytes such as phosphate (PO_4^-), calcium (Ca^{2+}) and magnesium (Mg^{2+}), sodium (Na^+), potassium (K^+), and chloride (Cl^-) are linked via regulatory systems with

overlapping effects and are also controlled by systems specific to each of them as shown in Table 2.3.

Table 2.3: Homeostatic balance of selected substances by kidney in the human body

Substance	Description	Proximal tubule	Loop of Henle	Distal tubule	Collecting duct
Potassium	Varies upon dietary needs.	Reabsorption (65%)	reabsorption (20%, thick ascending, Na-K-2Cl symporter)	–	secretion via Na+/K+-ATPase, increased by aldosterone), or reabsorption hydrogen potassium ATPase)
Phosphate	Excreted as titratable acid.	Reabsorption (85%) via sodium/phosphate cotransporter. Inhibited by parathyroid hormone.	–	–	–
Magnesium	Calcium and magnesium compete, and an excess of one can lead to excretion of the other.	reabsorption	reabsorption (thick ascending)	reabsorption	–
Glucose	If glucose is not reabsorbed by the kidney, it appears in the urine, in a condition known as glycosuria.	reabsorption (almost 100%) via sodium-glucose transport proteins and GLUT	–	–	–
Sodium	Uses Na-H antiport, Na-glucose symport, sodium ion channels	reabsorption (65%, isosmotic)	reabsorption (25%, thick ascending, Na-K-2Cl symporter)	reabsorption (5%, sodium-chloride symporter)	reabsorption (5%, principal cells), stimulated by aldosterone via ENaC
Chloride	Usually follows sodium. Active and passive	reabsorption	reabsorption Na-K-2Cl symporter	reabsorption sodium-chloride symporter	–

2.4.1 Potassium

Potassium is the most important cation of the intracellular fluid. Average concentration is 150mEq/L of intracellular fluid. Extracellular potassium is important for its controlling influence upon neuromuscular irritability, cardiac muscle and the operation of Na+/K+-ATPase (Na+ pump) against the concentration gradient (He and MacGregor, 2008). In cells there is a significant concentration gradient of sodium and potassium across cell membranes.

Maintenance of potassium balance depends primarily on excretion by the kidneys as shown in Figure 2.2, since the amount excreted in the fecal matter is only about 5 to 10 per cent of the potassium intake. Thus, the maintenance of normal potassium balance requires the kidneys to adjust their potassium excretion rapidly and precisely to wide variations in intake, as is also true for most other electrolytes (Guyton, 2006, Gibson, 2005). Potassium excretion is determined by the sum of three renal processes: (1) the rate of potassium filtration, (2) the rate of potassium reabsorption by the tubules, and (3) the rate of potassium secretion by the tubules.

Potassium (K^+) is freely filtered by the glomerulus. Severe decreases in glomerular filtration rate (GFR) in certain renal diseases, can cause serious potassium accumulation resulting in hyperkalemia (Giebisch, 2002). The bulk of filtered K^+ is reabsorbed in the proximal tubule and loop of Henle, such that less than 10% of the filtered load reaches the distal nephron. In the proximal tubule, K^+ absorption is primarily passive and proportional to Na^+ and water. Potassium reabsorption in the thick ascending limb of Henle occurs through both

transcellular and paracellular pathways. The transcellular component is mediated by K^+ transport on the apical membrane Na^+-K^+-$2Cl^-$ cotransporter. K^+ secretion begins in the early distal convoluted tubule and progressively increases along the distal nephron into the cortical collecting duct. Most urinary K^+ can be accounted for by electrogenic K^+ secretion mediated by principal cells in the initial collecting duct and the cortical collecting duct. An electroneutral K^+ and Cl^- cotransport mechanism is also present on the apical surface of the distal nephron (Velazquez et al., 1987). Under conditions of K^+ depletion, reabsorption of K^+ occurs in the collecting duct. This process is mediated by upregulation in the apically located H^+-K^+-ATPase on α-intercalated cells (DuBose et al., 1995).

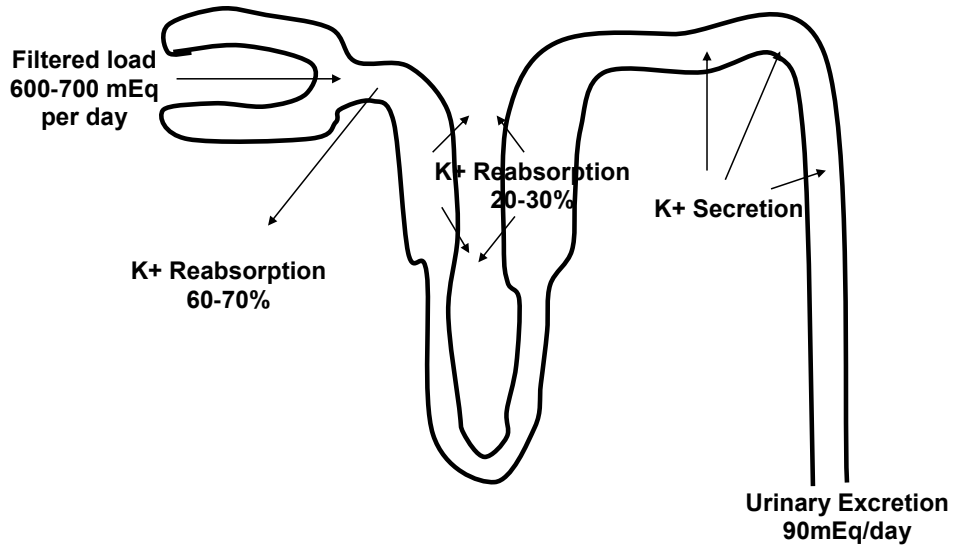


Figure 2.2: Handling of potassium by the nephron of the kidney in healthy individuals. About 65% of the filtered potassium is reabsorbed in the proximal tubule. Another 20 to 30% of the filtered potassium is reabsorbed in the loop of Henle, especially in the thick ascending part where potassium is actively co-transported along with sodium and chloride. In both the proximal tubule and the loop of Henle, a relatively constant fraction of the filtered potassium load is reabsorbed. (Source: modified from Giebisch, 2002).

The high intracellular potassium is maintained by an energy requiring extrusion of the 3 sodium ions out of the cell with the replacement by 2 potassium ions. Intracellular potassium is essential for a number of enzyme reactions for maintaining osmotic and acid-based balance. Potassium is required for the functioning of nerves, skeletal muscle and cardiac muscles. Either decreased or increased potassium levels finally cause cardiac arrest. Potassium is required as a cofactor in several enzymatic reactions in the body. Potassium is involved in acid-based balance. The potassium contained in a single meal is often as high as 50 milliequivalents, and the daily intake usually ranges between 50 and 200 mEq/day; therefore, failure to rapidly rid the extracellular fluid of the ingested potassium could cause life-threatening hyperkalemia. Likewise, a small loss of potassium from the extracellular fluid could cause severe hypokalemia in the absence of rapid and appropriate compensatory responses. The normal range of potassium in serum is 3.5 – 5.0 mEq/L. In plasma 3.5 – 4.5 mEq/L. serum potassium levels above 7 mEq/L and below 2.5 mEq/L is serious, life threatening and requires immediate attention. Control of potassium distribution between the extracellular and intracellular compartments also plays an important role in potassium homeostasis. Because over 98 per cent of the total body potassium is contained in the cells, they can serve as an overflow site for excess extracellular fluid potassium during hyperkalemia or as a source of potassium during hypokalemia. Thus, redistribution of potassium between the intra- and extracellular fluid compartments provides a first line of defense against changes in extracellular fluid potassium concentration (Guyton, 2006).

The most important sites for regulating potassium excretion are the principal cells of the late distal tubules and cortical collecting tubules. In these tubular segments, potassium can at times be reabsorbed or at other times be secreted, depending on the needs of the body. With a normal potassium intake of 100 mEq/day, the kidneys must excrete about 92 mEq/day (the remaining 8 milliequivalents are lost in the feces). About one third (31 mEq/day) of this amount of potassium is secreted into the distal and collecting tubules (Giebisch, 2002). With high potassium intakes, the required extra excretion of potassium is achieved almost entirely by increasing the secretion of potassium into the distal and collecting tubules. In fact, with extremely high potassium diets, the rate of potassium excretion can exceed the amount of potassium in the glomerular filtrate, indicating a powerful mechanism for secreting potassium (Guyton, 2006). When potassium intake is reduced below normal, the secretion rate of potassium in the distal tubules and collecting tubules decreases, causing a reduction in urinary potassium secretion. With extreme reductions in potassium intake, there is net reabsorption of potassium in the distal segments of the nephron, and potassium excretion can fall to 1 per cent of the potassium in the glomerular filtrate (to less than 10 mEq/day) (Rosa et al., 1990). With potassium intakes below this level, severe hypokalemia can develop. Thus, most of the day-to-day regulation of potassium excretion occurs in the late distal and cortical collecting tubules, where potassium can be either reabsorbed or secreted, depending on the needs of the body.

The cells in the late distal and cortical collecting tubules that secrete potassium are called principal cells and make up about 90 per cent of the epithelial cells in these regions. Secretion of potassium from the blood into the tubular lumen is a two-step process, beginning with uptake from the interstitium into the cell by the sodium-potassium ATPase pump in the basolateral membrane of the cell. This pump moves sodium out of the cell into the interstitium and at the same time moves potassium to the interior of the cell. The second step of the process is passive diffusion of potassium from the interior of the cell into the tubular fluid. The sodium-potassium ATPase pump creates a high intracellular potassium concentration, which provides the driving force for passive diffusion of potassium from the cell into the tubular lumen. The luminal membrane of the principal cells is highly permeable to potassium. One reason for this high permeability is that there are special channels that are specifically permeable to potassium ions, thus allowing these ions to diffuse across the membrane.

The fractional excretion of an electrolyte can be used as a biomarker to detect early stages of renal injury (Gheissari et al., 2011). For instance, the fractional excretion of potassium (FEK+) in normal subjects is 8% (range 4-16%). The work carried out by Elisaf in1995 showed that the mean FEK+ in patients with hypokalemia of extra-renal origin was 2.8% (range 1.5-6.4) while the mean FEK+ in hypokalemic patients in whom renal potassium loss was the main etiologic factor for the pathogenesis of hypokalemia was found to be 15% (range 9.5-24%) (Elisaf and Siamopoulos, 1995). Later it was discovered that

hypokalemia co-exist with hypomagnesaemia in certain type of patients such as alcoholic patients (Elisaf et al., 2002). The mechanism by which this occurs is not fully elucidated but it is now known that it is the inhibition of the sodium chloride co-transporter in the distal convoluted tubule that is associated with hypokalemia and hypomagnesaemia (Assadi, 2010).

2.4.2 Phosphate

The second most abundant electrolyte in the human body is phosphate. Phosphate accounts for 6.5 – 11g/kg body weight in adults and approximately 85% of phosphate is present in the bone matrix as calcium phosphate (Cogan, 1991, Gamblin et al., 2014, Oreffo et al., 1998, Weinges et al., 1953). Phosphate is an essential factor in all the energy-producing reactions of the cells in the body. Phosphate also plays vital functional roles in many physiological buffer systems and the activation of many catalytic proteins by the process of phosphorylation. An estimation of 55% - 70% of all phosphate from nutrition taken orally is absorbed by healthy adults. Most phosphate in the diet is absorbed by a passive, concentration dependent process, while a small portion is absorbed by active transport facilitated by 1,25-dihydroxyvitamin D (Blaine et al., 2015, Park et al., 2011).

The kidney has a major role in regulating phosphate balance as shown in Figure 2.3. More than 80% of absorbed phosphate is excreted by the kidney, so that in healthy adults urinary phosphate reflects the amount of absorbed dietary phosphate. When dietary intakes of phosphate are low, renal excretion of phosphate is reduced by a mechanism that is not well established. Dietary

deficiency of phosphate is rare, as phosphate intakes generally exceed requirements. Moreover, in the absence of disease, parathyroid and renal mechanisms function to conserve body phosphate. Depletion of phosphate generates hypophosphatemia. It is accompanied by a reduction in urinary phosphate excretion and an increase in urinary excretion of calcium, magnesium and potassium. As a result, phosphate deficiency can manifest in a wide variety of clinical complications. These include impairment of oxygen delivery, failure of muscle contractility, severe muscle weakness, anorexia, bone pain, and cardiac and respiratory failure.

Serum phosphate concentrations tend to fall within a narrow range (0.8 – 1.6mmol/L). They are regulated largely by the tubular reabsorptive capacity of the kidney under the influence of the parathyroid hormone (Figure 2.3) (Harrison, 1984), and other factors that stimulate change in renal phosphate clearance. Serum phosphate concentrations indicative of hyperphosphatemia are >1.5mmol/L in adults and >2.0mmol/L in children (Chen et al., 1974). A renal abnormality such as fanconi syndrome, renal tubular acidosis and connective tissue tumors may result in hypophosphatemia. Fanconi syndrome is a condition of the renal proximal renal tubules (Magen et al., 2010) in which, glucose, amino acids, uric acid, phosphate and bicarbonate are excreted into the urine, without being reabsorbed. Fanconi syndrome involves the renal proximal tubule, which is the first part of the nephron tubule to process fluid after it is filtered through the process of glomerular filtration. The condition may be inherited, caused by drugs or heavy metal toxicity.

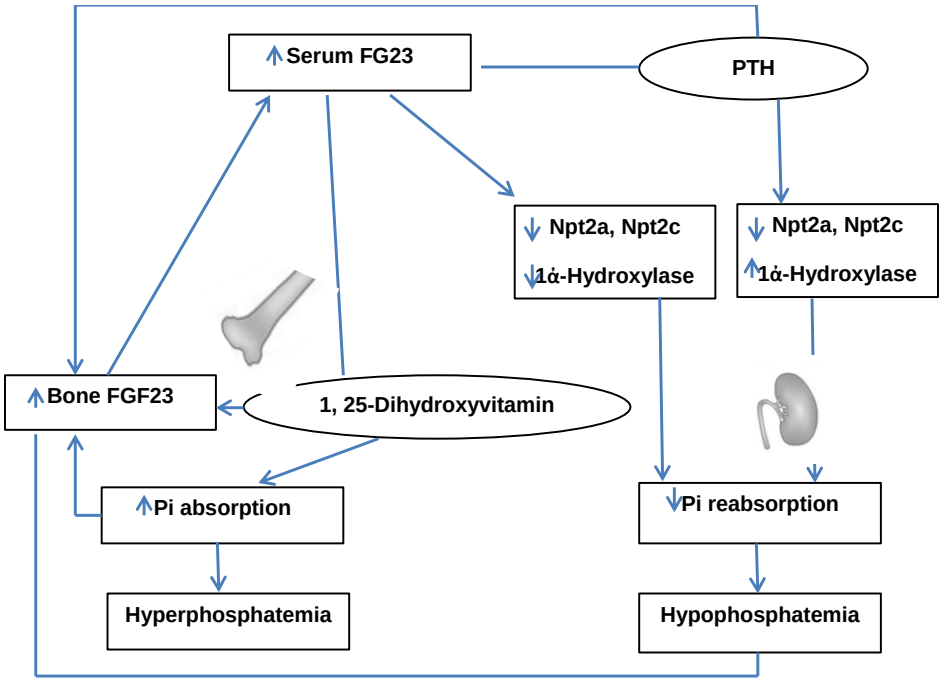


Figure 2.3: Renal handling of phosphate. The parathyroid hormone (PTH) regulates phosphate concentration through two effects: (1) PTH promotes bone resorption, thereby dumping large amounts of phosphate ions into the extracellular fluid from the bone salts, and (2) PTH decreases the transport maximum for phosphate by the renal tubules, so that a greater proportion of the tubular phosphate is lost in the urine. Thus, whenever plasma PTH is increased, tubular phosphate reabsorption is decreased and more phosphate is excreted (Source: Modified from Harrison, 1984).

The hypophosphatemia arises from excessive urinary losses of phosphate (Calvo, 1994). Phosphate excretion by the kidneys is controlled primarily by an overflow mechanism that can be explained as follows: The renal tubules have a normal transport maximum for reabsorbing phosphate of about 0.1 mM/min. When less than this amount of phosphate is present in the glomerular filtrate, essentially all the filtered phosphate is reabsorbed. When more than this is present, the excess is excreted. Therefore, phosphate normally begins to spill into the urine when its concentration in the extracellular fluid rises above a threshold of about 0.8 mM/L, which gives a tubular load of phosphate of

about 0.1 mM/min, assuming a GFR of 125 ml/min. Because most people ingest large quantities of phosphate in milk products and meat, the concentration of phosphate is usually maintained above 1 mM/L, a level at which there is continual excretion of phosphate into the urine. Changes in tubular phosphate reabsorption can also influence phosphate excretion. For instance, a diet low in phosphate can, over time, increase the reabsorptive transport maximum for phosphate, thereby reducing the tendency for phosphate to spill over into the urine.

The homeostatic maintenance of normal serum phosphate levels is primarily achieved through a tightly regulated process of phosphate reabsorption from the glomerular filtrate. In the nephron, approximately 85% of phosphate reabsorption occurs within the proximal tubule (Figure 2.4). The remainder of the nephron plays a minor role in phosphate regulation and the transporters involved have yet to be identified (Blaine et al., 2015). However, it is known that transport of ultrafiltered phosphate across the proximal tubule epithelium is an energy-dependent process that needs sodium to take place (Forster et al., 2006). The energy derived from the transport of sodium drives the inorganic phosphate from the lumen filtrate into the cell through the renal sodium phosphate cotransporters located in the apical side of the renal tubule cells. The abundance of these cotransporters determines the amount of phosphate reabsorbed from the filtrate (Forster et al., 2006).Thus, nutritional factors or hormones that alter phosphate reabsorption in the kidney do so by changing the abundance of the sodium phosphate cotransporters in the apical membrane of renal proximal tubule cells.

An increase in the brush border levels of the sodium phosphate cotransporters abundance results in increased phosphate absorption from the urine, whereas a decrease in cotransporter abundance leads to phosphaturia (Azzarolo et al., 1991).

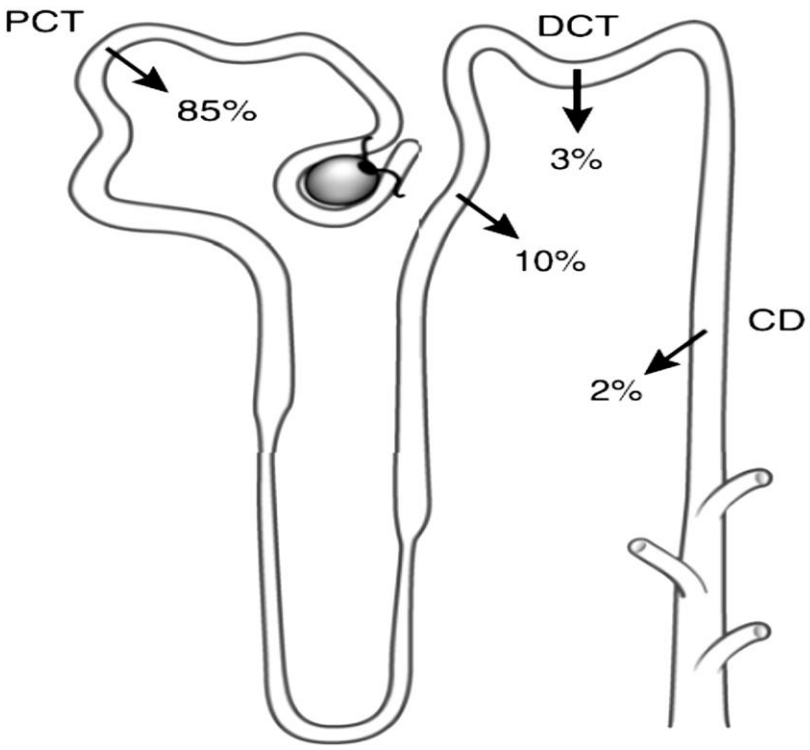


Figure 2.4: Phosphate reabsorption in the proximal tubule. Approximately 85% of phosphate reabsorption occurs in the proximal convoluted tubule. Approximately 10% of Pi reabsorption occurs in the loop of Henle, 3% occurs in the distal convoluted tubule, and 2% in the collecting duct via unidentified pathways. CD, collecting duct; DCT, distal convoluted tubule; PCT, proximal convoluted tubule (Source: Blaine et al., 2014).

2.4.3 Magnesium

Magnesium is the fourth most important cation in the body, with about 50% present in the bones associated with calcium and phosphate. Much of the remaining magnesium is intracellular and only a small amount is found in extracellular fluid (Elin, 1991). Magnesium functions as an activator for various physiochemical processes, including phosphorylation, protein synthesis, and DNA metabolism. It is also involved in neuromuscular conduction and excitability of skeletal and cardiac muscle. Ingested magnesium is absorbed in the intestine and the amount absorbed is inversely related to the total magnesium.

The kidneys effectively control magnesium homeostasis through tubular reabsorption, which conserves magnesium when intake is low and excretes excess when the intake is high. Increased serum magnesium concentrations occur in renal failure, acute diabetic acidosis, dehydration, and Addison's disease (Kyne and Lee, 1993). Hypermagnesemia has a depressing effect on the central nervous system, causing general anesthesia and respiratory failure. It alters the conduction mechanism of the heart, causing cardiac arrest. Hypomagnesemia may be observed in chronic alcoholism, malabsorption, severe diarrhea, acute pancreatitis, diuretic therapy, prolonged parenteral fluids therapy without magnesium supplementation, kidney disorders such as glomerulonephritis and tubular reabsorption defects. Decreased serum magnesium concentrations may result in tetany, convulsions and cardiac arrhythmias (Fetemi et al., 1991). Regulation of magnesium excretion is achieved mainly by changing tubular reabsorption. The proximal tubule usually reabsorbs only about 25 per cent of the

filtered magnesium. The primary site of reabsorption is the loop of Henle, where about 65% of the filtered load of magnesium is reabsorbed. Only a small amount, usually less than 5% of the filtered magnesium is reabsorbed in the distal and collecting tubules (Elin, 1991). In hypomagnesemic or hypocalcemic states, the rates of calcium and magnesium reabsorption in the loop of Henle are increased via Ca^{2+}/Mg^{2+}-sensing receptor-mediated stimulation of the Na^{+}-K^{+}-$2Cl^{-}$ cotransporter and the apical ROMK (renal outer medulla potassium) channel. By contrast, hypermagnesemia and hypercalcemia inhibit Na^{+}-K^{+}-$2Cl^{-}$cotransport and activity of the ROMK channel (Ryan, 1991). Assuming a normal GFR, the kidney filters approximately 2000–2400 mg of magnesium per day. This takes into account the fact that only 70% of total serum magnesium (30% is protein-bound) is available for glomerular filtration. Under normal conditions, 96% of filtered magnesium is reabsorbed in the renal tubules by several coordinated transport processes and magnesium transporters detailed below (Houillier, 2014, Ikari, 2013, Quamme and Dirks, 1983, Dimke et al., 2010, Haisch and Konrad, 2012).

As shown in Figure 2.5, 10%–20% of the filtered magnesium is absorbed in the proximal tubule. Although the exact mechanisms are not known, magnesium is believed to be absorbed *via* a paracellular pathway aided by a chemical gradient generated by Na gradient–driven water transport that increases intraluminal magnesium as well as lumen-positive potential.

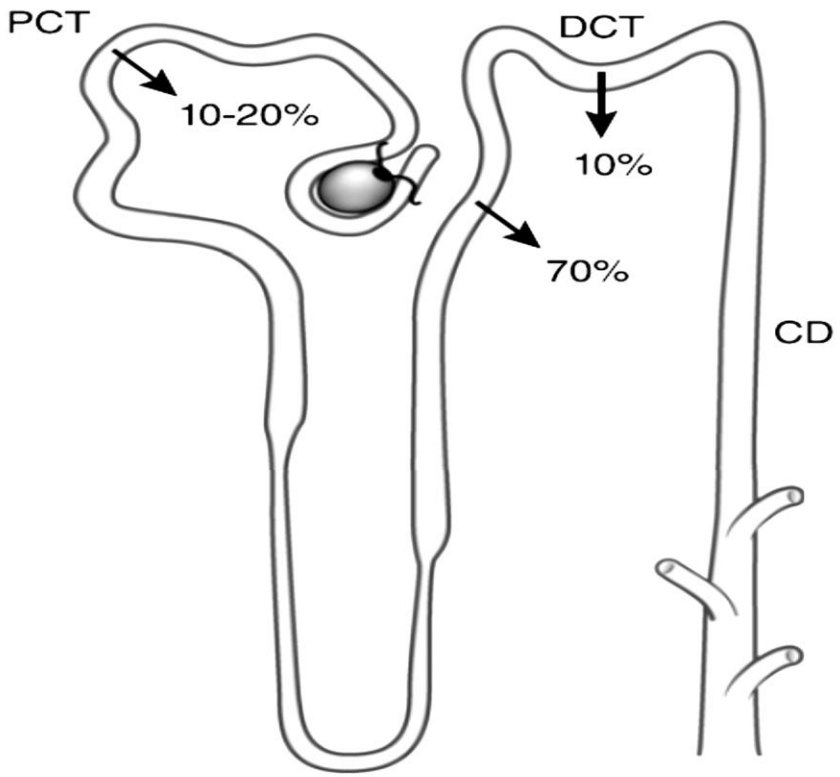


Figure 2.5: Magnesium handling in the renal tubules. Approximately 10%–20% of the filtered magnesium is absorbed in the proximal tubule, 70% of filtered magnesium is absorbed in the thick ascending limb, and the remaining 10% of magnesium is reabsorbed in the distal convoluted tubule. CD, collecting duct; DCT, distal convoluted tubule; PCT, proximal convoluted tubule (Source: Modified from Blaine et al., 2014).

A paracellular pathway in the thick ascending limb absorbs 40%–70% of filtered magnesium, mostly enhanced by lumen-positive transepithelial voltage, in which claudin-16 and claudin-19 play an important role. The NKCC2 cotransporter mediates apical absorption of Na, K, and Cl. The apical ROMK mediates apical recycling of K back to the tubular lumen and generation of lumen-positive voltage. The Cl channel ClC-Kb mediates Cl exit through the

basolateral membrane. Na, K-ATPase also mediates Na^+ exit through the basolateral membrane and generates the Na^+ gradient for Na^+ absorption. The tight junction proteins claudin-16 and claudin-19 play a prominent role in magnesium absorption (Figure 2.6) (Naderi and Reilly, 2008).

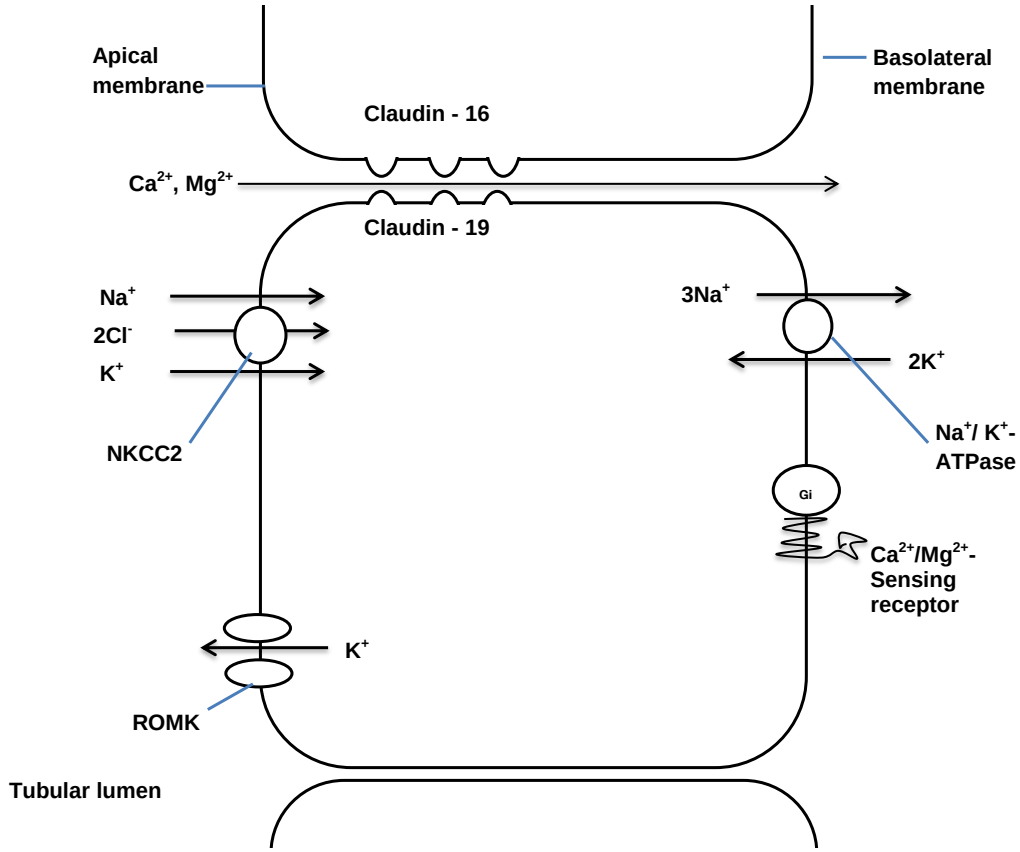


Figure 2.6: Magnesium transport in the thick ascending loop of Henle. Magnesium movement is a passive and paracellular transport mediated by claudin.16 and claudin-19. The lumen-positive electrical gradient is the driving force for paracellular magnesium transport and is dependent on potassium exit via renal outer medulla potassium channel (ROMK). Sodium entry and exit are mediated via the furosemide-sensitive Na^+-K^+-$2Cl^-$cotransporter (NKCC2) and the Na^+/K^+-ATPase, respectively. The Ca^+/Mg^{2+}-sensing receptor expressed in the basolateral membrane is an important regulator of ROMK and NKCC2 (Source: Modified from Naderi, 2008).

The Ca^{2+}/Mg^{2+}-sensing receptor has also been determined to regulate magnesium transport in this segment: upon stimulation, magnesium transport is decreased. Basolateral receptor activation inhibits apical K channels and possibly Na-2Cl-K cotransport in the rat thick ascending limb (Delpire et al., 1996, Hebert, 1995, Wang et al., 1996). This inhibition would be expected to diminish transepithelial voltage and, in turn, passive transport of magnesium within the cortical thick ascending limb. The remaining 5%–10% of magnesium is reabsorbed in the distal convoluted tubule mainly by active transcellular transport (Hou et al., 2009, Thumfart et al., 2008, Monnens et al., 2000).

More than one half of the body's magnesium is stored in the bones. Most of the rest resides within the cells, with less than 1 per cent located in the extracellular fluid. Although the total plasma magnesium concentration is about 1.8 mEq/L, more than one half of this is bound to plasma proteins. Therefore, the free ionized concentration of magnesium is only about 0.8 mEq/L (Assarzadegan et al., 2015, Bajpai et al., 1967, Harada et al., 1985, de Roij van Zuijdewijn et al., 2015). The normal daily intake of magnesium is about 250 to 300 mg/day, but only about one half of this intake is absorbed by the gastrointestinal tract. To maintain magnesium balance, the kidneys must excrete this absorbed magnesium, about one half the daily intakes of magnesium, or 125 to 150 mg/day. The kidneys normally excrete about 10 to 15 per cent of the magnesium in the glomerular filtrate (Quamme and Dirks, 1983). Renal excretion of magnesium can increase markedly during magnesium excess or can decrease to almost nil during magnesium depletion. Because magnesium is involved in many

biochemical processes in the body, including activation of many enzymes, its concentration must be closely regulated. The mechanisms that regulate magnesium excretion are not well understood, but the following disturbances lead to increased magnesium excretion: (1) increased extracellular fluid magnesium concentration, (2) extracellular volume expansion, and (3) increased extracellular fluid calcium concentration.

The kidney is the major regulator of total body magnesium homeostasis. Several mechanisms enable the kidney to regulate and maintain serum magnesium concentration within a narrow range. In the setting of hypomagnesemia, the kidney decreases magnesium excretion to as little as 0.5% of the filtered load. Conversely, in the setting of hypermagnesemia, up to 80% of the filtered load can be excreted. A proportion of circulating magnesium is protein bound, such that only 70% of total plasma magnesium is ultrafilterable (Guyton, 2006, Sands, 2015, Sgouralis and Layton, 2015). In adults, a small fraction of filtered magnesium is reabsorbed in the proximal tubule. In contrast to most other ions, which are primarily reabsorbed in the proximal tubule, the thick ascending limb of the loop of Henle is the main site of magnesium reabsorption. The Ca^{2+}/Mg^{2+}-sensing receptor, a member of the G-protein-coupled receptor family, is an important regulator of magnesium homeostasis. This receptor is located in the basolateral membrane of thick ascending limb cells and in the distal convoluted tubule, as well as in cells of the parathyroid glands that secrete parathyroid hormone (PTH). In hypomagnesemic or hypocalcemic states, the rates of calcium and magnesium reabsorption in the loop of Henle are increased

via Ca^{2+}/Mg^{2+}-sensing receptor-mediated stimulation of the Na^{+}-K^{+}-$2Cl^{-}$ cotransporter and the apical renal outer medulla potassium channel. By contrast, hypermagnesemia and hypercalcemia inhibit Na^{+}-K^{+}-$2Cl^{-}$ cotransport and activity of the renal outer medulla potassium channel channel (Blaine et al., 2015).

2.5 Antiretroviral Therapy in HIV Infection

Globally, treatment of HIV-infected individuals with highly effective ART has transformed the course of the disease (Simonetti and Kearney, 2015). Life expectancy for HIV-infected patients on therapy is now longer than previously reported (Ali et al., 2014). In an effort to mitigate the HIV/AIDS disease, provision of antiretroviral therapy (ART) for HIV-infected individuals has rapidly expanded in sub-Saharan Africa (WHO, 2011). For example, by 2005, ART coverage had expanded in Zambia to cater for approximately 12,000 patients as depicted in Figure 2.7. Figure 2.8, shows that tenofovir-based regimen has been one of the most efficacious in reducing viral load (De Clercq and Holy, 2005).

A study was designed to evaluate the anti-HIV-1 activity, safety, tolerance and pharmacokinetics of oral tenofovir-disoproxil Fumarate when administered as a single dose for 28 consecutive days. The study showed that the pharmacokinetic parameters were dose proportional. Reductions in plasma HIV-1 RNA were dose related at tenofovir DF doses of 75 to 300mg, but there was no increase in viral suppression between the 300mg and 600mg dose cohorts, despite dose proportional increases in drug exposure (Barditch-Crovo et al., 2001). In support of tenofovir-based regimen use in HIV- infected patients, a study on efficacy of tenofovir compared to stavudine was done. The study

showed HIV RNA less than 400 copies/ml at 48 weeks of treatment in both groups (Gallant et al., 2004), demonstrating the two drugs were bioequivalent. In the era of antiretroviral therapy, longer lifespans have been made possible in people living with HIV-infection but comorbid conditions are now more common due to a mix of chronic immune activation, medication side effects, co-infections and aging itself (Wools-Kaloustian et al., 2007). Medication related side effects include kidney diseases, neurological disorders and bone disorders (Narayan et al., 2014). Multiple studies have assessed the impact of ART initiation on bone mineral density (BMD) and have generally shown a 2% to 6% loss of bone BMD after 48 – 96 weeks of therapy regardless of the type of ART initiated (Brown et al., 2009). However, tenofovir accumulative exposure was associated with loss of bone BMD and increased risk of incident fracture (HR, 1.16; 95%CI, 1.24; $p<0.0001$) (Bedimo et al., 2015).

Weight gain during treatment in HIV patients is critical for survival. Studies have shown that weight loss and wasting are still common among HIV-infected person whose nutrition is not supplemented. One study confirmed that the risk of greater or equal to 5% unintentional weight loss over 6-month intervals is on the rise in HIV-infected patients despite better control of HIV infection in recent years (Tang et al., 2005a, Arentz et al., 2005, Martyn et al., 2015). This could be, in part, because among malnourished HIV-infected patients appetite for food increases several weeks after the initiation of antiretroviral therapy (Rehman et al., 2015a). In one of the studies, delayed supplementation showed that the three months' lipid-based nutrient supplementation at the start of ART did not only

improve weight in malnourished HIV-infected patients but lean body mass as well (Olsen et al., 2014).

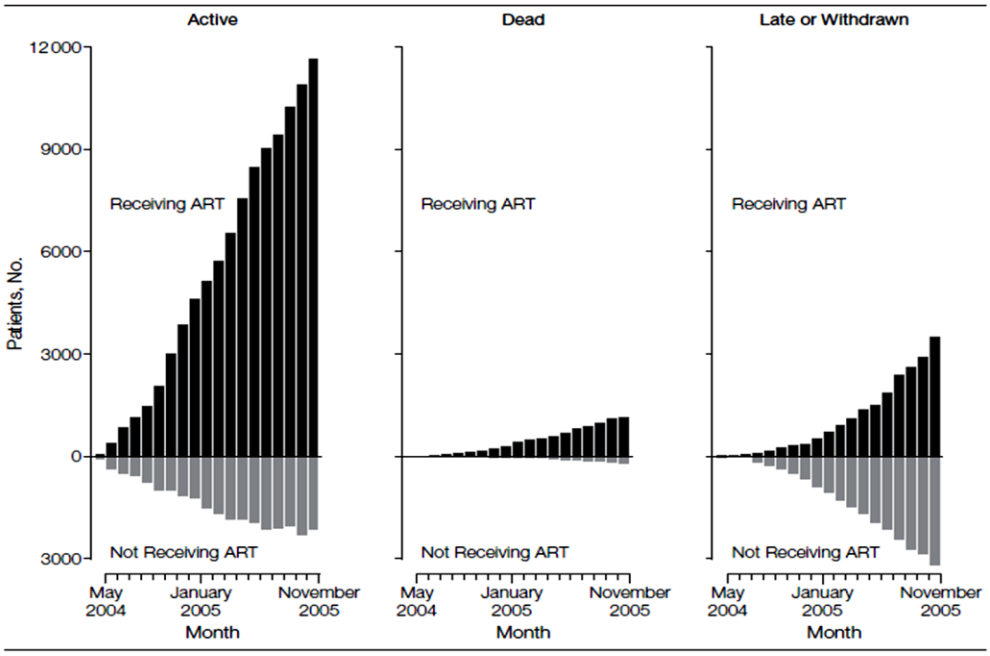


Figure 2.7: Cumulative status over time of participants in the antiretroviral therapy program in Lusaka, Zambia from April 2004 to November 2005. The number of participants in a given category indicated as active, dead, late, or withdrawn is presented at a given point in time. Of interest, because of the dynamic nature of the cohort, individuals may be categorized as late 1 month but return for follow-up the next and be categorized as active. The status of each participant in the study is presented as of a particular moment in time, at the end of each month. The first bar in each plot includes data from April 26 through May 1, 2004. The last bar in each plot includes data from October 2 through November 5, 2005. The other bars include data through the first of each month. The number of those not receiving ART in the late or withdrawn category in November 2005 is 3216 (Source: Stringer et al., 2006).

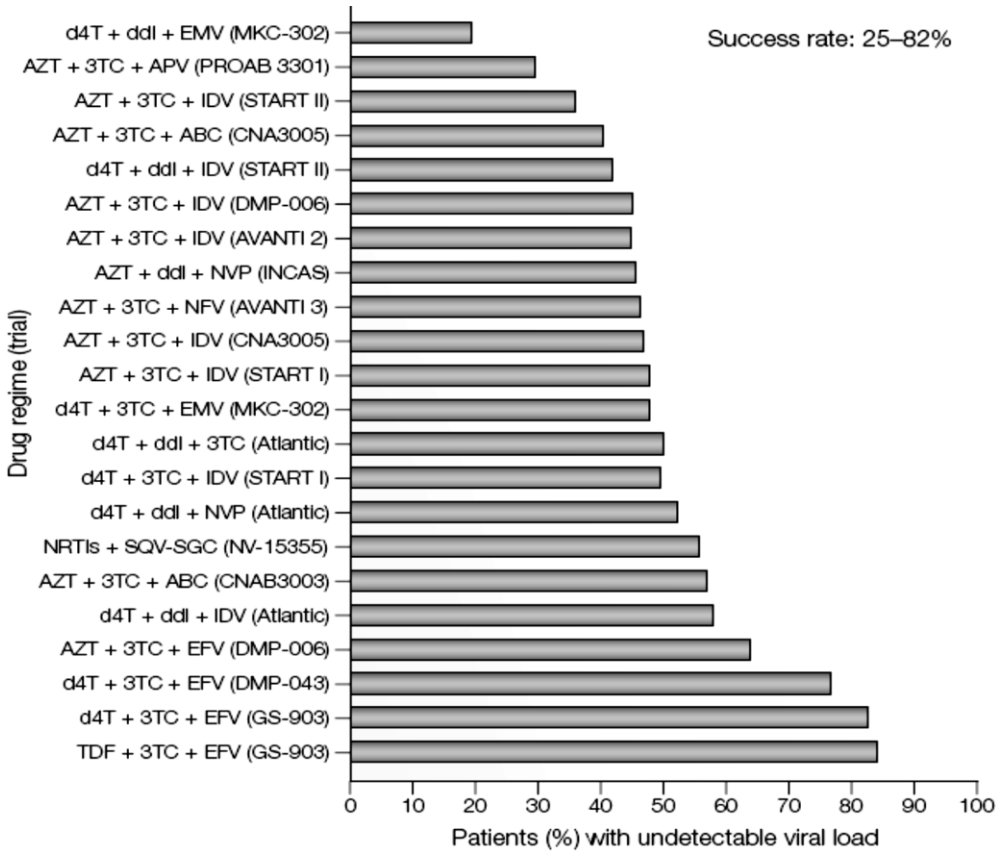


Figure 2.8: Efficacy of tenofovir-based regimen. Tenofovir disoproxil fumarate (TDF)-based ART in the treatment of human immunodeficiency virus-infected (AIDS) patients is used as first-line regimen in most countries. This is a comparison of success rates of different drug combination regimes with intention to treat analysis, as based on percentage of patients with HIV RNA copies less than 50 copies per ml at 48 weeks. Tenofovir-base ART is superior in viral suppression within 48 weeks of treatment. Abbreviations: ABC, abacavir; APV, amprenavir; AZT, zidovudine; ddI, didanosine; d4T, stavudine; EFV, efavirenz; EMV, emivirine; IDV, indinavir; NFV, nelfinavir; NRTIs, nucleoside reverse transcriptase inhibitors; NVP, nevirapine; SQV, saquinavir; SQV-SGC, saquinavir soft gell capsules; 3TC, lamivudine (Source: De Clerq & Holy, 2005).

A study done among Caucasian HIV-positive patients demonstrated that 32% of women with body weight ≤ 50kg versus 18% of women with body weight > 50kg experienced tenofovir-related toxicity. In this study women who experienced toxicity had plasma tenofovir concentrations significantly higher than

those who did not experience drug-related adverse events (173±131 versus 106±65 ng/ml, p=0.042). These results imply that HIV- infected women with low body weight are at risk to be exposed to high tenofovir plasma trough concentrations that eventually result in a significant hazard to develop drug-related complication. The risk could be handled by routine therapeutic drug monitoring of tenofovir plasma concentrations (Gervasoni et al., 2013). Tenofovir renal toxicity might not necessarily entirely be attributed to drug-drug interactions with other drugs. A study was done in Tanzania to evaluate the effect of rifampicin-based tuberculosis (TB) treatment on pharmacokinetics of tenofovir/emtricitabine/efavirenz in fixed dose combination tablet. It was found that co-administration of tenofovir, emtricitabine and efavirenz with standard first-line TB treatment regimen did not significantly alter the pharmacokinetic parameters of these drugs (Semvua et al., 2013).

Some studies have shown disparities in ART adherence across ethnic groups. A retrospective cohort study revealed that adherence levels to ART were higher among White (70.1%; ref) compared with Black (64.2%; $p < 0.001$) and Hispanic patients (65.2%; $p < 0.001$) (Silverberg et al., 2009). However, it is now known that adherence to ART has improved in black patients in settings where patients are supplied with food during treatment. In Zambia, the treatment adherence was shown to be of significant challenge. Social supports from spouses and people on ART could facilitate treatment adherence (Sasaki et al., 2012). In addition, poverty reduction strategies may help to reinforce adherence to ART and could mitigate the influence of HIV infection for poor patients and

those who fall into poverty after starting ART. In a study where multiple logistic regression analysis was used as indicated in Table 2.4, gender, food insufficiency and spouse were factors that affected treatment adherence in HIV-positive patients on treatment.

Table 2.4: Factors affecting adherence to ART

Variable	B	SE	β	p	AOR	95% CI
Gender (Females)	0.21	0.09	0.21	0.021*	3.26	1.20-8.90
Experience of food insufficiency in the previous 30 days	0.30	0.09	0.31	0.002**	5.00	1.81-13.76
Disclose HIV status to spouse	0.20	0.12	0.15	0.130	2.85	0.73-11.06
Spouse on ART	0.26	0.09	0.25	0.007**	4.44	1.50-13.12
R^2	0.28					

B: Unstandardized coefficient; SE: Standard error; β: Standardized coefficient; p value at <0.05* & <0.01**; A0R: Adjusted odds ratio; CI: Confidence interval; R^2: Coefficient of determination. The variables of which the associated p value level was less than 0.1 by an univariate analysis were entered into a multiple logistic regression model (Source**:** Sasaki et al. 2012).

2.6 Antiretroviral Therapy and Renal Function

Despite the fact that antiretroviral therapy has transformed the prognosis of HIV-1 infection, some of drugs have been associated with renal dysfunction and metabolic consequences (Guerri-Fernandez et al., 2014, Eckard and McComsey, 2014, Mpondo and Neilson, 2015, Hamzah et al., 2015). These

agents pose a risk of nephrotoxicity especially in susceptible individuals. Patients at higher risk include those taking nephrotoxic drugs and/or undiagnosed renal disorders. There are case reports with almost every ARV drug of inducing renal dysfunction but there are three agents with well-established associations with direct nephrotoxicity supported by numerous case reports and large cohort studies. These are indinavir [(2S)-1-[(2S,4R)-4-benzyl-2-hydroxy-5-[[(1S,2R)-2-hydroxy-2,3-dihydro-1H-inden-1-yl]amino]-5-oxopentyl]-N-tert-butyl-4-(pyridin-3-ylmethyl)piperazine-2-carboxamide], atazanavir [methyl *N*-[(1*S*)-1-{[(2*S*,3*S*)-3-hydroxy-4-[(2*S*)-2-[(methoxycarbonyl)amino]-3,3-dimethyl-*N′*-{[4-(pyridin-2-yl)phenyl]methyl}butanehydrazido]-1-phenylbutan-2-yl]carbamoyl}-2,2-dimethylpropyl]carbamate] and tenofovir [({[(2*R*)-1-(6-amino-9*H*-purin-9-yl) propan-2-yl] oxy} methyl) phosphonic acid] (Ryom et al., 2013, Ross et al., 2014). Of the three, tenofovir is the most implicated one. Tenofovir is one of the most attractive and most widely used antiretroviral agents because of its favorable profile in terms of high efficacy, low hepatotoxicity and once-daily dosing (Jimenez-Nacher et al., 2008b, Horberg and Klein, 2010, Sax et al., 2012). In addition, it is also formulated as a single, fixed dose combination tablet that contains efavirenz 600mg, emtricitabine 200mg and tenofovir Disoproxil Fumarate 300mg (Goicoechea and Best, 2007). However, cases of renal tubular dysfunction have increased and concern exists regarding the use of tenofovir especially in patients with predisposing factors for renal injury such as undernutrition (Rodriguez-Novoa et al., 2009b). The biological signs of renal

toxicity during tenofovir therapy occur within 5 to 64 weeks and resolve in less than 16 weeks after discontinuation of treatment (Peyriere et al., 2004).

A study on prevalence of proximal renal tubular dysfunction in HIV-infected French patients, found significant independent associations between proximal renal tubular dysfunction and age (odds ratio (OR) 1.28 per 5-year increase, p=0.17), atazanavir (OR 1.28 per cumulative year of exposure, p=021) and tenofovir (OR 1.23 per cumulative year of exposure, p=0.028) (Dauchy et al., 2011). Tenofovir has also been shown to induce proximal tubular injury and Fanconi syndrome (Hall et al., 2009a) and administration of tenofovir is associated with renal phosphate wasting (Izzedine et al., 2009a), an electrolyte essential for metabolic life processes in the body. Mechanism has been postulated though not clearly known by which tenofovir contributes to renal damage. It has been suggested that tenofovir induces oxidative stress, primarily due to mitochondrial reactive oxygen species (ROS) (Maria et al., 2011). Tenofovir induces oxidative stress by inhibition of mtDNA polymerase gamma which results in mitochondrial toxicity in the proximal tubular cells (Figure 2.9) (Hall et al., 2011). Dysfunctional mitochondria are central to the formation of excess reactive oxygen species (ROS) (Kohler et al., 2009a, Martin-Mateo et al., 1999). Normally, ROS are balanced by natural antioxidant enzymes. When mitochondria become dysfunctional, they produce less cell energy and more ROS. The imbalance between these ROS and the natural antioxidants create the condition of oxidative stress which is responsible for acute renal injury (ARI) (Figure 2.10) (Poljsak et al., 2013).

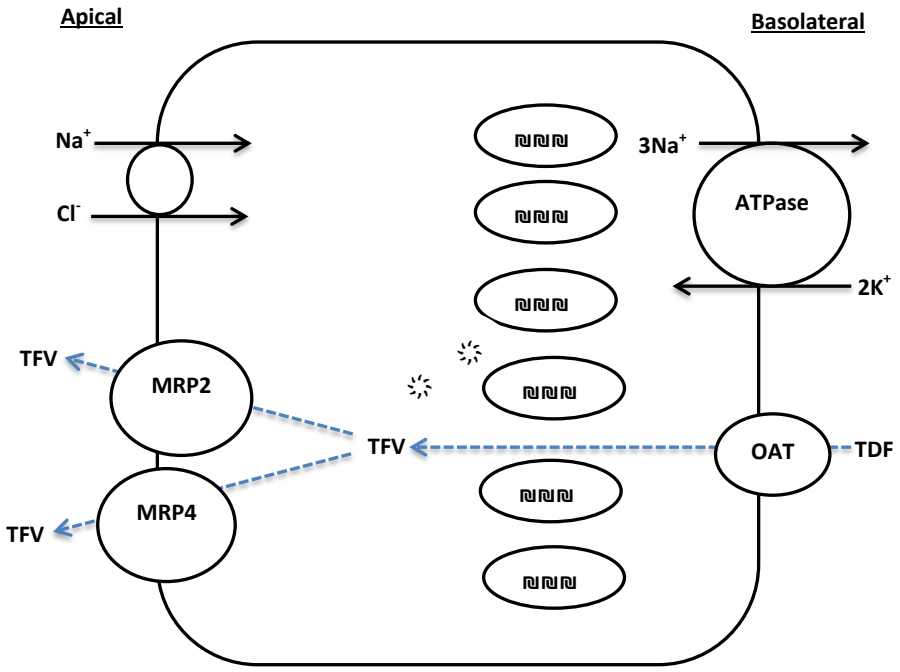


Figure 2.9: Transport of tenofovir disoproxil fumarate (TDF) in the renal proximal tubule. TDF enters proximal tubular cells across the basolateral membrane through organic anion transporters (OAT), where it competes for binding with molecules that are transported by the OAT. In the cell it is converted to tenofovir (TFV). TFV exits the tubule cells across the apical membrane through the multidrug resistance transporters (MRP2 and MRP4). In circumstances where MRPs do not extrude TFV enough, it accumulates in the proximal tubule cells. The proximal tubule contains a high density of mitochondria, which lie in a basolateral striated distribution, and evidence suggests that these organelles are the target of TFV toxicity. A variety of solutes are reabsorbed across the apical membrane through sodium (Na^+)-mediated cotransport, which is driven by the Na^+ gradients generated by the activity of the basolateral adenosine triphosphatase sodium-potassium pump (Na^+-K^+-ATPase). Mitochondrial toxicity in the proximal tubule leads to impaired reabsorption of electrolytes, with urinary wasting and the clinical features of renal Fanconi syndrome (Source: modified from Hall et al., 2011).

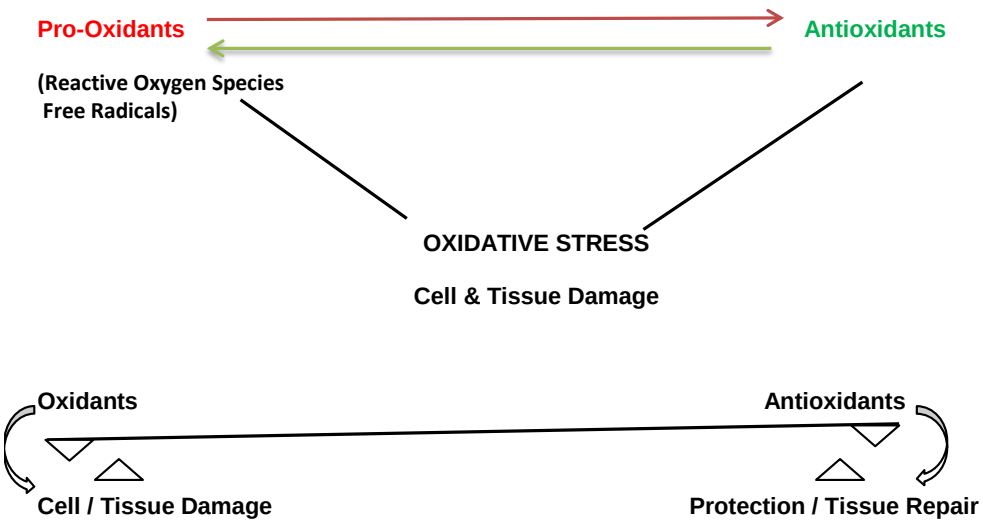


Figure 2.10: Balance between Pro-Oxidants and Antioxidants. An imbalance in pro-oxidant/antioxidant homeostasis leads to generation of toxic reactive oxygen species (ROS), such as hydrogen peroxide. The excess production of free radicals and/or deficiency of the antioxidant system result in oxidative stress. Oxidative stress causes tissue damage by reacting with important cellular components such as DNA, or the cell membrane that results in apoptosis (programmed cell death). To prevent free radical damage the body has a defense system of antioxidants (Modified from Poljsak et al., 2013).

The injured mitochondria through oxidative stress cause release of cytochrome-C and activation of caspase pathways that lead to apoptotic deletion of renal cells (Gobe and Crane, 2010, Money et al., 2015). Deficiencies of anti-oxidant vitamins and minerals also contribute to oxidative stress, a condition that may accelerate immune and renal cell death (Oguntibeju et al., 2009) and increase the rate of HIV replication. Renal proximal tubular cells are much more affected because production of ROS is higher in the proximal tubules than the distal cells (Hall et al., 2009b). It is now evident that there is lower antioxidant levels and high lipid peroxidation in HIV-infected patients on highly active antiretroviral therapy as compared to naïve HIV-infected patients (Awodele et al., 2012). In a related study, Akiibinu and Olusegun concluded that micronutrient

deficiency was possibly contributing disproportionately to the oxidative stress in AIDS patients (Akiibinu and Olusegun, 2012). They further recommended that adjuvant micronutrients therapy should be seriously considered in the management of AIDS patients to avert or ameliorate the complications of AIDS and slow the progression of the disease.

To counteract the effects of oxidative stress, some studies have suggested that the restoration of depleted micronutrients through supplementation may have cellular and clinical benefits in HIV-positive persons receiving ART (Paul and Rowland, 2011). Therefore, there have been recommendations that administration of nutrition therapy, in conjunction with an early start of ART, might increase the chances of nutritional recovery in severely malnourished HIV patients (Ahoua et al., 2011) and consequently improve the quality of life. In one of the pharmacogenetic studies, it was found that approximately 17% of HIV-infected patients treated with tenofovir-based regimen developed kidney tubular dysfunction and that homozygosity for the C allele at position -24 of the ABCC2 gene was a predisposing factor (Rodriguez-Novoa et al., 2009a). Apart from the role of genetics in renal dysfunction, others have suggested that drugs can cause acute kidney injury by altering the flow or distribution of blood in various parts of the kidney or direct blockage of free flow of urine out of the body through the kidney (Twombley et al., 2011). It is further reported that exposure to tenofovir is associated with an increased risk of kidney tubular abnormalities in the absence of significant

impaired glomerular function by measure of creatinine clearance (Labarga et al., 2009). Renal tubular injuries usually results in electrolyte disorders such as hypophosphatemia and hypomagnesemia (Izzedine et al., 2010). Figure 2.11, illustrates hypophosphatemia in patients taking tenofovir compared to the non-tenofovir group. In this cross-sectional study they observed a nonsignificant increase in the proportion of patients with hypophosphatemia in the TDF group. This warrants further exploration of renal reabsorption excretion of phosphate in clinical trials since it was not measured.

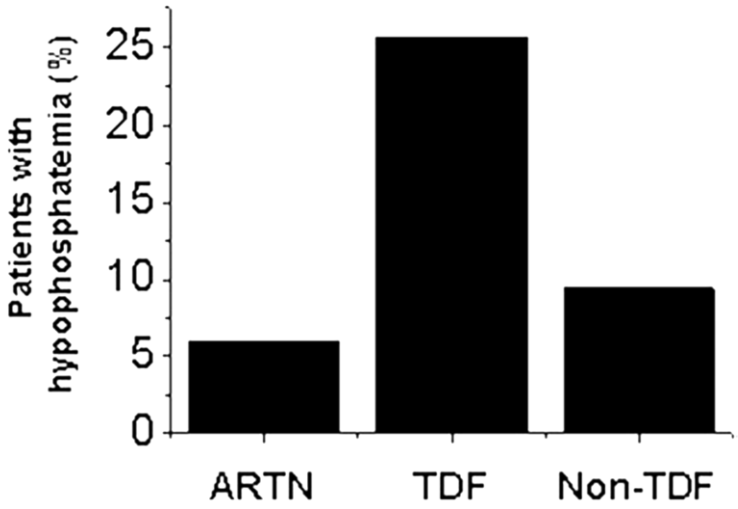


Figure 2.11: Hypophosphatemia in patients taking tenofovir. The proportion of patients in the group exposed to tenofovir (TDF group) who had a serum phosphate value less than the normal range was greater than in the antiretroviral treatment naive group (ARTN) and those with no exposure to tenofovir (non-TDF group), but this difference was not statistically significant (Source: Hall et al., 2009).

Animal models have demonstrated that tenofovir targets mitochondrial toxicity in the proximal tubule cells in HIV/AIDS models. In this study mitochondrial DNA (mtDNA) abundance in renal proximal tubules of HIV transgenic mice treated with tenofovir decreased compared with vehicle

transgenics and wild-type mice controls. Tenofovir treatment of wild-types also resulted in decreased mtDNA abundance compared with that of vehicle-treated wild-types, although the difference was not enough to be significant. In contrast, treatment with didanosine a similar drug to tenofovir had no effect on proximal tubular epithelial mtDNA abundance. Therefore tenofovir seems to specifically deplete mtDNA in proximal tubules (Kohler et al., 2009b). A follow up study by the same author revealed that disruption of organic anion transporter 1 (OAT1) activity prevents tenofovir toxicity but loss of multidrug-resistant protein type 4 (MRP4) can lead to increased renal proximal tubular toxicity. This was in a study to determine the role of OAT1 or MRP4 on tenofovir-associated mitochondrial toxicity. These studied elucidate drug-specific regulatory roles of OAT1 and MRP4 to renal proximal tubular mitochondrial toxicity from nucleoside analogs used to treat HIV-1. This evidence suggests that OAT1 regulates the transport of tenofovir from the blood into the proximal tubules, while MRP4 plays a critical role in the efflux of tenofovir out into the urine, preventing the accumulation of tenofovir inside the proximal tubules resulting in reduced renal toxicity (Kohler et al., 2011).

Tenofovir has been extensively studied and associated with a significantly greater risk of inducing proximal tubular dysfunction (Horberg et al., 2010) is overwhelming to the extent some studies recommend tenofovir renal dosing when glomerular filtration rate is below 50ml/min. (Labarga et al., 2009). By contrast, a retrospective cohort study in Singapore on renal safety of tenofovir containing ART showed that renal toxicity was uncommon and transient. The

patients had relatively normal creatinine clearance and only 2.2% of patients developed renal toxicity. Nevertheless, this study did not have a non-tenofovir comparator group and follow up time period was limited to 24 months with only 98 patients included in the analysis (Chua et al., 2012). In a cohort study of 56 HIV-1 infected adults a detailed comparison of the effect of tenofovir versus no-tenofovir use on calcium, phosphate and vitamin D was carried out. Tenofovir was found to be associated with renal phosphate wasting. Patients on tenofovir had higher fractional excretion of phosphate (median 26.1% versus 14.6; p=0.025) and lower serum phosphate (median 0.79mmol/L versus 1.02; p=0.040) than those not taking tenofovir. The same study showed that patients on tenofovir had significantly reduced urinary calcium excretion (median 3.01 mmol/24 hours) compared to non-tenofovir users (4.56; $p < 0.0001$) (Klassen et al., 2012).

The renal handling of calcium, magnesium and potassium are linked. For instance the Ca++/Mg++-sensing receptor, a member of the G-protein coupled receptor family is an important regulator of calcium and magnesium homeostasis (Ryan, 1991) and this was illustrated by Dai in 2001 as shown in Figure 2.12. This shows that what affects excretion of calcium might affect the excretion of magnesium as well. In this particular instance, insufficient evidence was available to implicate tenofovir as a causative agent in the urinary excretion of magnesium.

A study of 51 patients to assess the incidence of renal tubular dysfunction during long-term nucleotide therapy of chronic hepatitis B found that high-dose therapy of tenofovir was associated with renal tubular dysfunction (Gara et al.,

2012). All seven patients on tenofovir had low urinary percent maximal tubular reabsorption of phosphate (<82%). Patients with renal tubular dysfunction were older (58 vs. 44 years; p=0.01) and had lower baseline glomerular filtration rates (82 vs. 97 cc/min; p=0.08) compared to those without, but did not differ in other features. Six patients with renal tubular dysfunction were switched to entecavir, all subsequently had improvements in serum phosphate (2.0-3.0mg/dL). Figure 2.13 shows the incidence of significant tubular dysfunction over time in HIV-infected patients treated with tenofovir containing ART and non-tenofovir regimen.

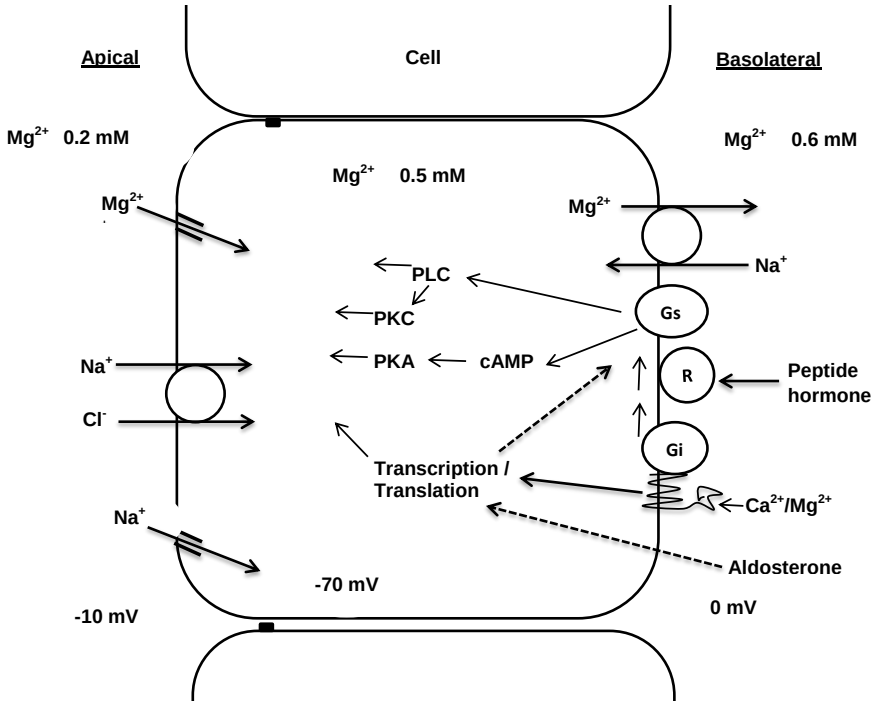


Figure 2.12: Schematic model of magnesium absorption in the distal convoluted tubule. Conductive pathways and carrier-mediated transport are denoted by solid arrows. Peptide hormones such as parathyroid hormone (PTH), calcitonin, glucagon, and arginine vasopressin (AVP) enhance magnesium reabsorption in the distal convoluted tubule (DCT). The extracellular Ca^{2+}/Mg^{2+}-sensing receptor modulates hormone-stimulated Mg^{2+} transport through $G\alpha_i$ protein coupling. (Source: Modified from Dai, 2001).

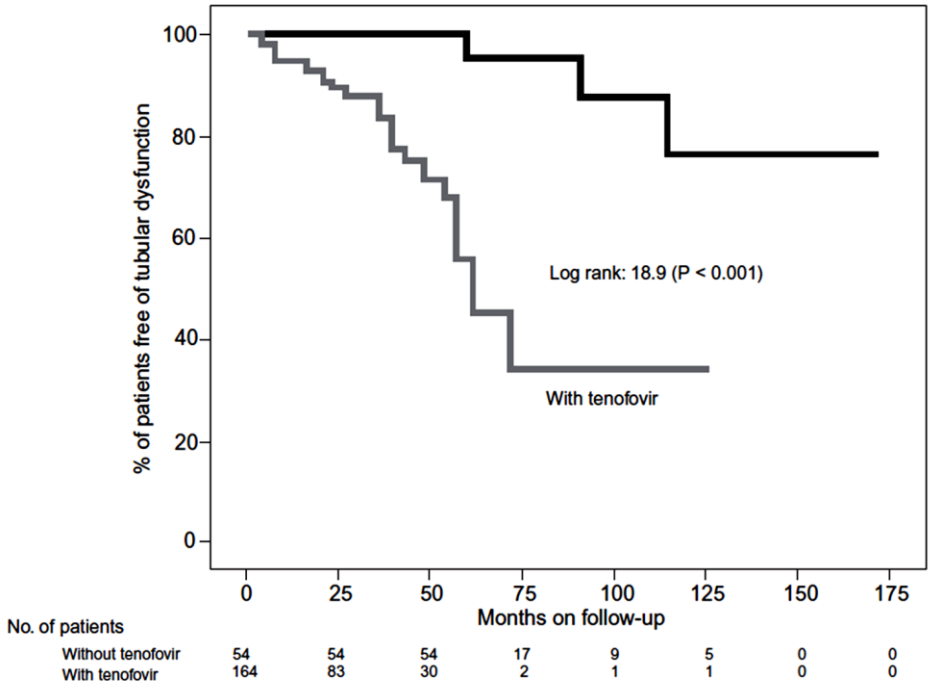


Figure 2.13: Development of tubular damage in patients on ART, with and without tenofovir. The incidence of significant tubular dysfunction over time was assessed. Although the study was prospectively done, the Kaplan–Meir curve shows the incidence of kidney tubular abnormalities in this population that took into consideration prior TDF exposure for each individual. After a median follow-up of 462 and 225 patients-year, those receiving TDF had significantly greater risk for tubular damage than patients never treated with TDF (odds ratio (OR) 18.9; P<0.001). According to this analysis, estimates for tubular dysfunction at 4 years were 25% for patients on TDF and null for the rest (Source: Labarga et al., 2009).

A study of 13 cases of tenofovir nephrotoxicity by way of renal biopsy revealed toxic acute tubular necrosis, with distinctive proximal tubular eosinophilic occlusions representing giant mitochondria visible by light microscopy. Electron microscopy showed mitochondrial enlargement, depletion and dysmorphic changes. Significant recovery of renal function occurred in all the patients after discontinuation of tenofovir. This study showed that tenofovir

nephrotoxicity is a largely reversible form of toxic acute tubular necrosis targeting proximal tubules and ultrastructural features of mitochondrial injury (Herlitz et al., 2010). The light microscopic and ultrastructural findings support the hypothesis that tenofovir toxicity primarily targets mitochondria, and as a result, the pathological changes are most prominent in the proximal tubules, leading to histological findings of toxic acute tubular necrosis and clinical finding of proximal tubular dysfunction with Fanconi syndrome. In post marketing safety data for tenofovir, the study identified advanced age, low body weight and low CD4 count as risk factors of development of renal dysfunction during tenofovir use (Nelson et al., 2007). The renal injury resembled Fanconi's syndrome and presented with variable degree of proximal renal tubular dysfunction, including hypophoshatemia, aminoaciduria and glucosuria. Pharmacokinetically, tenofovir undergoes renal clearance largely by active tubular secretion at the proximal renal tubules of the nephron where maximal reabsorption of phosphate occurs. This is the reason why when it induces renal impairment it is the proximal renal tubules that are mostly affected (Woodward et al., 2009) resulting in renal phosphate wasting.

A study that assessed the effect of tenofovir on renal tubules in chronic hepatitis B patients found that tenofovir induce renal dysfunction to the extent where all the seven patients had low urinary percent maximal tubular reabsorption of phosphate of less than 82% (Gara et al., 2012). Another study where tenofovir was compared with abacavir in as far as inducing renal tubular dysfunction was concerned tenofovir more than abacavir associated with

subclinical mitochondrial damage. Moreover, there was significant increase in urinary excretion of phosphate in patients on tenofovir compared to those on abacavir (Maggi et al., 2012b). This is consistent with findings in which a decrease in serum phosphate was associated with increase in years on tenofovir therapy as shown in Figure 2.14 (Soler-Palacı´n et al., 2011).

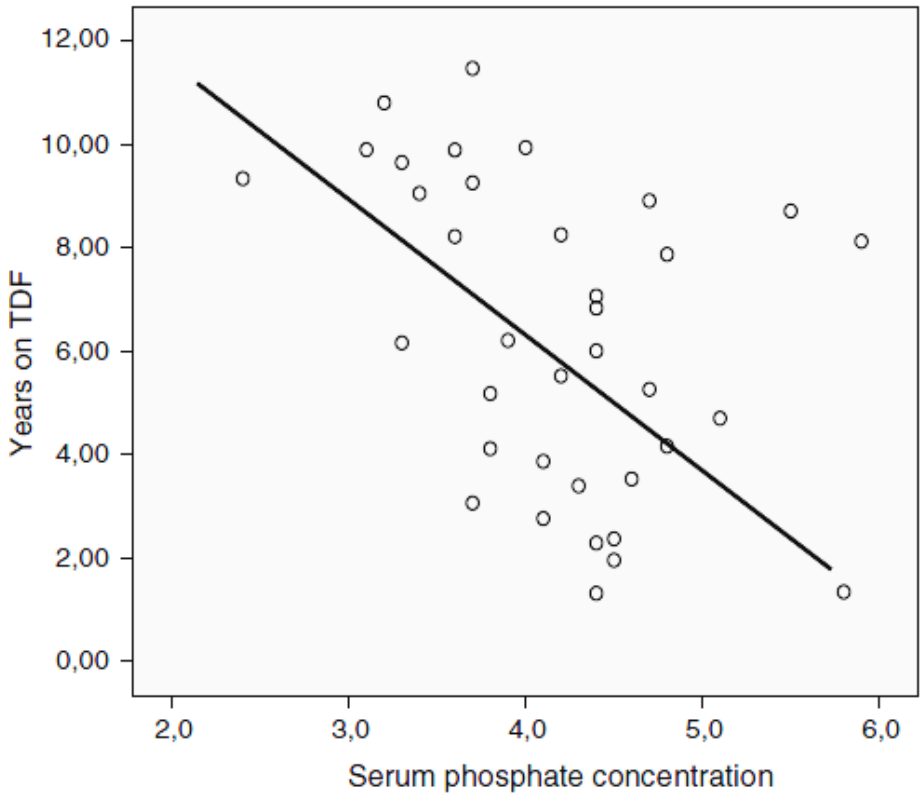


Figure 2.14: Correlation between serum phosphate and time on tenofovir. There was negative lineal correlation between serum phosphate concentration and time on tenofovir disoproxil Fumarate (TDF) treatment (r = -0.48, P=0.013). These were data analyzed from Spanish pediatric patients aged 18 years or less, receiving TDF as part of their antiretroviral treatment. TDF dosing was adjusted to body weight in all cases (Source**:** Soler-Palacı´n et al., 2011).

A study that involved 115 participants in the analysis of plasma tenofovir, found that mean (SD) tenofovir concentration was 68.8 (33) ng/ml in HIV-infected patients on tenofovir containing regimen. Plasma tenofovir concentrations were

correlated positively with vitamin D binding protein (r=0.26; p=0.007) and negatively correlated with free 1,250-OH (2) D (r=-0.34; p<0.001). The plasma concentrations of tenofovir were also correlated negatively with estimate glomerular filtration rate (eGFR) (r=-0.29; p=0.002). These data suggest that tenofovir-associated increased vitamin D binding protein is associated with decreased 1,25-OH(2)D and functional vitamin D deficiency (Havens et al., 2013). Studies in animal models have demonstrated that antiretroviral therapy in the setting of hypokalemia and hypomagnesemia predisposed rats to nephrotoxicity (Seguro et al., 2003). And it has been postulated that ART administration may produce acute renal failure in AIDS patients with hypokalemia and/or hypomagnesemia. Moreover, other studies have demonstrated that age, and estimated creatinine clearance influenced tenofovir plasma trough concentrations in HIV-infected patients (Baxi et al., 2014, Jullien et al., 2005) resulting in renal toxicity (Baxi et al., 2014). In agreement with these studies, tenofovir trough concentrations in 195 patients (median, 50ng/ml and interquartile range, 35 to 77ng/ml) were significantly associated with the estimated glomerular filtration rate.

Therefore, it is necessary that serum potassium and magnesium levels should be carefully monitored in HIV-infected patients on treatment. The schema in Figure 2.15 shows the drug-nutrient interactions:

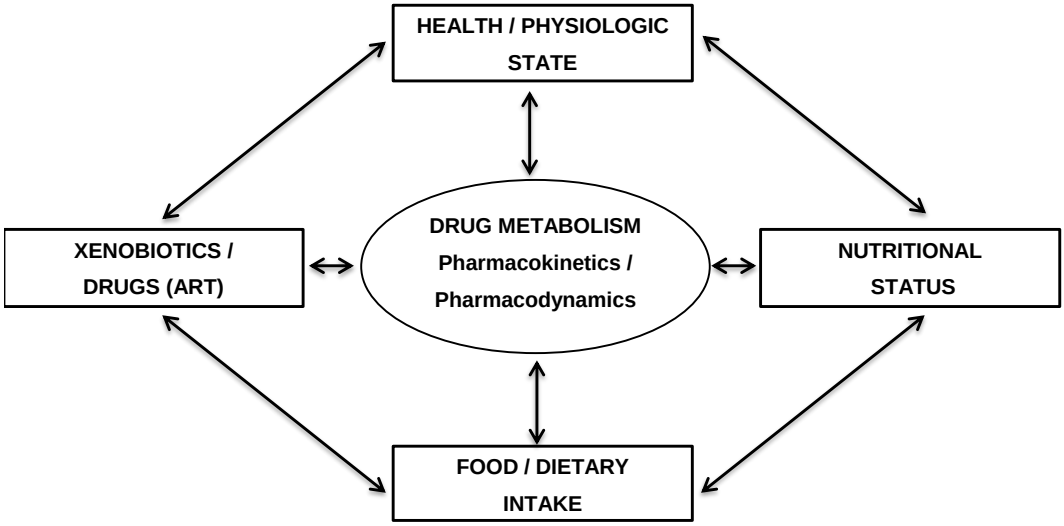


Figure 2.15: Conceptual model of drug-nutrient interaction. The drugs include antiretroviral drugs (ARVs). The conceptual model is within the context of pharmacokinetics and pharmacodynamics pathways in which nutrition might affect drugs and vice versa. Both drugs and disease cause changes in appetite and nutrient intake and the resultant malnutrition affects drug efficacy. Drugs and foods have a mechanical interaction, via binding or adsorption, that increases or decreases drug and nutrient absorption. Drugs and nutrients depending lipid solubility factors compete for amino acid transport systems resulting in competitive inhibition of each other. Drugs and nutrients can synergistically and competitively interact to cause increased or decreased excretion of electrolytes (Source: modified from Raiten, 2011).

A study in animal models demonstrated that tenofovir caused renal failure in a dose-dependent manner, leading to a greater reduction of renal transporters such as the endothelial nitric-oxide synthase expression and intense vasoconstriction. In addition, tenofovir-induced tubular toxicity involved down regulation of other renal transporters including sodium/hydrogen exchanger 3 (NHE3), sodium-phosphate co-transporter subtype IIa (NaPi-IIa), and aquaporin (AQP2). In this study rosiglitazone (RSG) reversed all mechanisms of tenofovir-

induced nephrotoxicity, re-establishing the expression of eNOS, NHE3, NaPi-IIa and AQP2 as well as normalizing biochemical parameters (Liborio et al., 2008).

A study conducted in Japan demonstrated a high incidence of tenofovir-associated renal dysfunction among Japanese HIV-infected patients. Tenofovir-related renal dysfunction occurred in 97 out of 495 (19.6%) patients. the incidence of tenofovir-related renal dysfunction was significantly associated with smaller body weight and body mass index, respectively (per 5kg decrement, HR=1.23: 95%CI, 1.10-1.37; p<0.001) (per 1kg/m^2 decrement, HR=1.14: 95%CI, 1.05-1.23; p=0.001). This study recommended the importance of close monitoring for renal function in patients with small body weight emphasizing early detection of tenofovir-associated renal dysfunction (Nishijima et al., 2011). Tenofovir renal toxicity studies have also been conducted in HIV-uninfected populations. A study in Thailand that recruited 2,413 HIV-uninfected participants randomized to receive tenofovir or placebo found significant decreases in creatinine clearance and glomerular filtration rate (GFR) in the tenofovir group compared with the placebo group. The study showed that creatinine clearance and GFR results were lower at 24, 36, 48 and 60 months in the tenofovir group compared with the placebo group. Results declined more in the tenofovir group than the placebo group during follow up using the Cockcroft-Gault (p,0.001) and Chronic Kidney Disease Epidemiological Collaboration (CKD-EPI) (p=0.007) equations (Dias et al., 2013). A study conducted in Zambia found that among HIV-infected patients with no or mild renal dysfunction (eGFR 60-89ml/min) at baseline, those receiving tenofovir based regimen were more likely to develop

moderate (AOR, 3.11; 95%CI 2.52-3.87) or severe (eGFR <30ml/min) (AOR, 2.43; 95%CI 1.80-3.28) estimates glomerular filtration rate decrease, although the incidence in such patients was low (Mulenga et al., 2014).

2.7 HIV and Antiretroviral Therapy in Zambia

The first HIV/AIDS case in Zambia was reported in 1984 (WHO, 2011), and by 2013, approximately 54,000 adults and 12,000 children were infected with HIV in Zambia (UNAIDS, 2014). HIV prevalence has been consistently high in Zambia, 14.3% in 2010 (Central Statistical Office et al., 2014) and 13.2 % in 2014 (UNAIDS, 2014). However there has been considerable increase in life expectancy in the country from 49.4 years in 2012 to 58.1 years in 2014 partly due to antiretroviral treatment (UNDP, 2014).

Antiretroviral therapy options for HIV-1 infection were very few before 2002 in Zambia (WHO, 2011). The clinical treatment of HIV-1 then, largely consisted of treatment of common opportunistic infections such tuberculosis, cryptococcal meningitis and related illnesses. The treatment of HIV-1 infection was revolutionized in 2004 when the State with the help of Non-governmental Organizations (NGOs) started providing treatment through antiretroviral therapy clinics. In fact in 2004 Zambia declared HIV/AIDS a national disaster and offered subsidized antiretroviral therapy to all those who could afford the subsidized treatment (WHO, 2005). Subsequently, in February 2005 in recognition of treatment of HIV/AIDS as a human right and moral issue, the government of Zambia committed its self to provision of free antiretroviral therapy to all persons

living in the country and in need of the treatment. Currently, everyone who is clinically eligible for antiretroviral therapy has access to available combinations of antiretroviral therapy options. The advent of treatment options for the treatment of HIV-1 infection has been seminal in reducing the morbidity and mortality associated with HIV-1 infection and AIDS. In HIV-infected patients with CD4 cells of less than 350 per cubic mm, a stage at which viral levels can often reach 10,000–100,000 copies per mL, combinations of antiretroviral therapy dramatically has proved to suppress viral replication and reduce the plasma HIV-1 viral load to below the limits of detection. .

In Zambia, approximately 95% of patients are started on a single fixed-dose antiretroviral therapy tablet containing tenofovir disoproxil fumarate (TDF) 300 mg, emtricitabine 200 mg, and efavirenz 600 mg as first-line of therapy in the management of HIV-infected patients. Tenofovir-based antiretroviral therapy is preferred because of the long half-life in vivo, high antiretroviral efficacy and cost effectiveness (Jimenez-Nacher et al., 2008b, Jimenez-Nacher et al., 2008a, Wandeler et al., 2012). Therefore, the following Chapter 3 discusses in detail the pharmacology of tenofovir.

CHAPTER 3

PHARMACOLOGY OF TENOFOVIR

3.1 Antiretroviral Drugs

Antiretroviral drugs (ARVs) are medications that are used to treat human immunodeficiency virus (HIV) and tenofovir is one of these drugs. If not treated, HIV can lead to the disease known as acquired immunodeficiency syndrome (AIDS) (Palella et al., 1998, Montaner et al., 2010, Mwagomba et al., 2010). AIDS is the final stage of HIV infection. The first case of AIDS appeared in the 1980s (Zhu et al., 1998, Pence, 1988, Sepkowitz, 2001). By 1987 zidovudine, also known as azidothymidine (AZT) was approved as the first drug for treatment of HIV (Wright, 1986, Jeffries, 1989). Thereafter, more antiretroviral drugs were approved. The antiretroviral drugs do not cure HIV but when taken in combination they prevent the proliferation of the virus. This slows down progression to HIV/AIDS disease (May and Ingle, 2011). Treatment with antiretroviral drug is referred to as antiretroviral therapy (ART). Combination antiretroviral therapy is referred to as highly active antiretroviral therapy (HAART). The highly active antiretroviral therapy has reduced the morbidity and mortality of HIV infection (Schackman et al., 2006, Schackman et al., 2015, Knoll et al., 2007) and the classification of antiretroviral drugs have been well categorized.

3.2 Classification of Antiretroviral Drugs

Classification of antiretroviral drugs is based on the mechanism of action which includes inhibition of various viral enzymes critical to the HIV replication cycle (Figure 3.1) and (Table 3.1). These include specifically, reverse transcriptase, integrase and protease**.** Nucleoside Reverse Transcriptase Inhibitors (NRTIs) act on the reverse transcriptase enzyme, incorporating themselves into the DNA chain created by the virus. They terminate the chain and thus prevent the virus from reproducing itself. Drugs in this class include: Zidovudine, Abacavir, Lamivudine and Tenofovir. Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs) directly block the action of the reverse transcriptase enzyme and the multiplication of the virus. Drugs in this class include: Efavirenz, Nevirapine and Etravirine. Protease Inhibitors act on the protease enzyme, blocking its action and preventing the production of new copies of HIV infected cells. Drugs in this class include: Amprenavir, Atazanavir, Darunavir, Indinavir, Lopinavir/r, Nelfinavir, Ritonavir and Saquinavir. Integrase Inhibitors block the activity of the integrase enzyme, which incorporates HIV's DNA into human DNA. In this way it inhibits the replication of the virus and its ability to infect new cells. Drugs in this class include: Raltegravir. Fusion inhibitors (FIs) prevent HIV from entering healthy CD4 cells, so that it cannot reproduce itself. These drugs bind to the HIV envelope protein gp41, which is involved in viral entry. Through blocking the interactions between regions of the gp41 molecule, FIs interfere with the conformational change of the envelope molecule required for fusion with the target cell membrane. Drugs in this class: Enfuvirtide.

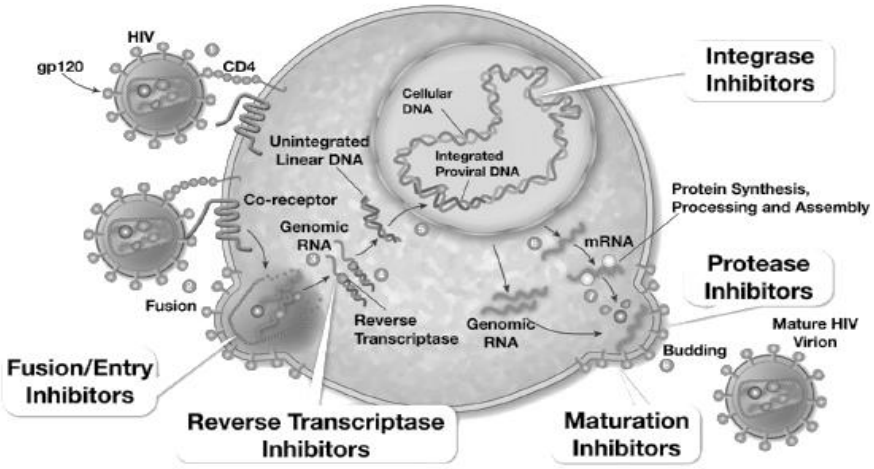


Figure 3.1: Antiretroviral drugs classified according to sites of action for human immunodeficiency virus infection. After the virus penetrates the host cell and become uncoated, the viral RNA is transcribed by reverse transcriptase to form viral DNA. Viral DNA is incorporated into the host genome in the cell nucleus by HIV integrase. The viral DNA is then transcribed to RNA. Viral RNA is incorporated into new virions and is translated to synthesize polyproteins. The polyproteins are cleaved into viral proteins by HIV protease as the new virions are released from the cell; drugs for HIV infection inhibit reverse transcriptase or protease (Source: Adapted from Palmisano and Vella, 2011).

Chemokine coreceptor blockers also prevent the HIV from entering CD4 target cells. These bind to either CCR5 or CXCR4 coreceptors on the surface of CD4 cells. When they attach, Chemokine coreceptor antagonists block a requisite step for the virus to enter the CD4 cells. Unlike nucleoside reverse transcriptase inhibitors (NRTIs), reverse transcriptase inhibitors (NNRTIs), protease inhibitors or integrase inhibitors which act on viral enzymes, chemokine coreceptor blockers bind human proteins. The rational use of these antiretroviral drugs in clinical practice is guided by their frequency of dosing, side effect profile, practice treatment guidelines, efficacy based on clinical evidence and patient clinical data.

Table 3.1: Current twenty six ARV medications

NRTI	PI	Integrase Inhibitor
▪ **Abacavir (ABC)**	▪ **Atazanavir (ATV)**	▪ **Raltegravir (RAL)**
▪ Didanosine (ddI)	▪ **Darunavir (DRV)**	▪ **Elvitegravir (EVG)**
▪ **Emtricitabine (FTC)**	▪ **Fosamprenavir (FPV)**	▪ **Dolutegravir (DTG)**
▪ **Lamivudine (3TC)**	▪ Indinavir (IDV)	**Fusion Inhibitor**
▪ Stavudine (d4T)	▪ **Lopinavir (LPV)**	▪ Enfuvirtide (ENF, T-20)
▪ **Tenofovir (TDF)**	▪ Nelfinavir (NFV)	
▪ **Zidovudine (AZT)**	▪ **Ritonavir (RTV)**	**CCR5 Antagonist**
	▪ Saquinavir (SQV)	▪ **Maraviroc (MVC)**
NNRTI	▪ Tipranavir (TPV)	
▪ Delavirdine (DLV)		
▪ **Efavirenz (EFV)**		
▪ **Etravirine (ETR)**		
▪ Nevirapine (NVP)		
▪ **Rilpivirine (RPV)**		

Abbreviations: Nucleoside Reverse Transcriptase Inhibitor (NRTI), Non-Nucleoside Reverse Transcriptase Inhibitor (NNRTI), Protease Inhibitor (PI).

3.3 Antiretroviral Drug Combinations

When HIV converts its RNA into DNA through reverse transcriptase, there are no proofreading enzymes to correct errors made in the process. The high error rate causes the virus to mutate. As more active copies of the virus are produced, there is a greater possibility that one will be resistant to antiretroviral drugs (Schmit et al., 1996). Therefore, antiretroviral drugs are used in combination to avoid resistance by suppressing the HIV replication at every

stage of retroviral replication process. This results in reduced potential for spontaneous resistance to therapy. Standard care requires the use of at least two classes of antiretroviral drugs. Three drug combinations are referred to as triple cocktail (Henkel, 1999). Presently, there are several options of fixed-dose combinations that combine 3 drugs into one pill taken once daily. For example commonly prescribed combinations in Zambia are shown in table 3.2 (Iyidogan and Anderson, 2014) and tenofovir-based regimen was used as first-line treatment at the time of this study.

Table 3.2: Commonly Prescribed ARV Regimens in Zambia

First line regimen	
	NNRTI – Based Regimen
	TDF/FTC/ EFV or NVP
	ABC/3TC/EFV or NVP
	AZT/3TC/NVP or EFV
Second line regimen	
	PI – Based Regimens:
	TDF/FTC/LPV/r
	AZT/3TC/LPV/r
	TDF/FTC/LPV/r

Abbreviations**:** Abacavir (ABC), Efavirenz (EFV), Emtricitabine, Lamivudine (3TC), Lopinavir (LPV), Nevirapine (NVP), Ritonavir (RTV), Stavudine (d4T), Tenofovir (TDF), Zidovudine (AZT).

Tenofovir-based regimen is a single fixed-dose tablet containing tenofovir disoproxil fumarate (TDF) 300 mg, emtricitabine 200 mg, and efavirenz 600 mg (Figure 3.2). This therapy is preferred because of the long half-life in vivo, high antiretroviral efficacy and cost effectiveness (Jimenez-Nacher et al., 2008b, Wandeler et al., 2012, Astuti and Maggiolo, 2014).

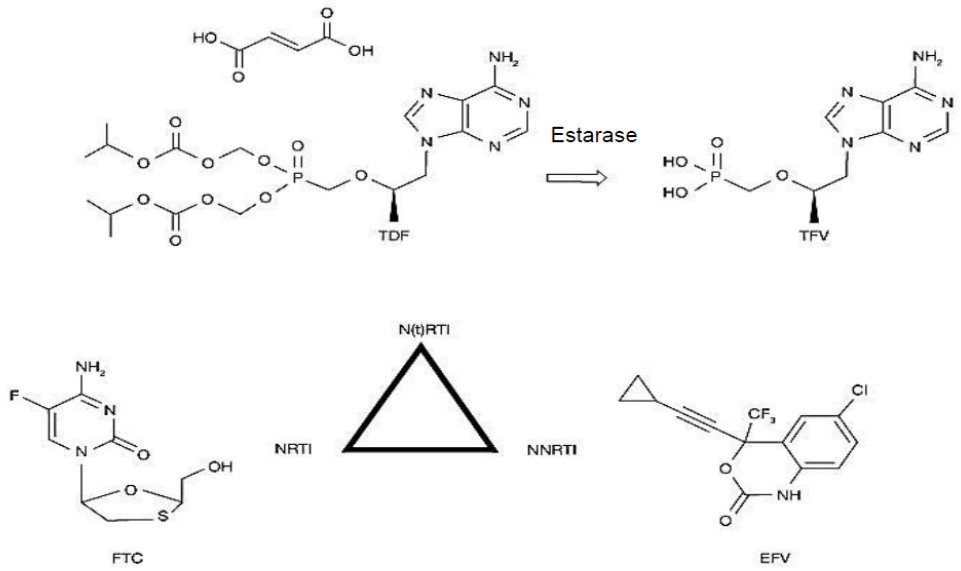


Figure 3.2: Components of Atripla® co-formulation. Chemical structures of tenofovir (TFV) tenofovir disoproxil fumarate (TDF), emtricitabine and efavirenz (EFV). Abbreviations: NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; N(t)RTI, nucleotide reverse transcriptase inhibitor (Source: Iyidogan and Anderson, 2014).

3.4 Pharmacokinetics of Tenofovir

Tenofovir {9-[(*R*)-2-(phosphonomethoxy)propyl]adenine}, formerly known as PMPA is an acyclic nucleoside phosphonate analogue of adenosine 5′-monophosphate with potent activity against retrovirus and hepadnaviruses (Emau et al., 2006, Ross et al., 2014). Nucleotide analogues possess distinct pharmacokinetic, toxicity and antiviral resistance profiles compared with nucleoside analogues, to which they are closely related. However, tenofovir demonstrated low oral bioavailability in animal studies. Therefore, a prodrug of tenofovir, tenofovir disoproxil fumarate (tenofovir DF) {9-[(*R*)-2-[[bis[[(isopropoxycarbonyl)oxy]methoxy]phosphinyl] methoxy]propyl]adenine

fumarate}; characterized by an advantageous pharmacokinetic profile and therapeutic index, was formulated for clinical use (Shaw et al., 1997, Geboers et al., 2015, Stella et al., 1985).

3.5 Absorption and bioavailability of tenofovir

Early studies showed poor bioavailability of tenofovir following oral administration. The ionic nature of tenofovir, at physiological pH, limits its permeation across epithelial barriers (Naesens et al., 1998) due to its hydrophilic nature (Stella et al., 1985, Malhotra et al., 2009). However, the addition of two alkyl methyl carbonate esters significantly enhanced lipophilicity of the drug, thus improving its permeability,(Van Gelder et al., 2002) biological stability and oral bioavailability of tenofovir (Shaw et al., 1997) allowing for its formulation into a single tablet for oral administration. Tenofovir disoproxil fumarate undergoes intestinal first-pass metabolism by estarases where it is converted to the mono(ester) and further to tenofovir during transepithelial transport (Van Gelder et al., 1999). Upon oral administration, it undergoes esterase hydrolysis, which removes the two ester groups as illustrated in Figure 3.3. Cleavage of the first ester group yields the monoester inter mediate, while cleavage of the second ester group yields tenofovir (Van Gelder et al., 2002, Shaw et al., 1997).

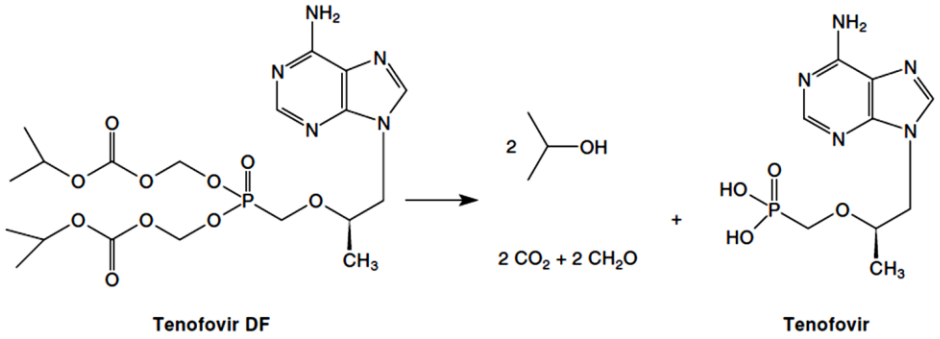


Figure 3.3: Conversion of Tenofovir Disoproxil Fumarate to Tenofovir. A pro-drug, tenofovir disoproxil fumarate (TDF) is converted to tenofovir (TFV) in plasma by serum esterases. Up to 25% of TDF is converted into TFV (Barditch-Crovo et al 2001).

After its conversion, tenofovir is taken up by cells, then phosphorylated by the cellular nucleotide kinase, adenylate kinase, to the monophosphate intermediate, and then rapidly converted by nucleoside diphosphate kinase to the active diphosphate form (Robbins et al., 2003) as shown in Figure 3.4.

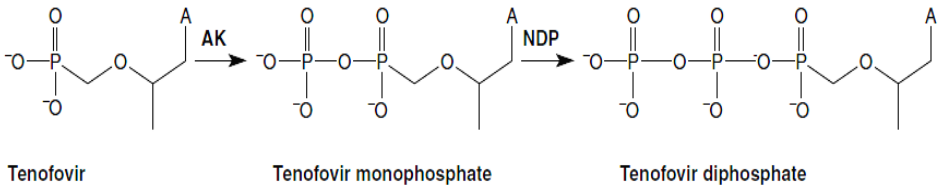


Figure 3.4: Intracellular anabolism of tenofovir to tenofovir diphosphate. Tenofovir diphosphate (active metabolite), has a prolonged intracellular half-life that ranges from 12 to 50 h (Robbins et al. 1998) while approximately 12-18 h is the plasma half-life of tenofovir that determines the dosing schedule of once daily administration. AK and NDPK are ubiquitous enzymes that are constitutively active in dividing and non-dividing cells. A = adenine; AK = adenylate kinase; NDP = nucleoside diphosphate kinase (Barditch-Crovo et al 2001).

Tenofovir is thought to enter the rest of body cells except renal cells via a passive process of fluid-phase endocytosis that is not believed to be transport-mediated or a saturable kinetic process (Kearney et al., 2004). Information currently available does not indicate transport of tenofovir or its anabolites by active transporters. What is known is that tenofovir is a substrate for the human organic anion transporters 1 and 3 located in the renal proximal tubules (Kohler et al., 2011) and these have been shown to be responsible for its transport into renal tubular renal cells while multidrug resistance-associated proteins secretion tenofovir into the urine tubules (Kohler et al., 2011). No clinical data are presently available in human subjects regarding tenofovir penetration into other various tissues and fluids after administration of tenofovir disoproxil fumarate. However, penetration into the cerebrospinal fluid is understood to be low because of the anionic charge of the molecule at physiological pH.

The orally bioavailable prodrug tenofovir disoproxil fumarate, formulated as an investigational 75mg tablet, was used in a phase I/II dose-escalating pharmacokinetic, safety and antiviral activity study in 38 HIV infected patients (Barditch-Crovo et al., 2001). Tenofovir maximum concentration (Cmax) and area under the curve (AUC) were dose-proportional following single and multiple doses of tenofovir disoproxil fumarate over the dose range of 75–600mg in HIV-infected adults. Following the achievement of Cmax, tenofovir concentrations declined in a biphasic manner, with an apparent $t_{1/2}\beta$ of 12–13 hours over 24 hours of observation. The oral bioavailability of tenofovir following administration of a 300mg dose of tenofovir disoproxil fumarate was approximately 25% in the

fasted state, based on urinary recovery data and intravenous tenofovir data in patients.

3.6 Distribution and Metabolism of tenofovir

Tenofovir protein binding was evaluated in human plasma and serum by ultracentrifugation over a range of concentrations of 0.01–25.01 mg/L. The extent of tenofovir binding to serum or plasma proteins was not concentration-dependent over the range tested. Overall, binding is low with less than 1% and 7.2% bound in human plasma and serum, respectively. After intravenous administration of single and multiple doses of tenofovir 1 mg/kg to HIV-positive patients mean steady-state volume of distribution was approximately 800 mL/kg, (Deeks et al., 1998) suggesting that tenofovir distributes into a volume that approximates total body water (TBW). Body water is the water content of a human or an animal body that is contained in the tissues, the blood, the bones and other compartments of the body. This water makes up a significant fraction of the human body, both by weight and by volume. Most of body water is contained in various body fluids. These include Intracellular fluid, Extracellular fluid, Plasma, Interstitial fluid, and Transcellular fluid (Buendia et al., 2015, Yuana et al., 2015). The studies have demonstrated that neither tenofovir disoproxil fumarate nor tenofovir are substrates for CYP enzymes. Studies with rat hepatocytes suggested a small degree of CYP1A and CYP2B induction. However these effects have not been observed in drug interaction studies in humans (Lacombe et al., 2008, Duarte-Rojo and Heathcote, 2010). Thus, tenofovir is neither a substrate nor an inhibitor of CYP enzymes, which suggests

a low potential for clinically important drug-drug interactions with drugs that are substrates or inducers/inhibitors of these enzymes. The use of tenofovir disoproxil fumarate in combination with various antiretroviral drugs and other drugs frequently used by HIV-infected patients has been evaluated, including drugs that are metabolized by CYP or may affect CYP metabolism of other drugs. The clinical pharmacokinetic drug-drug interaction potential of tenofovir disoproxil fumarate has been comprehensively evaluated with more than 15 antiretroviral and non-antiretroviral medications. Of all the evaluations performed, only didanosine (Kearney et al., 2005) and atazanavir (Taburet et al., 2004, Bifano et al., 2013, Kis et al., 2013) have demonstrated clinically relevant drug-drug interactions requiring an alteration in the dosage of the agent coadministered with tenofovir disoproxil fumarate. The interaction of tenofovir and didanosine in particular has been evaluated in detail. However, when tenofovir disoproxil fumarate is given with a reduced dosage of didanosine (250 mg), the didanosine AUC is comparable to the AUCs of didanosine administered at standard doses, regardless of whether the drugs are given concomitantly or separated by 2 hours, and regardless of whether they are taken with food or in fasted state (Kearney et al., 2005). Table 3.3 highlights the results of some clinical studies conducted demonstrating only minor alterations in the pharmacokinetic parameters of either tenofovir or the majority of coadministered medications, including drugs that are renally eliminated and subject to active tubular secretion.

Table 3.3: Summary of pharmacokinetic parameters for tenofovir disoproxil fumarate in the presence of co-administered drugs

Co-administered drug	Change [% (90% CI)] of pharmacokinetic parameters vs. co-administered drug alone		
	Cmax	AUC	Cmin
Abacavir	↑12 (↓1, ↑26)	↔	ND
Adefovir dipivoxil	↔	↔	↔
Atazanavir	↓20 (↓27, ↓14)	↓25 (↓30, ↓19)	↓40 (↓42, ↓32)
Efavirenz	↔	↔	↔
Emtricitabine	↔	↔	↔
Indinavir	↓11 (↓30, ↑12)	↔	↔
Lamivudine	↓24 (↓34, ↓12)	↔	↔
Lopinavir	↔	↔	↔
Methadonea	↔	↔	↔
Oral contraceptivesb	↔	↔	↔
Stavudine (extended release)	↔	↔	↔
Ribavirin	↔	↔	ND
Ritonavir (100mg)	↔	↔	↔

a Exposures to (*R*)- (active), (*S*)- and total methadone were equivalent when given alone or with tenofovir. Individual subjects were maintained on their stable methadone dosage. No pharmacodynamic alterations (opioid toxicity or withdrawal signs or symptoms) were reported.

b Exposures to ethinylestradiol and 17-deacetyl-norgestimate (pharmacologically active metabolite) were equivalent when given alone or with tenofovir disoproxil fumarate

AUC = area under the concentration-time curve; Cmax = maximum serum/plasma concentration; Cmin = trough serum/plasma concentration; ND = not determined; ↑ indicates increase; ↓ indicates decrease; ↔ indicates no change.

3.7 Renal Elimination of tenofovir

Most studies show that tenofovir is excreted unchanged in the urine. Renal excretion is the primary route of elimination in which 70-80% is excreted unchanged drug over 72h following IV administration and 32 ± 10% of administered dose over 24h following multiple oral dosing with food. Tenofovir clearance was shown to exceed the glomerular filtration rate, suggesting urinary excretion by active tubular secretion is the most important (Cundy et al., 1998, Cundy et al., 1996). Figure 3.5 shows how tenofovir is excreted by kidney.

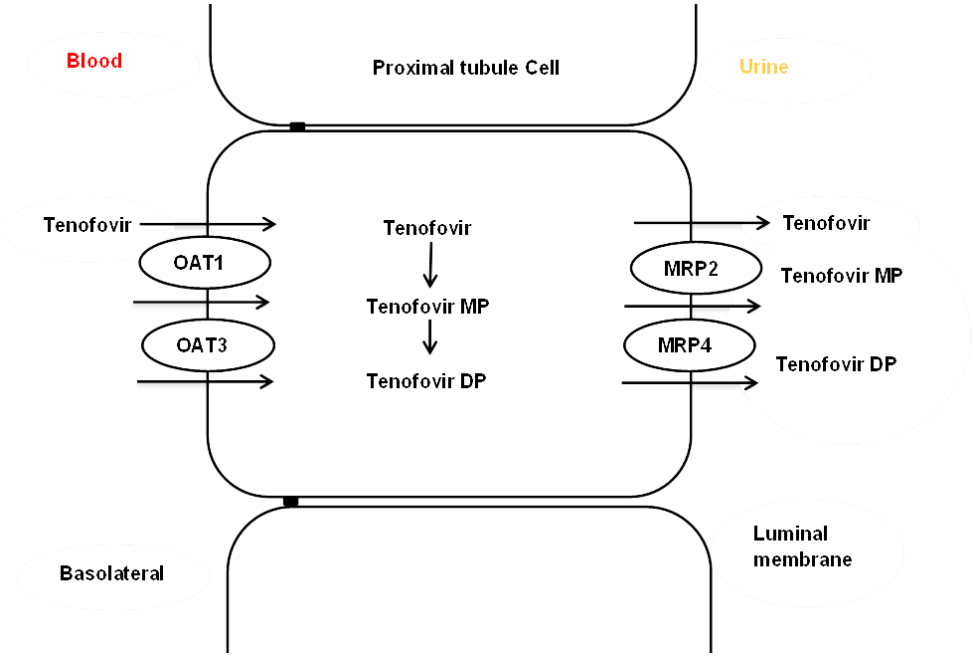


Figure 3.5: Elimination of Tenofovir in renal proximal tubule cells through active tubular secretion. On the basolateral side of the renal proximal tubule cell, tenofovir influx is primarily transported by hOAT1. Both MRP2 and MRP4 transport tenofovir. Tenofovir MP and tenofovir DP are both more toxic than tenofovir. Abbreviations: hOAT, human organic anion transporter; MRP, multidrug resistance protein; tenofovir DP, tenofovir diphosphate; tenofovir MP, tenofovir monophosphate (Source: Modified from Izzedine et al., 2009).

Tenofovir has been associated with kidney toxicity during the process of drug elimination. Some studies show that tenofovir contributes to renal dysfunction directly by inducing acute tubular necrosis, acute interstitial nephritis, crystal nephropathy, and renal tubular disorders or indirectly via drug interactions. In general tenofovir-anchored antiretroviral therapy is well tolerated in most patients but clinical cases of renal proximal tubular dysfunction (Horberg et al., 2010) including development of life-threatening Fanconi's syndrome have been overwhelmingly reported (Hall et al., 2009a, Baxi et al., 2014, Kalyesubula and Perazella, 2011). Thus, tenofovir- based antiretroviral therapy is known also to contribute to life-threatening renal electrolyte loss due to renal injury especially in patients with small body weight (Maggi et al., 2012a, Rodriguez-Novoa et al., 2009b, Nishijima et al., 2011, Gervasoni et al., 2013). Since tenofovir is eliminated as unchanged drug in the urine, a reduction in renal function is likely to result in higher drug exposure, and possibly drug-associated adverse effects, especially if the standard 300 mg/day dosage of tenofovir disoproxil fumarate was used in a patient with significant renal impairment.

Tenofovir pharmacokinetics were characterized after a single 300mg dose of tenofovir disoproxil fumarate in HIV negative individuals with mild (CLCR 50–80 mL/min; n = 10), moderate (CLCR 30–49 mL/min; n =8), and severe (CLCR <30 mL/min; n = 11) renal impairment, and in patients with end-stage renal disease receiving long-term haemodialysis (n = 9). Subjects with normal renal function (CLCR>80 mL/min; n = 3) were also included as controls. Pharmacokinetic parameters in serum and urine were evaluated from samples

collected over 96 hours and subjects with end-stage renal disease were studied during (intra-dialysis) and between (inter-dialysis) haemodialysis treatment sessions (Barditch-Crovo et al., 2001). Pharmacokinetic results in each of the study groups are summarized as shown in table 3.4. Compared with subjects with normal renal function, those with mild impairment had only slight increases in tenofovir AUC∞ with no appreciable differences observed in Cmax, tmax or $t_{1/2}\beta$. By contrast, subjects with moderate or severe renal impairment demonstrated substantial increases in tenofovir exposure. In moderately and severely impaired subjects, tenofovir AUC∞ increased with reductions in renal function as estimated by calculated CL_{CR} using the Cockcroft-Gault equation. Tenofovir renal clearance was 2- to 3-fold higher than CL_{CR} and decreased in direct proportion to reductions in both Cockroft-Gault calculated and urinary measured CL_{CR}, demonstrating preservation of active secretion of drug in the setting of renal impairment. When a single 300mg dose of tenofovir disoproxil fumarate was given to end stage renal disease patients the morning after a haemodialysis session, tenofovir serum concentrations increased progressively over time. Tenofovir concentrations decreased rapidly coinciding with a 4-hour haemodialysis session, rebounding immediately post-dialysis, and reaching a plateau thereafter, indicating the absence of an extrarenal route of elimination for tenofovir. Tenofovir was efficiently removed from serum by haemodialysis, as shown by a median extraction coefficient of 54% and median dialysis clearance of 134 mL/min (Barditch-Crovo et al., 2001).

Table 3.4: Single-dose tenofovir pharmacokinetic parameters in subjects with normal renal function and various degrees of renal impairment

Parameter	Renal function			
	normal (n = 3)	mild impairment (n = 10)	moderate impairment (n = 8)	severe impairment (n = 11)
Median renal clearance (mL/min)				
Creatinine[a]	86.5	64.2	33.8	18.6
Tenofovir	247	167	92	32
Tenofovir pharmacokinetic parameters [mean (CV %)]				
Cmax (µg/L)	335 (9.5%)	330 (18.5%)	372 (41.9%)	602 (30.8%)
AUC∞(µg . h/L)	2185 (11.8%)	3064 (30.3%)	6009 (41.7%)	15 985 (45.2%)

a Calculated by the Cockcroft-Gault method.

AUC∞ = area under the concentration-time curve from time zero to infinity; Cmax = maximum serum concentration; CV% = percentage coefficient of variation (Source: Kearney et al., 2001).

The pharmacokinetics of tenofovir, were not affected to an extent requiring dosage modification in participants with mild renal impairment (CL_{CR}50–80 mL/min). Subjects with moderate or severe renal impairment or those with end-stage renal disease requiring haemodialysis have substantial reductions in renal elimination of tenofovir and high systemic exposure requiring dosage adjustment. Pharmacokinetic modeling and simulations were conducted and used to develop dosage recommendations (Table 3.5) to limit unnecessary tenofovir

accumulation and result in steady-state trough concentrations (Cmin) more closely approximating those observed in patients with normal renal function. Although accumulation of tenofovir is reduced by extending the administration interval, Cmax remains elevated and the time to reach Cmin is prolonged relative to patients with normal renal function; therefore, monitoring of renal function is warranted for patients with renal disease treated with tenofovir disoproxil Fumarate (Barditch-Crovo et al., 2001).

Table 3.5: Recommendations for tenofovir 300mg to patients with renal impairment

Clinical condition	Administration interval
CLCR (mL/min)[a]	
≥50	Every 24h
30–49	Every 48h
10–29	Twice weekly
ESRD requiring haemodialysis	Every 7 days or after a total of approximately 12h of dialysis[b]

a Calculated using ideal bodyweight.

b Generally once weekly assuming three haemodialysis sessions a week of approximately 4h duration. Tenofovir should be administered following completion of dialysis.

CLCR = creatinine clearance; ESRD = end-stage renal disease (Source: Barditch-Crovo et al., 2001).

Notwithstanding the above, it is worth noting that the renal pharmacokinetic evidence and dosage modification have mostly been discussed on account of creatinine clearance as a marker of renal function. However, to

assess renal function along the length of the nephron may require assessment of other renal markers such as proximal tubular reabsorption of phosphate, fractional excretion of magnesium or fractional excretion of potassium etc.

3.8 Pharmacodynamics of tenofovir

Tenofovir is activated to tenofovir diphosphate in both active and resting cells (Robbins et al., 2003, Barditch-Crovo et al., 2001, Baheti et al., 2013). Tenofovir diphosphate inhibits HIV replication in macrophages and other non-dividing cells, a unique and potentially vital property that is not found with many members of the nucleoside analogue class particularly thymidine analogues such as zidovudine and stavudine. Tenofovir diphosphate competes with the natural deoxyadenosine 5′-triphosphate for incorporation into the growing viral DNA chain during HIV transcription. Unlike the natural deoxynucleotides substrates, tenofovir diphosphate lacks a 3′-hydroxyl group on the deoxyribose moiety. Therefore, after incorporation, tenofovir diphosphate blocks the action of HIV reverse transcriptase in that the next incoming deoxynucleotide cannot form the next 5′–3′ phosphodiester bond required to elongate the DNA chain (Robbins et al., 2003) as shown in Figure 3.6. Hence, when tenofovir is incorporated, viral DNA synthesis is terminated. Unfortunately, tenofovir terminates not only viral DNA but also host DNA synthesis. This is what results into viral suppression and drug toxicity or side effects of tenofovir at the same time.

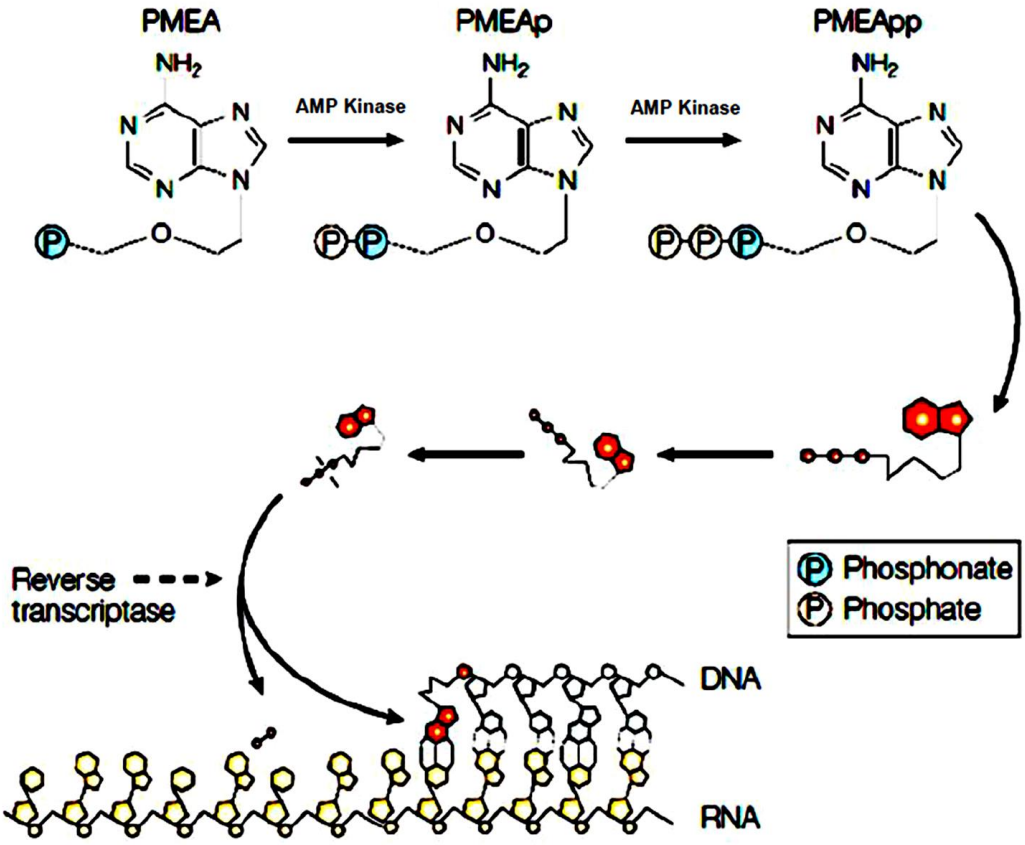


Figure 3.6: Mechanism of action of tenofovir. The mechanism of action of tenofovir (PMPA) is similar to that of adefovir (PMEA). As acyclic nucleoside phosphonates adefovir and tenofovir only need two phosphorylations (instead of three, as is the case for acyclic nucleoside analogues such as acyclovir and ganciclovir) to be converted to their active (diphosphorylated) forms. The diphosphorylated form then act as chain terminators of the DNA synthesis catalysed by their target enzyme, which is human immunodeficiency virus or hepatitis B virus RNA-dependent DNA polymerase (reverse transcriptase). The integration of one molecule of at the 3′-end of the growing DNA chain terminates further chain elongation. PMEA, 9-[2-(phosphonomethoxy)ethyl]adenine; PMPA, (*R*)-9-[2-(phosphonomethoxy)propyl]adenine; Adenosine monophosphate kinase, AMP Kinase (Source: Modified from De Clercq and Holy, 2005).

The pharmacodynamics of tenofovir, have been evaluated over a range of oral doses. One clinical pharmacology study with tenofovir disoproxil fumarate in humans was a randomised, double-blind placebo-controlled, dose-escalation study in antiretroviral-naive and -experienced HIV-1-infected participants. Thirty-eight patients received oral tenofovir disoproxil fumarate at dosages of 75, 150, 300 or 600mg once daily and 11 patients received placebo. Following 28 days of administration, median changes in HIV-1 RNA (log10 copies/mL) were –0.01 for the placebo group and –0.33, –0.44, –0.85 and –0.80 for the 75, 150 300 and 600mg dosage cohorts, respectively. Compared with placebo, the suppression of plasma HIV 1 RNA was significant for all four dosage groups with no additional increase observed in antiviral effect for the 600mg dosage compared with the 300mg dosage (Barditch-Crovo et al., 2001). Using a logistic model in the analysis, the dose-proportional relationship between dose of tenofovir disoproxil fumarate and change in log10 plasma HIV-1 RNA was demonstrated (Figure 3.7). The dose representing 50% of a maximal effect (effective dose, ED50) was estimated as 115mg. As the pharmacokinetics of tenofovir, are dose-proportional, similar relationships were also observed between Cmax and antiviral effect. Tenofovir disoproxil fumarate-based antiretroviral therapy has clearly demonstrated potency and efficacy in the management of HIV infection. Hence, it is not surprising that the Food and Drug Administration (FDA) approved the standard dose of tenofovir disoproxil fumarate 300 mg once daily for adults and children aged ≥12 years and weight ≥35 kg.

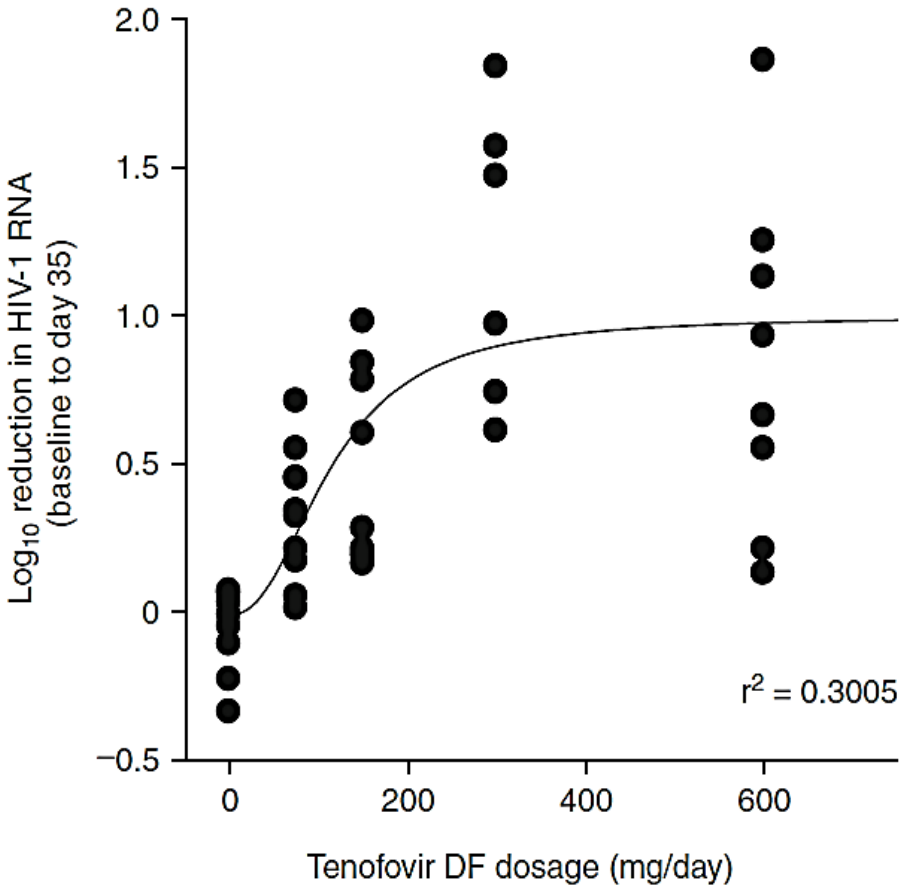


Figure 3.7: Relationship between dose of tenofovir and change in HIV-1 RNA. Pharmacokinetic-pharmacodynamics relationship indicates that the suppression of plasma HIV 1 RNA was significant for all four dosage groups with no additional increase observed in antiviral effect for the 600mg dosage compared with the 300mg dosage (Source: Barditch-Crovo et al., 2001).

3.9 Quantification of tenofovir

In early studies, concentrations of tenofovir in plasma and urine were determined by a validated high performance liquid chromatography (HPLC) assay using fluorescence detection (Deeks et al., 1998, Naesens et al., 1998) with a lower limit of quantification of 25 µg/L in serum and 1000 µg/L in urine. Subsequently, a more sensitive liquid chromatography tandem mass spectrometry (LC/MS/MS) assay was developed with a lower limit of

quantification of 0.5–3 µg/L in serum and plasma and 500 µg/L in urine (Hazra et al., 2004). Most stability studies have demonstrated that tenofovir is stable for extended periods of time in all matrices from all species tested. However, stability analyses revealed that tenofovir is unstable in strong acidic and alkaline environments (Agrahari et al., 2015).

Studies have demonstrated that the use of LC-MS/MS has high throughput in the determination of the antiretroviral drugs including tenofovir. The new method by Valluru was validated over a concentration range of 2-500ng/mL for tenofovir with relative recoveries ranging from 61 to 85% (Valluru et al., 2013). In this method the intra and inter batch precision (%CV) across four validation runs was less than 12.2%. In terms of accuracy for four quality control (QC) levels (lower limit of quantification (LLOQ), low quality control (LQC), medium quality control (MQC) and high quality control (HQC)) was within +/-8.5% in as far as relative error was concerned. In another experiment, Delahunty validated a tenofovir method using LC-MS/MS techniques. Hare, Chromatographic separation was achieved with a Polar-RP Synergi, 2.0 mm x 150 mm, reversed-phase analytical column. The mobile phase was 3% acetonitrile, 1% acetic acid aq. Detection of tenofovir and the internal standard (adefovir) was achieved by ESI MS/MS in the positive ion mode using 288/176 and 274/162 transitions, respectively. The method was linear from 10 to 750 ng/ml with a minimum quantifiable limit of 10 ng/ml when 250 microl aliquots were analyzed. The usefulness of this LC/MS/MS method to routinely monitor plasma concentrations of tenofovir was demonstrated along with its ability to assist in the performance of

pharmacokinetic studies (Delahunty et al., 2006). More and more validated methods using liquid chromatography-tandem mass spectrometry (LC-MS-MS) are being developed and used to determine various antiretroviral drugs other than tenofovir including efavirenz, emtricitabine and zidovudine (North et al., 2014).

Chromatographic methods such as liquid chromatography (LC) coupled with mass spectrometry (MS) largely provide better selectivity than high performance with UV or fluorescence methods. In the present study some samples for the analysis of tenofovir that were collected on HemaSpot dried blood spot were determined using LC-MS/MS methods. In the following section of this thesis, dried blood spot technology is discussed in detail.

3.10 Dried blood spot (DBS) Technology

The dried blood spot (DBS) in medicine was first introduced by Robert Guthrie in 1963 when he used the sample collection technique for screening of phenylketonuria in neonates (Guthrie and Susi, 1963). The usefulness of DBS as a simple approach to collect laboratory data became a topic of great interest owing to the numerous advantages it has over conversional whole blood and plasma sampling (Edelbroek et al., 2009, McDade et al., 2007, Mei et al., 2001). DBS has been used as a sampling technique for over 50 years now. The technique was also used by the father of Modern Clinical Microchemistry, Ivar Christian Bang with a co-worker, who successfully applied dried blood spot in the quantification of blood sugar for over a century (Bang and Bergmann, 1913). The technique has emerged as a pertinent method in both qualitative and quantitative

bioanalysis milieu (McDade et al., 2007, Wong et al., 2010, Wong et al., 2011, Kong et al., 2014). In DBS method, a spot of blood from a heel or finger stick is placed on filter paper and after drying it can be analyzed by modern analytical, immunological or genomic detection systems. A circular punch (about 3 mm) is removed, eluted with solvent and analyzed. More than 50 separate analytes can be measured from a quarter size spot of blood (Mei et al., 2001, Koulman et al., 2014, Uyeda et al., 2011, Applegarth et al., 1979), mainly due to adoption within the last 5 years of mass spectrometry for analysis.

The main advantages of DBS sampling method include minimal volume requirements of approximately 2 to 3 drops per spot, ease of sample attainment by finger or heel stick with minimal training required (Barfield et al., 2008, Beaudette and Bateman, 2004, la Marca et al., 2009), ease of transport and sample stability. Once dried, the sample is stable for months to years at ambient temperature or under refrigeration (McDade et al., 2007). The traditional blood sampling technique is made possible through Venipuncture, but this has the disadvantage of requiring large volume of blood. Furthermore, serial sampling has been shown to be difficult in infants during pharmacokinetic (PK) and toxicokinetic studies due to large volumes of blood required but with DBS this is easy through a heel bleed (Beaudette and Bateman, 2004). By contrast, blood sampling through DBS requires minimal amounts of blood. Following the collection of blood drops on a filter paper, drying and transportation, the blood spot is extracted and analyzed in the laboratory.

Another major advantage of DBS cards is that it is considered to be less biohazardous in comparison to liquid plasma samples in the sense that blood is in dried form is less infectious (Parker and Cubitt, 1999) since most proteins, pathogens and enzymes get inactivated on card and bacterial growth becomes dormant. For this reason it is considered as non-infectious material by International Air Transport Association regulations (ICAO, 2005, WHO/HSE/IHR, 2010, IATA, 2010). The less invasive nature of DBS and use of micro-volume blood samples have made it a very useful and promising sampling method especially for those in which it is not possible to collect larger volumes of blood such as neonates and very sick patients (Parker and Cubitt, 1999, Wilcken and Wiley, 2008). DBS does not need cold chain during transportation (Torok et al., 2002) and storage as well as centrifugation of sample thereby reducing the number of steps preceding drug sample analysis. This technique has also been reported to be suitable for drugs which are susceptible to photo-degradation (Alfazil and Anderson, 2008). Accelerated and natural photo-degradation experimental data has shown that some drugs exhibit higher photo-stability when spotted and stored on various DBS paper than that in water, plasma or whole blood (Bowen et al., 2010, Wiseman et al., 2010). DBS assisted analysis has extensive applications in PK and TK studies (Barfield et al., 2008, Spooner et al., 2009, Liang et al., 2009) therapeutic drug monitoring (TDM) (Edelbroek et al., 2009), disease screenings, testing of doping substances and metabolomic studies. It is being widely utilized in the pharmaceutical industries, hospitals and research centers, particularly where blood or plasma sample volumes are low,

difficult to collect, store, process or transport (Edelbroek et al., 2009, Garg and Dasouki, 2006, Wilcken and Wiley, 2008, Mei et al., 2001).

A recent study that characterize the pharmacokinetics of Tenofovir diphosphate and emtricitabine triphosphate in red blood cells versus peripheral blood mononuclear cells using DBS technique concluded that DBSs offer a convenient measure of recent adherence via tenofovir/emtricitabine levels and cumulative adherence over time via tenofovir diphosphate levels in red blood cells (Castillo-Mancilla et al., 2013, Zheng et al., 2014). This study supports the use of DBS as a suitable matrix for tenofovir testing. DBSs have been historically used for neonatal screening of inborn errors of metabolism with consistent results (Johannessen, 2010, Denes et al., 2012). Thus in the present study we used DBS technology in the quantitation of tenofovir in HIV-infected patients on tenofovir-based therapy.

The application of DBS technology is on the increase in various areas including research, clinical metabolic screening, TDM, preclinical and clinical PK, TK, forensic, biological and immunological sciences. Evidence is increasing in reported literature on the use of DBS in the investigations of drug (Saracino et al., 2012, Allanson et al., 2007, Damen et al., 2009, Calcagno et al., 2015, Ter Heine et al., 2011, la Marca et al., 2012, D'Apolito et al., 2012). Interestingly enough, DBS technology has evolved over the years and HemaSpot DBS is now believed to be the next generation for DBS collection technology (Figure 3.8).

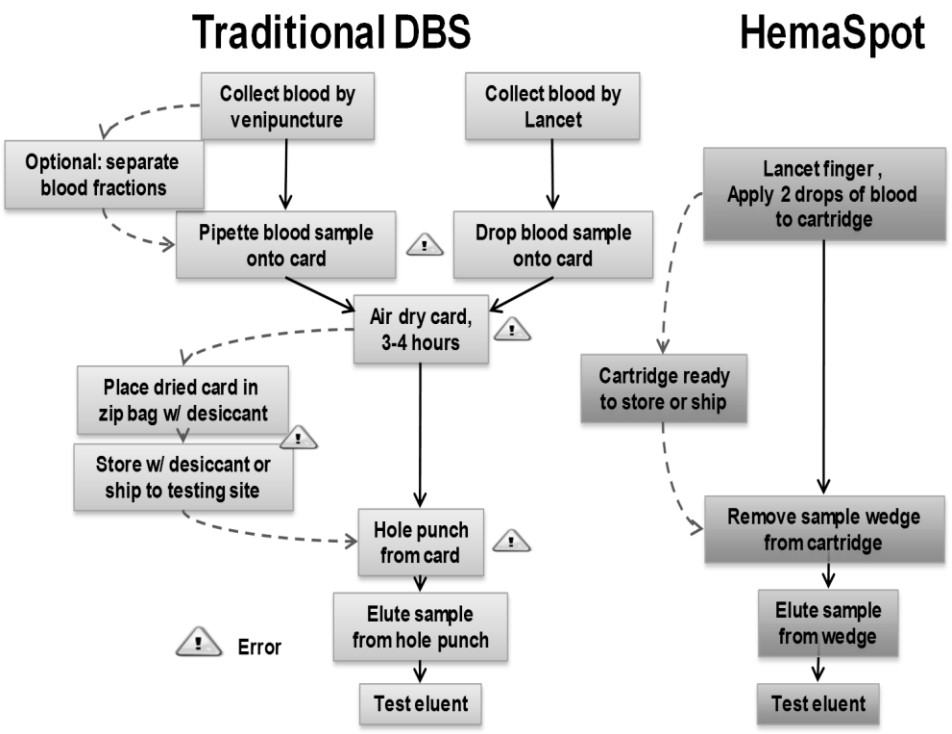


Figure 3.8: Compares Traditional-DBS with HemaSpot-DBS. HemaSpot is designed to streamline blood collection as compared to venipuncuture or to traditional dried blood spot, which can be problematic and errors can be made at multiple steps as denoted by orange triangle. With few steps and simple sampling, HemaSpot eliminates these problem steps (Source: Modified from Mei et al., 2001, Li and Tse, 2010).

HemaSpot DBS as an easy-to-use device utilizes all the beneficial properties of traditional DBS technology while improving, simplifying specimen collection and improving sample quality (Parker and Cubitt, 1999, Wilcken and Wiley, 2008). The additional features that make HemaSpot DBS distinctively different from other traditional DBS platforms include: a) the innovative fan-shaped form of absorbent material that improves biological sample collection and analysis as compared to traditional DBSs b) the patented cartridge that contains an absorbent paper and desiccant under an application surface with a small opening to allow entry for blood (Mei et al., 2001). The desiccant rapidly dries the

sample thereby maintaining sample stability unlike traditional DBS which requires 2-3 hours for blood to dry (Edelbroek et al., 2009, Mei et al., 2001, Li and Tse, 2010). Samples dry about 2 times faster with HemaForm as compared to traditional spots.

Therapeutic drug monitoring is the measurement of specific drug concentration in patients' blood with time to optimize dose regimen (Jang et al., 2015, Balakrishnan and Shorten, 2015, Ossa et al., 2015, Hiemke, 2015). It involves the use of drug concentration measurements in body fluids as an aid to the management of drug therapy for the cure, alleviation or prevention of disease. Several methods are reported for therapeutic drug monitoring using DBS as sampling technique. DBS method is suitable for therapeutic drug monitoring. In particular, HemaSpot DBS-LC-MS/MS is expected to play a progressively key role in the therapeutic drug monitoring especially in developing countries where such services are inadequate. A number of adult studies suggest that modified dose intervals and regimen choices based on therapeutic drug monitoring result in achievement of targeted ARV drug concentrations and are associated with improved clinical response and/or tolerability (Bossi et al., 2004, Nettles et al., 2006, Perrone et al., 2014). However, adverse drug events are common and attributable to a variety of factors, including inadequate medication adherence, pharmacokinetic attributes, and the drug-food interactions. One of the challenges in the management of HIV-infected patients eligible for ART is the institution of an optimized dose (Kearney et al., 2004) and dosing regimen that maximizes clinical response in a beneficial manner and

minimizes hepatic and renal toxicities for example (Gerard et al., 2007, Wools-Kaloustian et al., 2007). These challenges could be mitigated by assessing antiretroviral drug blood concentration levels so as to adjust doses or dosing intervals in order to optimize therapy (Kearney et al., 2004). Optimization of the use of antiretroviral drugs depends on some understanding of 1) the pharmacodynamics, (2) the pharmacokinetics, and (3) the interaction between these two, known as the time-dependent pharmacodynamics. In the antiretroviral therapy era, advances in drug quantification techniques have led to the development of assays for therapeutic drug monitoring but that is more available in developed countries than resource limited countries. The Quantification of antiretroviral drugs through therapeutic drug monitoring remains an important therapeutic challenge in Africa (Lamorde et al., 2014) and Zambia in particular. A study done in Netherlands that involved 147 patients starting nelfinavir and indinavir showed that TDM in treatment-naïve patients improves treatment response. In this study, after one year of follow up significantly fewer patients in the TDM group had discontinued therapy than the control group without TDM (17.4% versus 39.7%) (Burger et al., 2003). Clearly offers the possibility to detect patients with drug concentrations outside therapeutic ranges, who can subsequently benefit from dose modification.

CHAPTER 4

STATEMENT OF THE PROBLEM

The substantial evidence based data revealed in literature review of chapter 2 and 3 of this thesis regarding the pathophysiological overlaps of malnutrition, HIV-infection and/or tenofovir-based treatment, necessitated the undertaking of the present study. The problem investigated in this study is three fold, namely 1) there are disturbing reports of nutrient abnormalities in low body mass index (BMI) HIV-infected patients initiating tenofovir-based therapy, 2) high tenofovir concentration exposure has been reported in low BMI patients infected with HIV taking tenofovir-based regimen with resultant effect of renal dysfunction and 3) unavailability of user friendly robust methods for determining tenofovir serum concentrations in HIV-infected patient with low BMI in resource limited countries like Zambia.

Problem number 1: Malnutrition is a serious common health problem to the extent even post United Nations Millennium Development Goals (MDGs), 2015 era, under-nutrition remains a challenge in developing countries (Haddad et al., 2015, Hawkes et al., 2015) where HIV-infection is endemic. Under-nutrition has been linked to depletion or altered metabolism of vitamins and minerals . A study conducted in the South of India that involved a total of 464 HIV-infected ART experienced and naïve patients analyzed for abnormalities in potassium, sodium, chloride and bicarbonate levels. Of these 278 (60%), 312 (67.2%), 319 (68.7%) and 176 (38%) had abnormal potassium, sodium, chloride and carbonate levels respectively. A study in Zambia showed that low phosphate

serum levels in HIV-infected patients with body mass index (BMI) less than 18.5 Kg/m^2 predicted early mortality (Heimburger et al., 2010). This study showed that baseline serum phosphate was significantly higher among participants alive at 12 weeks of antiretroviral drugs (median 1.30 mmol/L; IQR: 1.04, 1.43), compared to those who died (median 1.06 mmol/L; IQR: 0.89, 1.27; $p < 0.01$). In Zambia, the great majority of patients are started on a single fixed-dose ART tablet containing tenofovir disoproxil fumarate (TDF) 300 mg, emtricitabine 200 mg, and efavirenz 600 mg as first-line of therapy. Tenofovir-based regimen has also been linked with renal phosphate wasting as a consequence of renal tubular injury (Izzedine et al., 2009a) and tenofovir-anchored ART can also exacerbate bone loss leading to electrolyte wasting (Cotter and Mallon, 2011). Moreover, renal injury and subsequent electrolyte wasting can occur with HIV infection per se (Kalyesubula et al., 2014). There is increasing evidence that despite efficacious ART, mortality rates for patients starting ART remains high in low BMI HIV-infected patients especially in the first three months of therapy (Stringer et al., 2006, Filteau et al., 2015, Bigna et al., 2015, Moore et al., 2011).

It is important to investigate the effect of lipid-based nutrient supplement containing vitamins and minerals (LNS-VM) compared to lipid-based nutrient supplement (LNS) alone on renal excretion of potassium, magnesium and phosphate in malnourished HIV-infected patients initiating tenofovir-based antiretroviral therapy.

Problem number 2: A study that evaluated the incidence and risk factors for acute kidney injury in HIV-infected patients found that Black race and low

body mass index (BMI) were among factors that had significant acute kidney injury associations. The study argues that although acute kidney injury incidence has decreased during the ART era, it remains common in low BMI HIV-infected patients (Li et al., 2012). Tenofovir-based ART is known to contribute to renal tubular injury resulting in life-threatening electrolyte loss (Maggi et al., 2012b). A study done among Caucasian HIV-positive patients demonstrated that 32% of women with body weight ≤ 50kg versus 18% of women with body weight > 50kg experienced tenofovir-related toxicity. In that study women who experienced toxicity had plasma tenofovir concentrations significantly higher than those who did not experience drug-related adverse events (173±131 versus 106±65 ng/ml, p=0.042). These results imply that HIV- infected women with low body weight are at risk to be exposed to high tenofovir plasma trough concentrations that eventually result in a significant hazard to develop drug-related complication. In post marketing safety data for tenofovir, the study identified advanced age, low body weight and low CD4 count as risk factors of development of renal dysfunction during tenofovir use (Nelson et al., 2007). A Zambian study involving nearly 30,000 patients showed that death rates in the first three months of starting antiretroviral treatment were highest among the most severely malnourished patients (Koethe et al., 2010), but mortality causes remain unexplained. Africa has reported increasing prevalence of renal dysfunction ranging from 6% in South Africa, 33.5% in Zambia and 48.5% in Uganda (Brennan et al., 2011, Mulenga et al., 2008, Izzedine et al., 2004). Studies have demonstrated that age, BMI and estimated creatinine clearance influenced

tenofovir plasma trough concentrations in HIV-infected patients (Jullien et al., 2005, Baxi et al., 2014) resulting in renal toxicity and subsequent mortality (Wyatt et al., 2006). In agreement with related studies the tenofovir trough concentrations in 195 patients (median, 50ng/ml and interquartile range, 35 to 77ng/ml) were significantly associated with the estimated glomerular filtration rate and BMI.

It is thus imperative to assess whether high tenofovir concentrations are associated with low BMI in malnourished HIV-infected patients initiating tenofovir-based antiretroviral therapy.

Problem number 3: Studies have demonstrated that therapeutic drug monitoring (TDM) offers the possibility to detect patients with drug concentrations outside therapeutic ranges, who can subsequently benefit from dose modification. A study done in Netherlands that involved 147 patients starting nelfinavir and indinavir showed that TDM in treatment-naïve patients improves treatment response. In this study, after one year of follow up significantly fewer patients in the TDM group had discontinued therapy than the control group without TDM (17.4% versus 39.7%). In a non-completer equals failure analysis, the TDM group showed a significantly higher proportion of patients with a viral load below 500 copies/ml after 12 months of treatment (78.2% versus 55.1%) (Burger et al., 2003). These data could be useful for designing strategies to manage tenofovir-associated toxicity, since this toxicity has been reported to be dose dependent (Calcagno et al., 2013). In the ART era, advances in drug quantification techniques have led to the development of assays for ARV

pharmacokinetic monitoring but that is more available in developed countries than resource limited countries (Mei et al., 2001, Perrone et al., 2014, Cattaneo et al., 2012).

Quantifying the ARVs by therapeutic drug monitoring (TDM) remains an important therapeutic challenge in Africa (Lamorde et al., 2014) and Zambia in particular. One of the challenges in the management of HIV-infected patients eligible for ART is the institution of an optimized dose (Kearney et al., 2004) and dosing regimen that maximizes clinical response in a beneficial manner and minimizes hepatic and renal toxicities for example (Jullien et al., 2005, Wools-Kaloustian et al., 2007, Gerard et al., 2007). These challenges could be mitigated by assessing ARV blood concentration levels so as to adjust doses or dosing intervals in order to optimize therapy (Kearney et al., 2004). The usefulness of DBS as a simple approach to collect laboratory data became a topic of great interest owing to the numerous advantages it has over conversional whole blood and plasma sampling (Edelbroek et al., 2009, McDade et al., 2007, Mei et al., 2001). For a long time now, immunoassay and other assay methods have been applied in quantitative analysis of drugs using DBS technology. Nonetheless, immunoassays require handling of radioactive materials and/or prolonged incubation, and are susceptible to cross reactivity by endogenous components and nonspecific binding (Chen et al., 2009, Gorsky et al., 1983). In contrast, chromatographic methods such as liquid chromatography (LC) coupled with UV or fluorescence detection; largely provide better selectivity than some immunoassay methods. This is achieved through chromatographic separation of

the analyte of interest from most interfering components in the sample matrix. DBS-LC-MS/MS has emerged as an important method for quantitative analysis of drugs. Its application in therapeutic drug monitoring is relatively new but the impressive expansion of the technique in quantitative analysis of drugs has been well demonstrated. In particular, HemaSpot DBS-LC-MS/MS has shown to be superior and may play a key role in the therapeutic drug monitoring especially in developing countries where such services are inadequate like Zambia. We propose to use TDM for tenofovir to monitor toxicity and adherence to the drug regimen in underweight HIV-infected patients.

Thus, it is important to quantify and compare tenofovir concentrations measured from HemaSpot dry blood spots versus fresh blood samples in malnourished HIV-infected patients initiating tenofovir-based antiretroviral therapy.

4.1 Research Questions

1. Does lipid-based nutrient supplement with added vitamins and minerals alter renal excretion of potassium, phosphate and magnesium and other electrolytes in underweight HIV/AIDS patients on tenofovir-based antiretroviral therapy?
2. Can dry blood spots be used to monitor tenofovir concentrations levels in underweight HIV-infected patients initiating tenofovir-based antiretroviral therapy?
3. Are underweight HIV+ patients starting the standard tenofovir-based ART prone to accumulation of toxic tenofovir concentrations?

4.2 Study Justification

In the last few years there have been numerous randomised clinical trials to explore the benefits of micronutrient supplementation in patients living with HIV/AIDS in many of the African countries (Nchito et al., 2004, Rehman et al., 2015b, Filteau et al., 2015). To date attention has focused mainly on micronutrient supplementation in relation to CD4+ cell count and mortality. Much has been learned from such studies, but much remains to be accomplished to ensure the effective and safe use of antiretroviral drugs in HIV-positive patient starting therapy (Raiten, 2011). In this regard, less attention has been paid to metabolic consequences of nutrients in HIV + positive patients and pharmacokinetic-pharmacodynamic interactions between nutrients and drugs. Nutrient-drug interactions in the kidney are important because they contribute to therapeutic outcomes of the patient. Currently there is insufficient available data on renal handling of micronutrients/electrolytes and nutrient-drug interactions in HIV-1 positive patients in Zambia and elsewhere. In addition quantification of antiretroviral drugs for therapeutic drug monitoring (TDM) remains an important therapeutic challenge in Africa (Lamorde et al., 2014) and Zambia in particular. This thesis sets out to investigate the effect of lipid-based nutrient supplement on renal excretion of electrolytes and determine tenofovir serum concentration levels among underweight Zambian HIV/AIDS patients initiating tenofovir-based antiretroviral therapy.

4.3 Study Rationale

Despite the fact that many studies have demonstrated low serum levels of electrolytes and equally more renal electrolyte wasting in HIV positive patients, little is known about the effect of lipid-based nutrient supplement on renal function in these patients. Most of the current and ongoing studies on nutrition and HIV/AIDS are not powered to examine pharmacokinetic profiles of both serum and renal electrolytes in nutrient supplemented versus non-supplemented patients. Therefore, the renal electrolytes mainly potassium, phosphate and magnesium were investigated in this sub-study because they are essentially dependent on the kidney for their excretion from the human body. This is because in people living with HIV/AIDS, factors such as malnutrition, HIV and drugs like tenofovir contribute to proximal renal tubular dysfunction resulting in loss in excessive loss of electrolytes via the kidney. The mechanisms that drive the potential wasting of vital renal electrolytes such as potassium, phosphate and magnesium in people living HIV/AIDS are not fully elucidated.

Dried blood spot (DBS) using LC-MS/MS has emerged as an important method for quantitative analysis of drugs. These advances in drug quantification techniques have led to the development of assays for ARV pharmacokinetic monitoring but that is more available in developed countries than resource limited countries (Cattaneo et al., 2012, Perrone et al., 2014). Quantifying the ARVs by therapeutic drug monitoring (TDM) remains an important therapeutic challenge in Africa (Lamorde et al., 2014) and Zambia in particular. TDM for tenofovir is useful to monitor toxicity and adherence to the drug regimen.

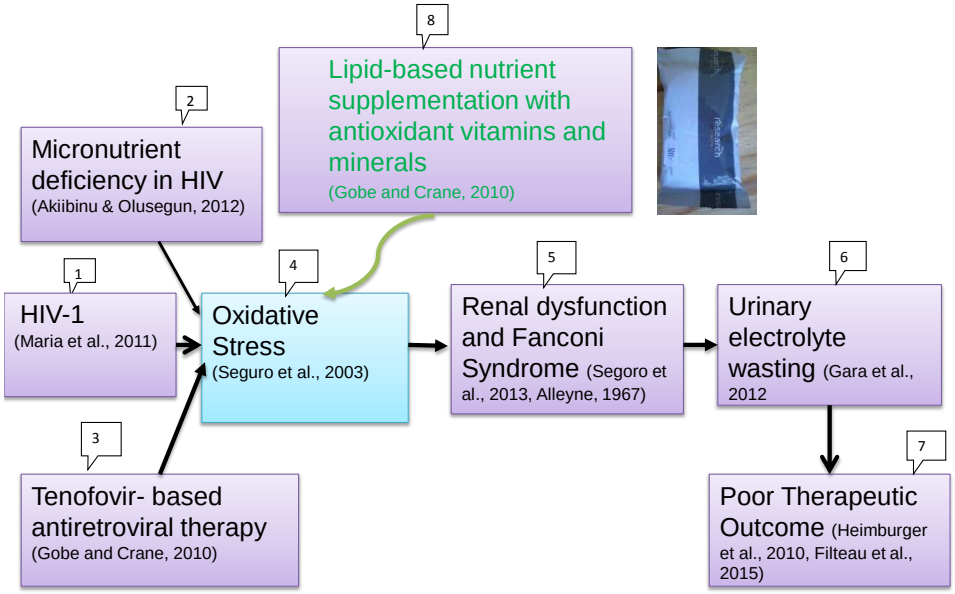


Figure 4.1: Problem analysis diagram for objective 1. Several factors including micronutrient deficiency, HIV-1 itself and tenofovir-based treatment contribute to oxidative stress. The oxidative stress is associated with acute renal injury with resultant loss of renal electrolytes such as phosphate culminating in poor therapeutic outcome e.g. mortality. It is envisaged that nutrient supplementation may mitigated the effect of oxidative stress through the provision of antioxidants to malnourished HIV-infected patients.

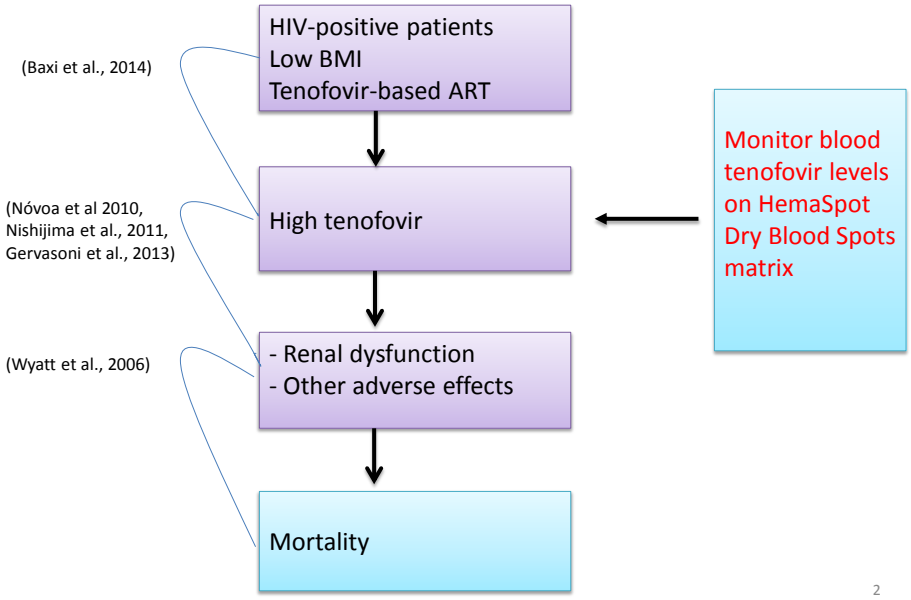


Figure 4.2: Problem analysis diagram for objective 2 & 3. Low BMI is a risk factor to high tenofovir exposure in HIV-infected patients taking tenofovir-based ART. And that high tenofovir exposure is associated with renal dysfunction which has been linked to increased mortality among these patients. It is thought that therapeutic drug monitoring via the implementation of HemaSpot DBS technology might improve therapeutic outcomes through dose modifications in HIV-infected patients presenting with low BMI. Abbreviations: Body mass index-BMI; Antiretroviral therapy-ART.

4.4 Hypotheses

1. Lipid-based nutrient supplement with added vitamins and minerals (LNS-VM) during tenofovir based therapy in HIV/AIDS attenuates renal loss of phosphate, potassium and magnesium compared to supplement (LNS) alone.
2. Low BMI is a significant risk factor for increased tenofovir exposure in HIV-infected patients initiating tenofovir-based ART.

4.5 General Objectives

To investigate the effect of lipid-based nutrient supplement on renal excretion of electrolytes and determine tenofovir blood concentration levels among underweight Zambian HIV/AIDS patients initiating tenofovir-based antiretroviral therapy.

4.6 Specific Objectives

1. To determine the effect of lipid-based nutrient supplement containing vitamins and minerals (LNS-VM) compared to lipid-based nutrient supplement (LNS) alone on renal excretion of potassium, magnesium and phosphate.
2. To quantify and compare tenofovir concentrations measured from HemaSpot dry blood spots versus fresh blood samples in malnourished HIV-infected patients initiating tenofovir-based antiretroviral therapy.
3. To assess whether high tenofovir concentrations are associated with low BMI in malnourished HIV-infected patients initiating tenofovir-based antiretroviral therapy.

CHAPTER 5

MATERIALS AND METHODS

This chapter describes in detail the materials and methods used in this study. It covers patient recruitment procedures, serum or urine collection and assays and chromatographic quantification of tenofovir from serum and HemaSpot dried blood spot. In addition statistical methods for data analyses and ethical approvals are discussed.

5.1 Site and Study Design

This was a nested study in a phase III randomized double-blinded clinical trial called Nutritional Support for Africans Starting Antiretroviral Therapy (NUSTART) (Filteau et al., 2015). Trial registration: PACTR201106000300631, registered on 1st June 2011. NUSTART was conducted from August 2011 to December 2013 at the University Teaching Hospital, Lusaka, Zambia and the National Institute for medical Research, Mwanza, Tanzania. The trial compared, in a 2-stage protocol, lipid-based nutrient supplement containing vitamins and minerals (LNS-VM) with control supplement (LNS) without added vitamins and minerals. The supplements were given to the study participants recruited at the two sites from recruitment (i.e. referral for ART) up to 6 weeks after starting ART. In the first stage, from referral until 2 weeks after ART initiation, the intervention and control supplements were given with minimal calories (30g/day, 100 kcal/day), then from 2-6 weeks after ART initiation, patients were given 250g/day, in two 125g sachets, comprising 1360 kcal/day in a calorie-rich supplement.

This sub-study was conducted at the University Teaching Hospital in Lusaka and participants were recruited between 13th May and 18th November, 2013 based on the inclusion and exclusion criteria of the parent study (Filteau et al., 2015). A total of 130 HIV-infected patients between ages 18-49 years were enrolled from six (6) health facilities of Zambia's capital city Lusaka. The health facilities included; Mtendere, Kanyama, Chilenje, Chawama, Chipata and Kamwala. These are peripheral clinics which manage antiretroviral support and refer complicated cases to the University Teaching Hospital (UTH). The antiretroviral therapy used as first-line was a single fixed-dose tenofovir-based antiretroviral therapy (ART) tablet containing tenofovir disoproxil fumarate (TDF) 300 mg, emtricitabine 200 mg, and efavirenz (EFV) 600 mg provided free for those with CD4 lymphocyte count <350 cells/μL or WHO stage 3 or stage 4.

5.2 Selection of Participants

Patients attending clinics for free HIV testing services for possible initiation of antiretroviral therapy in respective health facilities were approached and informed about NUSTART by study nurses. After informing them, patients were told that they were free to participant or not to take part in the study and that if they did not participate or withdrew their standard of care was not going to be affected. Those willing to participate were then referred and transported to the University Teaching Hospital NUSTART clinic for further assessment. The assessment and enrollment was done by NUSTART staff nurses.

Male and female patients were recruited into this sub study based on the NUSTART criteria at the UTH NUSTART clinic. Inclusion criteria were: at least

18 years old, ART-naive, BMI < 18.5 kg/m^2. In the presence of clinical oedema and a BMI < 20 kg/m^2, BMI was re-measured after loss of oedema, and the patient was considered eligible if BMI was < 18.5 kg/m^2 and antiretroviral therapy was not initiated, those requiring antiretroviral therapy as determined by CD4 count < 350/ µl or stage 3 or 4 disease, those willing to undertake intensive antiretroviral therapy follow-up in the study clinic, those willing to provide written and fully informed consent (thumbprint was accepted). Exclusion criteria were the non-fulfillment of inclusion criteria plus: participation in a potentially conflicting research protocol and pregnancy. Adult participants fulfilling the inclusion criteria were deemed eligible for the present study. This study then collected patient information including: date of recruitment, study identification number, age, sex, religion, occupation, education level, marital status, general health, current medication, dietary intake, and other lifestyle factors using a questionnaire. In addition, the patients had height and weight measured using calibrated scales and stadiometers (standardized techniques) respectively for calculation of body mass index. Weight was measured at all visits and height at recruitment. Measurements were taken in triplicate and the mean used in analyses. Body mass index was calculated by using patient body weight and height and cross checked these values with a pre-calculated body mass index reference table for height and weight in order to arrive at the individual patient's body mass index (BMI = wt/htxht) Kg/Msq).

The demographics and related data were collected at NUSTART clinic at the UTH during the time patients referred for assessment. After collection of

information at enrollment, patients were scheduled to be seen weekly from recruitment until the antiretroviral therapy initiation visit, then at 2, 4, 6, and 12 weeks after starting antiretroviral therapy. However, for the present study, samples were collected at only recruitment, 6 and 12 weeks of antiretroviral therapy. Patients were actively followed-up if they missed scheduled visits. Patients and relatives were contacted by phone.

5.3 Experimental Design

As part of standard care, before initiating antiretroviral therapy patients were screened, commenced on cotrimoxazole 960mg once daily for prevention of opportunistic infections and counseled regarding lifelong antiretroviral treatment adherence. During this pre-antiretroviral therapy period, the first stage of interventions was introduced.

Patients received a stepped nutritional supplement regimen of LNS or LNS with supplementary doses of vitamins and minerals (LNS-VM) developed by Nutriset, Malaunay, France (Table 5.1). Participants were randomly allocated to either LNS or LNS-VM. The supplementation started with small daily doses containing limited calories (30 g, 150 kcal), from time of referral for ART through the pre-ART preparation phase and until 2 weeks after start of ART. This was followed by larger daily doses containing greater calorie provisions (250 g, 1400 kcal) for four weeks. At a median interval of 3 weeks after referral, patients were started on a single fixed-dose antiretroviral therapy tablet that contained 300 mg tenofovir disoproxil fumarate (TDF), 200 mg emtricitabine , and 600 mg efavirenz (EFV).

Table 5.1: Nutritional composition of trial supplements – amounts per day

Nutrient	First phase supplement (from recruitment to 2 weeks of ART)		Second phase supplement (from 2 to 6 weeks of ART)	
	LNS-VM (30 g)	LNS (30 g	LNS-VM (250 g)	LNS (250 g)
Calories (kcal)	139	168	1397	1416
Protein (g)	2.4	2.3	55	55
Fat (g)	11.0	10.9	97.5	97.5
Potassium (mmol)	30	0.9	32	15.8
Phosphorus (mmol)	47	0.4	38	9.3
Magnesium (mmol)	16	0.3	17	5.7
Calcium (mg)	29.8	5.0	140	115
Iron (mg)	0.4	0.4	14.7	8.4
Zinc (mg)	21	0.2	21	3.8
Copper (mg)	3.6	0.06	3.6	1.2
Manganese (mg)	4.2	-	4.2	-
Iodine (µg)	420	-	420	-
Selenium (µg)	180	-	180	-
Chromium (µg)	75	-	75	-
Retinol (as palmitate) (µg)	1800	-	1800	-
Vitamin D (µg)	10	-	10	-
Vitamin E (mg)	45	-	45	-
Vitamin K (µg)	95	-	95	-
Vitamin C (mg)	120	-	120	-
Thiamin (mg)	2.4	-	2.4	-
Riboflavin (mg)	3.3	-	3.3	-
Niacin (mg)	39	-	39	-
Pyridoxine (mg)	3.6	-	3.6	-
Folate (µg)	600	-	600	-
Vitamin B12 (µg)	4.5	-	4.5	-
Pantothenic acid (mg)	9	-	9	-

Where nutrient contents are provided for both LNS and LNS-VM, these are values from analysis by the manufacturer, accounting for inter-batch variability; where values for only LNS-VM are given, these where not assessed in the prepared foods but refer to amounts added, that is, they do not include those intrinsic to the LNS.

5.4 Randomization and Blinding

The randomization and blinding was as the parent trial. Randomization was in blocks of 16 in the mother (NUSTART) trial. The statistician from the Data Safety and Monitoring Board generated and kept the code. Two codes were prepared: (1) an allocation code (letters A to H) indicating the contents of the packets (intervention vs. control) known only to the producer and the Data and Safety Monitoring Board (DSMB) statistician, and (2) a randomization code related to study ID numbers that was held by the DSMB statistician and study pharmacists. Packages of supplements were delivered by the producer in lots designated by allocation code. Packets were further labeled with the study ID numbers by the clinic pharmacists when they were dispensed. Participants were recruited to sequential IDs within clinics.

5.5 Adherence to Antiretroviral Therapy and Nutrients

As patients reported at each scheduled visit in the mother trial, they were given sufficient sachets of LNS (control) or LNS-VM to last until their next visit. Adherence to prescribed nutrients or antiretroviral therapy was determined by asking at each visit whether participant had missed or was late taking any supplement or medication since the last visit. Patients were asked to bring empty packages of nutrients or medication with them to the next visit and remaining sachets or tablets were counted before refilling them. Overall adherence was calculated as total empty packages or tablets returned divided by total packages or tablets expected to be consumed or taken for both the parent trial and the sub-study.

5.6 Sample size determination and Justification

The sample size of 130 patients was determined assuming renal fractional excretion standard deviation of 6% potassium, mean difference between experimental group and control group of 3.5% (Elisaf and Siamopoulos, 1995) 95% confidence,80% power and with 20% loss to follow-up as follows:

Table 5.2: Calculation of sample size considering alpha (a) and beta (b) errors

Study Characteristic	Assumptions
Type of study	Subset analysis randomized controlled trial of nutrient supplement
Data sets	Observations in cases and controls
Variable	Electrolyte urine levels
Losses to follow-up	20%
Fractional Excretion Standard deviation (s)	6% potassium
Variance $(s)^2$	36%
Data for alpha (Za)	P=0.05;95% confidence desired (two-tailed test); Za =1.96
Data for beta (Zb)	20% beta error;80% power desired (one tailed test); Za=0.84
Difference to be detected (d)	3.5% mean difference between the experimental group and control group

Calculation of sample size (N) $N = \frac{(Za+Zb)^2.2.(s)^2}{d^2}$ = $(1.96 + 0.84)^2$ x 2 x $(6)^2$ / $(3.5)^2$ = 7.84 x 2 x 36 / 12.25 = 46.08. Adjustment for expected lose to follow up is set at – 20% = 55.296, N = 56 participants per group x 2 groups = **112** participants total. The total number of participants need in this study is **112**. However the actual recruitment was **130** patients.

Table 5.3: Power Determination of Sample Size

	Significance	Loss to follow-up	Values in experimental group	Difference between control and intervention groups	N per group
Urine Electrolyte Excretion				**mmol/L**	
80%	20%	20%	24 (SD 6)	3.5	56
80%	20%	20%	24 (SD 6)	1.5	251

5.7 Standard of Care and ART

Standard of care was as predetermined in the parent study. Medical care, including choice of antiretroviral therapy regimen and other antimicrobial and supportive therapy, for study participants during both the pre- antiretroviral therapy and antiretroviral therapy phases were based on national treatment guidelines. The antiretroviral therapy regimens were those used routinely by the Zambian Ministry of Health which follows WHO guidelines. Usual first-line regimens including efavirenz or nevirapine in combination with tenofovir and emtricitabine were available. The medical care was provided at clinic level by study staff with referral to other facilities as was needed. The standard of care remained consistent with medical practice in Zambia. As per standard of care eligible participants were given co-trimoxazole to suppress Pneumocystis risk, tuberculosis treatment if they were sputum positive or diagnosed with TB by chest X-ray. Patients were also psychologically counseled and prepared for lifelong drug treatment and the need for high compliance was emphasized.

5.8 Collection of Samples

For the sub-study, the participants were given urine collection bottles for spot urine collection and had blood drawn. Venous blood specimens were collected at baseline, 6-weeks, and 12-weeks after starting antiretroviral therapy by the study nurses in the clinic. Approximately 3-5 ml of blood samples were collected. Only 6 weeks and 12 weeks blood samples were collected for fresh blood tenofovir samples. When samples arrived in the University Teaching Hospital (UTH) Internal Medicine Laboratory, the samples were entered the log book indicating the date, sample identification number, week and sex. The samples were then centrifuged to allow the separation of serum. The serum was transferred into tubes which were labeled according and stored at -80 degree Celsius (°C). In addition, 3 drops of blood were collected on HemaSpot dried blood spot devices through finger stick. Samples were then stored at fridge condition 4 degree Celsius (°C). The dried blood spot method was adopted for testing because it offered a simple and practical method to obtain blood samples in minute volumes from patients.

Approximately 20-30 ml of spot urine samples were collected at baseline, 6 weeks and 12 weeks after initiation of antiretroviral therapy. When urine samples arrived in the University Teaching Hospital (UTH) Internal Medicine Laboratory, the samples were prepared as follows: 750 µl of 10% nitric acid was pipetted into 1500µl storage tubes. To this solution was added 750 µl of urine sample. Then, 300 µl of second urine sample was pipetted into separate storage tubes without nitric acid. All the sample tubes were labelled with patient

identification number, date, gender and the week the sample was collected. The solutions were then vortexed. The prepared samples with nitric acid were then stored at fridge condition 4 degree Celsius (°C) in the refrigerator. These were stored in nitric acid condition to prevent calcium-phosphate precipitation that occurs when frozen. However, the other set of aliquots without nitric acid in them was stored at -80 degree Celsius (°C) to allow for future analyses.

In each case, the samples were collected in the morning between 9:00 Am and 10:00 AM each day and transported to the laboratory (Figure 5.1). Samples were collected approximately 14 hours after the most recent antiretroviral drug dose.

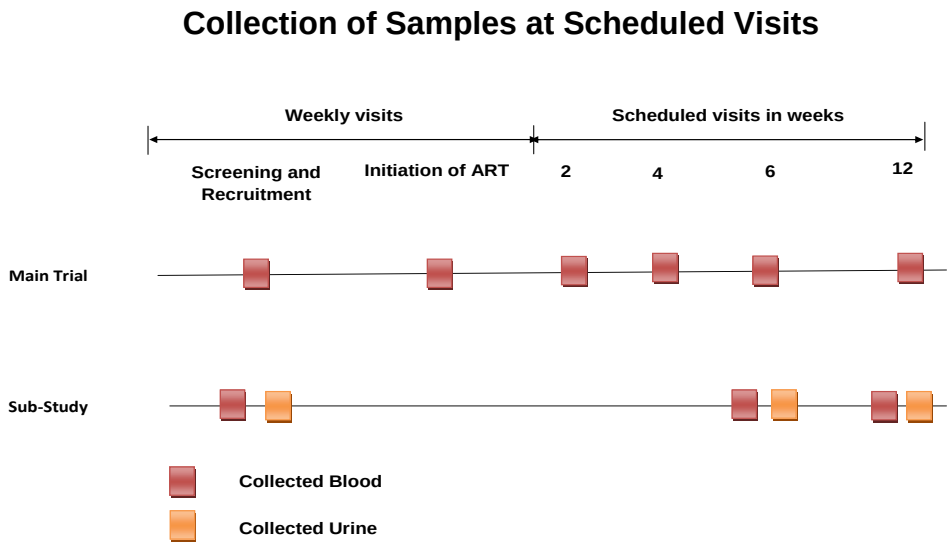


Figure 5.1: Collection of Samples at Scheduled Visits. Patients were scheduled to be seen weekly from recruitment until the ART initiation visit, then at 2, 4, 6, & 12 weeks after starting antiretroviral therapy. Urine & blood samples were collected at Baseline, 6 & 12 weeks of antiretroviral therapy for the sub-study.

5.9. Determination of CD4 cell count and Haemoglobin

Separate venous blood samples were collected for CD4 cell count and Hemoglobin assays. CD4 cell count was determined at recruitment and 12 weeks only. The CD4 cell data were acquired on an FC 500 MPL flow cytometer (Beckman Coulter, USA). 100 microliters of whole blood was stained, red blood cell were lysed, then without wash resuspended in 250 microliter paraformaldehyde or 10% cell fix before flow cytometric analysis. Intracellular Ki-67 staining was carried out after resuspansion in IX Per solution (BD) for nuclear membrane permeabilisation. Between 50,000 – 100,000 lymphocytes events were acquired per tube and fluorescence-minus-one (FMO) controls were used to set all gates. Analysis of markers was carried out using BD FACSuite^{TM} software v1.0.2. The measurements were done at Centre for Infectious Diseases Research (CIDRZ) laboratory in Lusaka. Hemoglobin measurements were undertaken immediately on whole blood. Hemoglobin was measured using a Sysmex xt 4000i automated hematology analyser and reported in grams per liter (g/L) at the University Teaching Hospital laboratory.

5.10 Determination of Creatinine, sodium, glucose and chloride

Creatinine, sodium, glucose and chloride were measured in blood and urine samples using standard clinical assays at the University Teaching Hospital Laboratory. Briefly these were measured using Beckman Coulter AU480 clinical chemistry analyser: (a) Creatinine was measured in urine and serum by the method of Jaffe (Jaffe, 1886). In this method creatinine forms a yellow-orange coloured compound with picric acid in an alkaline medium. The rate of change in

absorbance at 520/800 nm is proportional to the creatinine concentration in the sample. (b) Glucose was measured in urine and serum by hexokinase method (Barthelmai and Czok, 1962). This employs enzymatic ultraviolet method. Glucose here is phosphorylated by hexokinase in the presence of adenosine triphosphate (ATP) and magnesium ions to produce glucose-6-phosphate and adenosine diphosphate (ADP). Glucose-6-phosphate dehydrogenase (G6P-DH) specifically oxidises glucose-6-phosphate to gluconate-6-phosphate with the concurrent reduction of NAD+ to NADH. The increase in absorbance at 340 nm is proportional to the glucose concentration in the sample. (c) Sodium and Chloride were measured by the standard method that involves the quantitative (indirect) determination of Sodium (Na^+), and Chloride (Cl^-) concentrations in human samples. Here the Ion Selective Electrode (ISE) module for Na^+ and Cl^- employs crown ether membrane electrodes for sodium and a molecular oriented PVC membrane for chloride that is specific for each ion of interest in the sample. Thus, an electrical potential is developed according to the Nernst Equation for a specific ion. When compared to an internal reference, this electrical potential is translated into voltage corresponding to a specific ion concentration.

5.10.1 Calculation of Creatinine Clearance

A measure of glomerular filtration rate (GFR) was determined by calculating the estimated creatinine clearance (eCcr) using the Cockroft-Gault formula for men (15% less for women) using the following formula:

$$\text{eCcr} = \frac{[140 - Age\ (yrs)]\ x\ [Weight\ (kg)\ ]}{[72]\ x\ [Serum\ Creatinine\ (mg/dL)]}$$

5.11 Determination of magnesium and Potassium

The determination of potassium and magnesium was done using optical emission (Perkin Elmer Optima 7000 DV) at the University Teaching hospital Internal Medicine laboratory. The working diluent reagent was prepared as follows: 10 ml triton X100 in large bottle and 1 liter of deionized water was added and mixed I a volumetric flask. Then 10 ml ammonium hydroxide, 20 ml butanol and 140 µl of nitric acid were added. Finally, sufficient deionized water was added to make the final volume of 2 liters. For urine: the working diluent was prepared by adding 20 ml of concentrated nitric acid in 2 liters of deionized water in a large bottle to obtain a concentration of 1% nitric acid solution. The concentrated standard solutions were prepared to obtain 1 mmol/L of Mg^{++} and 4 mmol/L of K^{+}. Using an analytical balance (Ohaus, pioneer PA64C, New Jersey, USA) 250 µl Mg^{++} (MW=24.3051) stock solution, 1000 mg/L (41,144 mmol/L) was weighed into 20 ml vial. 1.6 ml (1600 µl) K^{+} (MW=39.098) stock solution, 1000 mg/L (25.577 mmol/L) was weighed into 20 ml vial. Then up to 10 ml with diluent was made. The summary is shown in the table below:

Table 5.4: Determination of Magnesium and Potassium Final Concentration

Step	Weight (g)	Final concentration in concentrated standard	
Tare vial	0.000	mg/L	mmol/L
Add 1 ml PO4	0.998	99.7	1.050
Add 250 µl Mg^{++}	1.246	24.775	1.019
Add 1.6 ml (1600 µl) K^{+}	2.842	159.44	4.078
Make up to 10 ml	10.010		

5.11.1 Serum K^{+} and Mg^{++} Standard and sample preparation

The blank, standard and sample solutions were prepared with each tube containing 4 ml volume as follows: Blank (80 µl dH_2O + 3920 µl diluent), standard 1 (40 µl standard solution + 3960µl diluent), standard 2 (80 µl standard solution + 3920µl diluent), standard 3 (160 µl standard solution + 3840 µl diluent) and internal quality controls, QCs and samples (80 µl sample + 3920 µl diluent). The set was thoroughly mixed and allowed to stand for 2 minutes at room temperature. The absorbance of the standards and samples were read against the reagent blank. Magnesium and potassium detected at 279.553 nm and 766.490 nm wavelengths respectively. See table of summary below:

Table 5.5: Serum K^+ and Mg^{++} standard and sample solutions dilutions

Blank, standard and sample type	Sample (µl)	Diluent (µl)	Dilution Factor
Blank	80 dH_2O	3920	
Standard 1	40	3960	1/100
Standard 2	80	3920	1/50
Standard 3	160	3840	1/25
Internal QC 1	80	3920	1/50
Sample 1	80	3920	1/50
Sample 2	80	3920	1/50
Sample 3	80	3920	1/50
Sample 4	80	3920	1/50
Sample ….	80	3920	1/50
QC 2	80	3920	1/50

The low and high cut-off serum values in mM were as follows: phosphate 0.8 - 1.5; potassium 3.5 - 5.5 and magnesium 0.7 - 1.1.

5.11.2 Urine K^+ and Mg^{++} Standard and sample preparation

The blank, standard and sample solutions were prepared with each tube containing 2 ml volume as follows: Blank (80 µl dH_2O + 1920 µl diluent), standard 1 (40 µl standard solution + 1960µl diluent), standard 2 (80 µl standard solution + 1920µl diluent), standard 3 (160 µl standard solution + 1840 µl diluent) and internal quality controls, QCs and samples (80 µl sample + 1920 µl diluent). The set was thoroughly mixed and allowed to stand for 2 minutes at room temperature. The absorbance of the standards and samples were read against the reagent blank. Magnesium and potassium detected at 279.553 nm and 766.490 nm wavelengths respectively. See table of summary below:

Table 5.6: Urine K^+ and Mg^{++} Standard and sample preparation

Blank, standard and sample type	Sample **(µl)**	Diluent **(µl)**	Dilution Factor
Blank	80 dH_2O	1920	
Standard 1	40	1960	1/50
Standard 2	80	1920	1/25
Standard 3	160	1840	1/12.5
Internal QC 1	80	1920	1/25
Sample 1	80	1920	1/25
Sample 2	80	1920	1/25
Sample 3	80	1920	1/25
Sample 4	80	1920	1/25
Sample ….	80	1920	1/25
QC 2	80	1920	1/25

Normal ranges in urine: K = 25 – 125 mmol/L, PO4 = 10 – 25 mmol/L and Mg = 0.85 – 9.5 mmol/L.

5.11.3 Principle of ICP-OES

The ICP-OES (Inductively Coupled Plasma-Optical Emission Spectrometry) is an analytical technique used for detection of trace elements in a given sample in solution form. When using the ICP-OES an aqueous sample introduced is converted to a mist of finely divided droplets called aerosols. These aerosols are then channeled to the inductively coupled plasma. The plasma is a highly energized “cloud” of gaseous ions and their electrons, which is a high temperature zone of about 8,000-10,000^{o}C. Argon gas is typically used to create the plasma. While in the plasma zone, the elements of interest known as the analytes undergo excitation resulting from collisions of analyte atoms with energetic electrons. Due to the thermic energy taken up by the electrons, they reach a higher "excited" state. When the electrons drop back to ground level energy is liberated as light (photons). Each element has its own characteristic emission spectrum. These emissions are then separated based on their specific

wavelengths by diffraction grating process. The subsequent intensities are measured by spectrometry. The intensities are proportional to the concentrations of the analytes in aqueous sample.

5.11.4 Calculations of fractional excretion of potassium and Magnesium

Fractional excretion of potassium percentages was calculated using the following equation:

$$\mathrm{FEK} = \frac{[Urine\ Potassium]\ x\ [Serum\ Creatinine]}{[Serum\ Potassium] x\ [Urine\ Creatinine]}\ x\ 100$$

Fractional excretion of potassium is a useful marker of potassium excretion. Unlike most electrolytes, urinary potassium excretion is governed by tubular secretion rather than reabsorption (Elisaf and Siamopoulos, 1995). In patients with normal GFR, FEK <6.4% is consistent with appropriate potassium conservation.

Fractional excretion of magnesium (FEMg) percentages was calculated using the following equations:

$$\mathrm{FEMg} = \frac{[Urine\ Magnesium]\ x\ [Serum\ Creatinine]}{0.7x[Serum\ Magnesium] x\ [Urine\ Creatinine]}\ x\ 100$$

FEMg is a marker of an intact tubulointerstitial structure (Gheissari et al., 2011) and values <4% reflect intact tubular function for reabsorption of filtered magnesium.

5.12 Determination of Phosphate

Phosphate was measured by the methods of Amador and Urban (Amador, 1972) using colorimetric procedures on Pointe 180 Plus 1907 (Baclabs, Nairobi, Kenya). The working reagent used was phosphorous reagent. The reagent was mixed with the standard and the sample. The blank, standard, pooled sample quality control (PSQC) and sample solutions were prepared with each tube containing 1020 µl volume as follows: Blank (1020µl dH_2O), standard 1(1010µl phosphorous reagent + 10µl phosphorous standard), standard 2 (1000µl phosphorous reagent + 20µl phosphorous standard), standard 3 (990µl phosphorous reagent + 30µl phosphorous standard), PSQC (1000µl phosphorous reagent + 20µl PSQC) and sample (1000µl phosphorous reagent + 20µl sample).

Table 5.7: Summary of preparation volumes for phosphate Standard and sample

Blank, standard and sample type	Sample (µl)	phosphorous reagent (µl)
Blank		1000 dH_2O
Phosphorous standard 1	10	1000
Phosphorous standard 2	20	1000
Phosphorous standard 3	30	990
PSQC	20	1000
Sample ID	20	1000

The set of tubes was thoroughly mixed and allowed to stand for 5 minutes at room temperature. The absorbance of the standards and samples were read at 340 nm against the blank. Phosphate in the sample reacts with ammonium molybdate and sulfuric acid forming a phosphomolybdate complex that can be

measured by spectrophotometer. The solutions in tubes were again vortexed before inserting tubes into the Pointe 180 Plus 1907 machine. In urine phosphate normal range was taken as 12 – 25 mmol/L while in blood 0.8 – 2.1 mmol/l was taken as normal range with reference to Division of AIDS (DAIDS) criteria.

5.12.1 Standard Operating Procedure (SOP) for Pointe 180 Equipment

To operate the Pointe 180 Plus 1907 machine required the following several steps to be exactingly followed. The steps involved; (1) To turn the machine on using the switch at the back on the left side, (2) To let the machine print the start-up data, (3) To press “Paper” a few times, at least 3 times and then rip off the print-out. This gave an allowance to save space in the laboratory book in which the print was secured and to only store relevant information in there, (4) To press “Test”, then key in “50” and press “Enter” again. This way the machine printed information about the test that was done, (5) To press “No” twice and then “Enter”, when it asked if you wanted to “Plot Curve Y/N”, (6) To follow the machine’s prompts when it asked you to read the blank tube, and then the three standards, (7) To press “Yes” when the machine asked if you would like to plot the curve, (8) To follows the machine’s prompts when it asked you to read the samples, and finally 9. To press “Clear” twice, when you were finished, this ended the test.

The day’s information including the date, blank content, PSQCs and sample IDs were recorded in the “PhD Lab Note Book” were copies of print out results were glued in. The Lab Book was essentially used and secured because the Pointe 180 Plus 1907 machine did not save data process and performed on

it, so this was the only way of saving these lab results. The maximum number of samples including the PSQC that was done per test run was 22. This was to allow the print-out to squarely fit in the lab book where data was stored. In an event where there was more than 22 samples the test were split accordingly.

5.12.2 Calculations of Tubular Reabsorption of Phosphate

The urine phosphate-creatinine ratio (concentrations measured in molar units) was used to correct for and estimate urinary phosphate excretion; tubular reabsorption of phosphate (TRP) was calculated as follows:

$$\mathrm{TRP} = 1 - \frac{[Urine\ Phosphate]\ x\ [Serum\ Creatinine]}{[Serum\ Phosphate] x\ [Urine\ Creatinine]}\ x\ 100$$

Tubular reabsorption of phosphate values >96% were taken to be normal .

5.13 Determination of Tenofovir in Fresh Blood

Tenofovir in fresh blood was measured using a validated high performance liquid chromatography (HPLC) method of Lunn George (Lunn et al., 1998). The assay was conducted at Zambia Bureau of Standards Laboratory in Lusaka.

5.13.1 Materials

Tenofovir Disoproxil Fumarate (TDF) USP Reference Standard (200mg) was purchased from Toronto Research, Toronto, Canada. Trifluroacetic acid and Acetonitrile HPLC grade were purchased from Merck, South Africa. Potassium dihydrogen phosphate HPLC grade and Sodium Hydroxide ACS reagent grade were obtained from Fluka (Sigma-Aldrich). 15 x 3.2 Brownlee RP-18 Newguard

and RP-18, spheri-5, C18 5µm, 25cm x 4.6mm internal diameter, Brownlee Conventional Column were purchased from Perkin Elmer.

5.13.2 Preparations of Standard stock solutions

Accurately weighed 30 mg of tenofovir disoproxil Fumarate (TDF) reference standard and transferred to 1000 ml volumetric flask by dissolving in small amount of HPLC grade water and complete to 600 ml with HPLC grade water to get 0.0226 mg/mL in tenofovir disoproxil fumarate stock solution. For the preparation of the standard solutions, serial dilution was done to get three different tenofovir disoproxil fumarate concentrations. This was done by accurately measuring 0.0078 ml, 0.061 ml and 1.0 ml from tenofovir disoproxil fumarate stock solution and adding buffer to make 50 mL of each with resultant concentrations of 3.52 ng/ml, 27.5 ng/ml and 452.0 ng/ml respectively. This was followed by measuring 50 uL of each diluted tenofovir disoproxil Fumarate standard solution to run HPLC to produce standard curves (Figure 5.2) and chromatograms. Tenofovir tenofovir disoproxil fumarate had a retention time of 3.010 minutes**.**

5.13.3 Sample Preparation

The samples were prepared with each tube containing 500 µL final volume by mixing serum sample solution with 0.1% trifluoroacetic acid in acetonitrile volume in a ratio of 1:2 v/v. The mixture of each serum sample contained 166.7 µl sample serum solution and 333.3 µl 0.1% trifluoroacetic. It was then

centrifuged for 5 min at 14 000 rpm, allowed to stand for 2 minutes at room temperature.

5.13.4 HPLC conditions

The separation was performed with guard column (15 × 3.2 Brownlee RP-18 Newguard) and column (250 × 4.6 5 μmZorbax RX-C18) and 50 μL aliquot of the supernatant was injected into the Shimadzu HPLC machine to produce chromatograms**.** The mobile phase consisted of two solutions. The HPLC grade water mixed with 0.5% formic acid and acetonitrile mixed with 0.5% formic acid run under isocratic conditions at 95% and 5% solutions respectively. The flow rate was 1 min/ml and the detection wavelength was 262 nm. All separations were performed at ambient temperature.

5.13.5 Interpretation of Chromatograms

Each chromatogram produced from sample run displayed 5 peaks but one peak only was identified as being tenofovir at a retention time of 2.854 minutes (Figure 5.4). Specifically, the peaks had retention times of 2.325, 2.540, 2.854 3.234 and 3.510 min. Prior to running samples, analyses of standards containing known amounts of tenofovir disoproxil fumarate were performed. Tenofovir disoproxil fumarate had a retention time of 3.010 minutes (Figure 5.3). The UV spectra identified with the HPLC Chemstation software was used to analyze tenofovir in the samples while running in the Spectral Task mode. Thus, the HPLC detector scanned for these spectra in the sample analysis phase. The confirmation was achieved by scrutinizing the UV spectra that had unique

signature for tenofovir and was the UV absorbance wavelengths along the x-axis that is relatively unique for tenofovir with UV detection of 262nm.

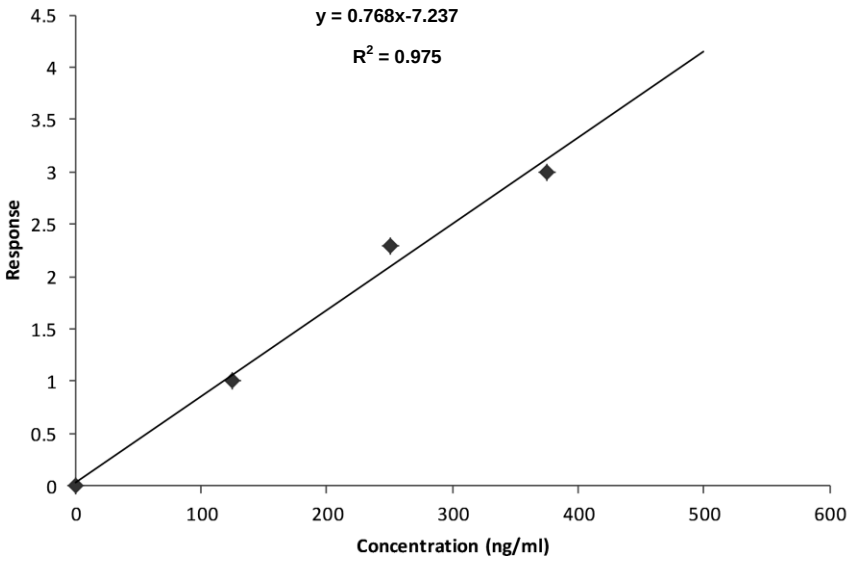


Figure 5.2: Standard curve for Tenofovir Disoproxil Fumarate (TDF). Showing a regression line plot (calibration curve) obtained from the standard solution; Compound name: Tenofovir Disoproxil Fumarate, Coefficient of Determination: 0.980632, Calibration Curve: 69:30290 *x 28.2624, Response Type: External Std. Area and Wave Type: Linear.

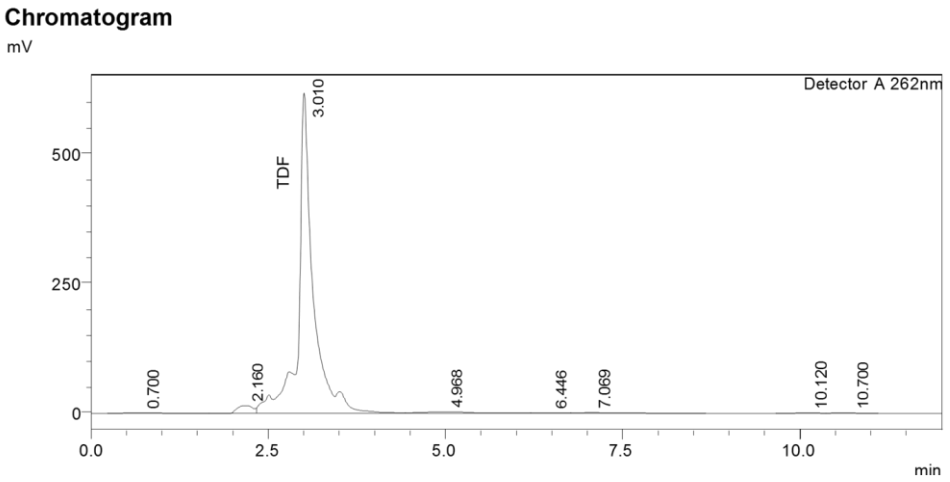


Figure 5.3: Chromatogram of tenofovir disoproxil fumarate standard. Tenofovir disoproxil fumarate had a retention time of 3.010 minutes. Abbreviations: Millivolt-mV, Time in minutes-min.

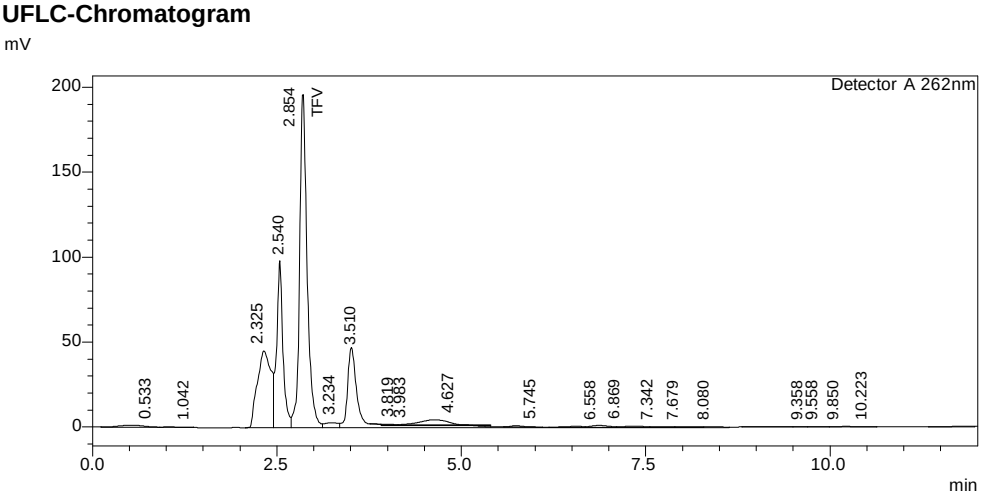


Figure 5.4: Chromatogram of tenofovir peak from serum sample. Tenofovir had a retention time of 2.854 minutes in the sample. Abbreviations: Millivolt-mV, Time in minutes-min.

5.14 Determination of Tenofovir in Dry Blood

Tenofovir in dry blood was determined using liquid chromatography coupled with mass spectrometry (LC-MS/MS) Austin, USA. Tenofovir (TFV) and tenofovir-d6 (TFV-d6) were purchased from Toronto Research, Toronto, Canada. Methanol and acetonitrile (HPLC grade) were bought from Sigma-Aldrich (St. Louis, MO). Water (HPLC grade) and formic acid were procured from Fisher Scientific (Fairlawn, NJ). HemaForm-80 and HemaSpot-HF's were obtained from Spot On Sciences, Inc. (Austin, TX). Dried blood spot cards were Munktell TFN cards from Lasec (Cape Town, SA).

5.14.1 Preparation of stock solutions and standard calibrators

Standard stock solutions of tenofovir and tenofovir-d6 were prepared at 1 mg/mL in HPLC grade water. The tenofovir stock solution was used to prepare a working standard curve beginning at 100 µg/mL in HPLC grade water. Ten microliters from each of the working standard solutions were combined with 490 microliters of fresh whole blood to give calibrations curves for tenofovir (2000, 1000, 500, 250, 125, 62.5, 31.25, 15.625, 7.8 and 3.9 ng/mL). Quality control samples were prepared from the same working standard solutions at three levels; 1000, 125 and 7.8 ng/mL. The internal standard solution was prepared from the stock solution in HPLC grade water at 50 ng/mL concentration. The standard curve is shown in Figure 5.5.

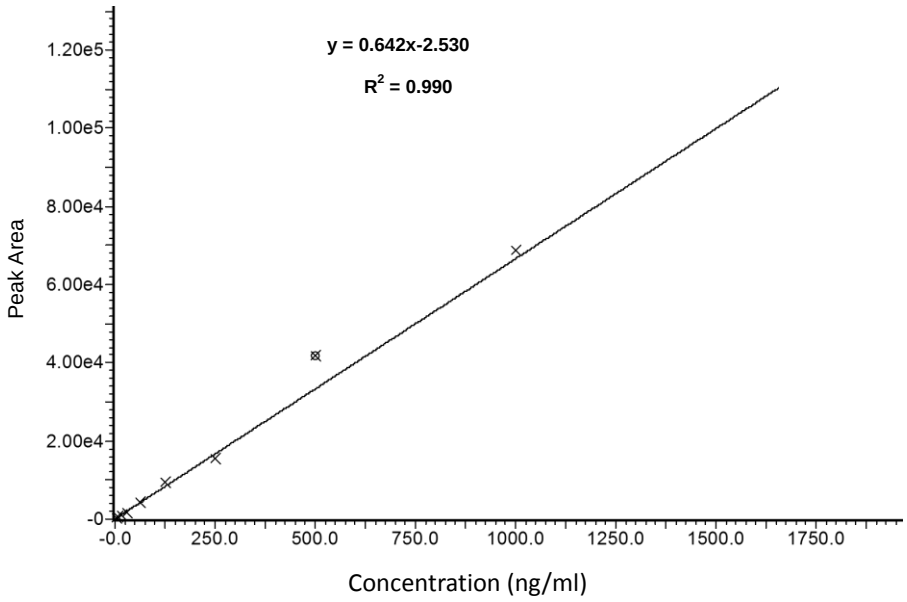


Figure 5.5: Standard Curve of tenofovir. Showing a regression line plot (calibration curve) obtained from the standard solution; Compound name: Tenofovir, Coefficient of Determination: 0.990408, Calibration Curve: 66:50960 *x 28.2624, Response Type: External Std. Area and Wave Type: Linear. The standard curve was linear from 3.9 ng/mL up to 2000 ng/mL for tenofovir calibrations. A single standard (500 ng/mL) in the curve deviated from the back calculated conconcentration by > 15% and was removed.

5.14.2 Preparation of dried blood spot calibration curves

Eighty microliters of each of these standard solutions were applied to HemaForm-80's (Figure 5.6) held in 12-well plates and allowed to air-dry overnight, protected from light. After drying, plates were covered with aluminum foil and stored at room temperature until used. An additional set of DBS' were made on HemaSpot-HF devices, sealed and stored at room temperature. A final portion 36.8 microliters from each fresh blood preparation (equivalent to 4 wedges of the HemaForm-80 when 80 microliters of blood is applied to the form)

was added directly to 500 microliters of methanol. 50 microliters of the surrogate standard was spiked in to each and the samples sonicated 30 minutes then vortexed overnight at moderate speed. The mother liquors were decanted into 1.5 mL matrix tubes and placed under a stream of nitrogen to evaporate to near dryness at 30 ^{o}C. Samples were then diluted with 70 microliters of HPLC grade water. After vortexing briefly, the samples were centrifuged at 10 kcps for 5 minutes before LC-MS/MS analysis. These samples were used to compare efficiency of the extractions from each of the HemaForm-80's (Figure 5.7).

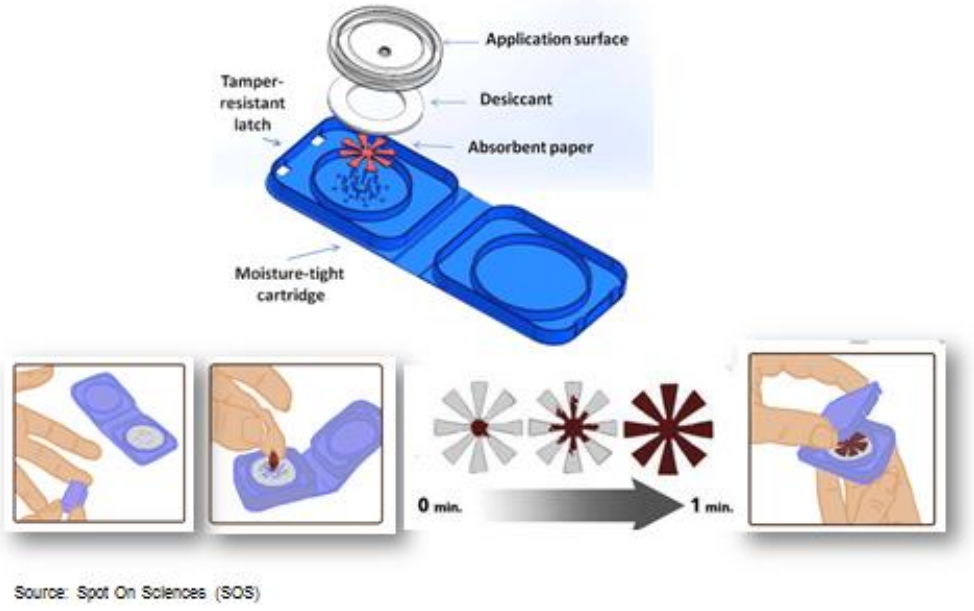


Figure 5.6: Application of HemaSpot Device. HemaSpot DBS matrix used to quantify TFV blood concentrations. Allow 80 microliters of blood to be used for analysis. HemaSpot samples are easy to store and transport than traditional venipuncture collected blood samples.

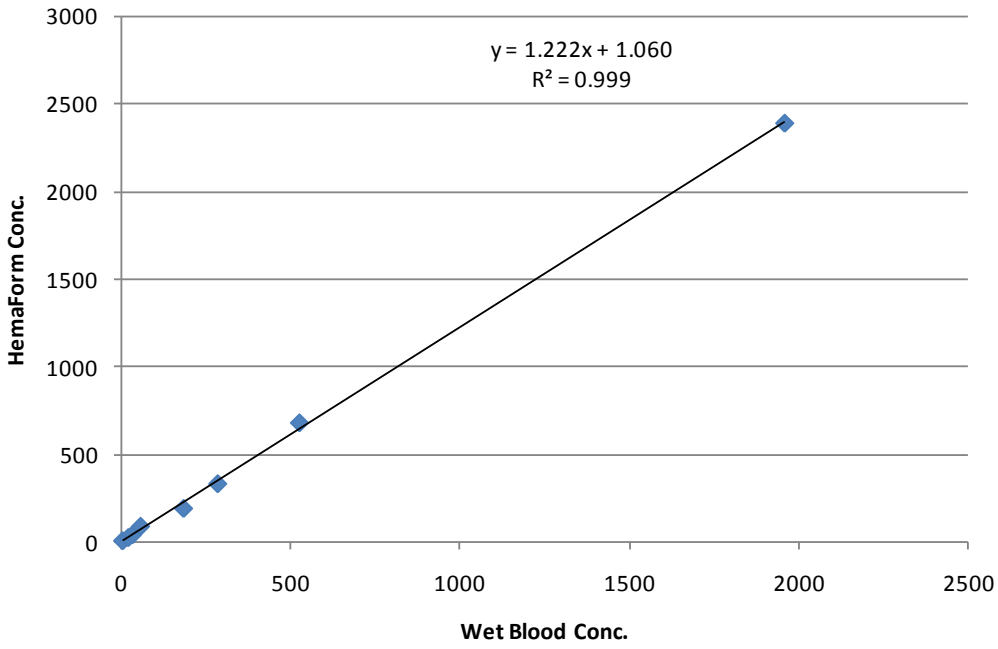


Figure 5.7: Correlation of Wet Blood Recovery to Hemaspot Recovery. The curve showing extraction recoveries of tenofovir from dried blood spot of >90% when compared to wet blood matrix. Lower limit of quantification (LLOQ) was 3.9 ng/mL and lower limit of detection (LOD) was under 1 ng/mL based on a S/N ratio > 3 using this method.

5.14.3 Sample extractions

Four wedges from each of the HemaForm-80's were pulled and placed in a 1.5 mL microfuge tube. To each tube was added 500 microliters of methanol and 50 microliters of the surrogate standard. Samples were sonicated 30 minutes then allowed to vortex overnight at moderate speed. After decanting, the mother liquor was evaporated at 30 oC under nitrogen then reconstituted with 70 uL HPLC grade water (0.1% FA). These solutions were then centrifuged at 10 kcps for 5 minutes before LC-MS/MS. Three 6 mm punches were collected from each blood spot from a dried blood spot card (Note 1) and extracted as described.

5.14.4 Analytical Method in LC-MS/MS

Quantitation of TFV and TFV-d6 was done using high performance liquid chromatography tandem mass spectroscopy (HPLC-MS/MS). The chromatographic analyses were conducted at Spot On Sciences laboratory in Austin, Texas, USA. The HPLC consisted of a Shimadzu SIL-HTA autosampler with dual LC-10AT pumps and a DGU-20Az Prominence degasser. Chromatography was performed on a Syneryi 2.5 micron Polar RP 100A 100 x 2 mm column from Phenomenex (Torrance, CA). The mobile phase consisted of a solution of A) HPLC grade water/0.5% formic acid and B) acetonitrile/0.5% formic acid run under isocratic conditions at 95% solution A and 5% solution B at a flow rate of 0.3 mL/min. 20 microliters of isolated sample were injected onto the column.

5.14.5 Tandem Mass Spectrometer

The mass spectrometer was a Micromass Quattro Ultima MS/MS operated in electrospray ionization (ESI) positive polarity mode using multiple reaction monitoring (MRM) transitions (m/z): TFV (287.8->176.3) and TFV-d6 (294.4 -> 181.6). Dwell time for each transition was 0.3 seconds. Cone voltage was 20 V and collision energy was 28 eV. A solvent delay of 1 minute was incorporated to divert waste while the total run time was 3 minutes. The retention time for TFV and TFV-d6 was 1.9 +/- 0.1 minutes (Figure 5.8).

Note 1: The three 6 mm punches were chosen to match the average area from both blood spot collection platforms. The average diameter of the blood spots on

the Munktell cards was found to be 15 mm. This gave an average area for each spot of 177 mm^2. Because four wedges from the HemaForm-80 is equivalent to 46% of the total form area, 46% of 177 mm^2 is 81 mm^2 or 95% of the area from three 6 mm punches.

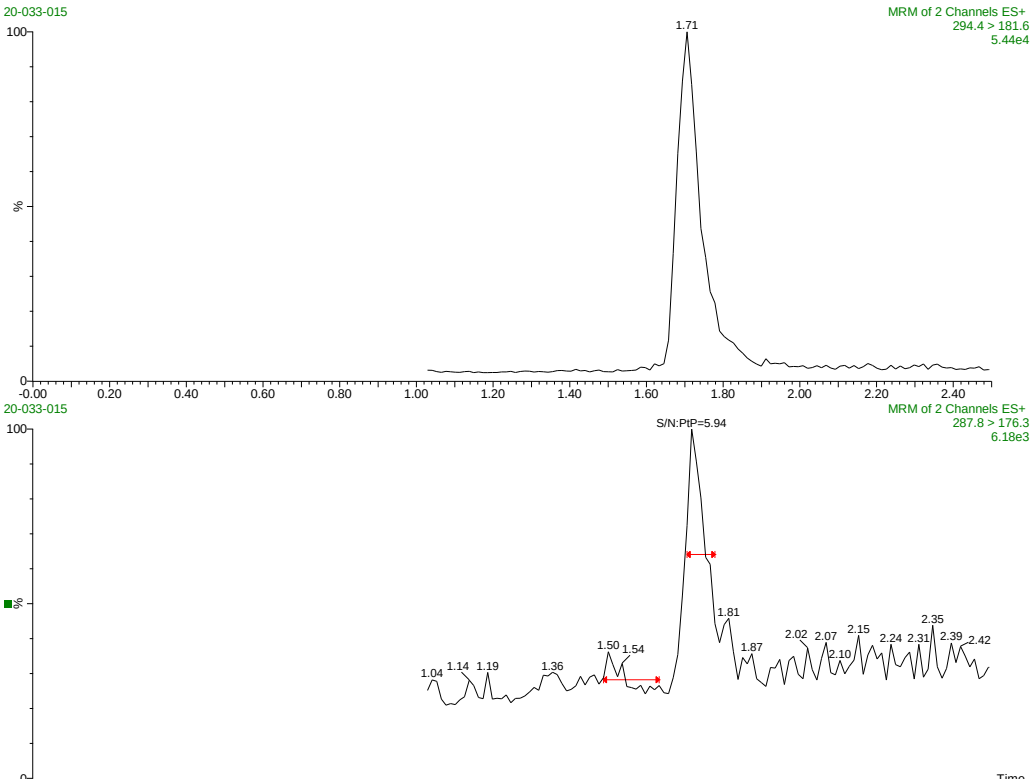


Figure 5.8: Tenofovir chromatograms. Upper chromatogram shows tenofovir-d6 at 50 ng/mL while the lower trace is tenofovir at 3.9 ng/mL. S/N = 6 is below the recommended lower limit of quantitation (LLOQ) of 10.

5.15 Statistical Data Analysis

The statistical analysis of data generated in the present study was carried out using Stata version 12.1 or GrapPad Prism 6 and values expressed as Mean ± SD unless otherwise stated. Between-group comparisons of continuous variables data were analyzed with t-tests, one-way ANOVA with Tukey's post-hoc test, or two-way ANOVA (Model I) with Bonferroni's post-hoc test. Data fitness for Gaussian distribution passed the normality and was verified with D'Agostino and Pearson Omnibus normality test. The Bland-Altman plot (Bland & Altman, 1986 and 1999) was used to determine limits of agreement of tenofovir assays in dried blood spot with fresh blood assays. Dichotomized variables such as age and sex were compared with Chi-Square tests. Multiple linear regressions were used to determine associations between tenofovir concentrations and body mass index and renal electrolytes after adjusting for potential covariates such as age and sex. P-values and 95% confidence intervals were reported. Tests were two-tailed with $\alpha = 0.05$.

5.16 Ethical Considerations

The present study complied with guidelines of the Declaration of Helsinki and was approved by the University of Zambia Biomedical Research Ethics Committee (Ref: 014-10-13). The main NUSTART trial was also approved by the ethics committee of the London School of Hygiene and Tropical Medicine. All patients gave written informed consent. Medical care of patients was according to national guidelines. The care was provided through the local health services where the participants were drawn from.

CHAPTER 6

RESULTS

This chapter describes in detail the findings of this study. It covers demographics, clinical electrolyte data and tenofovir blood concentration levels in adult HIV-infected Zambian patients.

6.1 Cohort of Study Participants and Intervention

Figure 6.1 shows the flow of participants through the study and allocation of participants to lipid-based nutrient supplement containing vitamins and minerals (LNS-VM, intervention arm) and supplement (LNS, control arm) without added vitamins and minerals. Of the 130 participants recruited into the study, 63 (48%) were assigned to LNS and 67 (52%) to LNS-VM. Loss to follow-up was similar in both groups. In the LNS research arm, 3 (5%) were loss to follow-up before 12 weeks of antiretroviral therapy and 5 (7%) were loss to follow-up in the LNS-VM before 12 weeks of antiretroviral therapy. Eighteen (29%) patients from the LNS and 16 (24%) from LNS-VM groups died, most of them during the referral interval before starting antiretroviral therapy. Before antiretroviral therapy initiation, 13 (4 females and 9 males) died in the LNS versus 8 (4 females and 4 males) in the LNS-VM. After initiation of antiretroviral therapy, 5 (3 females and 2 males) died in the LNS compared with 8 (3 females and 5 males) in the LNS-VM (Table 6.1). Therefore, of the 63 participants in the LNS, 42 completed the 12 weeks study follow-up time while of 67 in the LNS-VM, 46 completed the study.

Flow of participants through the sub-study

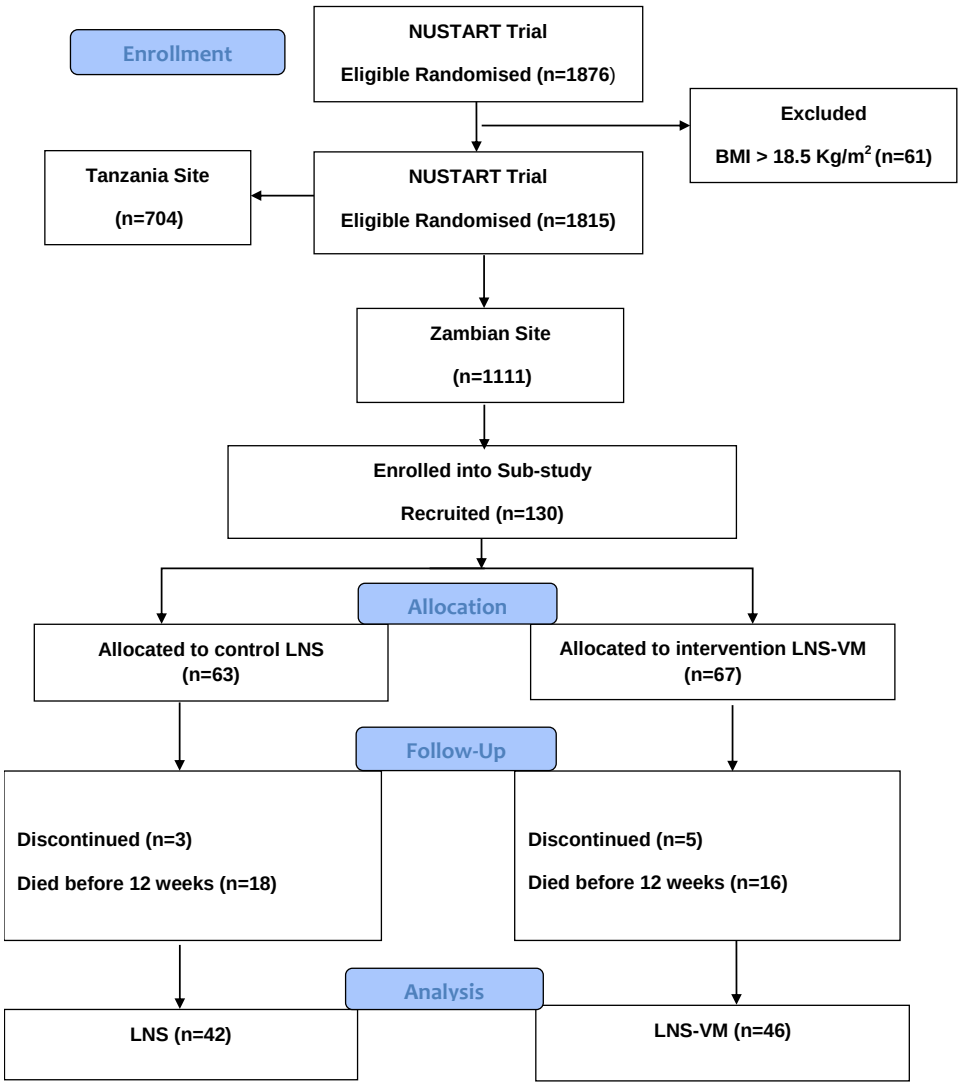


Figure 6.1: Flow of the participants through the sub-study. Patients were randomized in the parent Nutritional Support for African Adults Starting Antiretroviral Therapy (NUSTART) controlled trial. Recruitment was limited to the Lusaka site in Zambia and involved all HIV-infected patients referred for antiretroviral therapy with CD4 < 350 cells/μL and BMI <18.5 kg/m^2. The sub-study was conducted towards the end of the parent NUSTART study. Abbreviations: LNS, lipid-based nutritional supplement; LNS-VM, lipid-based nutritional supplement with vitamins and minerals.

Table 6.1: Mortality before and after initiation of antiretroviral therapy

	Assigned to LNS	Assigned to LNS-VM
	n=63	n=67
Loss to follow-up before week 12	3	5
Died before ART		
Females	4	4
Males	9	4
Died after ART		
Females	3	3
Males	2	5
Completers	42	46

Abbreviations: LNS -lipid-based nutritional supplement; LNS-VM -lipid-based nutritional supplement with vitamins and minerals; ART-antiretroviral therapy.

6.1.2 Demographics and Clinical Data of Study Participants at Baseline

The mean age, body mass index (BMI) and $CD4^+$ count for all patients at baseline were 36.5 ± 8.5 years, 16.6 ± 1.5 kg/m^2 and 127 ± 96 cells/µL respectively. The demographic and clinical data did not significantly differ between males and females. Table 6.2 shows baseline patient characteristics in the lipid-based nutrient without vitamins and minerals (LNS) and lipid-based nutrient containing vitamins and minerals (LNS-VM) research arms. Baseline data were similar between the two groups in terms of social and demographic profiles, biomarkers for renal homeostatic functions (i.e. estimated glomerular filtration rate (GFR), fractional excretion of potassium, fractional excretion of magnesium (FEMg) and tubular reabsorption of phosphate (TRP) and HIV treatment outcomes (i.e. CD4 cell counts and body mass index (BMI). Education level was low among the participants. Less than high school education was comparable between the two groups in terms of proportions as follows: LNS, 41 (65%); n = 63 and LNS-VM, 43 (64%); n = 67. Fifty nine patients (94%); n = 63 in the LNS versus 62 (93%); n = 67 in the LNS-VM research arm were on contrimoxazole prophylaxis. Table 6.3; shows baseline information between the survivors and those who died during the 12 weeks of follow-up study. The baseline data was also similar except for body weight (p=0.03), serum magnesium (p=0.02), serum creatinine (p=0.01) and creatinine clearance (p=0.04) by t-tests. The education and employment statuses were mostly less than secondary education and either self-employed or unemployed respectively (Figure 6.2). All the patients had $CD4^+$ cell count <350 cells/mm^3 and BMI <18.5

Kg/m^2. The group means for serum concentrations of phosphate, magnesium and potassium were within normal physiological ranges in the patients at baseline (Table 6.2), but there was wide variability in individual values as follows. At baseline 25 patients (19.2%) had low serum phosphate levels (LNS 20.6%, LNS-VM 17.9%) and 18 patients (13.8%) had high phosphate (LNS 12.7%, LNS-VM 14.9%). Similarly, 13 patients (10.0%) had low serum potassium levels (LNS 7.9%, LNS-VM 11.9%) and 11 patients (8.5%) had high potassium (LNS 9.5%, LNS-VM 7.5%). [NB: The low and high cut-off serum values in mmol/L were as follows: phosphate, 0.8 - 1.5; potassium, 3.5 - 5.5; and magnesium, 0.7 - 1.1].

Table 6.2: Baseline Characteristics of 130 HIV/AIDS Patients in the Study

Variable	LNS (n=63)	LNS-VM (n=67)	p-value
A. Demographics			
Age, years	35 (8)	38 (9)	0.18
Male, n (%)	36 (57)	41 (61)	0.48
Employed, n (%)	11 (17)	7 (10)	0.19
Education, < High school, n (%)	41 (65)	43 (64)	0.62
B. Clinical data			
Anti-TB drugs, n (%)	14 (22)	10 (15)	0.49
Cotrimoxazole, n (%)	59 (94)	62 (93)	0.53
CD4 count, cells/μL	135.9 (104)	119.4 (88)	0.33
Hemoglobin, g/dL	10.9 (2.1)	10.5 (1.9)	0.47
Body weight, Kg	46.9 (6.1)	45.9 (5.7)	0.28
Body mass index, Kg/m^2	16.7 (1.3)	16.3 (1.8)	0.06
Serum sodium, mmol/L	134.4 (3.3)	133.5 (3.6)	0.49
Serum potassium, mmol/L	4.5 (0.09)	4.5 (0.08)	0.64
Serum magnesium, mmol/L	0.8 (0.02)	0.8 (0.03)	0.15
Serum phosphate , mmol/L	1.0 (0.06)	1.1 (0.05)	0.60
Urine creatinine, mg/dL	81.4 (37.3)	90.5 (46.4)	0.63
Urine phosphate-Creatinine Ratio, mg/dL	1.2 (0.6)	1.1 (0.8)	0.86
Urine glucose , mmol/L	0.14 (0.21)	0.17 (0.23)	0.35
Serum glucose, mmol/L	4.5 (1.02)	4.7 (1.42)	0.26
Urine chloride, mmol/L	111.4 (46.9)	121.6 (41.6)	0.19
Serum chloride, mmol/L	102.3 (5.31)	102.61 (4.09)	0.67
Serum creatinine, μmol/l	59.4 (16.7)	63.7 (16.6)	0.22
C. Biomarker			
Creatinine clearance, ml/min	99.6 (23.3)	89.6 (24.9)	**0.01**
Tubular reabsorption of Phosphate (TRP) %	73.2 (16.3)	72.3 (17.6)	0.44
Fractional excretion of magnesium (FEMg) %	3.1 (1.9)	3.2 (1.7)	0.53
Fractional excretion of potassium (FEK) %	13.5 (2.5)	13.6 (2.7)	0.54

Description of characteristics of 130 participants at baseline in a study in Lusaka, Zambia between May 13 2013 and November 18, 2013 of a subset trial study. Values are mean ± SD unless stated otherwise. Chi-square tests were used for categorical variables and t-tests for continuous variables. Abbreviations: LNS -lipid-based nutritional supplement; LNS-VM -lipid-based nutritional supplement with vitamins and minerals; BMI-body mass index; Anti-TB drugs-anti tuberculosis drugs. Normal ranges: CD4 count (500 - 1500 cells/mm^3); Body mass index (18.5 - 24.9 Kg/m^2); Urine Creatinine (54 – 234 mg/dL); Urine Phosphate-Creatinine Ratio (0.6 – 09 mg/dl); Creatinine Clearance (88 - 137 ml/min); Serum creatinine (60 – 127 μmol/L); Tubular Reabsorption of Phosphate (>96%); FEMg (<4%); FEK (<6.4%). Serum in mmol/L: Potassium 3.5 – 5.5, Phosphate 0.8 – 1.5, Magnesium 0.7 – 1.1, Sodium 135 –146, Chloride 95 – 108, Glucose 3.3 – 7.8. Spot urine in mmol/L: Potassium 40 – 120, Phosphate 12 – 25, Magnesium 4 – 8, Sodium 20 – 230, Chloride 15 – 250, Glucose 0.0.

Table 6.3: Survival status baseline data of Zambian HIV-infected adult patients

Variable	Survived (n=96)	Died (n=34)	P value
A. Demographics			
Age, years	37 (8)	38 (9)	0.73
Male, n (%)	53 (55)	20 (59)	0.61
Employed, n (%)	18 (20)	6 (18)	0.73
Education, < High school, n (%)	84 (87)	32 (94)	0.72
B. Clinical data			
Anti-TB drugs, n (%)	24 (25)	4 (12)	0.07
Cotrimoxazole, n (%)	81 (84)	32 (94)	0.70
CD4 count, cells/μL	137.2 (98.7)	106.3 (92.9)	0.12
Hemoglobin, g/dL	10.9 (2.1)	10.0 (2.6)	0.19
Body weight, Kg	46.4 (5.6)	43.8 (5.9)	**0.03**
Body mass index, Kg/m^2	16.6 (1.5)	16.1 (1.5)	0.07
Serum sodium, mmol/L	134.4 (3.3)	130.6 (3.8)	0.49
Serum potassium, mmol/L	4.6 (0.06)	4.7 (0.10)	0.15
Serum magnesium, mmol/L	0.8 (0.01)	0.9 (0.02)	**0.02**
Serum phosphate , mmol/L	1.1 (0.04)	1.2 (0.06)	0.23
Serum creatinine, μmol/l	62.7 (16.6)	71.7 (17.1)	**0.01**
Urine Creatinine, mg/dL	87.1 (44.1)	91.6 (41.8)	0.65
Urine phosphate-Creatinine Ratio, mg/dL	1.1 (0.6)	1.1 (0.5)	0.69
C. Biomarkers			
Creatinine clearance, ml/min	94.0 (27.9)	78.2 (25.3)	**0.04**
Tubular reabsorption of Phosphate (TRP) %	75.8 (20.6)	72.4 (13.5)	0.40
Fractional Excretion of Magnesium (FEMg) %	3.6 (1.2)	3.0 (1.0)	0.52
Fractional Excretion of Potassium (FEK) %	13.5 (3.5)	13.6 (3.7)	0.56

Description of 130 participants at baseline for those that survived or died in a study in Lusaka, Zambia between May13, 2013 and November18, 2013 of a subset trial study. Values are mean ± SD unless stated otherwise. Chi-square tests were used for categorical variables and t-tests for continuous variables. Abbreviations: LNS -lipid-based nutritional supplement; LNS-VM -lipid-based nutritional supplement with vitamins and minerals; Anti-TB drugs-anti tuberculosis drugs. Normal ranges: CD4 count (500 - 1500 cells/mm^3); BMI (18.5 - 24.9 Kg/m^2); Urine Creatinine (54 – 234 mg/dL); Urine Phosphate-Creatinine Ratio (0.6 – 09 mg/dl); Creatinine Clearance (88 - 137 ml/min); Serum creatinine (60 – 127 μmol/L); Tubular Reabsorption of Phosphate (>96%); FEMg (<4%); FEK (<6.4%). Serum in mmol/L: Potassium 3.5 – 5.5, Phosphate 0.8 – 1.5, Magnesium 0.7 – 1.1, Sodium 135 –146, Chloride 95 – 108, Glucose 3.3 – 7.8. Spot urine in mmol/L: Potassium 40 – 120, Phosphate 12 – 25, Magnesium 4 – 8.

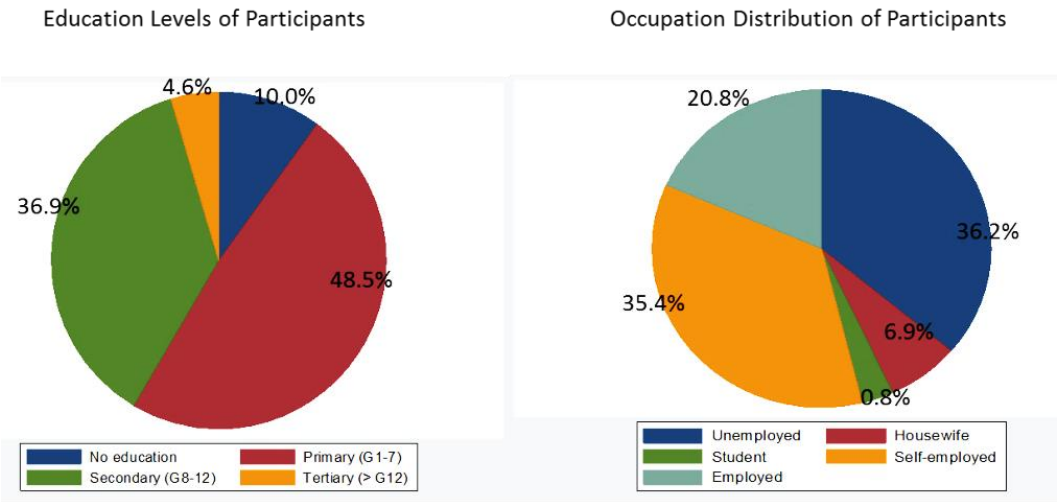


Figure 6.2: Education levels and Occupation Distribution of the study participants. Forty nine percent (49%) of participants had primary education while 37% had secondary education. There was no statistical difference between the LNS (65%) and the LNS-VM (64%) group in terms of those that had education less than secondary education. Participants were mostly either self-employed (35%) or unemployed (36%). Compared between groups, 26% in the LNS had salaried employment and 15% in the LNS-VM had salaried employment.

6.1.3 Effect of nutritional intervention on CD4 cell count and BMI

At baseline, $CD4^+$ Cell Count was comparable between the two research arms. However, from baseline to 12 weeks of antiretroviral therapy $CD4^+$ Cell Count was significantly increased in both arms; LNS baseline 136 ± 103 (95% CI, 110-162), n = 63 versus LNS 12 weeks 262 ± 119 (95% CI, 225-299), n = 42; $p < 0.001$ and LNS-VM baseline 119 ± 88 (95% CI, 98-141), n = 67 versus LNS-VM 12 weeks 271 ± 168 (95% CI, 221-321), n = 46; $p < 0.001$. At 12 weeks patients in the LNS-VM arm had insignificant higher $CD4^+$ Cell Count compared with the LNS, LNS 12 weeks 262 ± 119 (95% CI, 225-299), n = 42 versus LNS-VM 12 weeks 271 ± 168 (95% CI, 221-321), n = 46; $p = 0.78$ (Figure 6.3) by t-tests.

There was also corresponding significant increase in the body mass index from baseline to 12 weeks of antiretroviral therapy in both groups. However, at 12 weeks of antiretroviral therapy body mass index gain was comparable between the two groups (Figure 6.4). The efficacy of antiretroviral therapy regimen was evident from the significant increases in both $CD4^+$ Cell Counts and body mass index values obtained after 12 weeks of antiretroviral therapy relative to baseline values. There was significant increase in the body mass index from baseline to 12 weeks of antiretroviral therapy in both males and females. However, at 12 weeks of antiretroviral therapy body mass index was comparable between the two groups (Figure 6.5). All the patients recruited into the study had low $CD4^+$ Cell Count < 350 cells/mm^3 and BMI < 18.5 Kg/m^2. In the analysis, in those that survived or died there was no correlation between BMI and $CD4^+$ Cell Count at baseline (Figure 6.6).

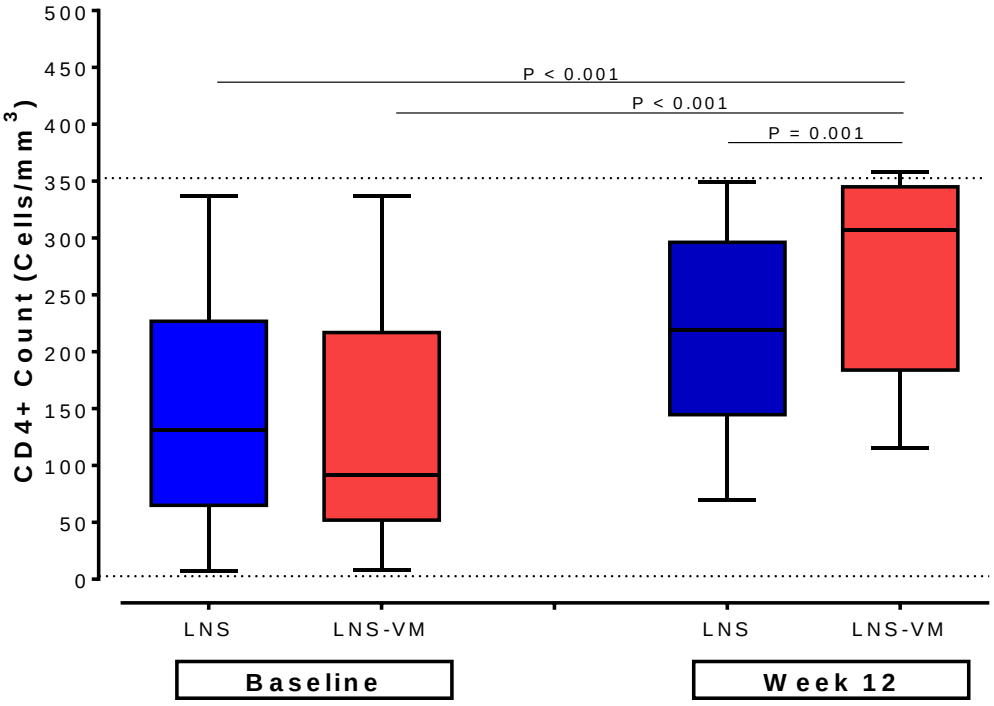


Figure 6.3: CD4+ Cell Count at Baseline and 12 weeks of Antiretroviral Therapy. Mann-Whitney test and Wilcoxon matched-pairs sign rank test were used to assess differences between unpaired and paired groups respectively. From baseline to 12 weeks of antiretroviral therapy $CD4^+$ Cell Count was significantly increased in both arms. At 12 weeks patients in the LNS-VM arm had insignificant higher $CD4^+$ Cell Count compares with the LNS. Abbreviation: Lipid-based nutritional supplement – LNS; Lipid based nutritional supplement with vitamins and minerals – LNS-VM. Dotted horizontal lines denote normal range: 500 to 1500 cells/mm^3.

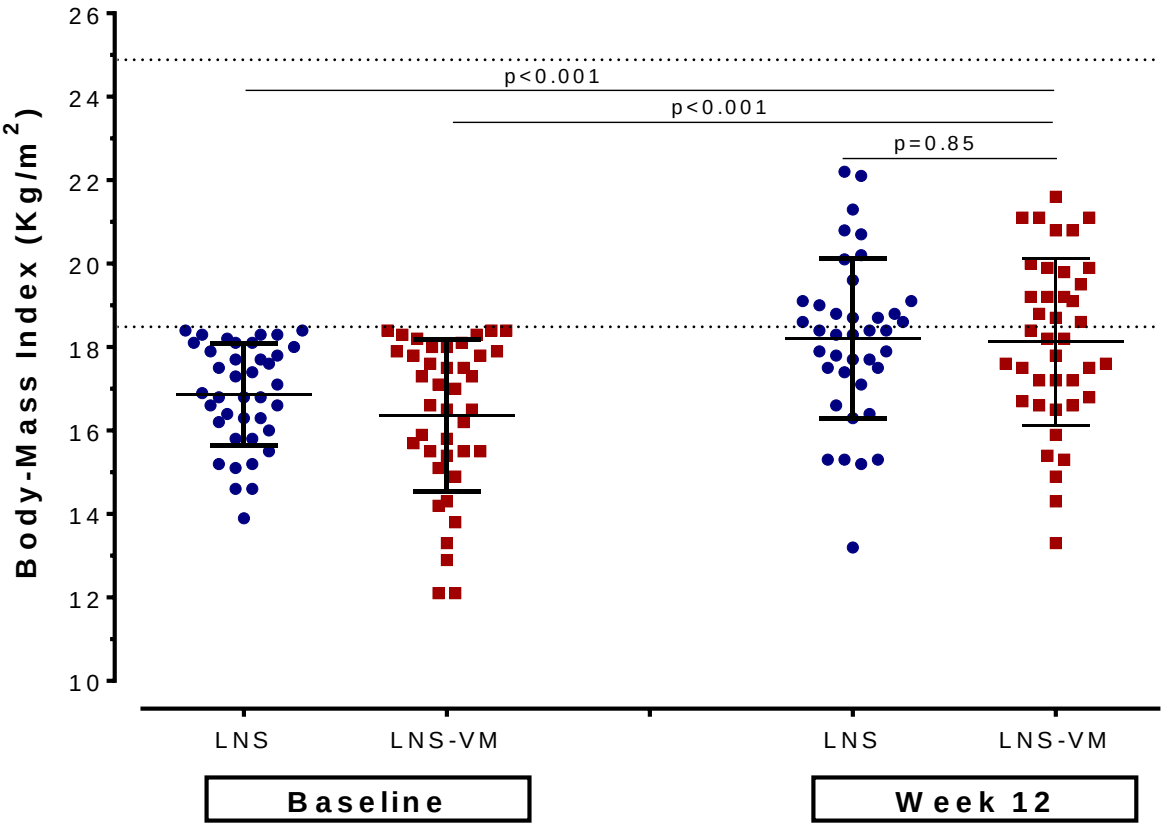


Figure 6.4: Body Mass Index at Baseline and 12 weeks of Antiretroviral Therapy. Values are mean ± SD and t-tests were used to assess differences between groups. There was significant increase in the body mass index from baseline to 12 weeks of antiretroviral therapy in both groups. However, at 12 weeks of antiretroviral therapy body mass index was comparable between the two groups. Abbreviation: Lipid-based nutritional supplement – LNS; Lipid-based nutritional supplement with vitamins and minerals – LNS-VM. Dotted horizontal lines denote normal range: 18.5 to 24.9 Kg/m^2.

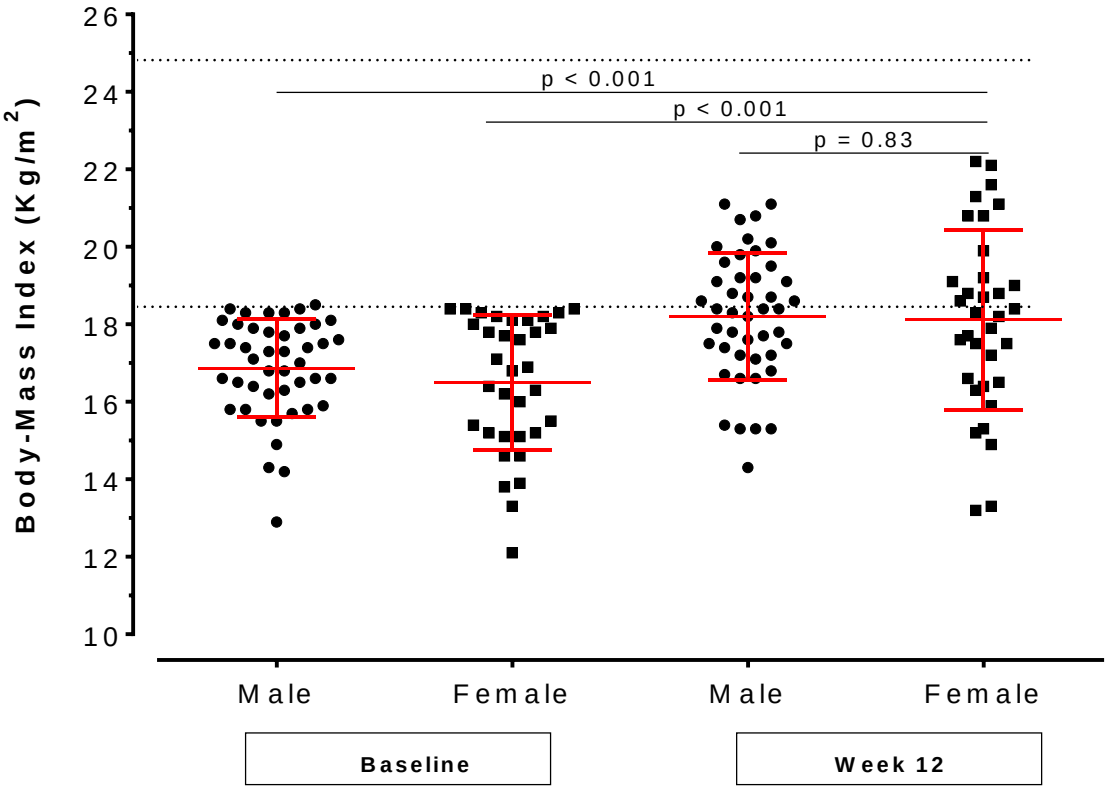


Figure 6.5: Body mass index in male versus female HIV-infected patients. Values are mean ± SD and t-tests were used to assess differences between groups. There was significant increase in the body mass index from baseline to 12 weeks of antiretroviral therapy in both males and females. However, at 12 weeks of antiretroviral therapy body mass index was comparable between the two groups. Dotted horizontal lines denote normal range: 18.5 to 24.9 Kg/m^2. The solid horizontal and vertical lines within the scatter points are mean ± SD.

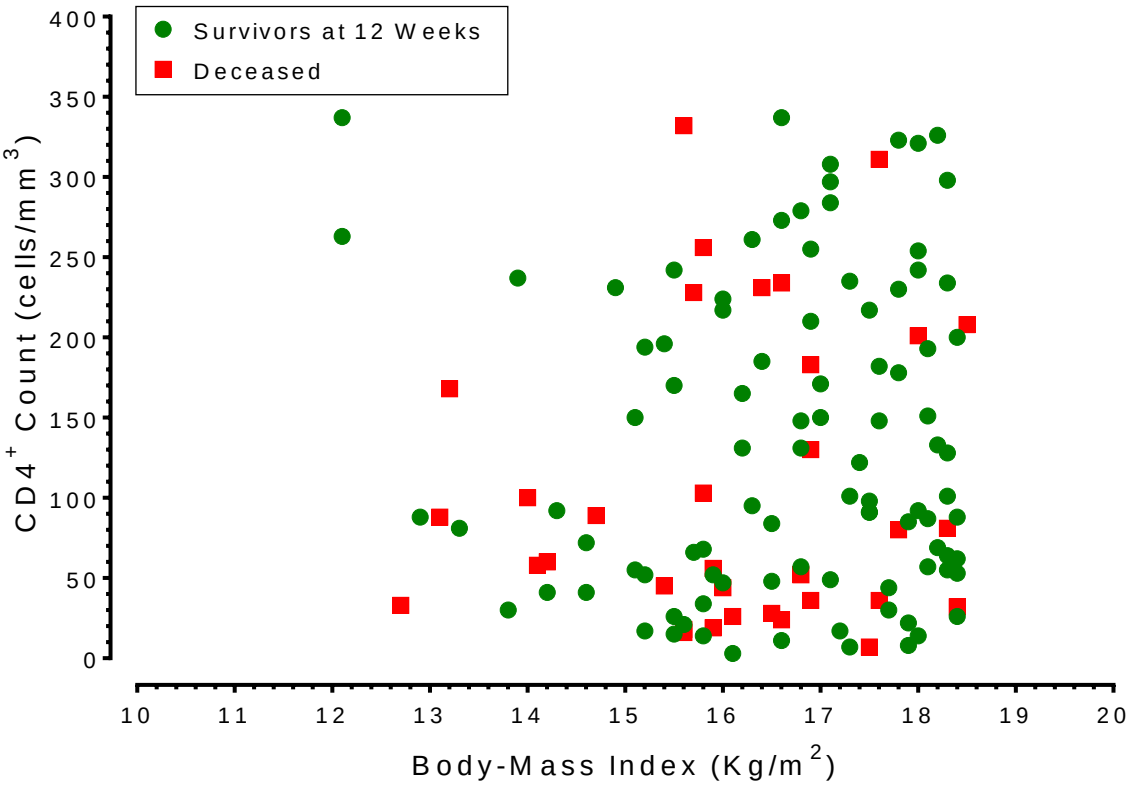


Figure 6.6: Correlates of CD4+ cell count with body mass index at baseline. All the patients had $CD4^+$ cell count <350 cells/mm^3 and BMI <18.5 Kg/m^2. In those that died or survived there was no correlation between BMI and $CD4^+$ cell count at baseline.

6.1.4 Serum Electrolytes and Creatinine Clearance

At baseline, estimated glomerular filtration rate (eGFR) from calculated creatinine clearance was within a reference range of 88 to 137 ml/min between the two research arms but significantly lower in the LNS-VM when compared to the LNS arm; LNS 100 ± 23 (95% CI, 94-106), n = 63 versus LNS-VM 90 ± 25 (95% CI, 84-96), n = 67; p = 0.012. However, by 12 weeks eGFR remained within normal range and was comparable between the two arms; LNS 101 ± 19 (95% CI, 96-107), n = 42 versus LNS-VM 103 ± 21 (95% CI, 97-109), n = 46; p = 0.71 using t-tests. Normal estimated creatinine clearance signified intact glomerular filtration rate of the kidneys (Figure 6.7).

At 12 weeks clinical data including serum electrolytes were also comparable between the two research arms. Serum phosphate; LNS 1.1± 0.3 mmol/L, n = 42 versus LNS-VM 1.1 ± 0.3 mmol/L, n = 46; p = 0.75 (Figure 6.8), serum potassium LNS 4.4 ± 0.5 mmol/L, n = 42 versus LNS-VM 4.3 ± 0.5 mmol/L, n = 46; p = 0.77 (Figure 6.9) and serum magnesium LNS 0.8 ± 0.1 mmol/L, n = 42 versus LNS-VM 0.79 ± 0.1, n = 46; p = 0.29 (Figure 6.8). The low and high cut-off serum values in mM were as follows: phosphate 0.8 - 1.5; potassium 3.5 - 5.5 and magnesium 0.7 - 1.1. In addition body weight; LNS 50.5 ± 7.3 Kg, n = 42 versus LNS-VM 51.0 ± 7.4 Kg, n = 46; p = 0.81, hemoglobin, LNS 11.6 ± 2.1 g/dL, n = 42 versus LNS-VM 10.6 ± 1.9 g/dL, n = 46; p = 0.051 by t-tests.

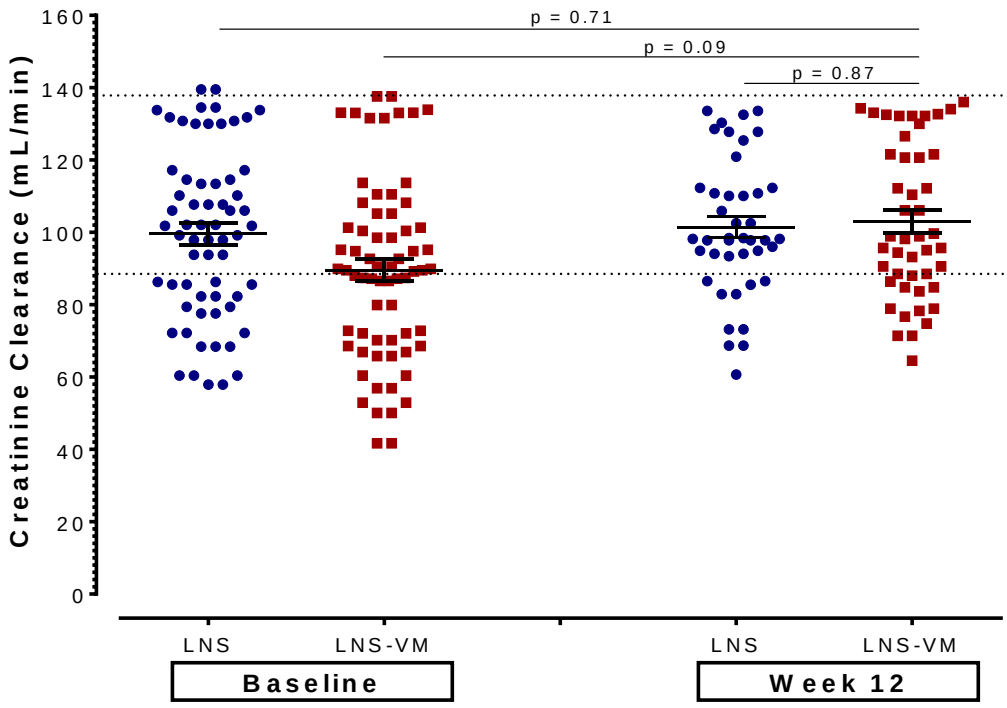


Figure 6.7: Estimated Creatinine Clearance at Baseline and Week 12 Antiretroviral Therapy. Values are mean ± SD and t-tests were used to assess differences between groups. At baseline creatinine clearance was significantly lower in the LNS-VM when compared to the LNS arm but at 12 weeks creatinine clearance was comparable between the two arms. The estimated creatinine clearance at both baseline and 12 weeks of antiretroviral therapy was within normal range and comparable only at 12 weeks. Estimated Creatinine Clearance was within normal ranges signifying intact glomerular filtration rate in the kidneys. Abbreviation: Lipid-based nutritional supplement – LNS; Lipid-based nutritional supplement with vitamins and minerals – LNS-VM. Dotted horizontal lines denote normal range: 88 to 137 ml/min.

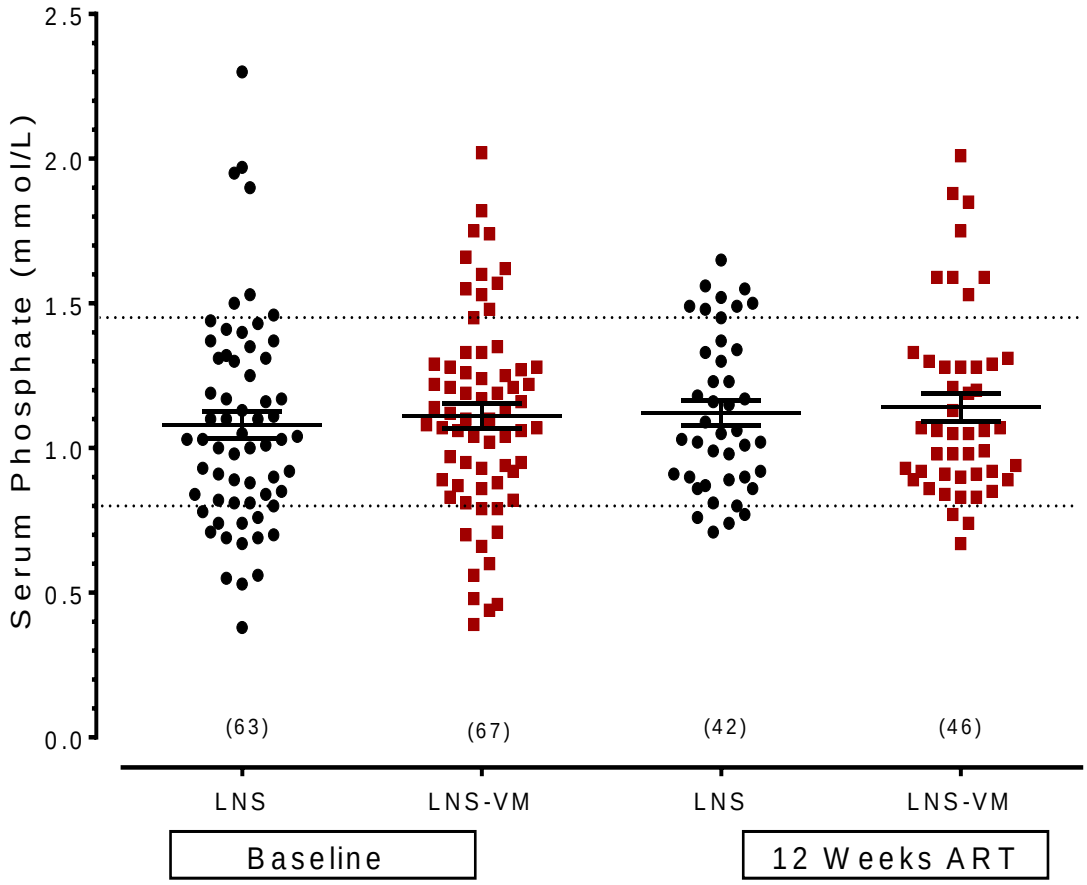


Figure 6.8: The effect of nutrient supplements on serum concentrations of phosphate. The group means for phosphate was within normal physiological ranges in the HIV infected patients at baseline and at 12 weeks of antiretroviral therapy. However, there was wide spread distribution of values particularly at baseline. The number of research subjects is shown in brackets but the plotted symbols reflecting individual patients may be less in number because some subjects had similar scores. Furthermore, the difference in sample sizes after treatment relative to baseline is due to loss to patient follow up as explained in main text. Horizontal dotted lines in each graph reflect the low and high cut-off concentrations for phosphates ions. The solid horizontal and vertical lines within the scatter points are mean ± SD, respectively. Abbreviations: ART, antiretroviral therapy; LNS, lipid-based nutritional supplement; LNS-VM, lipid-based nutritional supplement with vitamins and minerals.

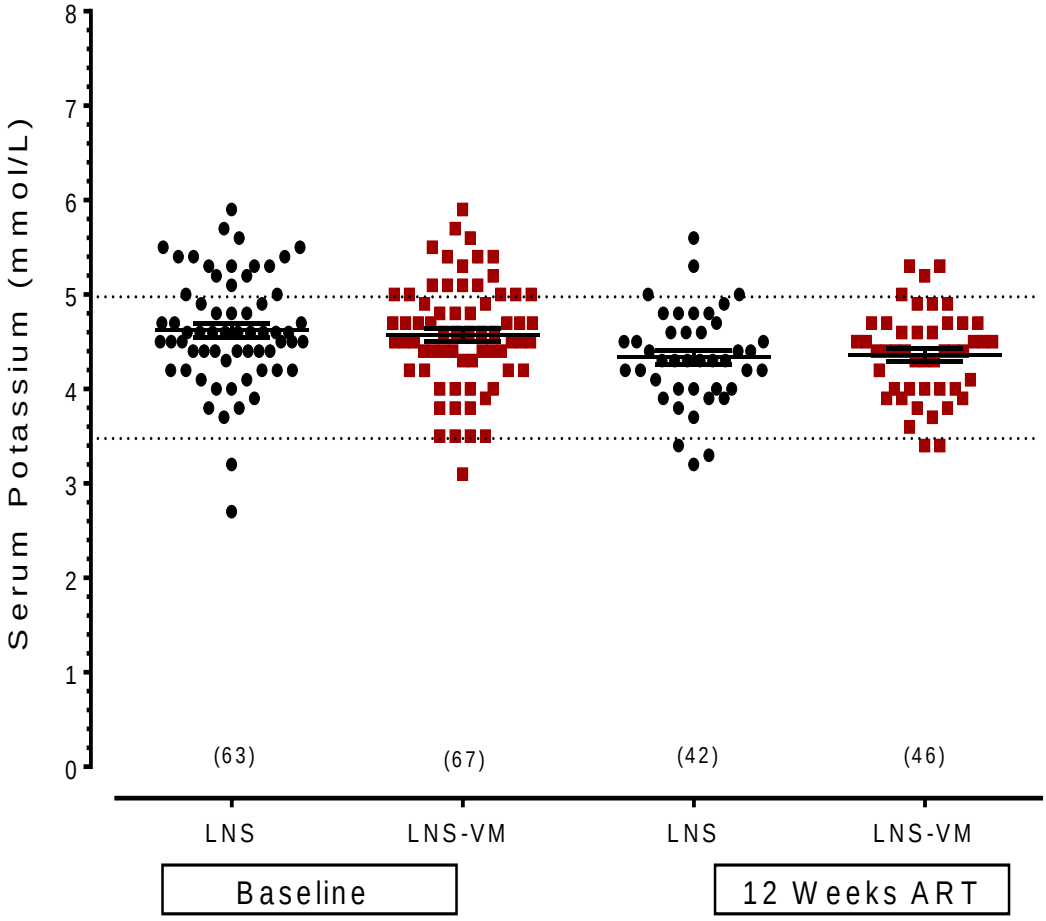


Figure 6.9: The effect of nutrient supplements on serum concentrations of potassium. The group means for potassium concentrations was within normal physiological ranges in the HIV infected patients at baseline and at 12 weeks of antiretroviral therapy. However, note the wide spread distribution of values particularly at baseline. The number of subjects is shown in brackets but the plotted symbols reflecting individual patients may be less in number because some subjects had similar scores. Furthermore, the difference in sample sizes after treatment relative to baseline is due to loss to patient follow up as explained in main text. Horizontal dotted lines in each graph reflect the low and high cut-off concentrations for phosphates potassium ions. The solid horizontal and vertical lines within the scatter points are mean ± SD, respectively. Abbreviations: ART, antiretroviral therapy; LNS, lipid-based nutritional supplement; LNS-VM, lipid-based nutritional supplement with vitamins and minerals.

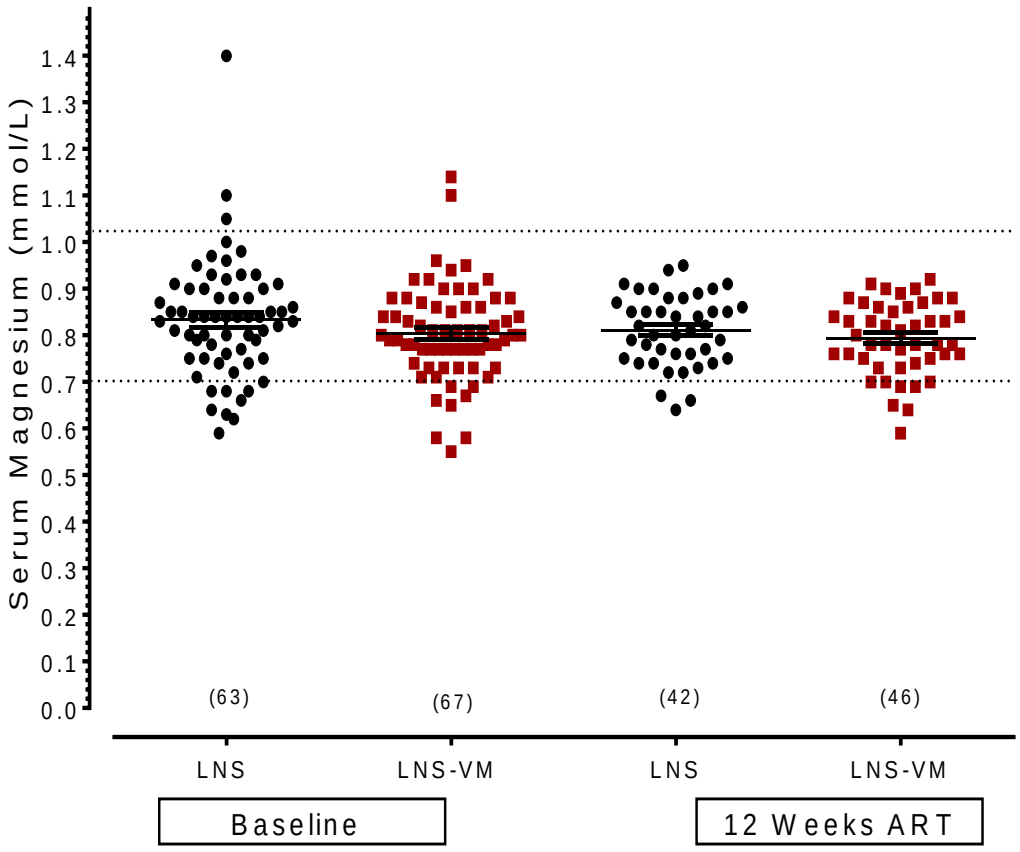


Figure 6.10: The effect of nutrient supplements on serum concentrations of magnesium. The group means for magnesium concentrations was within normal physiological ranges in the HIV infected patients at baseline and at 12 weeks of antiretroviral therapy. However, there was wide spread distribution of values particularly at baseline. The number of subjects per group is shown in brackets but the plotted symbols reflecting individual patients may be less in number because some subjects had similar scores. Furthermore, the difference in sample sizes after treatment relative to baseline is due to loss to patient follow up as explained in main text. Horizontal dotted lines in each graph reflect the low and high cut-off concentrations for ions. The solid horizontal and vertical lines within the scatter points are mean ± SD, respectively. Abbreviations: ART, antiretroviral therapy; LNS, lipid-based nutritional supplement; LNS-VM, lipid-based nutritional supplement with vitamins and minerals.

Table 6.4 Clinical characteristics of study participants at 12 weeks of ART

Variable	12 weeks of ART		
	LNS (42)	**LNS-VM (46)**	**P value**
Cotrimoxazole use, n (%)	38 (90.5)	43 (93.5)	0.88
Anti-TB drugs use, n (%)	11 (26.2)	13 (28.3)	0.75
Body weight, Kg	50.5 (7.3)	51.0 (7.4)	0.81
Hemoglobin, g/dL	11.6 (2.1)	10.6 (1.9)	0.051
Urine Creatinine, mg/dL	89.1 (23.5)	96.3 (27.2)	0.17
Serum creatinine, (µmol/L)	66.1 (11.9)	61.3 (13.8)	0.09
Serum sodium, mmol/L	131.9 (3.8)	130.7 (5.8)	0.25
Serum Phosphate (mmol/L)	1.1 (0.3)	1.1 (0.3)	0.75
Serum Potassium (mmol/L)	4.4 (0.5)	4.3 (0.5)	0.77
Serum Magnesium (mmol/L)	0.8 (0.1)	0.79 (0.1)	0.29

Chi-square tests were used for categorical variables and t tests for continuous variables. Abbreviations: Lipid-based nutritional supplement – LNS; Lipid-based nutritional supplement with vitamins and minerals – LNS-VM; antiretroviral therapy- ART. Values are mean ± SD unless stated otherwise. The low and high cut-off serum values in mM were as follows: phosphate 0.8 - 1.5; potassium 3.5 - 5.5 and magnesium 0.7 - 1.1. Creatinine 60 – 127 µmol/L.

6.2 Effect of nutritional intervention on renal electrolytes

At baseline phosphate excretion was high in both the LNS (mean ± SD in this and subsequent entries: 1.2 ± 0.6 mg/mg creatinine; n = 67) and LNS-VM (1.1 ± 0.8 mg/mg creatinine; n = 63) research groups. Excretion of phosphate remained high in the LNS group (1.1 ± 0.41 mg/mg creatinine; n = 42), whereas it was significantly decreased in the LNS-VM group (0.6 ± 0.28 mg/mg creatinine; n= 46; $p < 0.001$) by 12 weeks of antiretroviral therapy (Figure 6.11). This difference is probably explained by increased renal tubular reabsorption of phosphate in the LNS-VM group (88.3 ± 5.7%; n = 46) compared to the LNS group (76.6 ± 8.9%; n = 42; see Figure 6.12) after 12 weeks of antiretroviral therapy with intervention**.** Similarly, the fractional excretion of potassium values were high and exceeded normal physiological ranges (i.e. >6.4%) at baseline in both research groups and this indicated the presence of renal potassium wasting. However, the fractional excretion of potassium significantly decreased to within normal physiological ranges in the LNS-VM group (6.2 ± 3.4%; n = 46) but not in the LNS group (12.8 ± 4.7%; n = 42) after 12 weeks of antiretroviral therapy ($p < 0001$; Figure 6.13). Lastly, the results also showed that fractional excretion of magnesium was within normal physiological ranges at baseline. The renal fractional excretion of magnesium values remained unchanged and were within normal ranges after 12 weeks of antiretroviral therapy in both research groups (LNS, 3.3 ± 1.6; LNS-VM, 3.5 ± 1.3) relative to baseline values (LNS, 3.1 ± 1.9; LNS-VM, 3.2 ± 1.7; $p = 0.82$; Figure 6.14), respectively using t-tests.

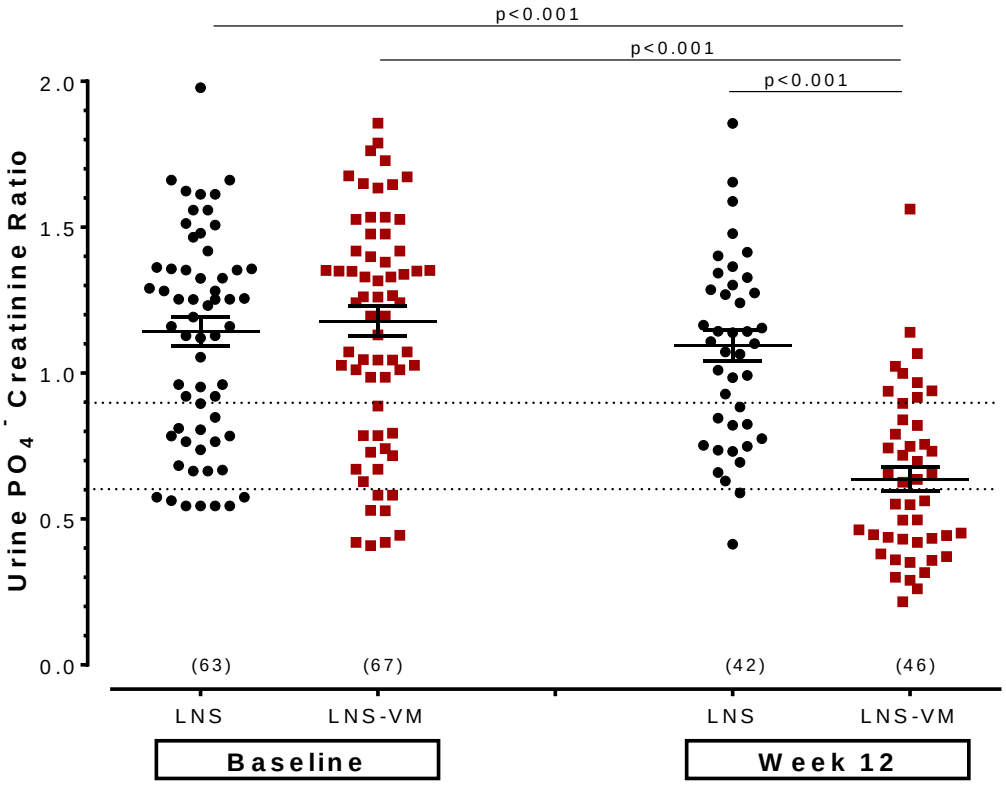


Figure 6.11: Urinary Phosphate Excretion at Baseline and Week 12. Urine phosphate-creatinine ratio was calculated to mimic the 24 hour urine. At baseline phosphate loss was high in both groups. By 12 weeks of antiretroviral therapy patients in the LNS-VM had significantly reduced phosphate loss compared to the LNS group in which urinary phosphate loss remained persistently high. Values are mean ± SD and t-tests were used to assess differences between groups. Abbreviation: Lipid-based nutritional supplement – LNS; Lipid-based nutritional supplement with vitamins and minerals – LNS-VM. Dotted horizontal lines denote normal range: 0.6 to 0.8 mg/dL.

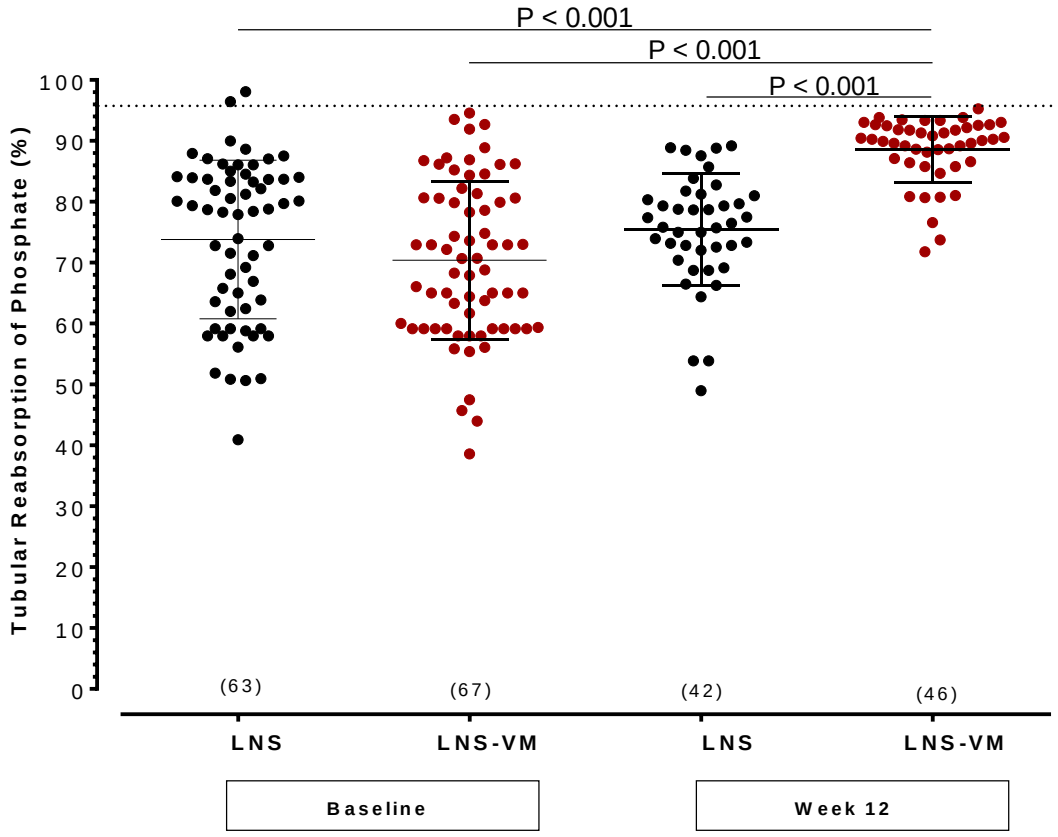


Figure 6.12: Assessment of renal handling of phosphate. Significant increase (p<0.001) in tubular phosphate reabsorption was only observed in LNS-VM but not LNS group after 12 weeks of antiretroviral therapy follow-up. The values are plotted as mean ± standard deviation (SD) and t-tests were used to assess differences between groups. Abbreviation: Lipid-based nutritional supplement – LNS; Lipid-based nutritional supplement with vitamins and minerals – LNS-VM. Renal tubular phosphate reabsorption values >96% reflect normal physiological values represented by dotted horizontal line.

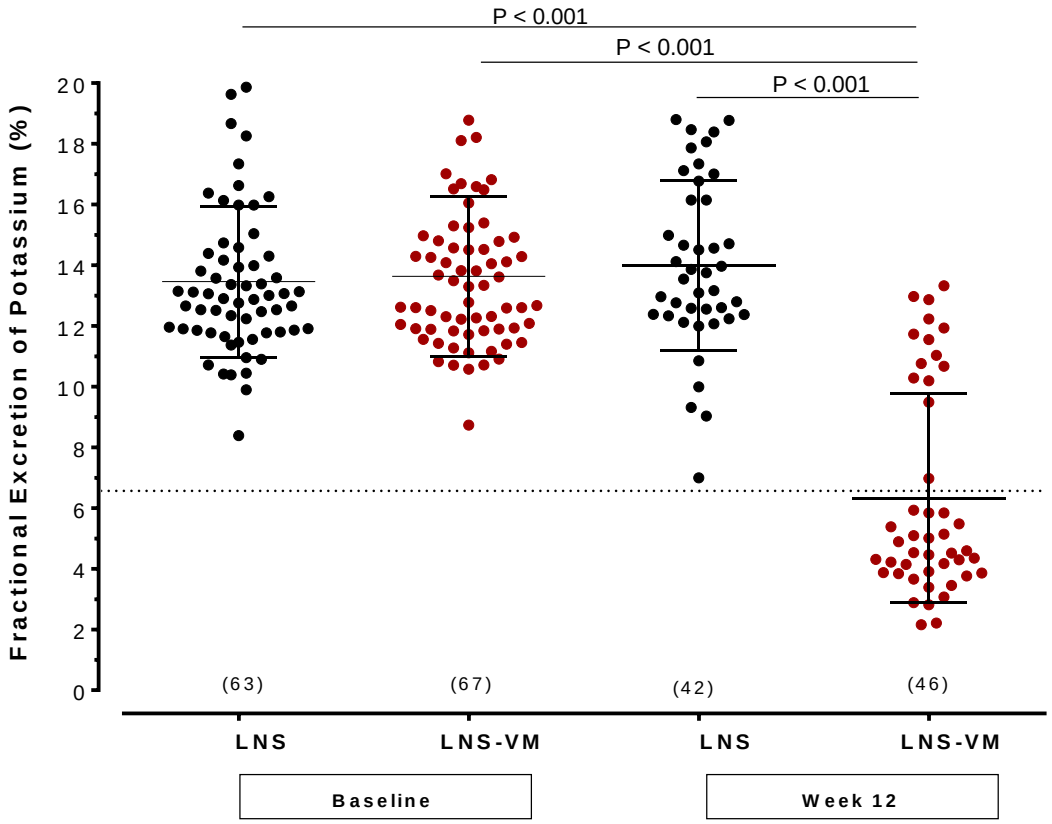


Figure 6.13: Assessment of renal handling potassium. Fractional excretion of potassium was significantly lowered only in LNS-VM group (p<0.001) but not in LNS group after 12 weeks of antiretroviral therapy. FEK is a useful marker of potassium excretion, and this is governed by tubular secretion rather than reabsorption. In patients with normal GFR, FEK <6.4% is consistent with appropriate potassium conservation represented by dotted horizontal line. The solid horizontal and vertical lines within the scatter points are mean ± SD. Paired and unpaired t-tests were used to compare within and between groups respectively. Abbreviation: Lipid-based nutritional supplement – LNS; Lipid-based nutritional supplement with vitamins and minerals – LNS-VM.

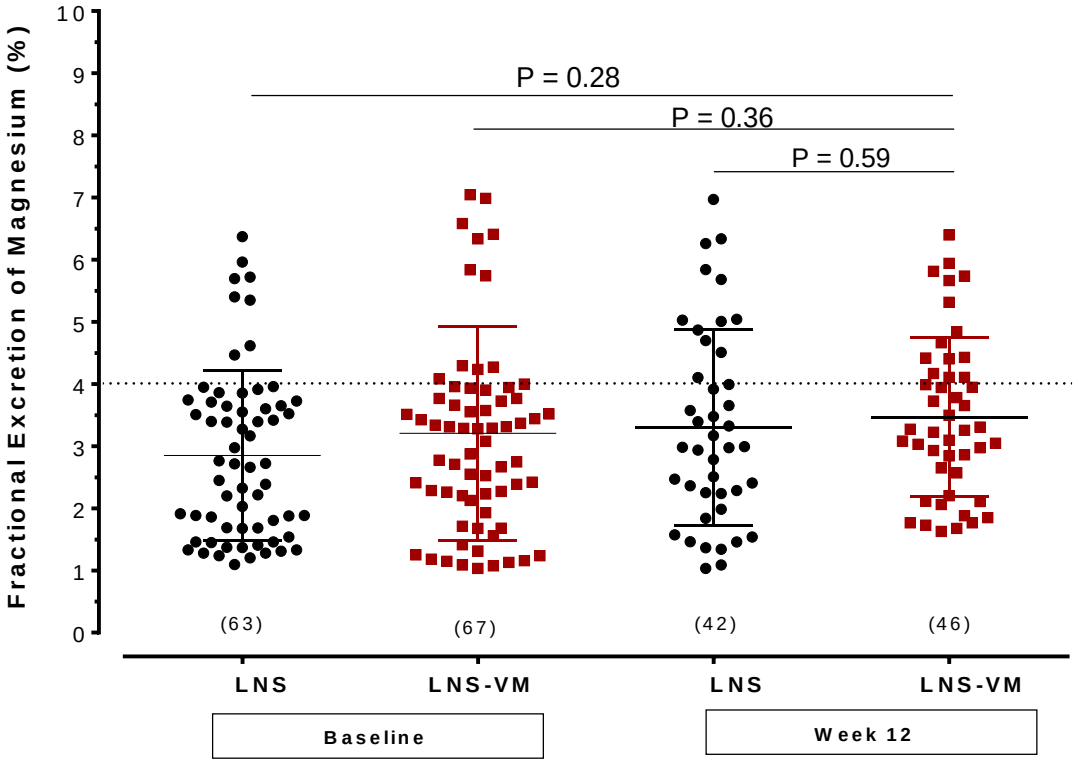


Figure 6.14: Renal Handling of Magnesium at Baseline and 12 Weeks. Neither LNS nor LNS-VM affected fractional excretion of magnesium ions. Fractional excretion of magnesium (FEMg) was comparable at baseline and unchanged after 12 weeks of antiretroviral therapy (p = 0.59) respectively. FEMg is a marker of intact tubulointerstitial structure and values <4% reflect intact tubular function for reabsorption of filtered magnesium represented by dotted horizontal line. The solid horizontal and vertical lines within the scatter points are mean ± SD and t-tests were used to assess differences between groups. Abbreviation: Lipid-based nutritional supplement – LNS; Lipid-based nutritional supplement with vitamins and minerals – LNS-VM.

6.3 Effect on Tenofovir concentrations of nutritional intervention

Since fat meals affect the intestinal absorption of tenofovir by resulting in increased systemic drug exposure that has been associated with renal dysfunction and low body weight, tenofovir blood concentrations were evaluated at 6 and 12 weeks to determine the effect of lipid-based nutrient supplementation on tenofovir levels in malnourished HIV-patients on treatment. The results from serum samples determined by using HPLC showed elevated levels relative to the minimum concentrations (Cmin = 64.4 ng/ml) as expected (Kearney et al., 2004). However, there were no differences in tenofovir blood concentrations between the two groups at 6 (LNS 208.4 ± 117.7 ng/ml (95% CI, 171.7-245.1); n = 42 versus LNS-VM 203.7 ± 102.3 ng/ml (95% CI, 173.3-234.1), n = 46; p = 0.84) and 12 weeks (LNS 207.4 ± 100.2 ng/ml (95% CI, 177.2-239.6); n = 42 versus LNS-VM 211.6 ± 103.4 ng/ml (95% CI, 180.9-242.3), n = 46; p = 0.88) of antiretroviral therapy (Figure 6.15).

Similarly, results from dried blood spot samples determined by using LC-MS/MS showed no differences in tenofovir levels between the two research groups at 6 (LNS 184.4 ± 96.7 ng/ml (95% CI, 151.6-217.1); n = 42 versus LNS-VM 188.7 ± 87.0 ng/ml (95% CI, 155.6-221.8), n = 46; p = 0.85) and 12 weeks (LNS 204.8 ± 85.1 ng/ml (95% CI, 178.3-231.3); n = 42 versus LNS-VM 213.7 ± 86.7 ng/ml (95% CI, 187.9-239.5), n = 46; p = 0.62) of antiretroviral therapy (Figure 6.16) by t-tests.

Hemoglobin was used to assess the integrity of the dried blood spot matrix. The results showed that hemoglobin levels in dried blood spot (HemaSpot) samples were comparable to those in fresh blood samples at both 6 (Fresh blood 10.5 ± 2.3 g/dL (95% CI, 9.9-11.1); n = 88 versus Dry blood 10.5 ± 1.9 g/dL (95% CI, 9.9-11.0), n = 88; p = 0.93) and 12 weeks (Fresh blood 11.1 ± 2.1 g/dL (95% CI, 10.2-12.1); n = 88 versus Dry blood 11.0 ± 2.1 g/dL (95% CI, 10.3-11.7), n = 88; p = 0.85 using t-tests) of antiretroviral therapy indicating intact dried blood spot samples after about fourteen (14) months of storage (Figure 6.17). Bland-Altman plots showed that the difference between measurements using both Fresh blood and dried blood spot samples were within the limits of agreement ~95% of the time (mean bias=213, blue lines) confirming that the two methods were producing the same results (Figure 6.14). Comparing fresh blood and dried blood spot samples revealed no signifincant difference between the two methods (Figure 6.18).

Hence, tenofovir concentration levels at 6 weeks(Fresh blood tenofovir 205.9 ± 109.3 ng/ml (95% CI, 182.8-229.1); n = 88 versus Dry blood tenofovir 184.5 ± 84.7 (95% CI, 164.8-204.3), n = 88; p = 0.17) and 12 weeks (Fresh blood tenofovir 210.1 ± 101.3 ng/ml (95% CI, 188.6-231.5); n = 88 versus Dry blood tenofovir 209.4 ± 85.6 ng/ml (95% CI, 191.3-227.6), n = 88; p = 0.96) of antiretroviral therapy were comparable in fresh blood and dried blood spot samples(Figure 6.19) by 1-way ANOVA.

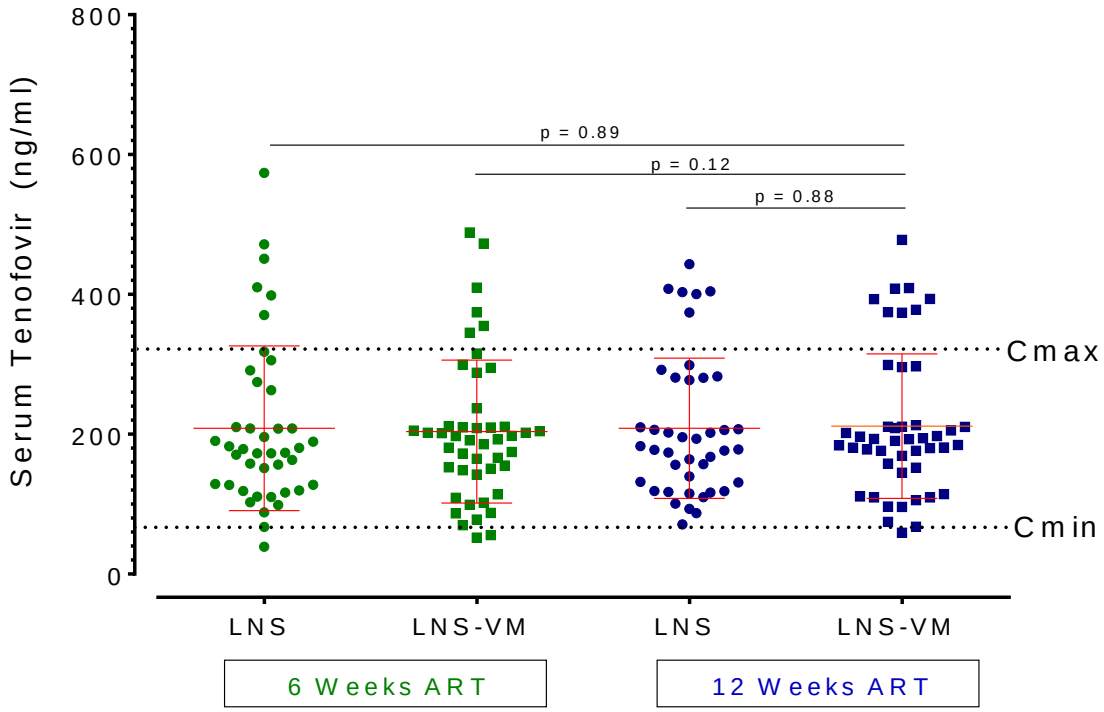


Figure 6.15: Serum tenofovir concentration levels at 6 and 12 weeks between LNS group and LNS-VM group. Tenofovir concentration levels were comparable at 6 and 12 weeks of antiretroviral therapy. At 12 weeks there was no signifincant difference between the two groups. Taking into account that blood samples were collected 14 hours after oral dose of tenofovir-based therapy, the mean tenofovir concentration levels were expected to be around the minimum concentration (Cmin), but the levels were way above the Cmin in both groups. This indicates potential for tenofovir toxicity in low body weight HIV-infected patients on treatment. Abbreviation: Lipid-based nutritional supplement – LNS; Lipid-based nutritional supplement with vitamins and minerals – LNS-VM; Maximum concentration-Cmax; Minimum concentration-Cmin. Cmax=326 ng/ml, Cmin=64.4 ng/ml represented by dotted horizontal lines (Kearney et al., 2004). The solid horizontal and vertical lines within the scatter points are mean ± SD and t-tests were used to assess differences between groups.

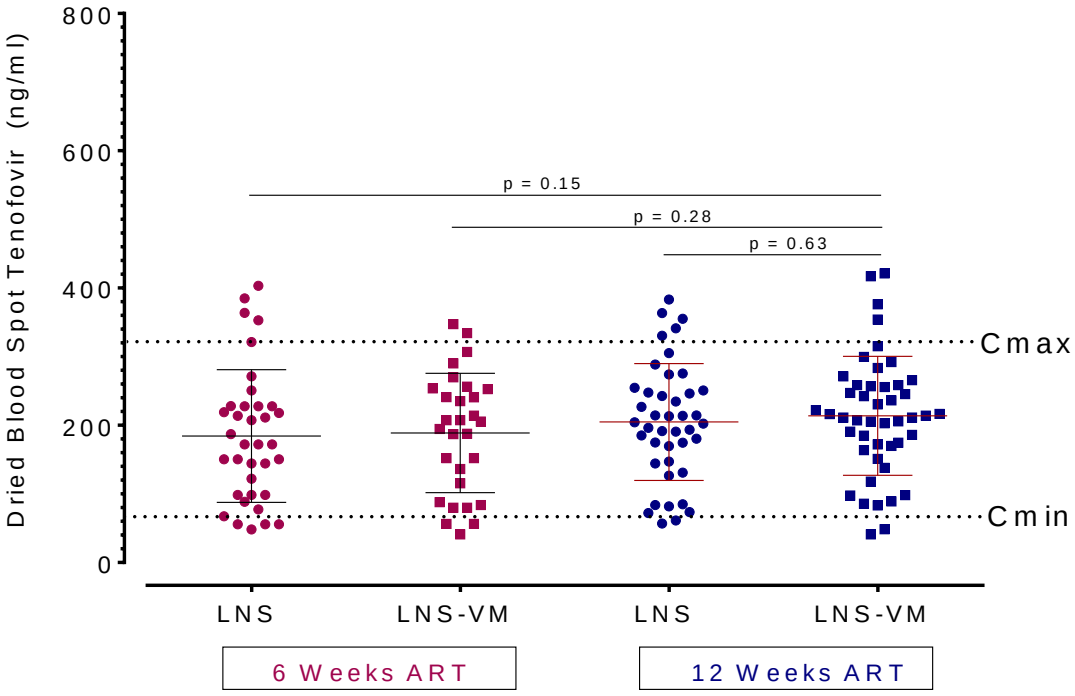


Figure 6.16: Dried blood spot tenofovir concentrations. Tenofovir concentration levels at 6 and 12 weeks of antiretroviral therapy were comparable. Taking into account that blood samples were collected 14 hours after oral dose of tenofovir-based therapy, the mean tenofovir concentration levels were expected to be around the minimum concentration (Cmin), but the levels were way above the Cmin in both groups. This indicates potential for tenofovir toxicity in low body weight HIV-infected patients on treatment. Abbreviation: Lipid-based nutritional supplement – LNS; Lipid-based nutritional supplement with vitamins and minerals – LNS-VM; Maximum concentration-Cmax; Minimum concentration-Cmin. Cmax=326 ng/ml, Cmin=64.4 ng/ml represented by dotted horizontal lines (Kearney et al., 2004). The solid horizontal and vertical lines within the scatter points are mean ± SD and t-tests were used to assess differences between groups.

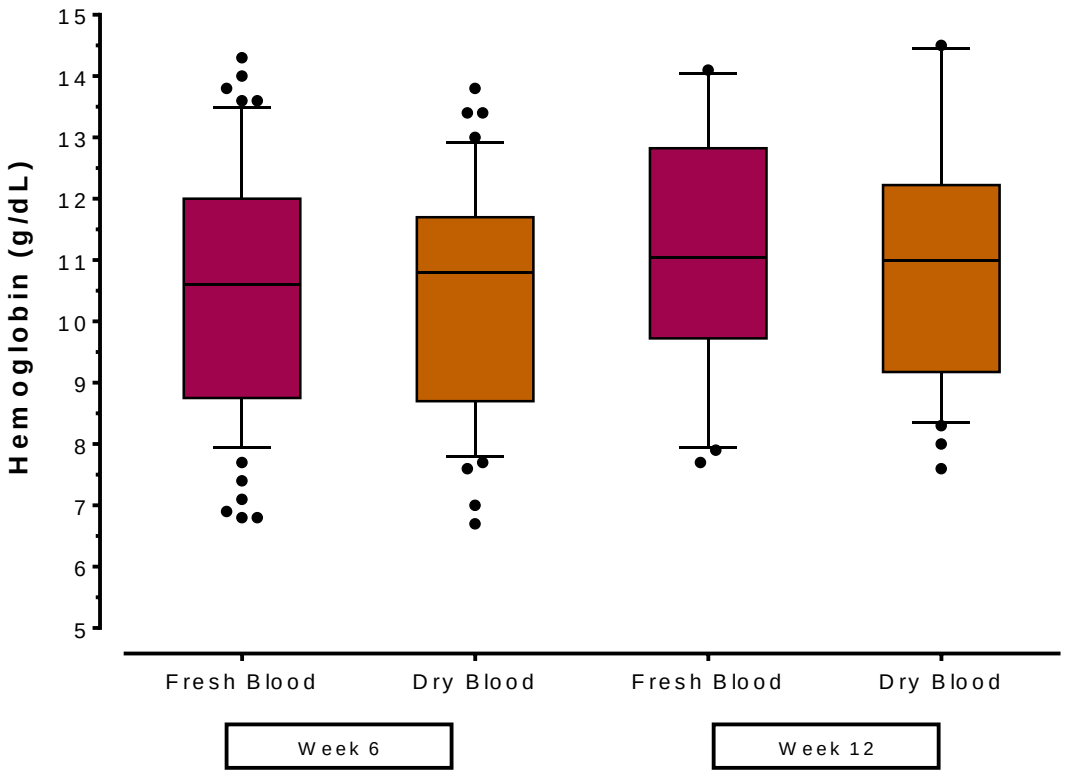


Figure 6.17: Hemoglobin in fresh blood versus dry blood at 6 and 12 weeks of antiretroviral therapy. Hemoglobin was used to assess the integrity of the dried blood spot matrix. The hemoglobin levels in dried blood spot samples were comparable to those in fresh blood samples indicating intact dried blood spot samples after storage. Values are mean ± SD and t-tests were used to assess differences between groups.

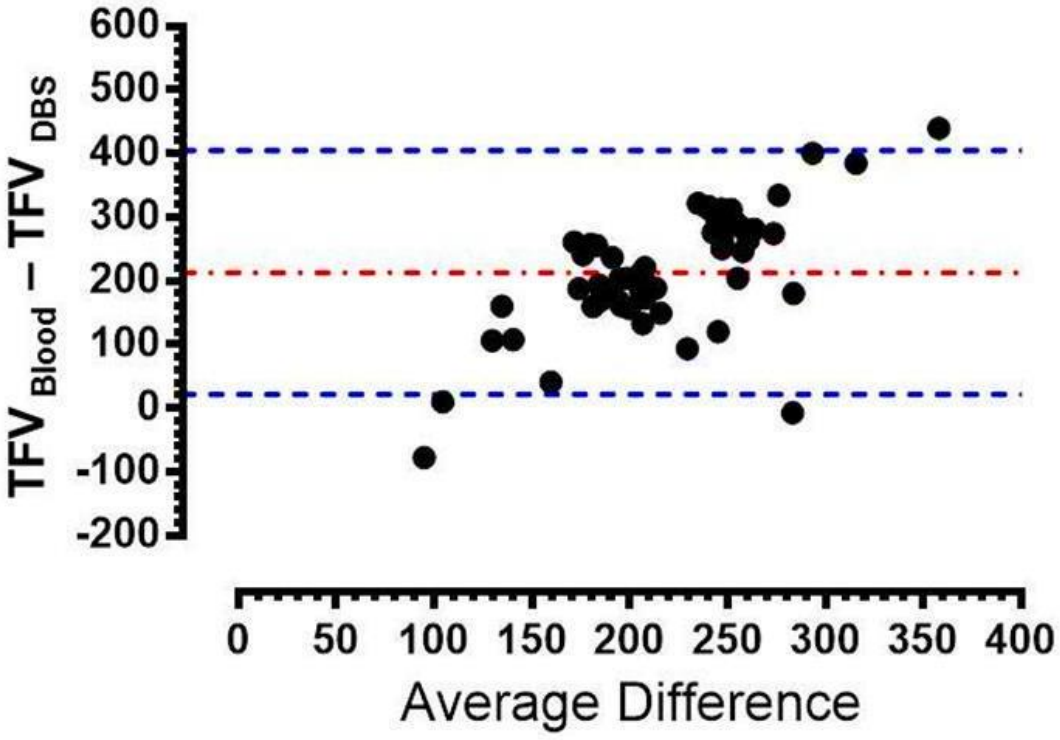


Figure 6.18: Bland-Altman plots showed that the difference between measurements using both Fresh blood and dried blood spot samples were within the limits of agreement ~95% of the time (mean bias=213, blue lines) confirming that the two methods were producing the same results. Clearly, the dried blood spot methods were valid and offer a robust method to collect specimen in resource-poor settings for transfer to analytical centers.

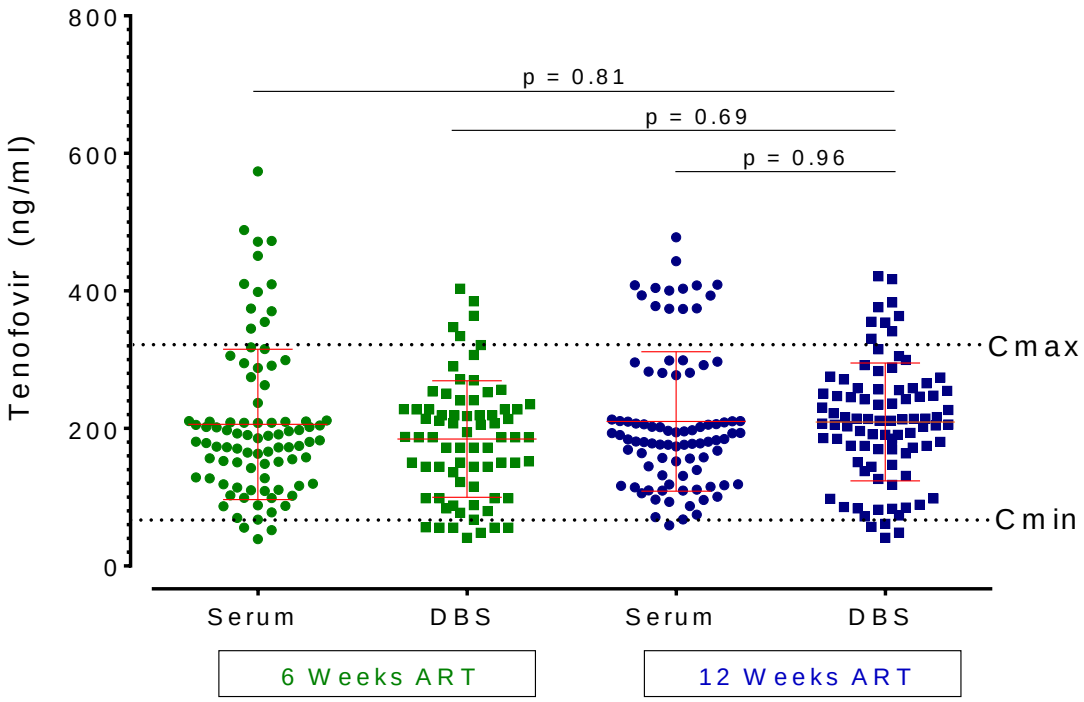


Figure 6.19: Serum versus DBS tenofovir Concentrations. Tenofovir concentration levels were comparable at 6 and 12 weeks of antiretroviral therapy. Comparing serum and dried blood spot samples revealed no signifincant difference between the two methods. Taking into account that blood samples were collected 14 hours after oral dose of tenofovir-based therapy, both serum and dried blood spot samples yielded tenofovir concentrations that significantly exceeded minimum concentration levels (dotted line) expected in HIV-infected patients stabilized on antiretroviral therapy. This was suggestive of potential for tenofovir toxicity in low body weight HIV-infected patients on treatment. Abbreviation: Dried blood spot-DBS; Maximum concentration-Cmax; Minimum concentration-Cmin. Cmax=326 ng/ml, Cmin=64.4 ng/ml represented by dotted horizontal lines (Kearney et al., 2004). The solid horizontal and vertical lines within the scatter points are mean ± SD and t-tests were used to assess differences between groups.

6.3.1 Correlation of Tenofovir concentrations with Body Mass Index

Having compared tenofovir and other clinical parameters between the two groups, correlates of tenofovir with other clinical variables were examined. Participant data at 6 weeks showed a significant negative correlation of tenofovir with body mass index only ($r = - 0.73$; $p = < 0.001$; Figure 6.20). When examined by research arm there was a stronger significant correlation in the LNS-VM group ($r = - 0.81$; $p < 0.001$) than the LNS group ($r = - 0.68$; $p < 0.001$) (Figure 6.21). Similarly, 12 weeks data also showed a significant correlation of tenofovir with body mass index ($r = - 0.66$; $p < 0.001$; Figure 6.22). When the evaluation was divided by research arm, correlates of tenofovir with body mass index were comparable between the arms (LNS-VM $r = - 0.63$; $p < 0.001$ versus LNS $r = - 0.69$; $p < 0.001$; Figure 6.23). Multivariable linear regression analysis of tenofovir concentration levels at 12 weeks of antiretroviral therapy with determinants such as age, sex, body mass index, CD4 cell count and renal electrolytes was performed. The regression analysis revealed body mass index as an independent predictor of serum tenofovir (coefficient -34.3; 95%CI (-41.43, -27.14), $p < 0.001$; Table 6.5).

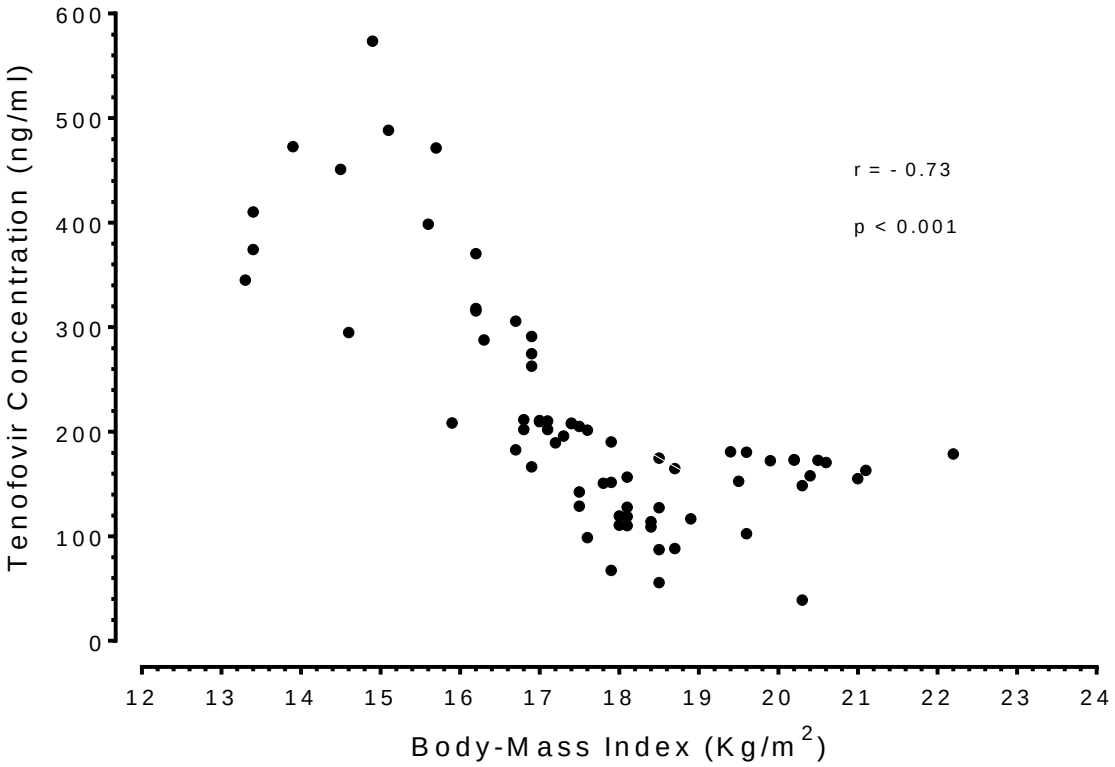
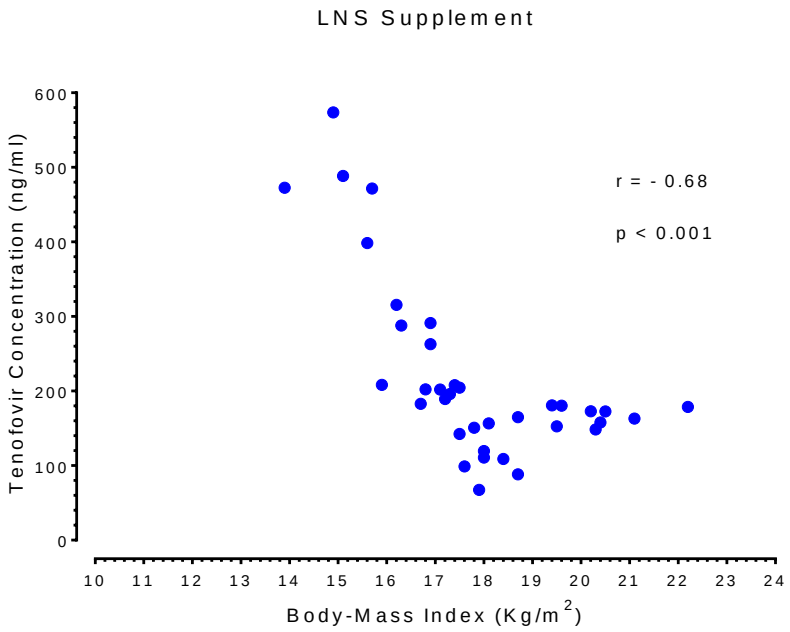


Figure 6.20: Correlation of tenofovir and body mass index at 6 weeks of antiretroviral therapy. There was a significant negative correlation between tenofovir concentration levels and body mass index at 6 weeks of antiretroviral therapy in the HIV-infected patients.

a



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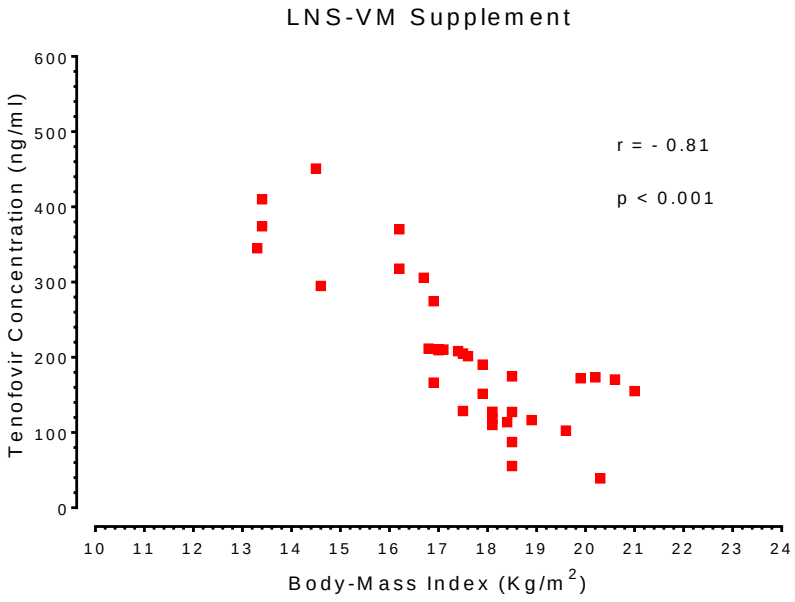


Figure 6.21: Correlation of tenofovir versus body mass index. There was a significant negative correlation between tenofovir concentration levels and body mass index at 6 weeks of antiretroviral therapy in the LNS group **(a)** as well as in the LNS-VM group **(b)**.

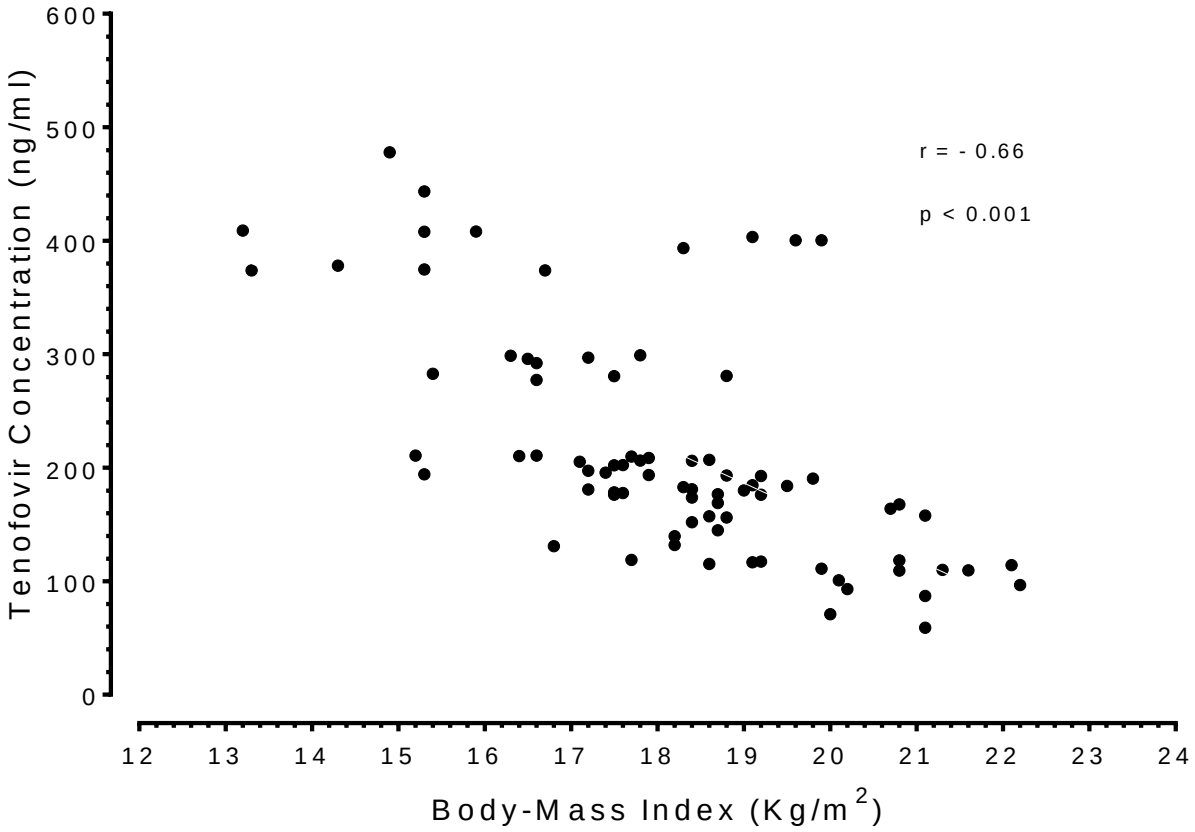
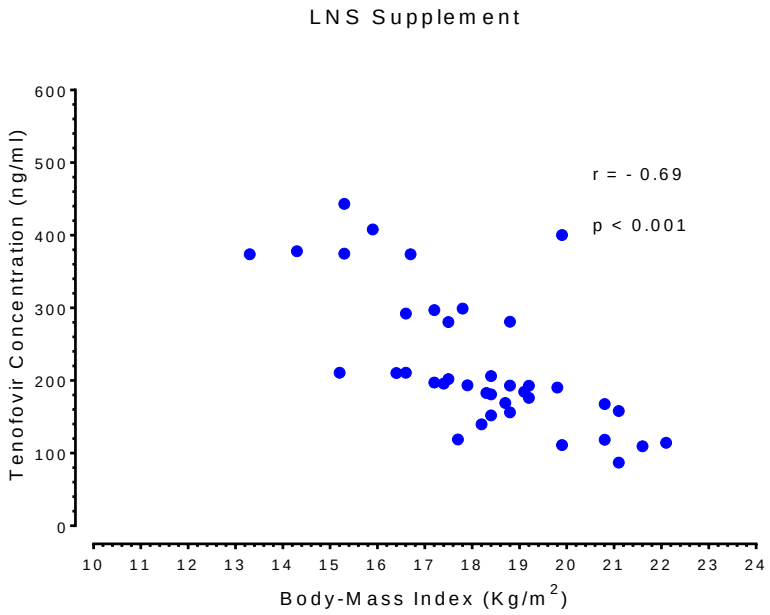


Figure 6.22: Correlation of tenofovir against body mass index at 12 weeks of antiretroviral therapy. There was a significant negative correlation between tenofovir concentration levels and body mass index at 12 weeks of antiretroviral therapy in the HIV-infected patients.

a



b

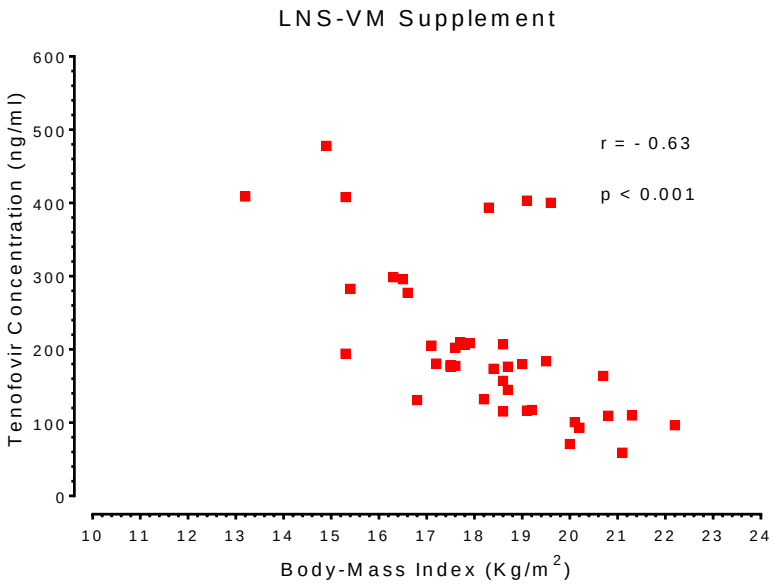


Figure 6.23: Correlation of tenofovir versus body mass index of LNS supplement arm and LNS-VM supplement arm at 12 Weeks ART. There was a significant negative correlation between tenofovir concentration levels and body mass index at 12 weeks of antiretroviral therapy in the LNS group **(a)** as well as in the LNS-VM group **(b)**.

Table 6.5: Multivariable linear regression analysis of Tenofovir levels at 12 weeks

Variable	Coefficient	95% CI		SE	P-Value
Sex	-2.10	-32.01	27.80	15.26	0.89
Age	0.30	-1.26	1.87	0.79	0.70
Renal Phosphate Reabsorption	-2.64	-5.73	0.46	1.58	0.09
Fractional Excretion of Potassium	-0.25	-3.61	3.10	1.71	0.88
CD4+ cell count	0.04	-0.06	0.14	0.05	0.45
Body mass index	-34.28	-41.43	-27.14	3.65	**< 0.001****
Fractional Excretion of Potassium	-0.35	- 6.53	7.22	3.51	0.92
Phosphate-Creatinine ratio	- 4.22	- 74.47	66.04	35.85	0.91
Serum Creatinine	- 0.04	- 1.66	1.59	0.83	0.97

**P-Value for Interaction Effect. Abbreviations: CI-Confidence Interval, SE-Standard Error. Linear regression model was used to estimate the effect of body mass index (BMI) on mean tenofovir concentrations adjusting for potential confounders such as age, sex, CD4 cell count and renal electrolytes. Confounders were identified using stepwise backward algorithms with probability of removal set at 0.2. P-values for removals were calculated using likelihood ratio test.

CHAPTER 7

DISCUSSION

7.1 Overview of the findings

This study investigated the effect of lipid-based nutrient supplement containing vitamins and minerals (LNS-VM) compared to lipid-based nutrient supplement (LNS) alone on: (a) renal excretion of electrolytes and (b) tenofovir blood concentration levels among underweight Zambian HIV/AIDS patients initiating tenofovir-based antiretroviral therapy. Baseline demographic, clinical and biomarker data were similar between the two research arms (Table 6.2). Specifically, the baseline, estimated glomerular filtration rate (eGFR) from calculated creatinine clearance was within a normal range of 88 to 137 ml/min between the two research arms and remained within normal range by 12 weeks of antiretroviral therapy in both groups. Normal estimated creatinine clearance signified intact glomerular filtration rate of the kidneys. Other studies have also reported no significant differences in creatinine clearance when comparing patients under a tenofovir-containing antiretroviral therapy; patients on antiretroviral therapy never exposed to tenofovir; and antiretroviral-naive individuals (Labarga et al., 2009). This is consistent with the findings in the present study. Similarly, in a study that assessed early markers of renal toxicity in patients taking either a tenofovir/emtricitabine regimen or an abacavir/lamivudine regimen, found no significant variation in estimated glomerular filtration rate (eGRF) even when there was a significant increase in

urinary excretion of phosphate in patients on tenofovir-based therapy compared to those on abacavir-based therapy (Maggi et al., 2012b).

In terms of renal tubule electrolytes handling, phosphate excretion was high in both the LNS and LNS-VM at baseline. However, phosphate excretion remained high in the LNS group whereas it was significantly decreased in the LNS-VM group (Figure 6.11). The above result was matched by the observed significant increase in phosphate reabsorption in the LNS-VM group and not the LNS group (Figure 6.12). Similarly, fractional excretion of potassium was significantly decreased in the LNS-VM group in relation to baseline levels in the same group and also versus the LNS group after 12 weeks of ART (Figure 6.13). The results also showed that fractional excretion of magnesium (FEMg) was unchanged in both LNS and LNS-VM groups (Figure 6.14).

In terms of tenofovir blood concentrations, since fat meal affects the intestinal absorption of tenofovir by resulting in increased systemic drug exposure (Kearney et al., 2004, Geboers et al., 2015) that has been associated with renal dysfunction and low body weight (Nishijima et al., 2011, Gervasoni et al., 2013), tenofovir blood concentrations were evaluated at 6 and 12 weeks to determine the effect of lipid-based nutrient supplementation on tenofovir levels in malnourished Zambian HIV-patients on treatment. The findings of this study showed that tenofovir blood concentrations were not statistically different between LNS and LNS-VM groups at 6 and 12 weeks of antiretroviral therapy but that were evaluated exceeding the expected minimum concentrations (Cmin = 64.4 ng/ml) (Kearney et al., 2004). Tenofovir mean concentrations were expected

to be at minimum level since blood samples were collected 14 hours after oral dose of tenofovir-based therapy that correspond with trough levels. Multivariable linear regression analysis of tenofovir concentration levels with determinants such as age, sex, body mass index, CD4 cell count and renal electrolytes revealed body mass index as an independent predictor of serum tenofovir. Tenofovir-based antiretroviral therapy has been associated with renal dysfunction and very high mortality rates particularly among HIV-infected patients with low body mass index at onset of treatment (Nishijima et al., 2011, Gervasoni et al., 2013). Consistent with this, results of this study showed high patient loss in both research arms due to mortality (total number deceased: LNS = 18 versus LNS-VM = 16). Interestingly, a greater number (13 of 18 subjects, i.e. 72.2%) became deceased in the lipid-based nutrient supplement without vitamins and minerals arm prior to initiation of antiretroviral therapy whereas only half the number (8 of 16 subjects, i.e. 50%) were lost during the same period in the lipid-based nutrient supplement containing vitamins and minerals group. However, there were insignificant differences in terms of demographic and clinical profiles at baseline between those who became deceased versus the survivors except in body weight, serum magnesium, serum creatinine and creatinine clearance which were statistically different between the two groups (Table 6.3). These could have contributed was to the observed differences in mortality profiles. Previously reported evidence shows weight loss as a negative prognostic indicator since early times in the HIV epidemic among HIV/AIDS patients (Babameto and Kotler, 1997, Grunfeld and Kotler, 1992).

7.2 Nutrition and Electrolytes

Micronutrient deficiencies or abnormalities have been increasing in recent years among HIV-infected patients partly due to the HIV disease and/or treatment modalities for HIV. The concept of marginal micronutrient deficiency was first proposed in the mid-1980s (Pietrzik, 1985). This shows that, long before the clinical symptoms of deficiency appear, micronutrient abnormalities develop progressively through several sub-clinical stages. Consistent with this, the present study has demonstrated deficits in renal phosphate reabsorption and potassium excretion in malnourished HIV/AIDS patients. It has been shown for the first time that subsequent administration of LNS-VM, but not LNS, improved phosphate reabsorption and significantly reduced potassium excretion. The group means for plasma concentrations of phosphates, potassium and magnesium were within normal physiological ranges and this was suggestive of 'compensatory' mechanisms at play. Plasma levels of phosphates, potassium, and magnesium are maintained within very narrow limits to sustain life (Cogan, 1991). Thus, loss of body electrolytes via excretion is expected to be counter-balanced by replenishment through nutritional intake. If this is inadequate, as may occur in malnutrition, then bone resorption and/or cell destruction is likely to occur to maintain electrolyte homeostasis. The finding that there was primarily potassium and phosphate wasting, but not magnesium, is suggestive of cell loss rather than bone resorption. By contrast, a finding of magnesium with phosphate wasting would have been suggestive of bone re-sorption more than cell loss (Cogan, 1991). Consequently, it is predicted that the improved renal electrolyte

retention observed in the LNS-VM group will be associated with a reduced cell wasting that occurs to maintain electrolyte homeostasis and this idea is supported by the recently reported findings of more rapid tissue deposition in the LNS-VM group relative to the LNS group (Rehman et al., 2015b).

It was unclear what mechanisms accounted for the benefits in renal function derived from LNS-VM co-administration with ART. However, it is feasible the benefits could be attributed to the effects of anti-oxidant vitamins and mineral replacement leading to improved kidney homeostatic functions. Other Studies also suggest that antioxidant supplementation may decrease markers of oxidative stress (Lanzillotti and Tang, 2005) which are linked to renal tubular dysfunction in individuals with HIV. First, phosphate homeostasis is balanced by daily phosphate intake and renal excretion. Serum levels are altered by intestinal phosphate absorption mediated by type 2b sodium-phosphate (Npt2b) co-transporters. Npt2b co-transporters are regulated by dietary phosphate intake as well as calcitrol derived from the metabolism of vitamin D (Marks et al., 2010, Cogan, 1991, Blaine et al., 2015). Calcitrol is a strong stimulant of both the rate and maximal capacity of intestinal absorption of phosphate. Probably LNS-VM exerted some of these effects because the product contained both vitamin D and a higher phosphorous content (see Table 5.1). Second, phosphate is freely filtered in the glomerulus but greater than 80% of the filtered load is reabsorbed in the renal proximal tubules mainly via type 2a and type 2c (Napt2a and Napt2c) co-transporters that move 3 sodium ions and one phosphate molecule (Cogan, 1991, Strimbu and Tavel, 2010, Bellorin-Font E, 1990). The abundance of

Napt2a and Napt2c in the apical membranes of proximal tubular cells is regulated by parathyroid hormone (PTH) secreted from the parathyroid gland (Bellorin-Font E, 1990, Forster et al., 2006). PTH inhibits the insertion of Napt2a and Napt2c into the proximal tubular cell apical membranes and this causes phosphaturia (Blaine et al., 2015). However, PTH also acts to increase the plasma concentration of calcium by promoting active absorption from the small intestines and mediating calcium resorption from bone (Blaine et al., 2015). But rising plasma calcium subsequently reverses PTH secretion and secondarily curtails the PTH-linked inhibition of Napt2a and Napt2b insertions resulting in increased phosphate reabsorption from the kidney. Third, dietary potassium deficiency leads to changes in brush border membrane lipid composition that are thought to inhibit sodium-phosphate co-transport (Breusegem et al., 2009). Taken together, nutritional supplements containing both phosphate and vitamin D would be expected to increase phosphate absorption from the intestines coupled with augmented reabsorption in the kidneys.

In terms of potassium excretion, the most parsimonious explanation is that the ingredients in LNS-VM augmented energy metabolism leading to increased production of cellular ATP in general (Huskisson et al., 2007) and this partly contributed towards the critical activation of sodium-potassium pumps (i.e. Na^+/K^+-ATPase). If this is valid then two important outcomes are predicted to occur as follows. First, Na^+/K^+-ATPases are powered by cellular ATP and they are responsible for maintaining the high potassium ions concentration and low sodium ions concentration in the cytoplasm relative to the extracellular levels,

respectively (Cogan, 1991). This is achieved via coupled transportation of 3 sodium ions from the cytoplasm versus cellular entry of 2 potassium ions. These 'unbalanced' ionic movements generate an electrical current that hyperpolarizes the cell membrane resulting in more negative resting membrane potentials that, in turn, further retard efflux of potassium ions from the cytoplasm to the extracellular space. Thus, potassium loss from body cells as may be occurring in malnourished HIV-1 infected patients would be minimized. Second, potassium is freely filtered in the glomerulus but 87-96% of the filtered load is reabsorbed primarily in the proximal tubule and ascending limbs of Henle (Cogan, 1991). However, potassium can also be secreted by the cells of the distal tubules and collecting duct system. In fact, potassium secretion from the blood into the tubular fluid by the cells of the distal tubule and collecting duct system is the major determinant of urinary potassium excretion (Cogan, 1991, Elisaf and Siamopoulos, 1995). This follows a 'two-step' process starting with potassium uptake from the blood across the basolateral membrane of the distal tubule and collecting duct system by the 'Na^+/K^+-ATPase' (Cogan, 1991). Then diffusion of potassium from the cells into the renal tubular fluids occurs through potassium channels driven by the high chemical gradient. In addition, the apical membrane contains sodium channels that increases the permeability to sodium ions whose entry across the apical membrane of distal tubule and collecting duct cells may decrease the resting membrane potential (i.e. make less polar). Such a depolarization of the cell membrane potential further increases the electrical driving force that promotes potassium secretion across the apical membrane into

the tubule fluid. However, activated Na^+/K^+-ATPase in the presence of ATP stores 'neutralizes' the depolarization due to the passive influx of sodium ions by actively transporting sodium ions out of these cells and even creating a hyperpolarization which prevents potassium secretion. Taken together, we contend that lipid-based nutrient supplements containing vitamins and minerals greatly augment ATP production some of which activates the Na+/K+-ATPase that maintains ionic gradients in cells. These actions would account for maintaining sodium and potassium gradients and prevent potassium loss from body cells, curtailing renal potassium secretion in particular. These ideas will need further experimental validation.

7.3 Nutrition and tenofovir

In the present study malnourished underweight patients received lipid-based nutrient supplementation and antiretroviral therapy containing efavirenz 600mg, emtricitabine 200mg and tenofovir disoproxil fumarate 300 mg. Tenofovir-disoproxil-fumarate (TDF) is a double- ester prodrug which enables intestinal uptake of tenofovir (TFV) after oral administration in humans. A prodrug is an inactive form of the drug, which has to be activated once taken up (Jarkko et al., 2008). TDF has two isopropyloxycarbo- nyloxymethyl moieties that mask the negative charges of TFV, to form a lipophilic ester prodrug that results in an increased transport across the intestinal epithelium. Studies have demonstrated that TDF bioavailability following oral administration in the fasted state is about 25% and the drug levels can be increased up to 40% with fat meals. This is because fat meals result in micellar formation and entrapment of tenofovir and

therefore increased systemic exposure. It is known that 70-90% of tenofovir and emtricitabine, but less than 3% of efavirenz, is excreted via the kidneys. However, emtricitabine is the least toxic drug to the kidney whereas tenofovir is linked to renal deficits. A recent meta-analysis of 17 studies found that TDF use was associated with a statistically significant loss of renal function compared to non- TDF containing regimens, but that the clinical magnitude of this effect was modest (Cooper et al., 2010). Therefore, this and the fact that intestinal absorption is affected by fat meals was the basis for monitoring tenofovir in the present study.

The elimination of tenofovir essentially takes place in the renal proximal tubules (Rodriguez-Novoa et al., 2009b); hence renal tubular dysfunction may either result in elevated systemic tenofovir exposure or increased elimination of tenofovir via the kidneys. High tenofovir blood levels have been associated with renal dysfunction in low body weight patients receiving treatment (Gervasoni et al., 2013, Kearney et al., 2006). Sub therapeutic levels of the drug could be used to monitor drug adherence in patients on treatment. The findings of this study showed that mean tenofovir blood concentrations from both serum and dried blood spot samples were evaluated and comparable at 6 and 12 weeks of antiretroviral therapy and exceeded the expected minimum concentrations of 64.4 ng/ml (Kearney et al., 2006). To assess the integrity of the sample in form of dried blood spot matrix hemoglobin was used. The hemoglobin levels in dried blood spot samples were comparable to those in fresh blood samples indicating intact dried blood spot samples after storage (Figure 6.17). Bland-Altman plots

showed that the difference between measurements using both Fresh blood and dried blood spot samples were within the limits of agreement ~95% of the time (mean bias=213, blue lines in Figure 6.18) confirming that the two methods were producing the same results. Clearly, the dried blood spot methods were valid and offered a robust method to collect specimen for transfer from Zambia to the analytical laboratory in Texas, USA where the experiments were performed. Comparing these results from serum and dried blood spot samples revealed no signifincant difference between the two methods (Figure 6.19). However, taking into account that both fresh blood samples and dried blood spot samples were collected 14 hours after oral dose of tenofovir-based therapy, the mean tenofovir concentration levels were expected to be around the minimum concentration (Cmin) of 64.4 ng/ml (Kearney et al., 2004), but the levels significantly exceeded the Cmin for lipid-based nutrient containing vitamins and minerals research arm and lipid-based nutrient alone research arm for both methods. This is suggestive of potential for tenofovir toxicity in low body weight HIV-infected patients on treatment regardless of treatment group. In the analyses, in part, there was observed association between lipid-based nutrient supplement and improved BMI by 12 weeks of antiretroviral therapy.

7.4 Body Mass Index and Tenofovir Concentrations

This is the first clinical trial in Zambia showing that malnourished HIV-infected patients with low body mass index have the high risk of being overexposed to tenofovir blood concentrations. Previously, a related study conducted in Zambia showed that among malnourished HIV-positive patients,

weight gain after antiretroviral initiation was associated with improved survival and decreased risk of clinical failure (Koethe et al., 2010). The following clinical evidence is consistent with the current findings in this present study. A retrospective study of male and female HIV-infected patients showed that low body weight patients were at increased risk of exposure to high tenofovir blood trough concentrations (Gervasoni et al., 2013). In this same study it was documented that patients treated with tenofovir disoproxil fumarate (TDF) at 300 mg/daily showed a wide distribution of tenofovir blood concentrations and confirmed that body weight was one of the major sources for the observed inter-individual variability of tenofovir (TFV) pharmacokinetics as previously reported (Jullien et al., 2005, Msango et al., 2011). Some TDF containing antiretroviral therapy regimens are first-line of therapy in the management of HIV-infected patients because of the long half-life in vivo, high antiretroviral efficacy and cost effectiveness (Jimenez-Nacher et al., 2008a, Astuti and Maggiolo, 2014, Lou, 2013). TDF is a prodrug that is converted to TFV in plasma and taken up in susceptible cells where it is rapidly converted into 'tenofovir diphosphate' (i.e. the active form) (Barditch-Crovo et al., 2001, Baheti et al., 2013). Administration of TDF 300 mg in fasted state results in up to 25% of TDF being converted into TFV (Barditch-Crovo et al., 2001) but how this varies in various physiological states including malnutrition is unclear. In clinical practice a standard dose of tenofovir-based ART is administered to adult patients without adjustments for factors such as gender or body weight. In the current study, high tenofovir blood trough concentration was independently associated with low body mass index in small

body weight patients. This was an interesting finding because low body weight patients present with significantly different 'apparent volume of distributions (denoted as V_D)' in relation to drug pharmacokinetics. For example, total body water (TBW) content is typically 60% of body weight in healthy adult men (~55% in female) (EFSA Panel on Dietetic Products, 2010). Thus, the average 70 kg male will have ~42 L of TBW, whereas a malnourished 45 kg male will have ~27 L TBW; this would reflect a 36% reduction in TBW capacity relative to the norm. This dramatic change in TBW will result in high concentrations of TFV that is attained by underweight patients relative to normal weight individuals because of the low V_D. This probably occurs with standard TFV dosing, a hydrophilic drug with a V_D of ~56 L in humans and findings of the present and previous studies support this idea. Therefore, it was not surprising to find that malnourished patients in the current study presented with elevated tenofovir blood concentrations relative to the trough concentration. A new prodrug termed tenofovir alafenamide fumarate (TAF) completed Phase I/II clinical trials and showed initial promise as an improvement over TDF in an attempt to curb increased TFV exposure in blood and subsequent toxicity (Markowitz et al., 2014). Unlike the TDF formulation, TAF is taken up into susceptible cells where it is converted to TFV and then tenofovir diphosphate (TFV-dp) (Markowitz et al., 2014, Lee et al., 2005, Birkus et al., 2008). Thus, plasma TFV levels are greatly reduced which allows reductions in TAF dose that needs to be administered (Markowitz et al., 2014). Consequently, it is theorized that the marked reduction in TAF will result in reduced TFV exposure and toxicity associated with TFV

(since conversion to TFV occurs in cells). Another benefit relates to the prospects that only a subset of body cells (key to HIV staging etc) have 'facilitated' transport mechanisms for intracellular accumulation of TAF. Thus, it is feasible many body cells will be ‘spared’ from increased TFV exposure and side effects/toxicity due to TFV (presuming it is the plasma TFV that is the main culprit mediating drug-related adverse events) but this needs clinical validation. The prospects still remain that TFV-related toxicity may reflect an ‘intracellular drug-related event’ and, therefore, TAF may actually cause even more adverse events in the long term (i.e. being a more potent drug which specifically accumulates in cells prior to conversion to TFV-dp including proximal renal tubules). Within this context, the proximal tubular cells responsible for renal excretion of TFV may become even more prone to ‘injury’ due to the extremely high density of OAT1 and OAT2 transporters. Moreover, whether TDF or TAF is utilized, the effective TFV therapeutic concentrations remain the same. Since the principle is still 'same dose for all HIV-infected patients', then underweight patients will still accumulate even greater intracellular TFV relative to normal/overweight HIV-infected patients following TAF administration. Consequently, the need for 'tailored therapy' in the early stages of antiretroviral therapy will not be resolved by TAF. Thus, for now the most important thing is identification of patients with increased tenofovir exposure. This should drive attention towards the possibility of reducing the TDF dose or the frequency of administration. This dose reduction of one tablet every other day has already been suggested in patients with significant renal impairment and could be justified for patients with low body mass index as well

because of the long intracellular half-life of TFV-dp. Therefore, therapeutic monitoring of tenofovir blood trough concentration, taken as surrogate marker of systemic drug expose, may allow the identification of patients with overexposure to tenofovir. In such patients modified dosing tenofovir-based regimens (i.e. dose-interval adjustments) could be recommended with the goal to reduce high levels of tenofovir exposure that may be associated with adverse events during treatment. Patients with low body mass index have substantial exposure of tenofovir and therefore may require more prolonged dosing interval. Similar to severe renal impairment, modeling data suggests a TDF 300 mg dose every 72 - 96 hours to achieve Cmax and Cmin ratios of 162 – 216%, and 64 – 121% respectively (Kearney et al., 2004). There has been prior demonstration of a dose-concentration relationship between tenofovir and renal toxicity. As a consequence, based on this knowledge, different strategies to reduce tenofovir exposure in HIV-positive patients on treatment can be suggested and warrants further clinical investigations across a wider range of BMI strata (Calcagno et al., 2013). These evidences, taken together, underscore the significance of close monitoring of TFV in HIV-infected patients with low body weight receiving treatment (Nishijima et al., 2011, Nishijima et al., 2012).

7.5 Dried Blood Spot and tenofovir Assay

This study also compared tenofovir concentrations in serum and dried blood spots (DBS) among HIV-positive patients receiving lipid-based nutrient supplement containing vitamins and minerals or lipid-based nutrient supplement alone. The applicability of the DBS method in general to routine clinical

investigation has long been demonstrated (Millership, 2011). However, DBS technology has not been widely applied in most of the developing countries for drug analysis due to several reasons including limited equipment and personnel with technical know-how. In the present study chromatographic-based microanalytical assays were used for the determination of tenofovir-level concentrations in dried blood spots. To achieve this, HPLC with UV detection was utilized in the determination of tenofovir in serum samples while LC-MS/MS was employed in determining tenofovir in HemaSpot DBS. The mean ± SD trough tenofovir concentrations in serum and DBS were approximately 210 ± 101 ng/ml and 209 ± 87 ng/ml respectively, which were higher than the previously reported mean trough concentrations (160 ± 93 ng/ml) among HIV-infected females shown to have increased risk for drug-related adverse events (Gervasoni et al., 2013). The results were comparable in the two methods. This was an intriguing finding but most importantly the study demonstrated the feasibility of using HemaSpot-DBS for monitoring tenofovir in HIV-positives receiving antiretroviral therapy in Zambia. The benefit of using DBS technology, and not fresh blood, to measure drug levels in patients is the easy and rapid estimation of drug bioavailability it provides especially for resource limited settings where storage of fresh samples that require refrigeration is a challenge. This implies that DBS might be applied to a wide array of remote research settings where skilled manpower is not available for sample collection meant for monitoring adverse drug events. A potential application for DBS would be to quantify drug exposures as routine measure to estimate adherence to

antiretroviral therapy over time and suspected drug toxicities. This possibility is necessitated by widespread use of drugs like tenofovir that have been reported to be efficacious but potentially nephrotoxic. Drug monitoring using HemaSpot-DBS in particular would streamline blood collection compared to traditional-DBS. This because HemaSpot-DBS utilizes all the beneficial properties of traditional-DBS technology while improving sample quality by facilitated sample drying thereby maintaining drug sample stability for drugs that easily undergo hydrolysis in aqueous samples.

The usefulness of HemaSpot-DBS as a simple approach to collect laboratory data has become a topic of great interest given the many advantages it has over traditional-DBS, which include the hard cartridge containing an innovative fan-shaped absorbent paper and desiccant that allow the sample to dry about 2 times faster than the traditional-DBS. It should also be noted that in this study, blood spotted on HemaSpot cards was obtained through finger-stick requiring only three drops of blood as opposed to venipuncture. So it permitted the use of not only small volume of sample but also less invasive procedure when compared to venipuncture procedure. This made the application of HemaSpot even more essential in collecting blood from the malnourished Zambian HIV-positive patients. HemaSpot-DBS in this study offered a unique, innovative and convenient sampling technique of blood for drug assays in the Zambian health care setting. The HemaSpot DBS technique also provided a suitable procedure for the storage and analysis of samples because it was easy to handle, transport and to store in the laboratory, in the absence of refrigeration.

Refrigeration can be a problem in most developing countries (Adawaye et al., 2013).

Generally, the HemaSpot DBS technique is suitable for drugs that are known to be rapidly degraded in biological blood samples. The water present in blood samples plays a very important role in hydrolysis reactions as an active reagent that cleaves drug molecules. The use of DBS results in enhancement of drug stability because dehydration of the sample on the card and consequent minimization of enzymatic and chemical hydrolysis of the drugs. Each HemaSpot DBS matrix is portable, compact, and self-contained, so it can be transported and operated by untrained users even in limited-resource settings. Since tenofovir toxicity in HIV-positive patients on antiretroviral therapy is dependent on level of drug concentration, recent guidelines recommend monitoring of drug levels in HIV-infected patients receiving tenofovir-containing regimen (Labarga et al., 2009). Unfortunately, frequent laboratory monitoring for toxicity in resource limited countries is a huge challenge due to paucity of cheaper monitoring tools and laboratory facilities among other things. This study has demonstrated that use of HemaSpot technology circumvents all these challenges.

7.6 Conclusion

In conclusion, in this study of the effects of a nutrition supplement with added minerals and vitamins on the renal excretion of electrolytes and tenofovir concentrations in blood among malnourished HIV/AIDS patients initiating tenofovir-based treatment, a controlled lipid-based nutrient with vitamins and minerals supplementation seems to reduce phosphate and potassium wasting in

comparison with the lipid-based nutrients alone. The lipid-based nutrients with vitamins and minerals may be useful in curtailing electrolyte loss in resource-limited settings where malnutrition and HIV/AIDS are common. These observations suggest presence of nutrition-driven opportunities to improve care and support of poorly nourished HIV- infected patients in resource-limited settings. Conversely, the lipid-based nutrients with vitamins and minerals did not alter tenofovir concentrations in blood compared to lipid-based nutrients alone but in both groups the levels appeared to significantly exceed the minimum concentration indicating increased risk of drug toxicity in underweight patients.

In recommendation, assessment of serum and spot urine electrolyte concentrations permit easy, rapid, and inexpensive estimation of kidney function and electrolyte loss in relation to HIV infection and antiretroviral therapy regimen. Clearly, more research is necessary for further evidence, but current findings support the use of supplement with high doses of minerals and vitamins as a low-cost adjunct to antiretroviral treatment of HIV/AIDS patients in resource limited settings. Equally, use of HemaSpot dried blood spot permits an easier, and more rapid estimation of bioavailability of tenofovir compared to conventional methods.

7.6.1 Study strengths and limitations

The current study's main strength was its randomized nested design, making it the first to investigate the effects of lipid-based nutrient supplement on the renal excretion of electrolytes and tenofovir concentrations in blood among HIV-infected patients initiating antiretroviral therapy in Zambia and elsewhere. Although mortality has been reported in this study, the present study was not

powered to detect differences in patient mortality but powered and detailed data analysis on fractional excretion of electrolytes and blood concentrations of tenofovir strengthened the evaluation of supplementation effects.

The present study was limited by including only patients with body mass index of less than 18.5kg/m^2 in the comparison between supplemented with additional vitamins and minerals and the supplemented without minerals and vitamins groups. The other limitation was that all the participants recruited were initiated on tenofovir-based regimen and so no comparison group was available for this set of data. However, the data on tenofovir comparisons were not the primarily the main target for analysis but rather the main focus was on the effect of nutritional supplement on renal excretion of electrolytes and blood tenofovir in which there were sufficient group data for comparison.

7.7 Future research direction

Further research is needed to verify links between tenofovir levels in underweight HIV-infected patients and development of adverse events in general and disrupted renal functions in particular. This is because low body mass index in the present study was an independent predictor of high blood tenofovir concentration levels. There is urgent need to strengthen capacity for chromatographic microassays at University of Zambia, School of Medicine in the Department of Pharmacy so as to open up more pharmacological-based training and research opportunities for Zambia and low-resource settings elsewhere in Africa.

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APPENDIXES

Appendix A: Ethical approvals for Sub-Study

THE UNIVERSITY OF ZAMBIA

BIOMEDICAL RESEARCH ETHICS COMMITTEE

Telephone: 260-1-256067
Telegrams: UNZA, LUSAKA
Telex: UNZALU ZA 44370
Fax: + 260-1-250753
E-mail: unzarec@unza.zm
Assurance No. FWA00000338
IRB00001131 of IORG0000774

Ridgeway Campus
P.O. Box 50110
Lusaka, Zambia

18th December, 2013.

Your Ref: 014-10-13.

Mr. Derrick Munkombwe,
University of Zambia,
Department of Pharmacy,
P.O Box 50110,
Lusaka.

Dear Mr. Munkombwe,

RE: RE-SUBMITTED RESEARCH PROPOSAL: **"THE EFFECT OF LIPID-BASED NUTRIENT SUPPLEMENTATION ON TENOFOVIR ASSOCIATED CHANGES IN RENAL EXCRETION OF PHOSPHATE, POTASSIUM AND MAGNESIUM AMONG ZAMBIAN HIV/AIDS PATIENTS" (REF. No. 014-10-13)**

The above mentioned research proposal was re-submitted to the Biomedical Research Ethics Committee with recommended changes on 12th December, 2013. The proposal is approved.

CONDITIONS:

- This approval is based strictly on your submitted proposal. Should there be need for you to modify or change the study design or methodology, you will need to seek clearance from the Research Ethics Committee.
- If you have need for further clarification please consult this office. Please note that it is mandatory that you submit a detailed progress report of your study to this Committee every six months and a final copy of your report at the end of the study.
- Any serious adverse events must be reported at once to this Committee.
- Please note that when your approval expires you may need to request for renewal. The request should be accompanied by a Progress Report (Progress Report Forms can be obtained from the Secretariat).
- **Ensure that a final copy of the results is submitted to this Committee.**

Yours sincerely,

Dr. J.C Murithali
CHAIRPERSON

Date of approval: 18th December, 2013. **Date of expiry:** 17th December, 2014.

Appendix B: UNZABREC Ethical approvals for NUSTART Main Trial

THE UNIVERSITY OF ZAMBIA

BIOMEDICAL RESEARCH ETHICS COMMITTEE

Telephone: 260-1-256067
Telegrams: UNZA, LUSAKA
Telex: UNZALU ZA 44370
Fax: + 260-1-250753
E-mail: unzarec@unza.zm
Assurance No. FWA00000338
IRB00001131 of IORG0000774

Ridgeway Campus
P.O. Box 50110
Lusaka, Zambia

12 April 2011.

Our Ref: 009-01-11

Dr. Lackson Kasonka,
University Teaching Hospital,
Department of Obstetrics & Gynaecology,
P/Bag RW 1X,
Lusaka.

Dear Dr. Kasonka,

RE: RE-SUBMITTED RESEARCH PROPOSAL: **"NUTRITIONAL SUPPORT FOR AFRICAN ADULTS STARTING ANTIRETROVIRAL THERAPY (NUSTART)"**

The above-mentioned research proposal was re-submitted to the Biomedical Research Ethics Committee on 09 March, 2011 with the recommended changes. The proposal is approved.

CONDITIONS:

- This approval is based strictly on your submitted proposal. Should there be need for you to modify or change the study design or methodology, you will need to seek clearance from the Research Ethics Committee.
- If you have need for further clarification please consult this office. Please note that it is mandatory that you submit a detailed progress report of your study to this Committee every six months and a final copy of your report at the end of the study.
- Any serious adverse events must be reported at once to this Committee.
- Please note that when your approval expires you may need to request for renewal. The request should be accompanied by a Progress Report (Progress Report Forms can be obtained from the Secretariat).
- **Ensure that a final copy of the results is submitted to this Committee.**

Yours sincerely,

Dr E. M. Nkandu
CHAIRPERSON

Date of approval: 12 April, 2011 **Date of expiry**: 11 April, 2012

Appendix C: LSTHM Ethical approvals for NUSTART Main Trial

LONDON SCHOOL OF HYGIENE
& TROPICAL MEDICINE

ETHICS COMMITTEE

APPROVAL FORM
Application number: **5876**

Name of Principal Investigator	**Suzanne Filteau**
Faculty	**Epidemiology and Population Health**
Head of Faculty	**Professor Laura Rodrigues**

Title: **Nutritional support for African adults starting antiretroviral therapy (NUSTART)**

This application is approved by the Committee.

Chair of the Ethics Committee

Date .....10 January 2011 .....

Approval is dependent on local ethical approval having been received.

Any subsequent changes to the application must be submitted to the Committee via an E2 amendment form.

Appendix D: Information Sheet

NUTRITIONAL SUPPORT FOR AFRICANS ADULTS STARTING ANTIRETROVIRAL THERAPY (NUSTART)-Effects of nutrient supplement on renal excretion of electrolytes.

Information sheet (to be kept by participant)

We have invited you to participate in a study that is designed to establish the effect of nutrient supplement on renal excretion of electrolytes in HIV-volunteers. Recent results of HIV-infected adults receiving antiretroviral therapy (ART) plus nutritional supplementation have shown an improvement in body mass index (BMI) and total CD4 count by 12 weeks. However, we don't have information regarding the effect of nutritional supplements on the renal function. Therefore, in this study we want to find out what effects nutritional supplementation has on the renal excretion of electrolytes in HIV-infected patients taking tenofovir-based antiretroviral therapy. Results from this study will be used to determine if nutritional supplementation attenuates renal excretion of electrolytes to clinically significant levels.

What are we asking you to do?

If you are willing to participate in this study, we will collect 5mls of blood (1 teaspoon) and 30mls of urine. During blood collection, you may experience a little discomfort (equivalent to a mosquito bite) at the time of pricking into the vein

on your arm. However, this pain will quickly disappear or disappear soon after. The whole process will require a maximum of 30 minutes of your time.

What do we do with the samples we collect?

The blood and urine samples that we will collect from you will be used for testing HIV, full blood count, CD4 cell count and for analysis of electrolytes and drugs.

What are the possible benefits to me?

Should we find anything that may require medical attention, this will be facilitated.

What are the possible disadvantages to me?

The only foreseeable disadvantage is the inconvenience of spending 30 minutes at our clinic for blood and urine collection. You may also experience a little discomfort at the site of a needle prick on your arm.

Confidentiality

Your details will be recorded on a paper form that will be locked away in your office within University Teaching Hospital (UTH). Furthermore, your details will be entered on a computer in a coded form and your name will not be included, only your enrolment number will be used. Any information and results obtained will remain absolutely confidential, and other family members and work colleagues will not be granted access to this information.

The study is voluntary

You do not have to participate in this study if you do not wish to, and even if you agree, you are free to change your mind at a later stage. The research

ethics committee of the University of Zambia has approved this study and their contact details are given below together with our contact details.

Contact details of the Principle Investigator:

Mr. Derick Munkombwe, Department of Pharmacy, School of Medicine, University of Zambia, P.O. Box 50110, Lusaka, Zambia (Phone: 0977704144).

Contact details of Research Ethics committee:

The Chairperson, UNZABREC office, Department of Anatomy, Ridgeway Campus, Nationalist Road, Lusaka, Zambia (Tel: 0211256067).

Appendix E: Consent form

NUTRITIONAL SUPPORT FOR AFRICANS ADULTS STARTING ANTIRETROVIRAL THERAPY (NUSTART)-Effects of nutrient supplement on renal electrolytes.

Consent record sheet (to be kept by study team)

I confirm that I have understood the information I have been given about the study. I agree to participate in the study. I confirm that I am joining the study of my free will and that I can withdraw at any time without affecting the care available to me. I understand what will be required of me.

Name…….

Signed (or thumbprint)

Date……

Signature (or thumbprint) of witness

Name……

Date……..

I confirm that I have explained the information fully and answered any questions

Signed for the study team

Name……

Date……..

Appendix F: Questionnaire

NUTRITIONAL SUPPORT FOR AFRICANS ADULTS STARTING ANTIRETROVIRAL THERAPY (NUSTART)-Effects of nutrient supplement on renal excretion of electrolytes and tenofovir in blood.

Questionnaire NO…… Study ID……..

1. Date…….
2. Age (years)…….
3. Sex Male
   Female
4. Religion None
   Christian
   Muslim
   Hindu
   Other……
5. Occupation Employed
   Student
   Other……

6. Highest education level completed
   - No education
   - Primary
   - Secondary
   - College
   - University

7. Marital status
   - Married
   - Widow/Widower
   - Divorced
   - Single-never married
   - Other ……

General Health and well-being

1. Have you been sick and or admitted to the hospital for at least overnight in the last three months? Yes/No
   a. If yes, for how long…….. (Days)
   b. What was the diagnosis (problem)?.....
2. Currently are you on any medication?

   If yes, indicate the type of medication
   a. Antibiotic (s)
   b. Other (Specify) ……

Urine Sample

1. Time of random urine collection (24 hour clock)……
2. Problems with urine sampling if any……
3. Time received in the lab (24 hour clock)…….

Blood sample

Time of phlebotomy and blood collection (24 hour clock)……

Problem with blood sampling if any……..

Time received in the lab (24 hour clock)……..

General Knowledge test

1. Have you ever heard of electrolyte testing?
2. Do you know what phosphate, magnesium, and potassium are?

Measure and record height in centimeters to the nearest 0.1 cm

……cm

Measure and record weight in kilograms to the nearest 0.1 Kg

…….Kg

Appendix G: Material Transfer Agreement Form

Serial number: E0098

NATIONAL HEALTH RESEARCH ETHICS COMMITTEE

Material Transfer Agreement Form
To be filled-in if exporting biological material

Section A.

1. Name of requesting organisation/individual: MUNKOMBWE DERICK
   UNIVERSITY OF ZAMBIA
2. Physical Address: P.O. BOX 50110
3. Contact details:
   - ✓ Telephone No: +260 211 257635
   - ✓ Fax No: +260 211 257635
   - ✓ Cell phone No: +260 977 70 41 44
   - ✓ Email address: hachuuludm@yahoo.co.uk

Name/s of Principal Investigator: MUNKOMBWE DERICK
Title: MR.
Title of Study: EFFECT OF NUTRIENT SUPPLEMENTATION ON TENOFOVIR EXCRETION OF ECTROLYTES
Signature: [signature]
Date: 10/11/2014

Please return a filled in copy of this Agreement to:

The National Health Research Ethics Committee, C/O Ministry of Health, P.O Box 30205, LUSAKA.
NHREC/MOH will be maintaining signed originals and the official list of signatory organizations.

The purpose of this agreement is to provide a record of the biological material transfer, to memorialize the agreement between the PROVIDER SCIENTIST (identified above) and the RECIPIENT SCIENTIST (identified below) to abide by all terms and conditions of the Material Transfer Agreement (MTA) and to certify that the RECIPIENT (identified below) organization has accepted and signed a copy of the MTA. All materials collected from Zambia shall remain the property of the Government of the Republic of Zambia. For any commercial product derived from the exported materials, the person from whom the samples were collected shall receive royalties. The RECEIPIENT agrees to acknowledge the source of the material in any publications reporting use of it. The RECEIPIENT agrees to use the MATERIAL in compliance with statutes and regulations.

Section B.

1. MATERIAL TO BE TRANSFERRED (full description and quantities)
HUNDRED AND FORTY (140) DRY BLOOD SPOT SAMPLES FROM PATIENTS ON TENOFOVIR-BASED TREATMENT.

2. PURPOSE OF EXPORT: DETERMINATION OF TENOFOVIR IN RESEARCH SAMPLES	3.DESTINATION OF MATERIAL (full details of institution and person responsible) PROF. SANIKA S. CHIRWA MCHARRY MEDICAL COLLEGE NASHVILLE, TENNESSEE, USA.
4. SOURCE OF MATERIAL (Study site) UNIVERSITY TEACHING HOSPITAL	
5. RECIPIENT ORGANIZATION CERTIFICATION (Organization receiving the ORIGINAL MATERIAL) MEHARRY MEDICAL COLLEGE / SPOT ON SCIENCES	
I hereby certify that the RECIPIENT organization has accepted and signed a copy of the MTA and will not outsource the material to another organization/laboratory without written approval from NHREC. I have read and understood the conditions outlined in this Agreement and I agree to abide by them in the receipt and use of MATERIAL.	
a. Name:	PROF. SANIKA S. CHIRWA
b. Title:	PROFESSOR & LAB DIRECTOR.
c. Organization/Laboratory:	MEHARRY MEDICAL COLLEGE/NEUROSCI. & PHARM. LAB
d. Physical Address:	RM 3234 BASIC SCIENCES, 1005 DB TODD BLVD
e. City and Country:	NASHVILLE TN, UNITED STATES OF AMERICA
f. Email and website:	schirwa@mmc.edu
g. Signature:	Date: 12 NOV. 2014

STATUS: (Recommended) / Not Recommended/Deferred

VICE-CHAIRPERSON NHREC: DR GERSHOM CHONGWE

Signature:

Date: 13.11.14

CHAIRPERSON NHREC: Professor Alfred M. Mwanza

Signature:

Date: 02/12/14

Official Stamp:

REPUBLIC OF ZAMBIA
MINISTRY OF HEALTH
OFFICE OF THE DIRECTOR
17 DEC 2014
DISEASE SURVEILLANCE CONTROL AND RESEARCH
P.O. BOX 30205, LUSAKA

VITAE

Derick Munkombwe was born on 18th November, 1974 in Choma District of Zambia's southern province. At the age of 6 years old he moved to Copper-belt province and started school at Mitobo primary in Kalulushi town. After primary school education he qualified to Kalulushi secondary school in the same town. Upon completing his secondary school in 1994, he joined and worked in the then Zambia Consolidated Copper Mines (ZCCM) Ltd as manpower development trainee. In 1998 Derick was awarded a Diploma in Education (Science) with merit by the University of Zambia. He was later appointed as a teacher of chemistry at Arakan High School in Lusaka by the Zambia ministry of education the position he held for about two years up until 2002 before entering the University of Zambia (UNZA) main campus where he studied natural sciences. Derick met the requirements to enter the UNZA-school of medicine where he pursued and received a Bachelor of Pharmacy degree with merit in 2007. Immediately after completing the pharmacy program he was appointed as pharmacist under the ministry of Health in Zambia. As pharmacist his job involved selection, procurement and rational use of therapeutic drugs at the University Teaching Hospital (UTH). In 2008 he was awarded staff development fellowship by UNZA and 2009 UNZA sponsored him to pursue Master of Public Health (MPH) at UNZA-school of medicine the program he completed successfully in 2011. Upon completing MPH he joined UNZA as full time lecturer in the department of pharmacy. The following year in 2012, he won a scholarship under London School of Hygiene and Tropical Medicine with European and Developing Countries Clinical Trials Partnership (EDCTP) fund to read for PhD in pharmacology and clinical nutrition at UNZA-school of medicine in collaboration with Vanderbilt University of Nashville, Tennessee, USA. His PhD work focused on **"The effect of lipid-based nutrient supplements containing vitamins and minerals on renal excretion of electrolytes and tenofovir concentrations in blood among Zambian HIV/AIDS patients"**. He was supervised by Dr. Tyson Muungo and Professor Charles Michelo both from the University of Zambia and Professor Sanika Chirwa from Meharry Medical College/Vanderbilt University (USA). Other eminent investigators who played pivotal roles in his training and development as an independent researcher include Professor Suzanne Filteau from the London School of Hygiene and Tropical Medicine (UK), Professor Paul Kelly from BARTS and London School of Medicine and Dentistry (UK)/UNZA-School of Medicine and Professor Douglas Heimburger from the Vanderbilt Global Health Institute, Vanderbilt University (USA).

Derick's study investigated the effect of lipid-based nutrient supplements containing vitamins and minerals (LNS-VM) on renal excretion of electrolytes and tenofovir concentrations in blood among low body mass index (BMI) Zambian HIV/AIDS patients initiating tenofovir-based antiretroviral therapy. The LNS-VM regimen appeared to offer protection against phosphate and potassium loss. This offers potential opportunities to improve care and support of poorly nourished HIV-infected patients. Conversely, LNS-VM did not alter tenofovir concentrations in blood but the levels appeared to exceed the expected minimum concentration indicating risk of tenofovir overexposure in patients with low BMI. Therapeutic drug monitoring (TDM) is recommended for low BMI patients on tenofovir-based antiretroviral drugs. This offers the possibility to detect patients with drug concentrations outside therapeutic ranges, who can subsequently benefit from dose modification.