EFFECT OF MICRONUTRIENT SUPPLEMENTATION ON SOME INDICES OF HIV INFECTION PROGRESSION AMONG ANTIRETROVIRAL NAÏVE PATIENTS ATTENDING HIV CLINICS IN ZARIA AND KADUNA, NIGERIA

BY

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FEBRUARY 2018

Declaration

I declare that the work in this thesis entitled ‘the effect of micronutrient supplementation on some indices of HIV infection progression among antiretroviral naïve patients attending HIV clinics in Zaria and Kaduna, Nigeria’has been carried out by me in the Department of Pharmacology and Therapeutics. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other Institution.

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Name Signature Date

Certification

This thesis entitled ‘EFFECT OF MICRONUTRIENT SUPPLEMENTATION ON SOME INDICES OF HIV INFECTION PROGRESSION AMONG ANTIRETROVIRAL NAÏVE PATIENTS ATTENDING HIV CLINICS IN ZARIAAND KADUNA’ by Onyeadumarakwe Reginald OBIAKO meets the regulationsgoverning the award of the degree of Doctor of Philosophy in Pharmacology of Ahmadu Bello University, and is approved for its contribution to knowledge and literarypresentation.

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Dedication

This thesis is dedicated to the memories of the victims of HIV/AIDS. The thesis is also dedicated to People Living with HIV who have to endure socialcastigation, discriminationand stigmatization, in addition to the systemic effects of HIV and its treatment.

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Abstract

Nigeria has the second highest HIV/AIDS burden in the world with an estimated 3,391,546 people living with HIV and AIDS; an estimated 239,155 people acquired new infections in 2013; and about 174,253 people died from AIDS related illness (s) in 2014. HIV infection causes a chronic disease that progresses from stage one (seroconversion or asymptomatic) to stage four (advanced disease/AIDS) over a median period of 5 years. The course of the HIV infection is characterized by a persistent pro-oxidant state that leads to programmed CD4+ cell apoptosis, elaboration of proinflammatory cytokines and acute phase proteins, HIV RNA replications, hyper-catabolism, increased utilization of antioxidants and micronutrients, and subsequent micronutrient deficiencies. The aim of this study was to evaluate the effect of micronutrient supplementationon some indices of HIV infection progression among ART-naïve patients. To achieve the aim of the study, 90 ART naïve HIV infected adult patients with CD4+ cell count ≥520/μL were enrolled. After obtaining informed written consent, their sociodemographic data, CD4+ cell counts (cc), body mass index (BMI), vital signs, symptoms and signs of HIV infection, were obtained, and blood samples for plasma HIV RNA load (pVL), haemoglobin, and serum C-reactive protein (CRP), interleukin-6 (IL-6), albumin, lipids and micronutrients (vitamin B_{12}, zinc, and copper) were collected. After these, each patient was given a daily dose of one capsule of Synovit^{TM} supplements and two tablets of co-trimoxazole. Eligible patients were re-evaluated every 12 weeks, and data onvital signs, BMI, symptoms and signs of HIV infection, CD4+ cc, and haemoglobin were obtained; while bloodsamples for pVL, CRP, IL-6, albumin, lipids and micronutrients werecollected at 24 weeks and 48 weeks

of Synovit^{TM} supplementation respectively.Patients who defaulted from one scheduled clinic visit, became pregnant, or became eligible for ART before the 48 weeks of Synovit^{TM} supplementation were dropped. The socio-demographic characteristics, vital signs, BMI, haemoglobin, and serum albumin, zinc, copper and vitamin B_{12} of the patients were compared with those of 90 age and sex-matched HIV negative apparently healthy volunteers from the same environments.Patients comprised 21 males and 69 females, aged 42.9±9.8 years and 34.6±8.6 years respectively ($p\leq0.05$). They were mainly low income earners and were enrolled within a median of 4 months of HIV infection diagnosis. Ninety (90) patients were initially evaluated, 78 and 68 were re-evaluated at 12 and 24 weeks respectively, while 57 patients were re-evaluated at 36 and 48 weeks respectively. The sociodemographic factors that determined retention of patients on micronutrient supplementation were found to be the age, occupation and source of income of the patients. The patients' baseline median CD4+ cc (704.5/µL) was within reference range(365-1571/µL) for healthy Nigerians. Their vital signs (temperature=38.2±0.7^{o}C; pulse rate=78.0±10.1 beats/minute; respiratory rate=12.0±3.0 breaths/minutes; systolic blood pressure=128.0±13.7 mmHg) were higher than those of healthy controls (temperature=36.8±0.2^{o}C; pulse rate=72.0±21.1 beats/minute; respiratory rate=16.0±1.0 breaths/minutes; systolic blood pressure=138.0±37.4 mmHg) ($p\leq0.05$). Although theywere overweight (median BMI of 25.8kg/m^2), their baseline median haemoglobin (11.0 g/dL), serum albumin (36.0g/L), zinc (0.01ppm), copper (-0.4ppm), vitamin B12 (15.0ng/L) and total cholesterol (4.6mmol/L)were significantly lower than those of healthy volunteers ($p\leq0.05$). Their serum CRP (27.8 ng/ml), and IL-6 (1.9 pg/ml) were also significantly higher than reference values

(*p≤0.05*).Micronutrient supplementation significantly decreased incidence of opportunistic illnesses (OIs), pVL, CRP and IL-6; and increased haemoglobin, serum zinc, copper, vitamin B12, total cholesterol and high density lipoproteins (*p≤0.05*); but did not increase CD4+ cc. The pVL maintained significant negative correlations with CD4+ cc, zinc and copper (*p≤0.05*), and positive correlations with IL-6 and CRP (*p≤0.05*). Significant predictors of HIV RNA load at baseline were zinc(*p*=0.00) and IL-6 (*p* =0.05); while serum IL-6 (*p=0.00*) and haemoglobin (*p=0.02*) were the predictors at 24 weeks and 48 weeks of micronutrient supplementation respectively. In conclusion,micronutrient supplementation enhanced retention of patients on the pre-ART care and support service programme and reduced the indices of progression of HIV infection with the exception of CD4+ cell counts.

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List of Abbreviations, Acronyms and Symbols

ABUTH ---------- Ahmadu Bello University Teaching Hospital

AIDS ---------- Acquired immune deficiency syndrome

ART ---------- Antiretroviral therapy

ARVs ---------- Antiretroviral drugs

BBFSW ----------Brothel-based female sex workers

BMI ---------- Body mass index

cals/g; kcal/g---------- Calories/gram; kilocalories/gram

CD4+/CD8+ T cell ---------- Cluster differentiated (T4 or T8) lymphocyte

CHO ---------- Carbohydrate

CI/SD ----------Confidence interval / Standard deviation

CRP / IL-6 ---------- C-reactive protein /interleukin-6

Cu^{2+} /Fe^{2+}/Zn^{2+} ----------Anions(Copper, ferrous, zinc)

CUNY ---------- City University of New York

FAO ----------Food and Agriculture Organization

GARPR ---------- Global AIDS Response Country Progress Report

GIT ---------- Gastrointestinal tract

GSH ---------- Glutathione

HAART ---------- Highly active antiretroviral therapy

H_20_2/ SOD----------Hydrogen peroxide / Superoxide dismutase

Hb ---------- Hemoglobin

HDL-C/LDL-C---------- Lipoprotein-cholesterol (high density/ low density)

HIV ---------- Human immunodeficiency virus

HCT/HTC/VCT	---------- HIV counseling & testing/ HIV testing & counseling/ voluntary counseling & testing
IFN-α / IFN-γ	---------- Interferon alpha / Interferon gamma
IeDEA	---------- International Epidemologic Databases to Evaluate AIDS
IQR	----------Interquartile range
LTNP	---------- Long- term non progressors
MDGs / SDGs	---------- Millennium(Sustainable) Development Goals
NACA	---------- National Agency for the Control of AIDS
NARHS	----------National HIV/AIDS and Reproductive Health Survey
NFκβ	----------Nuclear transcription factor kappa beta
ODs / Ois	---------- Opportunistic diseases/ infections
PLWHA	----------Persons living with HIV and AIDS
RNA	---------- Ribonucleic acid
RNI	----------Recommended nutrient intake
RNS /ROS	----------Reactive nitrogen species / Reactive oxygen species
SGCH	---------- St Gerard Catholic Hospital
SSA	----------Sub-Saharan Africa
SYNOVIT^{TM}	----------Synovit trademark
TGF-ß /TNF-α	---------- Tumour growth factor beta/ Tumour necrosis factor alpha
UNAIDS	---------- United Nations Programme on HIV/AIDS
UNICEF	---------- United Nations International Children Emergency Fund
US-CDC	---------- United States Centre for Diseases Control
WHO	---------- World Health Organization

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CHAPTER ONE

1.0INTRODUCTION

1.1 Human Immunodeficiency Virus(HIV)

1.1.1 Family and types

HIV belongs to the virus family Retroviridae and subfamily Lentiviridae. Lentiviruses typically cause a slowly progressive disease with prolonged subclinical infection (Yu*et al.*, 2005). HIV comprises HIV-1 and HIV-2. HIV-1 is closely related to a naturally occurring virus of sub-species of chimpanzees called SIVcpz, while HIV-2 is closely related to SIVsm, a virus that naturally infects sooty mangabey monkeys (Diop*et al.*, 2002). In contrast to HIV-1, there has been little international spread of HIV-2 despite the fact that the two viruses share similar modes of transmission. Most HIV-2 infections were reported in West Africa, and only few cases were identified in other countries (Kanki*et al.*, 1997). Co-infection of HIV-1 and 2 can occur but recombinants of the 2 types were reported to be rare (Kanki*et al.*, 1997).

1.1.2 Structure

Electronmicroscopicstudies showed that HIV has a cylindrical structure consisting of viral genome, core proteins and lipid bilayer membrane & envelopes (Yu *et al.*, 2005).

The viral genome is diploid, with a 60-70s complex of two identical RNA copies. The genome is flanked on both sides by long terminal repeat sequences that contain *gag, pol* and *env*structural genesthat code for capsid proteins, viral enzymes and envelopes respectively. The genome has five other regulatory & accessory genes: *tat, rev, nef, vif*&*vpu*(HIV-1) or *vpx* (HIV-2). The viral enzymes are the reverse transcriptase (RT),

protease (PR) & integrase (IN) which are encoded by *pol* geneThe core protein is made up of capsid protein (p24) and nucleocapsid protein (p18) encoded by the *gag* genes. The envelopes surround the core proteins that enclose the viral RNA genome and enzymes. The envelopes consist of external surface (gp 120) and transmembrane (gp 41) that connects to the core proteins. The gp 120 contains binding sites for CD4+ receptor and co-receptors. The gp41 tranverses through the lipid bilayer and both arepartially acquired from the surface of host cells as mature virions are released. Figure 1 below shows a schematic structure of HIV.

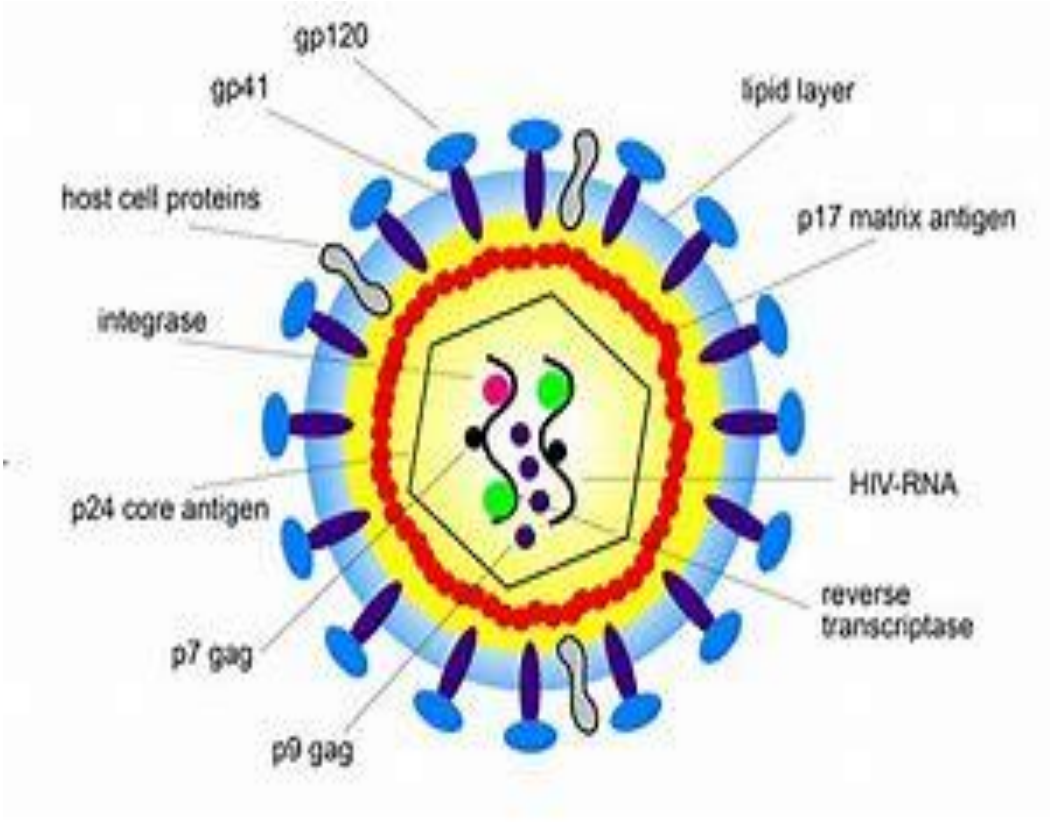


Figure 1.1: Human Immunodeficiency Virus

1.1.3 Genetic Diversity

HIV strains can be classified into three groups – major (M), non-major (N) and outlier (O) (McCutchan, 2000). Group M, the most common globally, is further divided into nine subtypes (A, B, C, D, F, G, H, J and K) and several circulating recombinant forms (CRFS). Subtype C has been documented to account for more than 50% of prevalent global HIV-1 infections and a higher proportion of new infections, particularly in China and the African continent; subtypes A, C and D were found more frequently in India and Central, Southern and the Horn of Africa; subtype B strains were only isolated from the USA and Western Europe through 1994, and subtype A/E recombinant were observed in South-East Asia (McCutchan, 2000). In Nigeria and West Africa, the dominant subtype was the CRF 02/A/G; while HIV-1 N and O groups were rare and limited to Cameroon and neighbouring countries (Kanki, *et al.*, 1997; McCutchan, 2000).

1.2Clinical Course of HIV Infection

1.2.1 Determinants of clinical course from infection to disease and AIDS

Entry of HIV into immune cells activates initial anti-inflammatory T cell response in an attempt to curtail HIV replication and viraemia. However, continued elaboration of anti-inflammatory cytokine cascade eventually becomes detrimental to the HIV infected person as HIV-1 stimulates CD40L on infected macrophages and monocytes to increase pro-inflammatory cytokines (such as interleukin-1 beta (IL-1ß), tumor necrosis factor- alpha (TNF-α) and interleukin (IL-6)), which in turn, directly increase the expression of interleukin-2 (IL-2) receptors on T cells and thymocytes leading to more T cell activation, growth, differentiation and programmed apoptosis (Poli*et al.*, 1990). IL-6, with or without

IL-1ß and TNF-α, mediate B cell differentiation, maturation and synthesis of antibodies. Antibodies indirectly stimulate T cell apoptosis, IL-6 secretion and HIV-1 replication. This vicious cycle between IL-6 secretion and HIV replication is responsible for increased viraemia, hypergammaglobulinaemia, lymphopaenia, and elevation of acute phase reactant proteins (Douek*et al.*, 2009). This phenomenon has also been linked to the immune reconstitution inflammatory syndrome (IRIS) and HIV encephalopathy (Conolly*et al.*, 2005). Thus the two antagonistic features of HIV infection, which are progressive increase in HIV RNA replication and a corresponding progressive decrease in the quantity and quality of the infected patient's CD4+ lymphocytes and other immune cells, are responsible for the cascade of events that are characterized by a profound cell mediated immune deficiency, cytokines dysregulation, inappropriately elevated acute phase proteins, gammaglobulins and superoxides, hypercatabolism and emergence of various forms of opportunistic infections (OIs) and diseases (ODs) (Longo and Fauci, 2010). The HIV infected person thus requires 10% to 15% more energy per day and approximately 50 to 100% more protein than the recommended 2070 kcal/ day of energy and 57 grams/day of protein for apparently active HIV negative adult (Piwoz and Preble, 2000; Marston *et al.*, 2004).

1.2.2 Types of HIV infection progressors

1.2.2.1Typical and rapid progressors

In more than 80% of HIV infected persons, programmed CD4+-cell apoptosis will persist from either the direct effect of HIV, or HIV-induced chronic inflammation and concomitant antioxidant imbalances in the host cells (Lelievre*et al.*, 2004); and the rate of CD4+- cell apoptosis will then determine whether theHIV infected individual can

develop AIDS within the median time of ten years (typical progressors) or within 2–3 years of infection (called rapid progressors) (Kirchhoff *et al.*, 1995).

1.2.2.2*Long- term non progressors*

In about 5–10 % of HIV-infected persons, the virus is contained in the lymphoid tissues with preservation of the lymph node architecture, and little or no viral replication and/or propagation of virions to the CD4+- cells (Sanchez *et al.*, 1997). Such individuals will, therefore, have stable CD4+-cell counts, low HIV RNA load in the blood, and slow disease progression despite years of HIV infection. These individuals, who are called long- term non progressors (LTNP), can remain asymptomatic for about 7–20 years after infection with stable CD4+ cell counts and low plasma HIV RNA, without been on ART (Sanchez *et al.*, 1997). Currently, they are divided into two groups based on viral load calculations/standards. Those with undetectable viremia (HIV RNA<50 copies/ml) are called elite controllers, while those with low but detectable viral load (HIV RNA <2000 copies/ml) are known as viremic controllers (Okulicz*et al.*, 2009).

1.2.3Inter-and intra-population variability

The course of HIV infection has been noted to vary within a population and between populations due to a combination of socioeconomic and immunological factors. These factors, particularly, genetic make- up, nutrition, and exposure to environmental pathogens, are responsible for inter- and intra-population variability and differences in CD4+ cell levels, both in HIV infected and HIV negative populations; and are the major limitations to the universal acceptability of the CD4+ for HIV classification and staging (WHO, 2005). This variability is demonstrated by the results of the reference CD4+

values of some healthy populations reported thus: 500/µl to 2,000/µl for WesternEuropean countries (Reichert *et al.,* 1991); 559/µl to 2,333/µl for Ugandans (Tugume*et al.,* 1995); 430/µl to 1740/µl for Indians (Uppal*et al.,* 2003); and 365/µl to 1571/µl for Nigerians (Oladepo*et al.,* 2009) respectively. In sub-Saharan Africa (SSA), clinical latency and progression to AIDS have been reported to be shorter due to constant exposure to many pathogens and malnutrition (Enwonwu, 2006).

1.3 Micronutrient Supplementation in HIV infection

Micronutrients function mainly as anti-oxidants and immune-modulators in HIV infection. Vitamin A, in the form of retinol or retinoic acid, improves immunity by modulating the growth and function of T-cells, B-cells and natural killer cells and their products. Vitamin C (ascorbate) is a potent antioxidant which can resonate between the free radicals (Asc-) and ascorbate (AscH-)(Nikiet *al.,* 1995). Ascorbate is readily regenerated from Asc- through NADH or NADPH-dependent reductases. Ascorbate can also regenerate other antioxidants such as glutathione (GS·) and vitamin E by neutralizing their radical form (Nikiet *al.,* 1995; Durak, 2014). Vitamin E is also an antioxidant which inhibits CD95 (APO–1/Fas) ligand expression (part of TNF receptor which T-cell uses to undergo apoptosis) and protects T-cell from activation-induced cell death of the CD95/CD95 ligand system of T-cells (Li-Weber *et al.,* 2002). It therefore stimulates CD4+-cell and IL-2 proliferation and completely blocks NFκB induced programmed T cell apoptosis (Li-Weber *et al.,* 2002, Durak, 2014). Trace elements such as copper assist vitamin E in down-regulating T-cell destruction by stimulating maturation and mobilisation of granulocytes in peripheral blood and proliferation of T-cells (Percival, 1998). Selenium enhances synthesis and expression of

selenoproteinsantioxidants such as thioredoxin reductase, glutathione peroxidase, and phospholipids hydroperoxide in T-cells, thereby boosting cellular immunity (Baum *et al.,* 2000; Moghadaszadeh*et al.,* 2006; Kamwesiga*et al.,* 2011). It binds to and activates thymulin, a thymic peptide responsible for the maturation and differentiation of immature thymocytes thereby enhancing expression of IL-2, T-cell proliferation and function (Baum *et al.,* 2000; Moghadaszadeh*et al.,* 2006). Zinc influences gene expression by structural stabilisation of different immunological transcription factors (Prasad, 2007). For example, it induces cytokines, including IL-1, IL-6 and TNF- α and influences NK cell-mediated killing (Fleiger*et al.,* 1995). It also inhibits NFκβ and TNF-α thereby modulating cytolytic T-cell activity. It stabilizes the thiol groups and phospholipids in biological membranes and protects them against oxidative stress (Fleiger*et al.,* 1995). Zinc therefore has both an enhancing and inhibiting activity depending on its concentration in the surrounding tissues.

The importance of nutritional interventions in the form of micronutrient supplementations as a tool to combat malnutrition and encourage patients to remain on treatment was recognised in 1984 by the Food and Nutrition Division, Food and Agriculture Organization (FAO) of WHO (William, 1985). This made the United Nations to add the following Millennium Development Goals (MDGs) to the HIV public health response (United Nations, 2006):

- MDG 1 (reducing extreme poverty and hunger),
- MDG 4 (reducing child mortality),
- MDG 5 (reducing maternal mortality) and
- MDG 6 (halting and beginning to reverse the spread of HIV by 2015).

At the expiration of MDGs, the following Sustainable Development Goals (SDGs) were also pursued as part of the HIV public health response (IISD, 2016; WHO/UNAIDS, 2016):

- SDG 1 (end poverty in all its forms everywhere),
- SDG 2 (end hunger, achieve food security and improved nutrition and promote sustainable agriculture),
- SDG 3 (ensure healthy lives and promote well-being for all at all ages), and
- SDG 12 (ensure sustainable consumption and production pattern by 2030)

1.4 Statement of the Research Problem

The fight against HIV/AIDS has posed enormous challenges worldwide, generating fears that success in its control may be too difficult or even impossible to attain (Barnet and Whiteside, 2002; NACA, 2015). By the year 2000, more than 70% of the PLWHAs live in Africa, but less than 20% were receiving HIV treatment (WHO/UNAIDS, 2016). But between 2005 and 2014, many countries in the African Region overcame formidable constraints to build and sustain national public health programmes powerful enough to turn the tide against the HIV pandemics by bringing HIV services closer to communities and scaling up HIV treatment programmes, using the public health approach recommended by WHO (WHO/UNAIDS, 2016). Yet, major challenges remain in SSA, particularly in some large countries with high HIV prevalence, because both the coverage and quality of HIV services are still insufficient(WHO/UNAIDS, 2016). For instance, in Nigeria where large scale use of ART started in 2000 (Idigbe*et al.*, 2003), in 2014, about 1,665,403 HIV infected persons (1,454,565 adults and 210,838 children

respectively) were in need of ART using the eligibility criteria of CD4 of 350/µL (NACA, 2015). In addition, the number of persons who do not know their HIV status, or the number of pre-ART and post-ART patients who default from care, follow-up and treatment respectively remain high (WHO/UNAIDS, 2016). These gaps in the HIV prevention and treatment response have been attributed to factors such as socio-cultural beliefs and practices, low literacy level, poverty, ignorance (Obiako *et al.*, 2010; Obiako *et al.,* 2012) , ART-related toxicities and ART-failures (Obiako *et al.,* 2011; Obiako*et al.*, 2012; Ogoina*et al.*, 2012).

The greatest challenges to the HIV prevention and treatment response today are: how to get individuals to do periodic HIV screening test for early diagnosis; how to persuade all HIV infected persons to start ART and remain on ART to avoid development of ARV resistance; and how to retain pre-ART HIV infected persons on care until they are ready for ART. Closing these observed gaps will require action and innovative goals such as those enunciated and crystallized into a set of global Fast-Track targets by the Joint United Nations Programme on HIV/AIDS and WHO (WHO/UNAIDS, 2016).These actions include: getting 90% of PLWHAs to know their HIV status; making 90% of those diagnosed with HIV to initiateand remain on ART, making 90% of HIV infected persons not ready to initiate ART to remain on care until they are ready to start ART, and getting 90% of the people receiving ART to have suppressed viral loads by 2020. To achieve the first target will require extensive HIV testing and counseling (HTC) services in communities through provision of free condoms and services such as sexual transmitted infection(STI) clinicsand marriage counseling. To achieve the second, third and fourth targets will require provision of facilities for pre-ART and ART services. These services which include nutritional intervention services, micronutrient

supplementation, prophylaxis and treatment of opportunistic infections/ diseases, ARV adherence counseling and toxicity monitoring willincrease adherence and prevent or reduce development of ARV resistance.

1.5 Justification for the Study

Micronutrients (essential trace elements and vitamins) have been shown to be important in maintaining immune function and neutralizing the reactive oxygen intermediates produced by activated macrophages and neutrophils in their response to microorganisms (Oguntibeju*et al.,* 2009). Their role as antioxidants and immune boosters for the optimization of health and prevention and/or treatment of chronic diseases such as HIV/AIDS was recognised from observational studies in some developed countries,which showed that deficiencies of vitamins and trace elements were common among HIV-infected patients (Sullivan *et al.,* 1998; Tang *et al.,* 2002). Studies also showed that diet rich in carbohydrates, proteins, fats, and antioxidant vitamins and minerals were associated with reduced risk of HIV progression to AIDS and death (de Luis Roman *et al.,* 2001).Other studies in some developing countries such as Thailand (Jiamton*et al.,* 2003), Malawi (Bowie *et al.,* 2005), and Vietnam (*FANTA III Project, 2014)*, also supported the beneficial effects of micronutrient supplementation in HIV/AIDS. These studies revealed that HIV infected patients who were treated with a multi-micronutrient supplement (Jiamton*et al.,* 2003), or were given food rations (Bowie *et al.,* 2005), or Ready-to-Use Therapeutic Food (RUTF) (Weiringa*et al.,* 2013; *FANTA III Project, 2014) showed* significant improvement in nutritional status, weight gain and reduction in morbidity and mortalitythan those who were given placebo.

A report of the global analysis of delays from ART eligibility to ART initiation among adults with HIV/AIDS from 1996 to 2006, revealed that nutritional intervention programmeshelped to retain many HIV positive persons who could have dropped-out of care before they became eligible for ART(CUNY, 2015). These studies,therefore, suggest that food supplementation could offer a simple and relatively inexpensive strategy to slow HIV progression, and improve quality of life of HIV infected persons, particularly in resource-limited countries where poverty and hunger are prevalent (de Lius Roman *et al.*, 2001; Marston *et al.*, 2004).The importance of food supplementation in HIV/AIDS was summarised by Clay William of the Food and Nutrition Division, Food and Agriculture Organization (FAO) of WHO, who said that "Food isn't a magic bullet. It won't stop people from dying of AIDS but it can help them live longer, more comfortable and productive lives" (William, 1985).

Although malnutrition among HIV infected patients have been documented in Nigeria (Obi *et al.*, 2010; Sudawa*et al.*, 2013; Anyabolu*et al.*, 2014), and other African countries such as South Africa (Evans *et al.*, 2012), Ethiopia (Gedle*et al.*, 2015), and Uganda (Lwanga*et al.*, 2015); and despite abundant evidence that nutritional intervention reverted malnutrition (Jiamton*et al.*, 2003; Bowie *et al.*, 2005; Weiringa*et al.*, 2013; FANTA III Project, 2014), and helped to retain many HIV positive persons on pre-ART care (CUNY, 2015);there is dearth of data on the effect of micronutrient supplementation on HIV infected adolescent and adults in Nigeria. Similarly, the lack of data on the effect of micronutrient supplementation on indices of HIV infection progression among antiretroviral –naïve adult patients necessitated the rationale for this study among ART- naïve patients attending HIVclinics in Zaria and Kaduna.

1.6Aim and Objectives of the Study

1.6.1Aim

The aim of the study is toevaluate the effect of micronutrient supplementation on some indices of HIV infection progression in HIV infected ART-naïve patients attending HIV clinics in Ahmadu Bello University Teaching Hospital (ABUTH) Shika Zaria and St Gerard's Catholic Hospital (SGCH)Kakuri Kaduna.

1.6. 2 Specificobjectives

i. To determine the sociodemographic characteristics of HIV infected ART-naïve patients and the effect of these on their retention on pre-ART care with micronutrient supplementation

ii. To determine the effect of micronutrient supplementation on vital signs, BMI and incidence of opportunistic diseases and infectionsin HIV infected ART-naïve patients

iii. To determine the baseline nutritional status (zinc, copper, vitamin B_{12}, haemoglobin, albumin and lipids) of HIV infected ART-naïve patientsin comparison with those of age-and sex-matched HIV negative healthy volunteers

iv. To determine the effect of micronutrient supplementation on some nutritional indices(zinc, copper, vitamin B_{12}, haemoglobin, albumin and lipids) of HIV infection progression in HIV infected ART-naïve patients

v. To determine the effect of micronutrient supplementation on plasma HIV RNA loadand some immunological indices (CD4+ cc, IL-6 and CRP) of HIV infection progressionin HIV infected ART-naïve patients

vi. To determine the relationships between HIV RNA load and other indices of HIV infection progression before and with micronutrient supplementationin HIV infected ART-naïve patients

1.7 Research Question/Hypothesis

Micronutrient supplementationdoes not significantly affectthe progression of HIV infection in HIV infected ART-naïve patients.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Epidemiology of HIV Infection

2.1.1Global epidemiology of HIV infection

There has been an astronomical increase in the global number of cases of HIV infection during the past two decades, of which 70% occurred in sub-Saharan Africa (WHO/UNAIDS, 2016). According to the WHO/UNAIDS, 2016 report, approximately 36.9 [34.3–41.4] million people were living with HIV globally by end of 2014. Of these, 2.0 [1.9–2.2] million people were newly infected with HIV, and 1.2 [980 000–1.6 million] million people died from HIV-related causes. The report also noted that since the beginning of the epidemic to 2014, HIV/AIDS has claimed more than 34 million lives globally.

2.1.2Epidemiology of HIV infection in Nigeria

According to NACA, Nigeria has the second highest HIV/AIDS burden in the world, after South Africa, with an estimated 3,391,546 people living with HIV and AIDS (PLWHAs), although new infections had declined from an estimated 316,733 in 2003 to 239,155 in 2013(NACA, Nigeria GARPR 2015). The report also noted that the number of AIDS related deaths declined from a total of 210,031 people in 2013 to 174,253 people in 2014 (NACA, 2015). This trend is clearly evident from the result of sentinel sero-prevalence study in Nigeria since the first official report of HIV/AIDS in 1986. The prevalence had steadily risen from 1.8% in 1991, 3.8% in 1993, 4.5% in 1996, 5.4% in 1999, 5.8% in 2002, and 7.8% in 2003, before declining progressively to 5% in 2004,

4.4% in 2005,3.6% in 2007, 4.1% in 2010 and 3.4% in 2012 (NACA, 2015). The results of 2013 ANC Sentinel Survey are being awaited (NACA, 2015).

2.1.2.1HIV prevalence by Geo-political Regions and States

Although Nigeria's epidemic is generalized, with wide variation in prevalence within the country, the overall national prevalence usually masks several variations in Nigeria's epidemic at the states and among different population groups. In 2007 the prevalence rates in Nigeria were as low as 2.5% in Kebbi and Katsina States, 5.1% in Kaduna State, and as high as 7.6% and 8.4% in Adamawa State and Federal Capital Territory Abuja respectively (FMOH, 2010). When the 2007 data was compared with the 2012 National HIV/AIDS and Reproductive Health Survey (NARHS) prevalence data in the country's six geopolitical zones, the South Southand South East Zones had the highest and lowest prevalence of 5.5% and 1.8% respectively (NARHS, 2012; NACA, 2015). The report also observed that while the HIV prevalence decreased in the North-Central, South-East and South-West Zones, it increased in the three other zones: South-South, North-East and North-West (NACA, 2015). Fourteen states reported a prevalence that was higher than the national prevalence of 3.0% while nine other states had a low prevalence ranging from 0.2% to 0.9% during the 2012 NARHS (NARHS, 2012; NACA, 2015). The four states with the highest prevalence were Rivers (15.2%), Taraba (10.5%), Kaduna (9.2%) and Nasarawa (8.1%) respectively. Ten States had prevalence ranging from 3.3% to 6.5%, and Ekiti state had the lowest prevalence (1.2%) among the states in the country. There were also differences between urban and rural areas with prevalence figures in urban 3% and 4% in rural area (NACA, 2015).

2.1.2.2HIV prevalence by age and sex

The pattern of distribution of HIV prevalence by sex showed that, irrespective of sex disaggregation, the prevalence pattern was the same across all selected background characteristics. The 2012 NARHS HIV prevalence was highest among those aged 35 to 39 years (4.4%), 20-24 years (3.2%) and lowest among the 15-19 age group (2.9%). The prevalence for males, aged 35 to 39 years was highest at 5.3%, while women aged 30 to 34 years was 4.2% (NARHS, 2012; NACA, 2015).

2.1.2.3HIV prevalence by educational levels and occupation

The 2012 NARHS showed that women, youths, and people with low level of formal education were worst affected by the epidemic. HIV prevalence among Most-at-Risk-Populations showed that the brothel-based female sex workers (BBFSW), had the prevalence of 27.4%, non-brothel based female sex workers (NBBFSW) rank next with an estimated prevalence of 21.7%, followed by men who have sex with men (MSM) with an estimated prevalence of 17.2% (NARHS, 2012; NACA, 2015).

2.1.3Factorsresponsible for HIV pandemic in Nigeria

The HIV pandemic in Nigeria has been traced to factors many of which are intertwined with socio-cultural practices, and related behavioral and biological indices (Adeokun, 2006). Key drivers of the HIV pandemic in Nigeria include low level of condom use and general lack of personal risk perception, customary and social practices of multiple marriages, serial marriages and marriages by proxy, including keeping of concubines, transactional and inter-generational sex, ineffective and inefficient services for STIs, and inadequate access to quality healthcare services(Orubuloye*et al.*, 1992). In addition, the

long latency between HIV infection and the development of AIDS- related conditions reduces the likelihood that people will associate a particular sexual contact with the time of transmission, thus compromising the role of voluntary counseling and testing (VCT) or HIV counseling and testing (HCT) in helping to prevent transmission (WHO/UNICEF/UNAIDS, 2015). Homosexual transmission, especially among incarcerated populations, and within some traditional and religious frameworks or in response to demands created by sex tourism in the metropolitan centers; nonsexual traditional practices- particularly male and female circumcision and the custom of creating facial and body markings among children with shared, non-sterile skin-piercing instruments also expose another significant number of persons to infection (WHO/UNICEF/ UNAIDS, 2015). Poor levels of HIV screening services, particularly of blood and blood products, exposed significant number of the urban poor and rural population to unscreened blood and blood products, while inadequate levels of services for prevention of mother to child transmission of HIV (PMTCT) was responsible for the increasing rate of infections in infants and children (WHO/UNICEF/UNAIDS, 2015).

2.2The Relationship between HIV Infection and the Immune System

2.2.1The immunopathogenic mechanismsof HIV infection

2.2.1.1HIV entry and acute viraemia

A complex array of multiphasic and multifactorial immunopathogenicmechanisms are involved in the establishment and progressionof HIV disease (Oxenius*et al.,* 2004). Soon after HIV enters the body, acute viremia occurs with wide dissemination and trapping of the virus predominantly withinthe processes of follicular dendritic cells in the germinalcenters of lymphoid tissue. The dendritic cells then initiate the

propagationof the virions to CD4+-lymphocytes (T4-helper cells) in thymus, bone marrow and other reticuloendothelial tissues. This process may be down-regulated by inappropriate immune activation of certain subsetsof $CD8^+$ T lymphocytes (T8-cytotoxic/suppressor cells) and elaboration of certain anti-inflammatory cytokines leading to elimination of the virus from the blood(Oxenius*et al.,* 2004). However, with rare exceptions,the virus is not completely eliminated from the body, and a stateof chronic, persistent viral replication ensues. The transition by HIVfrom acute to chronic infection with persistent replicationof virus is unique among viral infections in humans(Oxenius*et al.,* 2004). HIV tat protein may down-regulatefirst line radical scavengers such as Cu^{2+}/Zn^{2+}dependent superoxide dismutase (SOD), Mn^{2+}dependent SOD, Cu^{2+} binding proteins such as albumin, ceruloplasmin and metallothionein, and Fe^{2+} binding proteins such as ferritin, transferrin,haptoglobin and myoglobin (Aquaro*et al.*, 2008).

2.2.1.2CD4+-cell depletion

The CD4+cells are the principal cellular target of the HIV and their depletion is central to the progression of the infection to AIDS (Douek*et al.,* 2002). According to Lelievre*et al.*, (2004), HIV depletes CD4+ cells by at least four mechanisms:First, HIV can destroy CD4+ cells directly. Second, HIV-induces elaboration of CD8+T cells to either suppress the activities of infected CD4+ cells or destroy them directly. Third, HIV-induces elaboration of cytokines by infected immune cells (including CD4+-cells, macrophages and natural killer cells) to destroy uninfected bystander CD4+ cells. Fourth, after binding to the CD4+ receptor sites, the virus rapidly takes over the genome of the host cell, replicates and cause accumulation of infected cells in the lymph nodes. It then

elaborates a network of cytokines that enables it to generate reactive oxygen species (ROS) that enhances its mutation into so many different strains, the net result being to confuse, elude and overpower the immune system and destroy T4 cells through programmed apoptosis (Wainberg*et al.*, 2004).

2.2.1.3Immune dysfunction

Because of their pivotal role in regulating and amplifying the immune response, abnormalities of CD4+T cell functions render natural killer (NK) cells, macrophages and other innate immune cells less efficient, so that collectively the severe immunosuppression allows the virus and other pathogens to replicate unchecked within the individual (Lelievre*et al.*, 2004). Also, death of various uninfected immune cells, through excessive immune activation by a high persistent viral antigen loadresults in deficits of both cell mediated and humoral immune response to OIs and malignancies.

2.2.1.4Elaboration of cytokines

Infection of immune cells with CD4+ receptor/CCR5 co-receptors (e.g., T4 helper cells) and CD4+ receptor/CXCR4 co-receptors (macrophages, microglial cells, dendritic cells, and Langerhans cells) (Alimonti*et al.*, 2003) elicits release of a cascade of pro-inflammatory cytokines that promote virus replication, immune cells depletion and dysfunction, and subsequent disease progression (Poli*et al.*, 1990). The pro-inflammatory cytokines such as interleukin-1 beta (IL-1ß), tumor necrosis factor- alpha (TNF-α) and interleukin-6 (IL-6) directly or indirectly mediate B cell differentiation and maturation into plasma cells that are responsible for synthesis of antibodies. Antibodies, in turn, stimulate T cell activation, growth and differentiation by increasing the

expression of interleukin-2 (IL-2) receptors on the T cells and thymocytes (Poli*et al.*,1990). The T cell proliferation initially leads to elaboration of CD8+ cytotoxic cells which either suppress the activities of infected immune cells or kill them directly. However, the CD8+ cells are eventually overwhelmed because HIV-1 stimulates CD40L on infected macrophages and monocytes to increase IL-6 secretion, and IL-6 in turn, with or without IL-1ß and TNF-α, increase HIV-1 replication and secretion (Douek*et al.*,2009). Thus the positive feedback mechanism between HIV-1 and IL-6 increases HIV-1 replication in macrophages and other immune cells. The positive relationship between IL-6 secretion and HIV replication has been supported by results of various studies(Longo and Fauci, 2010;Nixon and Landay, 2010; Salter *et al.*, 2013). One result showed that cytokines such as TNF-α, IL-1β and IL-6 induce HIV replication through activation of nuclear factor-kappa beta (NF-kβ) (Longo and Fauci, 2010), another demonstrated higher circulating levels of IL-6 in HIV infected patients compared to controls (Salter *et al.*, 2013), while Nixon and Landay, (2010) further demonstrated that the levels of circulating IL-6 were directly proportional to the viral load and inversely proportional to the patients CD4+-cell count.

2.2.1.5 Elaboration of acute phase proteins

Acute- phase proteins (reactants) such as CRP, fibrinogen, D-dimer, serum amyloid A and amyloid P are recognised as important indicators of inflammatory response from infection, immune activation or injury (Nixon and Linday, 2010; Salter *et al.*, 2013). Macrophages engulf CRP and other inflammatory proteins bound to LDL cholesterol to become foam cells; and engorged foam cells discharge the reactive fats into damaged vascular endothelium further attracting formation of more foam cells. By causing LDL

cholesterol to oxidize into a more reactive, abrasive form, these reactants become initiators of a vicious cycle which may be responsible for atherosclerosis, coagulopathy, vasculitis, atheroma formation, stroke and cardiovascular events in HIV disease (Feldman *et al.,* 2003; Lau *et al.,* 2006; Salter *et al.,* 2013).

Many studies have demonstrated a strong positive relationship between CRP and HIV RNA load (Lau *et al.,* 2006; Nixon and Linday, 2010; Salter *et al.,* 2013). Thesestudies showed that levels of CRP change in response to proinflammatory cytokines, IL-1 and IL-6, whose expressions are induced by infections and tissue necrosis (Salter *et al.,* 2013); that lower levels of CRP predict longer survival within HIV-infected individuals, and vice versa (Lau *et al.,* 2006; Nixon and Linday, 2010); and that CRP concentrations were inversely correlated with CD4+- cell counts (r=−0.17; P_.001) and directly correlated with HIVRNA levels (r=0.20; P_.001), and duration of HIV disease and progression to AIDS (Lau *et al.,* 2006).

2.2.2The response of the immune system to HIV infection

2.2.2.1The response of CD8+ cytotoxic/suppressor T-cells

The CD8+ T-cells either suppress the activities of infected immune cells or kill them directly. Oxenius*et al.,* (2004), proposed that the CD8+ T- cells play a key role in the immunologic defense against HIV by controlling viral replication through at least two mechanisms, eitherthrough direct antigen-specific cytolysisor indirectly through release of soluble antiviral factors or chemokines.

2.2.2.2The response of natural immune cells and oxidative system

Since the T- cells play a vital role in cell mediated immune responses and help B- cells in humoral antibody production, binding of HIV on CD4+-cells induce the other cells of the immune system (such as macrophages, microglial cells, dendritic cells, and Langerhans cells) to perform their functions either by direct phagocytosis or by secretion of cytokines to activate their maturation and proliferation (Alimonti*et al.,* 2003). Antibodies when produced eliminate pathogens through opsonization, complement activation and antigen-antibody complex formation. These processes require oxidasesand other catalytic enzymes (such as NAD (P) H oxidase, xanthine oxidase, SODs, amino acid oxidase, peroxisomes, glutathione (GSH)peroxidasesand mitochondrial electron transport system) found in polymorphonuclear leukocytes, monocytes,and macrophages (Awodele*et al.,* 2012). Upon phagocytosis, these cells producea burst of superoxides ($O^{2-\cdot}$), hydroxyl radical (_OH), hydrogen peroxide (H_2O_2), and nitrogen radicals (polynitrites, nitric oxide, peroxynitrite) that lead to antiviral and bactericidal activities (Elbim*et al.,* 2001).At the initial stages, the pro-oxidant processes are effective in combating the virus, but because antioxidant defenses are not completely efficient in removing excess radicals, increased free-radical formation in the body leads to increased demand for radical scavenging anti-oxidants; which if not adequately met results in the state of 'oxidative stress' responsible for several pathophysiological phenomena involved in progression of HIV infection to HIV disease (Pasupathi*et al.,* 2009;Awodele*et al.,* 2012). First,free radical production increases from stage one to a maximum in stage two, and persist in different stages of the disease with or without ART (Ibehand Nwabunni, 2012). The more intense overproduction of ROS, particularly hydrogen peroxide H_2O_2, at the various stages, especially in stage two, is associated with reduced expression of the

antiapoptotic/antioxidant compounds Bcl-2 and thioredoxin (Elbim*et al.,* 2001). Second, chronic persistent oxidative stress changes DNA structure, modifies proteins and lipids, activates several stress-induced transcription factors, and leads to production of proinflammatory and anti-inflammatory cytokines and increased HIV replication. Specifically, TNF-α is elaborated by activated macrophages to activate NF-κβ induced HIV transcription and replication (Awodele*et al.,*2012; Birben*et al.,* 2012).Third, free-radical-induced cell death can proceed as T-cell necrosis or apoptosis, and immune dysfunction, lipid peroxidation, endothelial damage, atherosclerosis and tissue toxicity(Elbim*et al.,* 2001).It is estimated that these processes are responsible for the death of more than one billion CD4+ cells andthe replication ofover 50 million HIV on a daily basis in AIDS(Awodele*et al.,* 2012; Birben*et al.,* 2012).

2.2.2.3The response of endogenous and exogenous anti-oxidants

The defense mechanisms against free radical-induced oxidative damage are elaborated in HIV induced chronic pro-oxidant state(Elbim*et al.,* 2001). The first line scavengers are the endogenous enzymes such as glutathione (GSH), selenium dependent-glutathione peroxidase, thioredoxin (TRX), Cu^{2+}/Zn^{2+} dependent SOD, Mn^{2+}dependent SOD, and Fe^{2+}dependent catalase, and molecules such as albumin, bilirubin, uric acid, lipoic acid, N-acetylcysteine, ubiquinone (coenzyme Q10), and NADPH/NADH (Biswas *et al.*,2005, Birben*et al.,* 2012). The activity of some of them leads to elevation in the levels of malondyaldehyde (MDA), and 8-hydroxylguanosine in urine of affected individuals (del Valle *et al.,* 2013). The glutathione redox cycle is a major source of protection against low levels of oxidant stress, whereas catalase becomes more significant in protecting against severe oxidant stress (del Valle *et al.,* 2013; Durak,

2014). Glutathione reductase enzyme which reduces glutathione disulphide (GSSG) to the sulphhydryl form GSH, inhibits the reverse transcriptase (RT) enzyme of HIV (Palamara*et al.,* 1996), and blocks,in a concentration-dependent manner, the activation of HIV proteases, which is essential for splitting of viral proteins (Birben*et al.,* 2012). The selenium dependent glutathione peroxidase also inhibits the NFκB light chain induced activation of viral replication (Baum*et al.,* 2000; Kamwesiga*et al.,* 2011). In *vitro* studies have also shown that a decrease in GSH levels not only promotes HIV expression but also impairs T-cell function, which helps explain the link between GSH depletion and HIV disease progression(Palamara*et al.,* 1996; Biswas *et al.,* 2005).

Protection against macromolecular damage by stress or heat shock proteins leads to increased demand and subsequent deficiencies of exogenously derived second-line scavenging anti-oxidants such as ascorbic acid, α-tocopherol, tocotrienols, β-carotene, carotenoids and oxycarotenoids (lypene and lutein), and polyphenols such as flavonoids, flavones, flavonols and proanthocyanidins (Aquaro*et al.,* 2008). The B vitamins and essential trace elements, particularly selenium and zinc, enhance the activities of third-line scavenging anti-oxidants, called repair and denovo enzymes. These are lipases, proteases, DNA repair enzymes, transferases, and methionine sulphoxide reductase. These enzymes try to repair damaged DNA, damaged proteins, oxidized lipids and peroxides and stop peroxidation (Stone *et al.,* 2010).

2.3Classifications of HIV Infection for Initiation of Antiretroviral Therapy

2.3.1United States Centre for Diseases Control(US-CDC) 1993 classification

Since the hallmark of HIV infection is the progressive decline in the quantity and quality of the patient's CD4+- cell count (Longo and Fauci, 2010), and progression to AIDS,

theUS-CDC developed a staging system based on 3 ranges of CD4+ counts and 3 clinical categories (giving a matrix of 9 exclusive categories).

The 3 ranges of CD4+ counts were:

- 1 (≥ 500 cells/µL);
- 2 (200- 499 cells/µL) and
- 3 (< 200 cells/µL, defined as AIDS),

The 3 ranges of clinical categories were:

- A (asymptomatic HIV infection);
- B (symptoms, not A or C) and
- C (AIDS defining illnesses or presence of opportunistic infections).

Patients were started on ART if they fell into either category A3 and B3 or C1, C2, and C3 (CDC, 1993). However, this system was less valuable in clinical decision analysis when HIV RNA measurement became available.

2.3.2 The WHO 2005 revised CDC classification

The WHO 2005 revised the US-CDC classification, and classified HIV into five stages based on clinical signs and CD4+ cell counts. The five stages were:

- I (acute sero-conversion syndrome, > 1000 CD4+ cells/µL);
- II (early disease, > 500 CD4+ cells /µl);
- III (middle-stage disease, 200- 500 CD4+ cells /µl);
- IV (late disease, 50-199 CD4+ cells /µl); and V (advanced disease, < 50 CD4+ cells /µl).

The CD4+ cell count was used because it was affordable and cost effective for many resource-limited countries, who could not afford the cost of the HIV RNA load, the surrogate marker and most direct measurement of HIV disease progression and response to ART (Idigbe, 2003; Rosen et al., 2011; CUNY, 2015). Under this classification (WHO, 2015), patients with any of the underlisted criteria were commenced on ART:

- CD4+ of ≤ 200 cells/μL, irrespective of WHO clinical staging;
- WHO clinical stage IV or V disease, irrespective of CD4 cell count or total lymphocyte count;
- WHO clinical stage II or III disease with total lymphocyte counts of 1,200/μl and haemoglobin of 10 g/dl (when CD4+ cell count cannot be performed or was unavailable).

2.3.3 The WHO 2007 and 2010 revised HIV treatment guidelines

In 2007, when CD4+ cell count became universally available, the WHO modified the criteria to include patients with WHO clinical stage III disease and CD4+ of ≤350 cells/μL (WHO, 2007), and further modified in 2010, to include patients with CD4+ of ≤350 cells/μL, irrespective of WHO clinical staging (WHO, 2010).

2.3.4The WHO 2013 treatment guideline

Wide variability in results of CD4+ levels observed in different laboratories, and other factors affecting CD4+ cell counts in individuals (Rosen et al., 2011), made the WHO in 2013, to adopt a public health approach in the HIV treatment response by recommending

initiation of ART based only on clinical features. A typical HIV infection was therefore divided into four stages (WHO, 2013):

- I (primary infection or acute sero-conversion syndrome; clinical latency or asymptomatic infection);
- II (early symptomatic infection or mild disease);
- III (intermediate or moderate disease) and
- IV (late symptomatic infection or AIDS defining illness).

However, cost and other logistic challenges forced the WHO to modified the criteria for initiation of ART to include patients with CD4+ counts <500 cells/µL irrespective of WHO clinical staging (WHO, 2013).The WHO clinical staging and US-CDC1993 revised classification are attached as appendices II and III.

2.3.5The WHO 2015 treatment guideline('Test and Treat guideline')

The 2015 WHOtreatment guideline recommended that ART should be initiated on anyone diagnosed of HIV (based on HIV positive test and HIV RNA load), irrespective of WHO clinical stage and CD4+ cell count (WHO, 2015). The recommendation was based on results of clinical trials and observational studies, which showed that people who started ART immediately after HIV diagnosis, while their CD4+ cell count was high, had a significantly lower risk of HIV-related illness and death (INSIGHT START Study Group, 2015; TEMPRANO ANRS 12136 Study Group, 2015). Other considerations were: the ultimate need to suppress HIV RNA in order to stop the progression of HIV-related disease and drastically reduce the risk of onward transmission; the need to reduce lengthy and unnecessary delays in determining

eligibility for ART and initiating ART; the need to reduce rate of drop-out or loss to follow-up between HIV testing and ART initiation (Bastard *et al.,* 2013) , and evidence from some studies which showed that about 22% of adults were lost to follow-up before ART initiation at four years (IeDEA, 2010; CUNY, 2015).

The latest recommendation presents the major challengeof diagnosing greater numbers of people living with HIV and linking them successfully and rapidly to HIV treatment and care services, particularly in resource-limited countries. Some studies have reported the following low HCT/HTC activities, and treatment coverage for persons eligible for ART in many of these countries viz: slightly more than 20% in Cameroon, the Democratic Republic of the Congo and Nigeria and as low as 6% in South Sudan and 2% in Madagascar (Rosen and Fox, 2011; Kranzer, *et al.,* 2012).Systemic review of global data (IeDEA, 2010; CUNY, 2015), and other studies in sub- Sahara Africa (Kranzer, *et al.,* 2012) and Asia (WHO/UNICEF/UNAIDS, 2015),showed that the phenomenon of default, loss to follow-up and stopping ART before achieving sustained viral suppression among HIV infected persons was very common. For instance, in October 2014, Thailand adopted a national HIV treatment policy of initiating ART for all people living with HIV regardless of CD4 cell count. At the beginning, the ART coverage was 61% [55–66%], among the highest in the world, but there was a progressive decline in the proportion of people retained in treatment, from 83% at 12 months, 78% at 24 months to 75% at 60 months. The high attribution in the HIV care, support and treatment response in the African Region and Asia, which have the tendency to reduce the treatment and prevention benefits of ART and increase drug resistance and the cost of care (Bennett *et al.,* 2012; Vinikoor*et al.,* 2014; Kuznik*et al.,* 2016), have been attributed to factors such as transport costs and distance to the facility, hunger,

poverty, stigma, fear of disclosure, unreliable referral systems, staff shortages and long waiting times, as well as policy and legal barriers, especially for adolescents and incarcerated populations (Govindasamy*et al.,* 2012; Bastard *et al.,* 2013; Mugglin*et al.,* 2013). Inspite of above challenges, the Nigerian Government has adopted 2015 WHO 'Test and Treat' HIV treatment guidelines (FMOH, 2016).

The solutions to the above challenges lie in addressing factors that cause poor health-seeking behaviour, and integrating HIV care with screening for STIs and tuberculosis (TB); maternal, newborn and child health care services; opioid substitution therapy, and provision of food and micronutrient supplementation, in order to improve pre-ART, ART and post-ART retention (Fatti*et al.,* 2010; Kohler*et al.,* 2011; WHO/ UNICEF/UNAIDS, 2013). Other measuresinclude: continuous HCT/HTC, introduction of point-of-care diagnostics to minimize the number of clinic visits (Faal*et al.,* 2011), cutting waiting times at clinics by streamlining work flows, and spreading workloads through task-shifting (Govindasamy*et al.,* 2012; 2014), provision of intimate-partner notification by health providers to reduce self-denial and stigmatization (Brown *et al.,* 2011; Medley *et al.,* 2015), provision of diary cards, and psycho-social support, ART adherence counseling and use of mobile-phones text message reminders (Maduka*et al.,* 2013; Mills *et al.,*2014).

2.4Human Nutrition in Health and during HIVInfection

2.4.1Scope of human nutrition and balanced diet

Normal nutrition is defined as the absence of any or all of the following: low body weight, weight loss, anaemia, low serum albumin, macro- and micro-nutrient deficiencies, and deficiencies of other nutrients that affect the immune system (Ajala,

2006).Normal nutrition depends on a balanced diet which is 'the combination of different food groups in proportions enough to meet the macro- and micro-nutrient needs of an individual for cellular metabolism, energy expenditure, tissue growth and maintenance'. The United Kingdom (UK) National Food Guide (the Balance of Good Health) (Hunt *et al.,* 1995)recommends that a 'balanced diet' should consist of a pyramid of five food groups: - core [cereals, roots and tubers (33%)], secondary [fruits and vegetables (33%), meat and milk (26%)], and peripheral/ non-essential foods [fats and fatty foods/sugars and sugary foods such as biscuits, cakes, alcoholic beverages, confectionery and sauces (8%)]. The balanced diet supplies two to three kilocalories to adult males and about one to three kilocalories to adult females respectively(Hunt *et al.,* 1995). The nutritional details of the five food groups used in the Balance of Good Health are shown in appendix IV.

2.4.2 Components and functions of human food

According to J.A Ajala (2006), human food is made up of five biochemical groups: macronutrients (carbohydrates, proteins, fats or lipids); micronutrients (vitamins and minerals); electrolytes; phytochemicals, and water, as discussed below.

2.4.2.1Macronutrients

These are carbohydrates (CHO), proteins and fats (lipids), and are the commonest source of energy in humans. The CHO are chemical compounds composed of carbon, hydrogen and oxygen in varying combinations; and are divided into simple sugars (e.g., glucose, fructose or honey/fruit sugar, galactose/ lactose or milk sugar and sucrose or table sugar); and complex sugar (e.g. Starch, glycogen, cellulose and pectin). They are especially abundant in grains or cereals, fruits and vegetables, and are usually processed

into continental products such as bread, cakes, pasta, jams, jellies, rice; and some Nigerian staples (fufu, gari, agbu, tuwo). The proteins are chemical compounds formed from varying combinations of 21 specific amino acids, each amino acid being composed of carbon, hydrogen, oxygen and nitrogen. Nine of the amino acids (histidine, leucine, isoleucine, valine, lysine, tryptophan, threonine, methionine and phenylalanine) are "essential" because they must be derived from the diet as the body cannot synthesize them, while twelve of the amino acids are called "non-essential" because they can be synthesized by the human body (Jackson, 2003). Proteins are the major constituents of the human muscles, enzymes, antibodies, and other components of the immune system. They are involved in tissue-building, growth, maintenance, repair and regulation of metabolic processes, as well as serving as sources of energy for the body. These functions are optimally performed by proteins derived from animals (such as beef, fish, lamb, poultry, milk, eggs) than those derived from plants sources (such as beans, rice, and peanut)(Jackson, 2003). Lipids are chemical compounds thatare composed of carbon, hydrogen, and oxygen atoms linked together in specific ways. 95% of all fat consumed in food are triglycerides, and the other 5% are phospholipids and sterols. Each triglyceride is made up of three fatty acid molecules to one glycerol molecule. The nature of chemical bonding between carbon atoms of each fatty acid determines whether it is saturated or unsaturated fat. Majority of saturated fats are derived from animal fats (e.g., beef, chicken, lamb, milk, pork); and some plant fats (e.g., coconut oil, palm oil, and palm kernel oil); while plant fats derived from olive oil, peanut oil, cotton seed oil, sunflower oil, corn oil, soya bean oil contain mostly mono- and poly-unsaturated fatty acids.Lipids are beneficial to the body in many ways (DEFRA, 2001). Fat cells and adipose tissues are the major energy stores in the body and provide the richest source of

energy (9 calories per one gram, as against 4 calories per one gram each of carbohydrate and proteins), as well as providing protective padding and insulating the body from cold. They make food more tasteful and also enhance the absorption of fat-soluble vitamins (A, D, E, and K) (DEFRA, 2001).

2.4.2.2Micronutrients

These are vitamins and trace elements which must be obtained in minute quantities from food as they cannot be produced by the human body. They do not provide calories but are important for normal metabolism, growth, immunity, and psychomotor developmentwhich are critical to human health (Barasi, 2003, ed; Joint FAO/WHO, 1998/FAO/WHO/UN, 2004).Vitamins are organic compounds grouped into fat-soluble vitamins (A, D, E and K) and water-soluble (eight B vitamins and vitamin C).Although vitamins vary in their chemical compositions, they complement one another to produce similar metabolic effects, but the deficiency of one may affect the utilization of another. Also, vitamins synthesized in the laboratory are chemically identical to those found in foods, and function in similar ways when taken as supplements.The fat-soluble vitamins can be lost in fat malabsorption, liver, biliary and pancreatic diseases; and during prolonged use of mineral oil laxatives and antibiotics; while water-soluble vitamins are more readily lost during food preparation processes or are excreted in the urine (DEFRA, 2001). Occasionally, the fat-soluble vitamins can accumulate in adipose tissues and produce harmful effects (Joint FAO/WHO, 1998/FAO/WHO/UN, 2004).

Trace elementsare inorganic substances which make up about 5% of body tissues as structural components of teeth, muscles, blood cells, and bones; and are also essential to muscle contraction, blood clotting, protein synthesis, cell membrane permeability and

immunity (Joint FAO/WHO, 1998/FAO/WHO/UN, 2004). They occur naturally in water, soil and rocks, and are usually taken up by roots of plants. Ruminant animals consume minerals from plants and humans obtain them when they consume plants and/or animals. Essential trace elements are ferritin (Fe^{2+}), zinc (Zn^{2+}), manganese (Mn^{2+}), copper (Cu^{2+}), iodide (I^{-}), chromium (Cr^{2+}), cobalt (Co^{+}), selenium (Se^{2-}) and fluoride (F^{-}). The total body trace elements contents are shown in appendix V.

2.4.2.3Electrolytes

Electrolytes are essential for maintenance of normal water and fluid balance, and regulation of physiological activities in the body, particularly in HIV infection. Electrolytes such as calcium (Ca^{2+}), phosphorus (P^{2-}), magnesium (Mg^{2+}), potassium (K^{+}), sodium (Na^{+}), chloride (Cl^{-}) and sulphur (S^{2-}) work in conjunction with the trace elements for effective performance (Barasi, 2003, ed).

2.4.2.4Phytochemicals

These are substances of plant origin which, when consumed in the human diet, have anti-cancer, antioxidant and antitoxin properties. Examples are: isoprenoids (anticancers) found in fruits, vegetables, cereals and citrus oils; flavonoids (antioxidants) found in tea, onions and apples; phytosterols and tocotrienols (antioxidants) found in barley, wheat and corn (Barasi, 2003, ed; Durak, 2014).

2.4.3Effect of HIV infectionon human nutrition

2.4.3.1Effect of HIV infection on food acquisition, intake, absorption and distribution

Intake of food can be affected by mouth, throat and esophageal ulcers resulting from herpes simplex virus and candidiasis that cause eating and swallowing discomfort. Reduced intake of food can also result from anorexia, nausea and vomiting; from fevers or side effects of medications; or malaise and lethargy from depression or abnormal immune response (Enwonwu, 2006). Absorption and distribution of food can be affected by changes in the GIT as HIV causes direct involution of Peyer's lymphoid tissues, crypt hyperplasia, flattening of the intestinal villi, decreased luminal defensins, enterocyte apoptosis, leading to destruction of epithelial barrier, increased GIT permeability and vulnerability to OIs (Guarino*et al.,* 1993). Recurrent diarrhea also contributes to loss of nutrients, particularly carbohydrates, fats, vitamins A and E, bile acid and vitamin B12 (Guarino*et al.,* 1993). The end results of HIV/AIDS related morbidity are prolonged hospitalization leading to decline productive capacity and lack of resources to buy food, malnutrition, and increased healthcare costs, (Gillespie and Kadayila, 2005; Colecraft, 2008). Studies in some developing countries showed that poverty, decreased calorie and protein consumption, and severe wasting were independently associated with antiretroviral non-adherence, and rapid progression of HIV infection to AIDS (Tang *et al.,* 2002; Jiamton*et al.,* 2003; Taye*et al.,* 2010). In addition to affecting HIV disease progression, decreased caloric and protein consumption in sub-Saharan Africa has been found to be highly correlated with increased HIV/AIDS prevalence (Stillwaggon, 2000).

2.4.3.2. Effect of HIV infection on food metabolism and utilisation

Factors affecting metabolism and utilization of nutrients in HIV are heterogeneous and complex. Metabolic changes lead to increased energy and nutrients requirements, inefficient utilization of nutrients and consequently massive nutrient losses (Enwonwu,

2006). Energy requirements are elevated at different phases of the infection. Even during the asymptomatic phase, the energy requirements are increased by 20-30% (i.e., additional 400 kilocalories/day) above the minimal requirement of 2,500 kilocalories /day recommended for a healthy HIV negative individual of the same age, gender and physical activity level (Piwoz and Preble, 2000; Marston *et al.,* 2004). Recurrent fevers and OIs induce several catabolic changes in the body leading to breakdown of carbohydrates, proteins and fat. This is achieved through elaboration of pro-inflammatory cytokines (IL-1β, IL-2, IL-6, IL-8, TNF-α, soluble receptors of TNF-p55 and p75 (sTNF-p55 and sTNF-p75, interferon-gamma (IFN-γ) and CRP, and down regulation of anti-inflammatory cytokines (IL-1 receptor antagonist, IL-4, IL-10 and IL-13), which in turn, induces whole-body protein turnover, skeletal muscle breakdown for hepatic gluconeogenesis, urinary nitrogen loss, negative nitrogen balance and wasting (Chandra, 1999; Lau *et al.,* 2006; Nixon and Linday, 2010; Salter *et al.,*2013). Other catabolic changes are increased hepatic de novo fatty acid synthesis, decreased peripheral lipoprotein lipase activity, hypertriglyceridemia, hyperglycemia and insulin resistance (Tang *et al.,* 2002).

2.4.4Mechanisms and effects of HIV induced malnutrition

2.4.4.1Mechanisms of HIV induced malnutrition

In response to acute HIV-1 viraemia, activated phagocytes release reactive oxygen molecules and pro-inflammatory cytokines which cause oxidative stress, chemo-attraction and subsequent increased demand and consumption of antioxidants (vitamins E and C, and *β*-carotene, iron, selenium, copper, manganese and zinc) to counteract the pro-oxidant state (Fawzie, 2005;Pasupathi*et al.,* 2009;Birben*et al.,*2012). Persistent

oxidative state, arising from consequent deficiency of antioxidants, leads to increased HIV replication via activation of nuclear transcription factor cell gene (NF-kB), T cell apoptosis and dysfunction of cell-mediated immunity (Westendorp*et al.*,1994; Nicolini,*et al.*,2002; Birben*et al.*,2012).Oxidative burst may also induce secondary non-AIDS malnutrition (NAIDS) (Piwoz and Preble, 2000). HIV also incorporates host selenium into viral selenoprotein that has glutathione peroxidase activity for its own antioxidant protection, thereby inducing or exacerbating a selenium deficiency with increasing disease progression (Baum *et al.*, 2000; Stone *et al.*, 2010; Kamwesiga*et al.*, 2011).

2.4.4.2 Effects of HIV induced malnutrition

The deleterious effects of HIV induced malnutrition on the immune system are synergistic and occur in a vicious cycle such that both lead to decreased immunity and promote increased susceptibility of the affected individual to infections, that in turn, leads to increased nutrient requirements, which if not adequately met will lead to more malnutrition (Babamento and Kotler, 1997; Chandra, 1999; Enwonwu, 2006). The net effects of HIV-induced malnutrition and micronutrients deficiencies are widespread atrophy of lymphoid and reticuloendothelial tissues, and consequent depletion of total lymphocytes, particularly T4 and T8 cells, impaired B-cell activity, and antibody responses to T-dependent antigens, and ultimate depression of cell-mediated immunity (Patki*et al.*, 1997; Piwoz and Preble, 2000). IFN-γ and TNF-α secreted by both T4 and T8 cells in response to antigen-specific stimulation have been suggested as potential etiological factors in HIV wasting syndrome as they have been incriminated as appetite inhibitors (Macallan, 1999). Studies among HIV-infected individuals in some developed

countries showed that poor nutritional status (as assessed by weight loss, low BMI, low albumin, reduced serum concentrations of zinc, vitamin B12, and some fat-soluble vitamins) was an independent predictor of immunologic decline, OIs, and shorter survival time in both ART-naive and ART-experienced individuals (Chandra, 1999; Jiamton*et al.,* 2003; van der Sande *et al.*,2004).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1Research sites

3.1.1.1Ahmadu Bello UniversityTeaching Hospital (ABUTH), Shika Zaria

ABUTH is a 750-bed tertiary health institution with facilities for both inpatient and outpatient specialised care. The hospital has a monthly patient population of more than 15,000 spread across 10 states in northern Nigeria, including the Federal Capital, Abuja. Nasara Treatment and Care Centre (NTCC), where the research took place, was established as a unit of the hospital in 2006 by APIN/ HARVARDPEPFAR, to provide free ARTto eligible patients, and supportive care to ART-ineligible patients. The Centre also monitors patients' CD4+ cell count, plasma HIV RNA load (pVL), blood chemistry and hematology, and provides drugs for OIs prophylaxis, all at no cost to the patients.

3.1.1.2 St Gerard Catholic Hospital (SGCH), Kakuri- Kaduna

SGCH is a 100-bed secondary tier health institution that specialises in general and family medicine. It has a monthly patient population of about 10,000 spread across Kaduna State and surrounding states in northwestern and northcentral zones of Nigeria.

Caritas Catholic Relief Foundation (CCRF) HIV Centre, is a unit of the hospital established in 2000 to provide free ARTto eligible patients, and supportive care to ART-ineligible patients. CCRF also monitors patients' CD4+ cell count, pVL, blood chemistry and hematology, and provides drugs for OIs prophylaxis, all at subsidized rates to the patients. It has a good adherence and advocacy subunit that track defaulters, thus minimizing default among the patients.

3.1. 2Ethical certification of the research proposal

The research proposal was reviewed and approved by Institutional Health Research Ethical Committee (IHREC) of ABUTH Shika Zaria, Kaduna State Ministry of Health, Kadunaand St Gerard's Catholic Hospital, Kakuri Kaduna (appendices VI, VII and VIIIrespectively).

3.1.3 Written consent form

A consent form written in English language and translated in Hausa, Igbo and Yoruba languages to be signed by all research participants was provided in the protocol (appendixIX).

3.1.4 Research populations

3.1.4.1Sample size

A minimum sample size of 88 was determined from the Fisher's Sample size formula for longitudinal studies (Araoye, 2004): **N=** Z^2 P (1-P)/ d^2 x 1(1-f),

Where **N** = desired sample size when population is over 100,000;

Z = confidence interval at 95% (1.96);

P = prevalence rate of HIV infection in Kaduna State, Nigeria (5. 1%) (Federal Ministry of Health, Abuja, 2010);

d= sampling error at 5% (0.05);

f = attrition rate of 10%.

However, in order to increase power of the study, 90 HIV positive (HIV infected) ART-naïve adult patients, aged 18 to 59 years were recruited as cases, while 90 age-and sex-matched HIV negative apparently healthy volunteers were recruited as the controls.

3.1.4.2Inclusion criteria for recruitment of 90 HIV infected ART-naïve patients were:

- Adolescents and adults, aged 18- 59 years
- WHO clinical stage I and II
- CD4+ counts of ≥520.0 cells/ µl.

3.1.4.3 Inclusion criteria for recruitment of 90 HIV negative apparently healthy volunteer (controls) are:

- Adolescents and adults, aged 18 to 59 years,
- Seronegative to HIV antibodies
- Mentally alert and physicallyfit.

3.1.4.4Exclusion criteria for all study participants(HIV infected ART-naïve patients and age/sex-matched HIV negative apparently healthy controls) were:

- Children (aged ≤ 17 years)and the elderly (≥ 60 years);
- history of alcohol and substance abuse,
- evidence of chronic hepatitis B or C or HIV-2 infection,
- evidence of immunosuppressive illnesses (such as diabetes mellitus, chronic renal disease, malignancy and sickle cell anaemia),

- history of vegetarian diet or micronutrient supplementation,
- history or evidence of chronic hypertension, stroke or psychiatric disease
- pregnancy
- refusal to give consent
- In addition to the above criteria, the HIV infected patients were excluded if they became eligible for ART, or defaulted from scheduled one clinic visit

3.1. 5Laboratory materials

3.1.5.1General materials

- Distilled or deionized water
- Calibrated adjustable precision pipettes: 5 µL, 10 µL, 50 µL, 100 µL, 200 µL, 400 µL, 500 µL, 1000 µL (1.0 mL)and disposable pipette tips
- Calibrated adjustable 10mL – 100mL pipettes for reagent preparations
- EDTA vacutainers with venepuncture tubes and needles
- Calibrated serum separator tubes and Squirt bottles
- Vortex mixer (Labnet International Inc.)
- Eppendorf 5702 Centrifuge at 500 x g to 2000 x g rpm
- Refrigerator freezer (4^0C to -80^0c)
- Polypropylene test tubes for dilution of standards and samples
- Technico 85604, 100mL and 1,000mL graduated cylinders (Made in England)
- Adhesive strips (microplate sealers) and Vacuum aspirator for wash steps
- VWRStop clock (Timer) and Absorbent paper towels

- Microplate reader capable of measuring absorbance at 450nm, 540nm, 570nm, 650nm or 690nm
- GenLabIncubator

3.1.5.2Materials for humanserum vitamin B_{12}

Human vitamin B_{12}ELISA kit was a product of WKEA Medical Supplies Corps, 206 Building 6, ChenguangGardon, Qianjin Street Changchun 130012 China (stored at 2-8^0C). Components are listed in appendix X.

3.1.5.3Materials for humanserum CRP

These were obtained from Quantikine® ELISA Human C-Reactive Protein/CRP Immunoassay ™ kit(HSCC: 30021098).The ELISA kit was a brand of biȣtechne ® R& D Systems Europe Ltd, 19 Barton Lane, Abingdon Science Park, Abingdon UK OX14 3NB. The components are listed in appendix XI.

3.1.5.4Materials for human serum IL-6

These were obtained from Quantikine® HS ELISA High Sensitivity Human IL-6 Immunoassay ™ kit (HSCC: 30021098) brand of biȣtechne ® R& D Systems Europe Ltd, 19 Barton Lane, Abingdon Science Park, Abingdon UK OX14 3NB. The components are listed in appendix XII.

3.1.5.5Materials forCD4+ cell count

These were from the Partec flow cytometry (CD4 easy count kit). Product of SYSMEX Healthcare/ Immunolgy; SysmexPartecGmbH.AmFltgplatz 13.02828. Görlitz. Germany, listed in appendix XIII.

3.1.5.6Materials forplasma HIV-1

These were from the AMPLICOR® HIV- 1 MONITOR Test, version 1.5 (Roche Molecular (Diagnostic) Systems, Branchburg, NJ 08876 USA), as shown in appendix XIV.

3.1.5.7Materials forhuman serum zinc (Zn^{2+}) and copper (Cu^{2+}) using Atomic Absorption Spectrophometer (AAS) model number AA240FS (Varian, UK).

These are listed in appendix XV.

*3.1.5.8Materials for human serum lipids (total cholesterol, triglycerides, high density lipoprotein and low density lipoprotein) using automated Chemistry Analyser (Chemray 120, Kobe-Japan)*are listed in appendixXVI.

*3.1.5.9Materials for human serum albuminusing automated ChemistryAnalyser (Chemray 120, Kobe-Japan)*are listed in appendix XVII.

*3.1.5.10Materials for haemoglobin*using *Sysmex XN* automated Haematology Analyser(Sysmex, Kobe- Japan) are listed in appendix XVIII.

3.1.6Research drug:Synovit™micronutrient supplement capsules

The Synovit™micronutrient supplements were manufactured by Universal Medicare Pvt. Ltd, Plot No. 811, G.I.D.C. Sarigam, Dist. Valsad-396 155, India; for: V.S. International Pvt. Ltd, A-204, Neelam Centre, Hind Cycle Road, Worli, Mumbai-400

030, India. Manufacturing license No.00008901302010253157 7; NAFDAC Registration No.A4-6201; Batch No., 6914 SHL; Manufacture Date.08/2014; and Expiry Date.07/2017.The capsules are marketed in Nigeria by Synergy Healthcare Limited Lagos, as supplements for immunodeficiency states, malnutrition, and pregnancy. Synergy Healthcare Limited Lagos donated the Synovit™ capsules used for this research (appendix XIX). Each Synovit™ capsule is made up of a mixture of 11 vitamins (A, B1, B2, B3, B6, B12, biotin, folate, pantothenate, C and E), 6 trace elements (selenium, copper, zinc, ferrous, molybdenum and manganese), 2 electrolytes (potassium and magnesium), 2 essential amino acids (L-glycine and L-lysine) and 1 peptide (choline bitartarate). The concentration of each of these components was equal to or more than the United Nation Food and Agricultural Organization recommended nutrient intake (RNI) per day requirements of human nutrition (Joint FAO/WHO, 1998/FAO/WHO/UN, 2004). This is shown in appendix XX.

3.2 Methods

3.2.1Enrolment and evaluationsof HIV infected ART-naïve patients

At the various enrollment centres, patients were educated and counseled on various aspects of HIV care and support by Nurse HIV counselors, before the research objectives and protocolwere explained to them, with emphasis on the voluntary nature of the study and assurance of the confidentiality of their bio- data. After signing an informed written consent, each patient was enrolled into the research as outlined in Table 3.1 below.

The protocol consisted of clinical evaluations, laboratory evaluations, medications and clinic visit schedules respectively. Clinical evaluations comprised collection of sociodemographic data(at baseline only), and other patient data at baseline, and at 12th, 24^{th}, 36^{th} and 48^{th}week clinic visits. The patients' data include:evidence of OIs/ODs and other nonspecific illness (es), and vital signs(temperature, respiratory and pulse rates, blood pressures and BMI;usually performedafter 15 to 20 minutes rest. Blood sampleswere collected for haemoglobin estimation and CD4+ cell count at the quarterly clinic visits; and for pVL, serum IL-6, CRP, albumin, lipids, zinc, copper and vitamin B_{12} at baseline and at $24^{th}$and 48^{th}weeks respectively. At scheduled clinic visits, each eligible patient was given daily doses of one Synovit^{TM}capsule and two Co-trimoxazole 450mg tablets. Any patient who became eligible for ART, defaulted from one clinic visit, or became pregnant was dropped from the research at the next scheduled clinic visit.

Table 3.1: The procedure for evaluations of the HIV infectedART-naïve patients

Procedure	**Parameter for assessment**	**Scheduled time of clinic visit**				
		Baseline	**12th week**	**24th week**	**36th week**	**48th week**
Clinical evaluation	*Socio-demographic data	Yes	No	No	No	No
	Symptoms/ signs of OI or other diseases	Yes	Yes	Yes	Yes	Yes
	**Vital signs	Yes	Yes	Yes	Yes	Yes
	***BMI	Yes	Yes	Yes	Yes	Yes
Laboratory evaluation	Haemoglobin	Yes	Yes	Yes	Yes	Yes
	CD4+ cell count	Yes	Yes	Yes	Yes	Yes
	HIV RNA load	Yes	No	Yes	No	Yes
	IL- 6	Yes	No	Yes	No	Yes
	CRP	Yes	No	Yes	No	Yes
	Albumin	Yes	No	Yes	No	Yes
	Lipid profiles	Yes	No	Yes	No	Yes
	Zinc	Yes	No	Yes	No	Yes
	Copper	Yes	No	Yes	No	Yes
	Vitamin B_{12}	Yes	No	Yes	No	Yes
Medications	One Synovit™capsule daily	Yes	Yes	Yes	Yes	Yes
	Two Co-trimoxazole tablets daily	Yes	Yes	Yes	Yes	Yes
Clinic visits	****Number of patients (100%)	90 (100)	78 (86.7)	68 (75.6)	57 (63.3)	57 (63.3)

*sociodemographic data include: age, sex, marital status, religion, educational attainment, occupation, income level in Naira, WHO clinical stage and duration of HIV diagnosis before enrollment into research (in months);
**Vital signs include: temperature, respiratory rate (RR), pulse rate (PR), blood pressure (BP);
***BMI is calculated as: weight (kg)/height (m^2)
**** 33 patients did not complete the study for following reasons: CD4+ cell count decreased to ≤500.0/μL (13); became pregnant (11) and defaulted from clinic visits (9).
The research lasted for three years, from September 2013 to September 2016.

Yes =procedure was done; No= procedure was not done

3.2.2 Evaluation of HIV negative healthy controls

A control group comprising 90 age- and sex- matched HIV seronegative healthy volunteers were enrolled from communities in Kaduna and Zaria zones during awarenesscampaigns for HIV Counseling and Testing programmes. At the various enrollment centres, participants were educated on the research objectives and protocol with emphasis on the voluntary nature of the study and assurance of the confidentiality of their bio- data. After signing an informed written consent, their sociodemographic data, vital signs and BMI were obtained, followed by collection of blood samples for haemoglobin, and serum zinc, copper, vitamin B_{12}, albumin and lipids.

3.2.3 Blood collection, preparation, storage and analysis

3.2.3.1Laboratory analyses for HIV infected ART-naïve patients

A total of twenty milliliters (20.0ml) of whole venous blood samples was collected from each patient for pVL, CD4+ cell counts, haemoglobin, and serum IL-6, CRP, albumin, zinc, copper and vitamin B_{12} concentrations. Ten (10.0) ml of blood for pVL was collected into a sterile EDTA vacutainer specimen bottle and spun for 20 minutes at 16,000rpm, then frozen at -70^0C (for storage in weeks) or at 20^0C-8^0C (for storage lasting < 5 days), before analysis using COBAS AmpliPrep and COBAS Taqman machines. Five (5.0) ml of blood for CD4+ cell count and haemoglobin was collected into a sterile EDTA vacutainer specimen bottle and used within 3 hours. CD4+ cell count and haemoglobin were measured using CyflowPartec counter and the *Sysmex XN* Automated Haematology Analyser (Sysmex, Kobe- Japan)respectively. 5.0ml of blood specimen was collected into a sterile plain vacutainer specimen bottle, and allowed to clot at room temperature and spun for 5 minutes at 3000rpm to obtain a clear

unhaemolyzed serum. The sera were harvested into sterile serum-separation tubes and rapidly stored at -20^{o}C until assayed in batches; for vitamin B_{12} using human vitamin B_{12} ELISA kits; for zinc and copper concentrations using Atomic Absorption Spectrophometer (AAS) model number AA240FS (Varian, UK); and for lipids and albumin concentrations, usingAutomated Chemistry AnalyserChemray 120, Kobe-Japan (colorimeter reader).

3.2.3.2*Laboratory analyses for HIV negative healthy controls*

The procedures for the determination of haemoglobin, vitamin B_{12}, zinc, copper, lipids and albumin concentrations were as discussed in 3.2.3.1 above.

The details of procedures for the various laboratory analyses are shown in appendices X-XVIII.

3.3Methods of Statistical Analysis

3.3.1 Data entry

Quantitative data were entered into Excel sheet and qualitative data were entered into IBM Statistical Package for Social Sciences (SPSS) version 20 for Windows. Parallel entry anddouble checking of data was done to eliminate errors.

3.3.2 Data analysis

Descriptive and inferential analyses were performed using various statistical tools with two-tailed significance level of equal or less than 0.05 ($p \leq 0.005$).

3.3.2.1Descriptive statistics

These were used to determine frequencies and percentages, means and standard deviation, and confidence interval for the mean at 95%, for parametric variables. Nonparametric variables were analysed using median, extreme ranges (minimum and maximum), and interquartile ranges.

3.3.2.2.*Inferential statistics*

These were used to describe relationships between variables. They include:

- Chi-square which was used to determine relationships between proportional variables
- Independent- Samples mean T test: used to determine the relationship between the means of two independent samples
- Independent Samples median test: used to determine the relationship between the medians of two independent samples
- Independent Samples Moses test of extreme reaction: used to determine the relationship between extreme ranges of two independent samples
- Independent Samples Mann-Whitney U Test: used to determinethe relationship in the distribution of continuous variables (usually the interquartile ranges) between two independent samples
- One-way ANOVA Independent Samples Kruskal Wallis Test: used to determine the relationship in the distribution of continuous variables (usually the interquartile ranges) between three or more independent samples

- Pearson Correlation Coefficient: used to determinethe relationship between a variable of interest and other variables of an independent sample or samples.E.g., plasma HIV RNA load and CD4+ cell count; plasma HIV RNA load and serum IL-6; plasma HIV RNA load and serum CRP etc.
- Spearman Correlation Coefficient: used to determinethe bivariate relationships between the variables of interest.E.g., plasma HIV RNA load and CD4+ cell count; CD4+ cell count and IL-6; IL-6 and zinc etc.
- Multinominal Logistic Regression (Custom/ Stepwise Model): used to determine significant predictor (s) of a variable of interest such as plasma HIV RNA load.

CHAPTER FOUR

4.0RESULTS

4.1. Sociodemographic Characteristics of HIV Infected ART-Naïve Patients and Matched HIV Negative Healthy Controls

The study participants comprised 90 ART-naïve HIV infected patients and 90 HIV negative controls, each group consisting of 21 (23.3%) males and 69 (76.7%) females. The mean ages of males (40.0 ±2.2 years) and females (39.0 ±4.0 years) in the two groups were also similar, the males being slightlyolder than the females ($p > 0.05$).Majority of participants in both groups were married (46.7% in patients: 66.7% in healthy controls); and most of them attained secondary education (51% in patients: 76% in healthy controls). More than 70% of patients were employed and 49% earned monthly income of above 19,000 naira, while 51% of healthy controls were employed and 28% earned monthly income of above 19,000 naira. However, the percentage of persons with no source of income was higher among the healthy controls (38%) than the patients (24%). Majority (70 %)of the patients were in WHO clinical stage 1 at time of enrollment and more than 50% were enrolled within a median of 4 months of HIV diagnosis, within a minimum of one day and maximum of 36 months of HIV infection diagnosis respectively, with an interquartile range of one to 12 months.The sociodemographic details of HIV infected ART-naïve patients and HIV negative healthy controls are shown in Table 4.1 below.

Table 4.2: Sociodemographic characteristics of HIV infected ART-naïve patients and matched HIV negative healthy controls

Variable	**HIV infected ART-naïve patients, n=90 (100.0%)**	**HIV negative healthy controls, n=90 (100.0%)**	**Statistical test, *p-value***
Ages (years)	39.0 ±4.0	40.0 ±2.2	□ □ t=0.761, p=0.7
Sex			□ □ □□ □χ^2, p-value
Males	21 (23.3)	21 (23.3)	NS
Females	69 (76.7)	69 (76.7)	NS
Marital status			
Married	42 (46.7)	60 (66.7)	0.03□
Single	26 (28.9)	13 (14.4)	0.05□
Widow	14 (15.6)	10 (11.1)	NS
Divorced	8 (8.9)	7 (7.7)	NS
Educational level			
Primary	10 (11.1)	12 (13.3)	NS
Secondary	46 (51.1)	68 (75.6)	0.01□
Tertiary	34 (37.8)	10 (11.1)	0.04□
Occupation			
Employed	62 (69.0)	56 (62.2)	0.03□
Not employed	28 (31.1)	34 (37.8)	NS
Income per month			
≤18, 000.00 naira	18 (20.0)	31 (34.4)	0.03□
≥19,000.00 naira	44 (49.0)	25 (27.8)	0.05□
WHO clinical stage			
I	66 (73.3	-	
II	24 (26.7)	-	

Statistical analysis with □ □Independent samples T test and □ □□ □chi-square (χ^2);
□ Statistical significance (*p≤0.05*); NS= not statistically significant (*p> 0.05*).

4.2Sociodemographic Factors that Influenced the Retention ofART-Naïve HIV Patients on Micronutrient Supplementation

Sociodemographic factors that influenced the retention of the patients on micronutrientsupplementation at 48 weeks were age(21-40 years old) (*p=0.05*),being employed (*p=0.03*) and having a monthly income of ≥ 19,000.00 naira (*p=0.04*). All other characteristics, such as sex, marital status, religion, educational attainment, WHO clinical stage of HIV infection, and duration of diagnosis of HIV before enrolment into the research were not statistically significant. The details are shown in Table 4.2 below.

Table 4.2: Effect of sociodemographic characteristics on retention of HIV infected ART-naïvepatients oncare with micronutrient supplementation at 24 and 48 weeks respectively

Characteristics	**At baseline n=90 (100.0%)**	**At 24weeksn=68 (75.6%)**	**Chi-square, *p value***	**At 48 weeks n=57 (63.3%)**	**Chi-square, *p value***
Age group (years)					
≤ 20-40	56 (62.2)	40 (44.4)	2.284,	31 (34.4)	5.84,
41-60	34 (37.8)	28 (31.2)	0.3	26(28.9)	0.05□
Gender					
Females	69 (76.7)	51(56.7)	0.432,	42 (46.7)	0.773,
Males	21 (23.3)	17 (18.9)	0.5	15 (16.7)	0.4
Religion					
Christianity	73 (81.1)	57 (63.3)	1.336,	49 (54.4)	2.390,
Islam	17 (18.9)	11 (12.2)	0.3	8 (8.9)	0.1
Marital status					
Married/widowed/ divorced	64 (71.1)	48 (53.3)	1.101, 0.8	40 (44.4)	0.784, 0.9
Never married	26 (28.9)	20 (22.2)		17 (18.9)	
Education					
Primary/ Secondary	56 (62.2)	44 (48.9)	1.391, 0.5	36 (40.0)	1.352, 0.5
Tertiary	34 (37.8)	24 (26.7)		21 (23.3)	
Occupation					
Employed	68 (75.6)	52 (57.8)	9.315,	44 (48.9)	12.12,
Not employed	22 (24.4)	16 (17.7)	0.1	13 (14.4)	0.03□
Income per month in naira					
None-18, 000.00	46 (51.1)	24 (26.7)	11.59,	13 (14.4)	9.43,
≥19,000	44 (49.0)	44 (49.0)	0.03□	44 (49.0)	0.04□
WHO clinical stage					
1	66 (73.3)	56 (62.3)	0.036,	44 (48.9)	0.137,
2	24 (26.7)	12 (13.3)	0.9	13 (14.4)	0.5
HIV diagnosis in months					
≤ 3-12	58 (64.4)	42 (46.7)	4.204,	32 (35.6)	3.168,
≥13	33 (35.5)	26 (28.8)	0.3	25 (27.7)	0.4

*p- values*were calculatedusing Chi square; □statistically significant (*p≤0.05*)

4.3 Clinical Features of HIV Infected ART-Naïve Patients

4.3.1Vital signs and body mass index (BMI)of HIV infected ART-naïve patients at baseline compared withmatched HIV negative healthy controls

The HIV infected patients had significantly higher axilla temperature (38.2±0.7^{0}C), pulse rate (78.0±10.1beats/minute), respiratory rate (16.0±1.0 breaths/minutes) and systolic blood pressure (138.0±37.4 mmHg) than the HIV negative healthy controls, with 36.8±0.2^{0}C, 72.0±21.1 beats/minutes, 12.0±3.0 breaths/ minutes and 128.0±13.7 mmHg ($p \leq 0.05$). Conversely, the HIV infected patients had significantly lower BMI (25.8 kg/m^2) anddiastolic blood pressure (72.0±18.4 mmHg) than HIV negative controls' BMI (27.5 kg/m^2) and diastolicblood (87.0±10.2 mmHg) ($p > 0.05$).The details of these are shown in Table 4.3 below

Table 4.3: Vital signs and BMI of HIV infected ART-naïve patients at baseline compared withmatched HIV negative healthy controls

Variables	**HIV negative healthy controls n=90 (100%)**	**HIV infected patients at baseline n=90 (100%)**	***p- value***
Temperature (^{0}C)	36.8±0.2 (35.8-37.6)	38.2±0.7 (37.3-39.8)	0.04□ (0.04)□
Pulse rate (beats/minutes)	72.0 ±21.1 (68.0-96.0)	78.0 ±10.1 (64.0-108.0)	0.04□ (0.23)
Respiratory rate (breaths/minutes)	12.0 ±3.0 (13.0-16.0)	16.0 ±1.0 (14.0-19.0)	0.01□ (0.53)
Systolic blood pressure (mmHg)	128.0 ±13.7 (104-138.0)	138.0 ±37.4 (114.0-152.0)	0.05□ (0.02)□
Diastolic blood pressure (mmHg)	87.0 ±18.4 (64.0-104.0)	72.0±18.4 (66.0-120.0)	0.05□ (0.42)
□***BMI (kg/m^2)**	27.5(18.6-30.2)	25.8 (17.4-41.7)	0.03□ (0.08)

□statistically significant (*p≤0.05*); □*median value, interquartile rangesand their *p-values* (in parentheses) were calculated using Independent Samples median test and Independent Samples Mann-Whitney U Test respectively; mean ±SD, extreme range (minimum-maximum) and their p-values (in parentheses) were calculated using Independent Samples mean t test and Independent Samples Moses test of extreme reaction respectively.

4.3.2 Effect of micronutrient supplementationon vital signs and BMIof HIV infected ART-naïve patients

Following micronutrient supplementation, BMI of HIV infected patients increased progressively from 25. 8 kg/m^2 to 26. 5 kg/m^2 at 24 weeks ($p>0.05$) and to 27.1kg/m^2 at 48 weeks ($p\leq0.05$) to almost same level with healthy controls ($p>0.05$); while thevital signs, particularly the temperature, pulse rates, and diastolic blood pressures reduced significantly at 24 and 48 weeks ($p\leq0.05$), to be at almost same levels with healthy controls ($p>0.05$). The details of these are shown in Table 4.4 below.

Table 4.4: Effect of micronutrient supplementation on vital signs and BMI of HIV infected ART-naïve patients

Variables	**HIV negative healthy controls n=90 (100%)**	**HIV infected patients at baseline n=90 (100%)**	**HIV infected patients at 24 weeks n=68 (75.6%)**	***p-value* for HIV infected patients at baseline vs at 24 weeks**	**HIV infected patients at 48 weeks n=57 (63.3%)**	***p-value* for HIV infected patients at baseline vs at 48 weeks**	***p-value* for HIV infected patients at 48 weeks vs healthy controls**
Temperature (^{0}C)	36.8±0.2 (35.8-37.6)	38.2±0.7 (37.3-39.8)	36.4±1.2 (35.8-37.2)	0.08 (0.78)	37.2±0.9 (35.6-37.9)	0.04□ (0.05)□	0.99
Pulse rate (beats/minutes)	72.0 ±21.1 (68.0-96.0)	78.0 ±10.1 (64.0-108.0)	72.0 ±10.0 (60.0-90.0)	0.03□ (0.01)□	72.0±2.0 (60.0-84.0)	0.00□ (0.00)□	0.87
Respiratory rate (breaths/minutes)	12.0 ±3.0 (13.0-16.0)	16.0 ±1.0 (14.0-19.0)	14.0 ±1.0 (13.0-16.0)	0.04□ (0.07)	16.0±0.0 (14.0-18.0)	0.04□ (0.00)□	0.05□
Systolic blood pressure (mmHg)	128.0 ±13.7 (104-138.0)	138.0 ±37.4 (114.0-152.0)	142.0 ±16.0 (100.0-140.0)	0.00□ (0.7)	138.0±42.0 (90.0-142.0)	0.9 (0.00)□	0.04□
Diastolic blood pressure (mmHg)	87.0 ±18.4 (64.0-104.0)	72.0±18.4 (66.0-120.0)	71.8±12.1 (64.0-100.0)	0.8 (0.07)	80.0±12.0 (60.0-132,0)	0.06 (0.7)	0.06
□***BMI (kg/m^2)**	27.5 (18.6-30.2)	25.8 (17.4-41.7)	26.5 (17.8-43.8)	0.75 (0.47)	27.1 (19.4-44.1)	0.05□ (0.05)□	0.98

*statistically significant ($p\leq0.05$); □*median value, interquartile ranges(IQR) and their *p-values* (in parentheses) were calculated using Independent Samples median test and Independent Samples Mann-Whitney U Test respectively; mean ±SD, extreme range (minimum-maximum) and their *p-values* (in parentheses) were calculated using Independent Samples mean t test and Independent Samples Moses test of extreme reaction respectively; *p-values* for mean±SD, □ □ medianJQR and ranges of patients at 48 weeks vs healthy controls were calculated using Independent Samples Kruskal Wallis test.

4.3.3Effect of micronutrientsupplementation on the incidence of opportunistic infections/ diseasesin HIV infectedART-naïve patients

As shown in Table 4.5 below, the incidence of symptoms and signs of illnesses attributable to HIV infection or disease decreased significantly from 16 (17.8%) at baseline to 6 (5.9%) (*p=0.003*) and3 (5.3%) (*p=0.002*) at 24 and 48 weeks of micronutrient supplementation. Inspite of these reductions, one patienteach developed pneumonia and peripheral neuropathy respectively at 24 weeks of micronutrient supplementation.

Table 4.5:Effect of micronutrient supplementation on the incidence of opportunistic infections/ diseasesin the HIV infected ART-naïve patients

Opportunistic infections/ diseases	**At baseline n=90 (100.0%)**	**At 24 weeks n=68 (75.6%)**	**Chi-square, *p-value***	**At 48 weeks n=57 (63.3%)**	**Chi-square, *p-value***
Herpes zoster	4 (5.9)	1 (1.4)		0 (0.0)	
Weight loss	2 (2.2)	0 (0.0)		1 (1.8)	
Diarrhea	2 (2.2)	0 (0.0)		1 (1.8)	
Vaginal discharge	2 (2.2)	1 (1.5)		0 (0.0)	
Boils	2 (2.2)	0 (0.0)		0 (0.0)	
Bell's palsy	2 (2.2)	0 (0.0)		1 (1.7)	
Skin rash	2 (2.2)	0 (0.0)		0 (0.0)	
Peripheral neuropathy	0 (0.0)	1 (1.5)		0 (0.0)	
Pneumonia	0 (0.0)	1 (1.5)		0 (0.0)	
Total	16 (17.8)	4 (5.9)	11.15, 0.00□	3 (5.3)	17.14. 0.00□

*p- values*were calculatedusing Chi square; □statistically significant (*p≤0.05*);
□Statistically significant (p≤0.05)

4.3.4Effect of micronutrient supplementation on the incidence of non HIV-specific symptoms in HIV infected ART-naïve patients

At baseline, 28 (31.0%) of patients had non HIV-specific symptoms. The frequency of the symptoms were as follows: body weakness [11 (12.2%)], body itching [6 (6.7%)], headache [4 (4.4%)], body pains [2 (2.2%)], fever [2 (2.2%)], cough [2 (2.2%)] and eye pains [1 (1.1%)]. However, the frequency of symptoms reduced significantly to 20 (22.2%) at 24 weeks($\square^2$=13.6, *p=0.00*), and to 9 (10.0) at 48 weeks ($\square^2$ =21.703, *p=0.00*) respectively; as shown in Table 4.6 below.

Table 4.6: Effect of micronutrient supplementation on the incidence of non HIV-specific symptoms in HIV infected ART- naïvepatients

Non HIV-specific symptoms	**At baseline, n=90(100.0)**	**At 24 weeks, n=68(75.6%)**	**Chi-square, *p-value***	**At 48 weeks, n=57(63.3%)**	**Chi-square, *p-value***
Body weakness	11 (12.2)	11 (12.2)		4 (4.4)	
Body itching	6 (6.7)	2 (2.2)		1 (1.1)	
Headache	4 (4.4)	4 (4.4)		3 (3.3)	
Body pains	2 (2.2)	1 (1.1)		1 (1.1)	
Fever	2 (2.2)	1 (1.1)		0 (0.0)	
Cough	2 (2.2)	1 (1.1)		0 (0.0)	
Eye pains	1 (1.1)	0 (0.0)		0 (0.0)	
Total	28 (31.0)	20 (22.2)	13.6, 0.01□	9 (10.0)	21.7, 0.00□

*p- values*were calculatedusing Chi square; □statistically significant (*p≤0.05*)

4.4Concentrations of Nutritional Indices inHIV Infected ART-Naïve Patients Compared with HIV Negative Healthy Controls

4.4.1Concentrations of haemoglobin, serum albumin, zinc, copper and vitamin B12 inHIV infected ART-naïve patients compared with HIV negative healthy controls

HIV infected ART-naïve patients had lower baseline nutritional indices than matched HIV negative controls; all at significant levels except haemoglobin and serum vitamin B_{12} concentrations. The patients' haemoglobinlevels (median=11.0 g/dL, IQR=11.2-13.0g/dL)were slightly lower than healthy controls (median=12.3g/dL, IQR=11.6-13.0g/dL) (*p>0.05*), but their extreme ranges (patients' range=7.4-15.0 g/dL vs healthy controls' range=11.3g/dL-15.8g/dL) were significant (*p=0.01*). Conversely, patients' serum vitamin B_{12}(median=15.0 ng/L, IQR=9.9-39.4 ng/L) were significantly lower than healthy controls (median=315.0 ng/L, IQR=231.7- 465.2 ng/L) (*p≤0.05*), while their respective extreme ranges (patients' range=1.0-346.1ng/L vs healthy controls' range =111.7- 1292.0 ng/L) were insignificant (*p=0.9*).The values of patients' serum albumin (median=36.0g/L, IQR=33.0-40.0g/dL, range=30-44.0g/dL) vshealthy controls (median=39.0g/L, IQR=37.0-41.0g/dL, range=33.0-48.0g/dL) (*p≤0.05*); patients' serum zinc (median= 0.01ppm, IQR= 0.01-0.03ppm, range=0.0 -0.83ppm) vs healthy controls (median =0.16, IQR=0.14 - 0.23ppm, range=0.06 - 0.3ppm) (*p≤0.05*); patients' serum copper (median= -0.4ppm, IQR= -0.5 - 0.03 ppm, range= -0.8 - 0.05ppm) vs healthy controls (median=0.04 ppm, IQR=0.04 - 0.05ppm, range=0.1 - 0.23ppm) (*p≤0.05*) are as shown in Table 4.7 below.

Table 4.7: Concentrations of nutritional indices in HIV infected ART-naïve patients at baseline compared with HIV negative healthy controls

Nutritional variable	**HIV negative healthy controls N=90 (100%)**	**HIV infected patients at baseline N=90 (100%)**	***p-value* for median (IQR)**
Haemoglobin (g/dL)	12.3 (11.6-13.0)	11.0 (11.2-13.0)	0.39 (0.89)
Albumin (g/L)	39.0 (37.0-41.0)	36.0 (33.-40.3)	0.00□ (0.04)□
Zinc (ppm)	0.16 (0.14-0.23)	0.01 (0.01-0.03)	0.00□ (0.00)□
Copper (ppm)	0.04 (0.04-0.05)	-0.4 (-0.5-0.03)	0.00□ (0.00)□
Vitamin B12 (ng/L)	315.0 (231.7-465.2)	15.0 (9.9-39.4)	0.00□ (0.00)□

□statistically significant (*p≤0.05*); median value, interquartile ranges(IQR) and their *p-values* (in parentheses) were calculated using Independent Samples median test and Independent Samples Mann-Whitney U Test respectively. *p-values* for HIV negative healthy controls vs HIV infected ART-naïve patients at baseline.

4.4.2Concentrations of serum lipidsin HIV infected ART-naïve patients compared with HIV negative healthy controls

The serum lipids profile consists of total cholesterol (TC), high density lipoprotein (HDL-C), low density lipoprotein (LDL-C) and triglycerides (TG). There were no significant differences between baseline lipid profile of patients and healthy controls; except the extreme ranges of TC (patient=3.8-6.2mmol/L vs healthy=3.0-8.1mmol/L)(*p=0.01*). The lipid profiles of patients' and healthy controls' are as follows: patients' TC (median=4.6 mmol/L; IQR=4.4-4.8 mmol/L vs healthy controls (median=4.7 mmol/L and IQR=4.5- 4.8 mmol/L) (*p>0.05*); patients' serum HDL-C (median=1.6mmol/L, IQR=1.3-1.9 mmol/L, range=0.7- 2.5mmol/L) vs healthy controls' (median=1.7mmol/L, IQR=1.3-2.0mmol/L, range=0.4-3.0 mmol/L) (*p>0.05*); patients' LDL-C(median=2.4 mmol/L, IQR=2.1-2.8 mmol/L, range=0.9-3.9 mmol/L) vs healthy controls' (median=2.6 mmol/L, IQR=2.2-2.9 mmol/L, range=1.3-3.7 mmol/L) (*p>0.05*); patients' serum TG(median=1.1 mmol/L, IQR=0.9-1.2 mmol/L, range=0.7-2.3 mmol/L) vs healthy controls (median=1.1 mmol/L, IQR=0.9-1.2 mmol/L, range=0.6-2.1 mmol/L) (*p>0.05*). The details of the lipid concentrations are summarized in Table 4.8 below.

Table 4.8: Concentrations of lipids among HIV infected ART-naïve patients compared with HIV negative healthy controls

Nutritional variable	HIV negative healthy controls n=90 (100%)	HIV infected patients at baseline n=90 (100%)	*p-value* for HIV infected patients at baseline vs healthy controls
Total cholesterol (mmol/L)	4.7 (4.5-4.8)	4.6 (4.4-4.8)	0.4 (0.01)□
High density lipoproteins (mmol/L)	1.7 (0.4-3.0)	1.6 (0.7-2.5)	0.4 (0.8)
Low density lipoproteins (mmol/L)	2.6 (1.3-3.7)	2.4 (2.1-2.8)	0.1 (0.5)
Triglycerides (mmol/L)	1.1 (0.6-2.1)	1.1 (0.9-1.2)	0.1 (0.2)

□statistically significant (*p≤0.05*); median value, interquartile ranges(IQR) and their *p-values*(in parentheses) were calculated using Independent Samples median test and Independent Samples Mann-Whitney U Test respectively.

4.5Effect of Micronutrient Supplementation on Nutritional Indices

ofProgression of HIV Infection among HIV infected ART-Naïve Patients

4.5.1Effect of micronutrient supplementation on haemoglobin, serum albumin, zinc, copper and vitamin B_{12} in HIV infected ART-naïve patients

Following micronutrient supplementation, the concentrations of the above nutritional indices increased progressively to reach almost same levels as the healthy controls at 48 weeks as shown in Table 4.9 below. The patients' haemoglobin concentrationsrose to became equal with those of healthy controls at 24 weeks (median=12.0g/dL, IQR=11.7-13.0g/dL, range=9.0-15.3g/dL) ($p>0.05$), and48 weeks (median=12.3g/dL, IQR=11.4-13.5g/dL, range=10.0-17.0g/dL)($p>0.05$); serum albumin increased to normal levels at 24 weeks (median=38.0g/dL, IQR=35.0-40.0g/dL, range=30.0-48.0g/dL) ($p>0.05$), and at 48 weeks (median=38.0g/dL, IQR=34.0-42.0g/dL, range=31.0-55.0g/dL) ($p>0.05$)); serum zinc increased significantly to surpass levels in healthy controls at 24 weeks (median=0.23ppm, IQR=0.21=0.27ppm, range=0.2-0.6ppm) ($p\leq0.05$) andat 48 weeks (median=0.3ppm, IQR=0.23=0.33ppm, range=0.2-0.8ppm)($p\leq0.05$); serum copper increased significantly at 24 weeks (median=0.03ppm, IQR=0.02- 0.03ppm, range=0.01 - 0.05ppm) ($p\leq0.05$) and at 48 weeks (median=0.05ppm, IQR=0.04 - 0.06ppm, range=0.06 - 0.2 ppm)to slightly surpass levels in healthy controls (p>0.05); serum vitamin B12 values increased significantly at 24 weeks (median=30.0ng/L, IQR=17.8 - 50.7ng/L, range=2.8 - 452.5ng/L), ($p\leq0.05$), but remained significantly lower than those of healthy controls (($p\leq0.05$); though extreme range(2.8 -452.5ng/L) was similar to the healthy controls' range of 111.7- 1292.0 ng/l ($p>0.05$).

Table 4.9: Effect of micronutrient supplementation on nutritional indices of HIV infection progression in HIV infected ART-naïve patients

Nutritional variables	**HIV negative healthy controls n=90 (100%)**	**HIV infected patients at baseline n=90 (100%)**	**HIV infected patients at 24 weeks n=68 (75.6%)**	***p-value* for HIV infected patients at baseline vs at 24 weeks**	**HIV infected patients at 48 weeks n=57 (63.3%)**	***p-value* for HIV infected patients at baseline vs at 48 weeks)**	***p-value* for HIV infected patients at 48 weeks vs healthy controls**
Haemoglobin (g/dL)	12.3 (11.6-13.0)	11.0 (11.2-13.0)	12.0 (11.7-13.0)	0.8 (0.8)	12.3 (11.4-13.5)	0.7 (0.7)	0.38
Albumin (g/L)	39.0 (37.0-41.0)	36.0 (33.-40.3)	38.0 (35.0-40.0)	0.32 (0.1)	38.0 (34.0-42.0)	0.32 (0.08)	0.21
Zinc (ppm)	0.16 (0.14-0.23)	0.01 (0.01-0.03)	0.23 (0.21-0.27)	0.00 (0.00)	0.3 (0.23-0.33)	0.00 (0.00)	0.00
Copper (ppm)	0.04 (0.04-0.05)	-0.4 (-0.5-0.03)	0.03 (0.02-0.03)	0.00 (0.00)	0.05 (0.04-0.06)	0.00 (0.00)	0.89
Vitamin B12 (ng/L)	315.0 (231.7-465.2)	15.0 (9.9-39.4)	30.0 (17.8-50.7)	0.02 (0.00)*	-	-	-

statistically significant (*p≤0.05*); interquartile ranges (IQR) and their *p-values* are in parentheses; *p-values* for median and IQR at baseline vs 24 weeks, baseline vs 48 weeks were calculated using Independent Samples median test and Independent Samples Mann-Whitney U test respectively. *p-value* for median, IQR and ranges at 48 weeks (*24 weeks for vitamin B12) vs healthy controlswere calculated using One way AnovaKruskal Wallis test; vitamin B_{12}wasnot analysed at 48 weeks due to lack of reagents

4.5.2 Effect of micronutrient supplementation on the concentrations of lipidsin HIV infected ART-naïve patients

Micronutrient supplementation produced variable changes in the baseline lipid profile of the patients. Serum TCconcentrations reduced significantly at 24 weeks (median=4.2mmol/L, IQR=4.0 - 4.5mmol/L) ($p \leq 0.05$), and at 48 weeks (median=4.3mmol/L, IQR=4.1- 4.7mmol/L) ($p \leq 0.05$), to below those of healthy controls (median=4.7 mmol/L and IQR=4.5- 4.8 mmol/L) ($p \leq 0.05$); serum HDL-C concentrations reduced significantly at 24 weeks (median=1.3mmol/L,IQR= 1.2 - 1.6mmol/L, range= 0.8-2.1mmol/L) ($p \leq 0.05$), and at 48 weeks (median=1.2mmol/L, IQR=1.1- 1.5mmol/L, range=0.7-2.0mmol/L) ($p \leq 0.05$), below those of healthy controls ($p \leq 0.05$). However, TG concentrations reduced significantly at 24 weeks (median=1.0 mmol/L, IQR=0.7-1.0 mmol/L) ($p \leq 0.05$) but increased at 48 weeks (median=1.1 mmol/L, IQR=1.0-1.2) to levels similar to the healthy controls (median=1.1 mmol/L, IQR=0.9-1.2 mmol/L)($p > 0.05$). Serum LDL-C reduced insignificantly at 24 weeks (median=2.3 mmol/L, IQR=2.0-2.7 mmol/L) ($p > 0.05$), and increased at 48 weeks (median=2.6 mmol/L, IQR=2.3-2.8 mmol/L) to levels similar to the health controls (median=2.6 mmol/L, IQR=2.2-2.9 mmol/L) ($p > 0.05$). The trend of lipid changes with micronutrient supplementation is shown in Table 4.10 below.

Table 4.10: Effect of micronutrient supplementation on lipids in HIV infected ART-naïve patients

Nutritional variables	**HIV negative healthy controls n=90 (100%)**	**HIV infected patients at baseline n=90 (100%)**	**HIV infected patients at 24 weeks n=68 (75.6%)**	***p-value* for HIV infected patients at baseline vs at 24 weeks**	**HIV infected patients at 48 weeks n=57 (63.3%)**	***p-value* for HIV infected patients at baseline vs at 48 weeks**	***p-value* for HIV infected patients at 48 weeks vs healthy controls**
Total cholesterol (mmol/L)	4.7 (4.5-4.8)	4.6 (4.4-4.8)	4.2 (4.0-4.5)	0.00□ (0.00)□	4.3 (4.1-4.7)	0.00□ (0.00)□	0.05□
High density lipoproteins (mmol/L)	1.7 (1.3-2.0)	1.6 (0.7-2.5)	1.3 (1.2-1.6)	0.00□ (0.00)□	1.2 (1.1-1.5)	0.00□ (0.00)□	0.00□
Low density lipoproteins (mmol/L)	2.6 (1.3-3.7)	2.4 (2.1-2.8)	2.3 (2.0-2.7)	0.4 (0.8)	2.6 (2.3-2.8)	0.09 (0.9)	1.0
Triglycerides (mmol/L)	1.1 (0.6-2.1)	1.1 (0.9-1.2)	1.0 (0.7-1.0)	0.00□ (0.00)□	1.1 (1.0-1.2)	1.0 (0.9)	1.0

□statistically significant; *p-values* for median and interquartile ranges (IQR) at baseline vs 24 weeks, baseline vs 48 weeks were calculated using Independent samples median test and Independent Samples Mann-Whitney U Test respectively. *p-value* for median, IQR and ranges at 48 weeks vs healthy controls were calculated using Kruskal Wallis test; Independent Samples Moses test of extreme reaction was used to calculate *p-values* for ranges at baseline vs 24 weeks, and baseline vs 48 weeks in the text. IQR and their *p-values* are in parentheses.

4.6Effect of Micronutrient Supplementation on Plasma HIV RNA Load and Immunological Indices of HIV Infection Progression inHIVInfected ART-Naïve Patients

4.6.1Effect of micronutrient supplementation on plasma HIV RNA load in HIVinfected ART-naïve patients

Micronutrient supplementation resulted in progressive reduction of patients' baseline plasma HIV RNA load (median=27,105.0 copies/mL, IQR=6,346.0 - 955,514.0 copies/mL, range= 100.0 -8,313,012.0 copies/mL). The reduction was insignificant at 24 weeks (median=16,227.5copies/mL, IQR=6,266.0-73,146.0 copies/mL, range=28.0-1,000,000.0 copies/mL) ($p>0.05$)but became significant at 48 weeks (585.0 copies/mL, IQR=20.0- 2,606.5 copies/mL; range=0.0- 16, 394.0 copies/mL) ($p\leq0.05$); as shown in Table 4.11 below.

4.6.2Effect of micronutrient supplementation on immunological indicesof HIV infection Progression in HIVInfected ART-naïve patients

4.6.2.1CD4+ cell counts

Micronutrient supplementation did not lead to increases in CD4+ cell count. Instead, there were progressive and significantdecrease of patients'CD4+ cell counts from baseline (median=704.5 cells/µL, IQR=600- 880, range=520.0 to 1552.0 cells/µL) to 24 weeks (median=561.0 cells/µL, IQR= 500-700; range=339.0 to 1477 cells/µL) ($p\leq0.05$); and 48weeks (median=595.0 cells/µL, IQR=496-710; range=257.0 to 1198.0 cells/µL) ($p\leq0.05$). Details of the CD4+cell count are shown in Table 4.11 below.

4.6.2.2 Serum interleukin (IL-6)

Micronutrient supplementation resulted in progressive reduction of the patients' serumIL-6 concentrationsfrom baseline (median=1.9 pg/mL, IQR=1.0 -3.8 pg/mL, range= 0.2-16.4 pg/mL)to 24 weeks (median= 1.3pg/mL, IQR= 0.7 -4.3 pg/mL, range=0.4- 12.6.pg/mL) ($p>0.05$); and 48 weeks (median=0.5 pg/mL, IQR=0.3-0.9 pg/mL, range=0.1-4.6 pg/ml) ($p\leq0.05$). Details of the serum IL-6 are shown in Table 4.11 below.

4.6.2.3 Serum C-reactive protein (CRP)

Micronutrient supplementation resulted in significant reductions inthe patients' CRP concentrations from baseline values (median= 29.4 ng/mL, IQR= 12.3-68.8 ng/mL) to 24 weeks' values (median=26.2 ng/mL and IQR=8.8-46.8ng/ml) ($p\leq0.05$). There was also a significant reduction in the median value (median=25.8ng/mL) at 48 weeks (($p\leq0.05$). The baseline range (0.5- 99.0 ng/mL) did not change significantly at 24 weeks (range=0.9-75.0 ng/mL) ($p>0.05$); and both the range (0.4- 51.2ng/mL) and IQR (15.2-44.0 ng/mL) were not significantly different from baseline at 48 weeks ($p>0.05$).The changes in values of serum CRP are shown in Table 4.11 below.

Table 4.11: Effect of micronutrient supplementation on plasma HIV RNA load and immunological indices of HIV infection progression

HIVRNA load and immunological variables	**HIV infected patients at baseline N=90 (100%)**	**HIV infected patients at 24 weeks N=68 (75.6%)**	***p-value* baseline vs 24 weeks**	**HIV infected patients at 48 weeks n=57 (63.3%)**	***p-value* for baseline vs 48 weeks**
	Median (IQR)				
HIV RNA (copies/mL)	27,105.0 (6,346.0-955,514.0)	16,227.5 (6,266.0-73,146.0)	0.32 (0.35)	585.0 (20.0-2,606.5)	0.00□ (0.00)□
CD4+count (cells/µL)	704.5 (600.0- 880.0)	561.0 (500.0-700.0)	0.00□ (0.00)□	595.0 (496.0-710.0)	0.00□ (0.00)□
IL-6 (pg/mL)	1.9 (1.0.-3.8)	1.3 (0.7-4.3)	0.6 (0.8)	0.5 (0.3-0.9)	0.00□ (0.02)□
CRP (ng/mL)	29.4 (12.3-68.8)	26.2 (8.8-46.8)	0.04□ (0.9)	25.8 (15.2-44.0)	0.03□ (0.7)

□statistically significant (*p≤0.05*); *p-values* for median and interquartile ranges (IQR) at baseline vs 24 weeks; baseline vs 48 weeks were calculated using Independent Samples median test and Independent Samples Mann-Whitney U Test respectively. Independent Samples Moses test of extreme reaction was used to calculate *p-values* for ranges at baseline vs 24 weeks, and baseline vs 48 weeks in the text. IQR and their *p-values* are in parentheses.

4.7Bivariate Relationships of Indicesof HIV Infection Progression inART-Naïve PatientsBefore Micronutrient Supplementation

Spearman's correlation coefficients analysis was done to determine significant bivariate relationships between indices of HIV infection progression before micronutrient supplementation with the following results.

4.7.1 Relationship between plasma HIV RNA andnutritional indices

Plasma HIV RNA load had inversecorrelations with zinc (rho= - 0.349, *p=0.00*) and copper (rho= - 0.471, *p=0.00*).

4.7. 2 Relationship between plasma HIV RNA and immunological indices

Plasma HIV RNA load had an inversecorrelation with CD4+ cell count (rho= -0.152, *p=0.04*);and direct correlations withinterleukin-6 (rho=0.332, *p=0.00*)and C-reactive protein (r=0.250, *p=0.04*).

4.7.3Bivariate relationships between immunological indices

CD4+ cell count was inversely correlated with C-reactive protein (rho= -0.166, *p=0.02*), while interleukin-6 was directly correlated with C-reactive protein (rho=0.137, *p=0.06*).

4.7. 4Bivariate relationships between nutritional indices

Zinc was directly correlated with copper (rho=0.672, *p=0.00*) and albumin (rho=0.806, *p=0.00*) and inversely correlated with total cholesterol (rho= -0.253, *p=0.00*); while copper was inversely correlated with total cholesterol (rho= -0.253, *p=0.00*).

4.7.5 Relationships between immunological indices andnutritional indices

C-reactive proteinwas inversely correlated with copper (rho= -0.134, *p=0.06*) and zinc (rho= -0.132, *p=0.07*); CD4+ cell count was inversely correlated with zinc (rho= -0.264, *p=0.00*) and copper (rho= -0.471, *p= 0.00*); and directly correlated with total cholesterol (rho=0.158, *p=0.04*); while interleukin-6 was directly correlated with total cholesterol (rho=0.158, *p=0.04*), and HDL-C (rho=0.181, *p=0.01*), and inversely correlated with zinc (rho= -0.302, *p=0.00*) and copper (rho= -0.355,*p=0.00*).

4. 8Effect ofMicronutrient Supplementation on the Relationships BetweenHIV RNA Load and Other Indices of HIV Infection Progression

Pearson's Correlation coefficients analysis was done to determine therelationship between HIV RNA load and other indices of HIV infection at 24 weeks and 48 weeks of micronutrient supplementation respectively.

4.8.1Relationships between HIV RNA load and other indices at 24 weeks

HIV RNA load was directly correlated with interleukin-6 (r=0.35, *p=0.00*) and inversely correlated with zinc (r= -0.917, *p= 0.00*). HIV RNA also had insignificant inverse correlation with BMI (r= -0.176, *p= 0.15*), haemoglobin (r= -0.024, *p= 0.85*), vitamin B_{12} (r= -0.180, *p=0.89*), albumin (r= -0.164, *p= 0.18*), and copper (r= -0.156, *p= 0.21*).

4.8. 2 Relationships between HIV RNA load and other indices at 48 weeks

HIV was inversely correlated with haemoglobin (r= -0.31, *p= 0.02*) and zinc (r= -0.27, *p= 0.04*).

4.9Significant Predictor (s) of Plasma HIV RNA Load and HIV Infection Progression in HIV infected ART-naïve Patients

Stepwise logistic regression of all the nutritional and immunological indices of HIV infection progression against plasma HIV RNA load was performed to determine the most significant predictor (s) of HIV RNA load before and with micronutrient supplementation.

4.9.1Predictor (s) of plasma HIV RNA load in HIV infected ART-naïve patients before micronutrient supplementation

The result showed that serum zinc(Beta=0.927, *p=0.00*),and interleukin-6 (Beta=0.094, *p=0.05*) were significant predictors of HIV RNA load before micronutrient supplementation. The relationship between plasma HIV load and serum zinc (Figure 4.1), and between plasma HIV load and serum interleukin -6(Figure 4.2) respectively, are shown below.

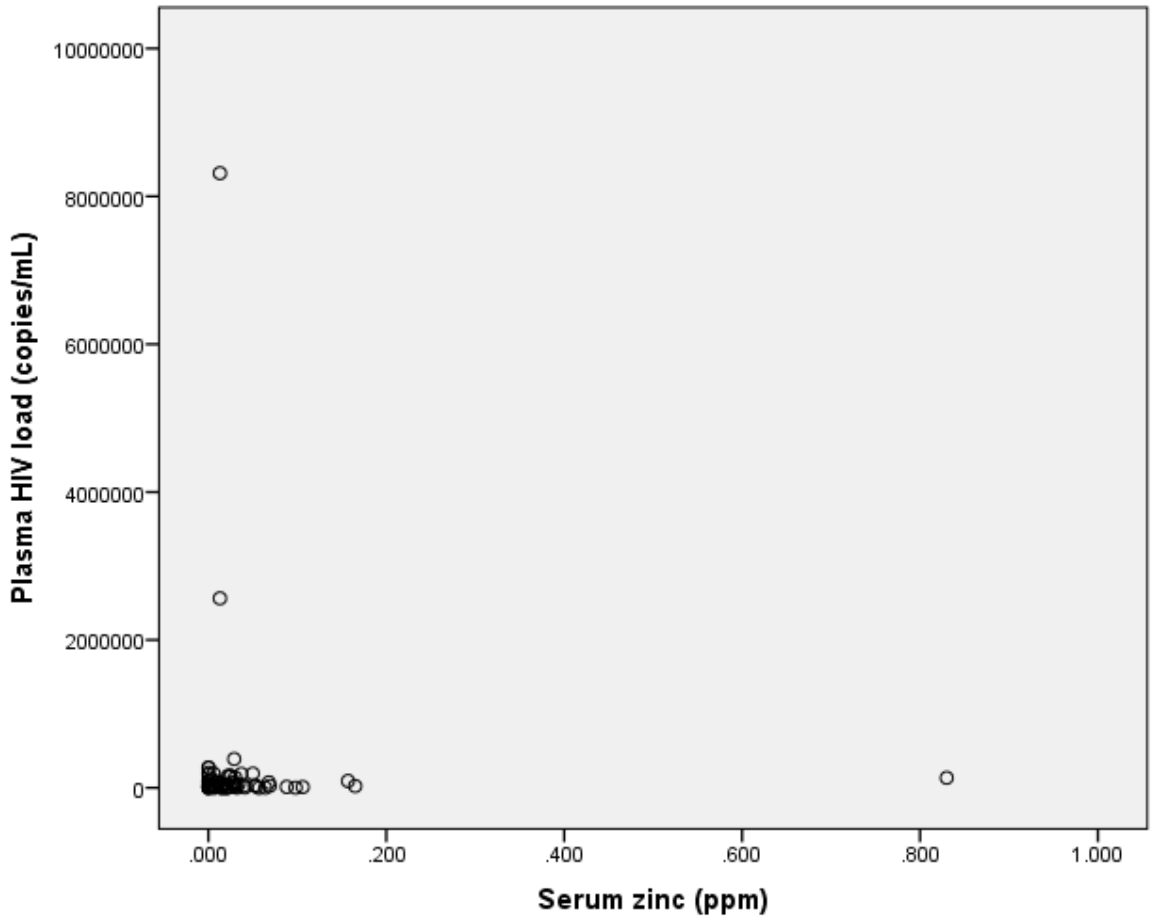


Figure 4.1: Relationship between plasma HIV RNA load and serum zinc in HIV infected ART-naïve patients before micronutrient supplementation

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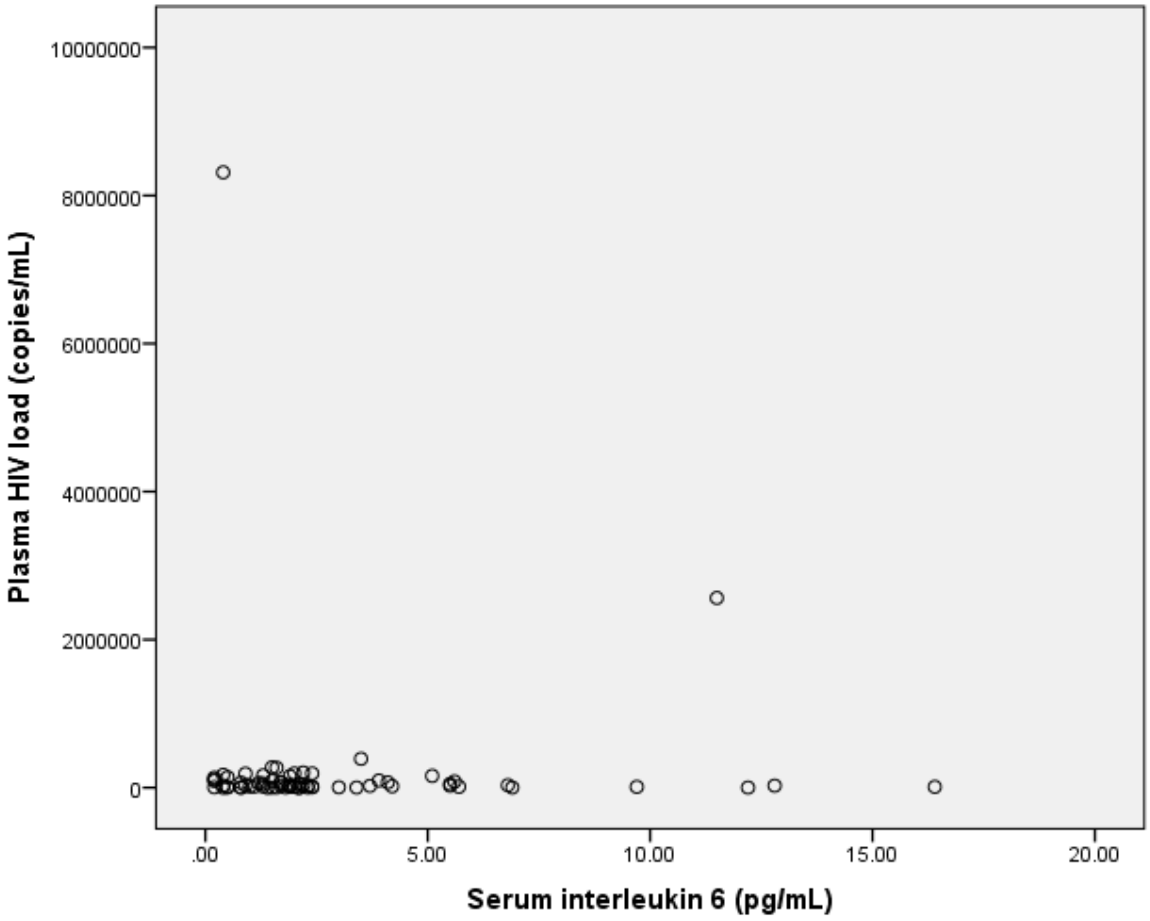


Figure 4.2: Relationship between plasma HIV RNA load and serum interleukin-6in HIV infected ART-naïve patients before micronutrient supplementation

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4.9.2 Predictor (s) of plasma HIV RNA load at 24 and 48 weeks of micronutrient supplementation

The result showedthat interleukin-6 (Beta=0.354, *p=0.00*) and haemoglobin (Beta=0.306, *p=0.02*) were the most significant predictors of plasma HIV RNA load at 24 and 48 weeksof micronutrient supplementation respectively. The relationship between plasma HIV RNA load and interleukin at 24 weeks of micronutrient supplementation (Figure 4.3); and between plasma HIV RNA load and haemoglobinat 48 weeks of micronutrient supplementation(Figure 4.4) respectively are shown below.

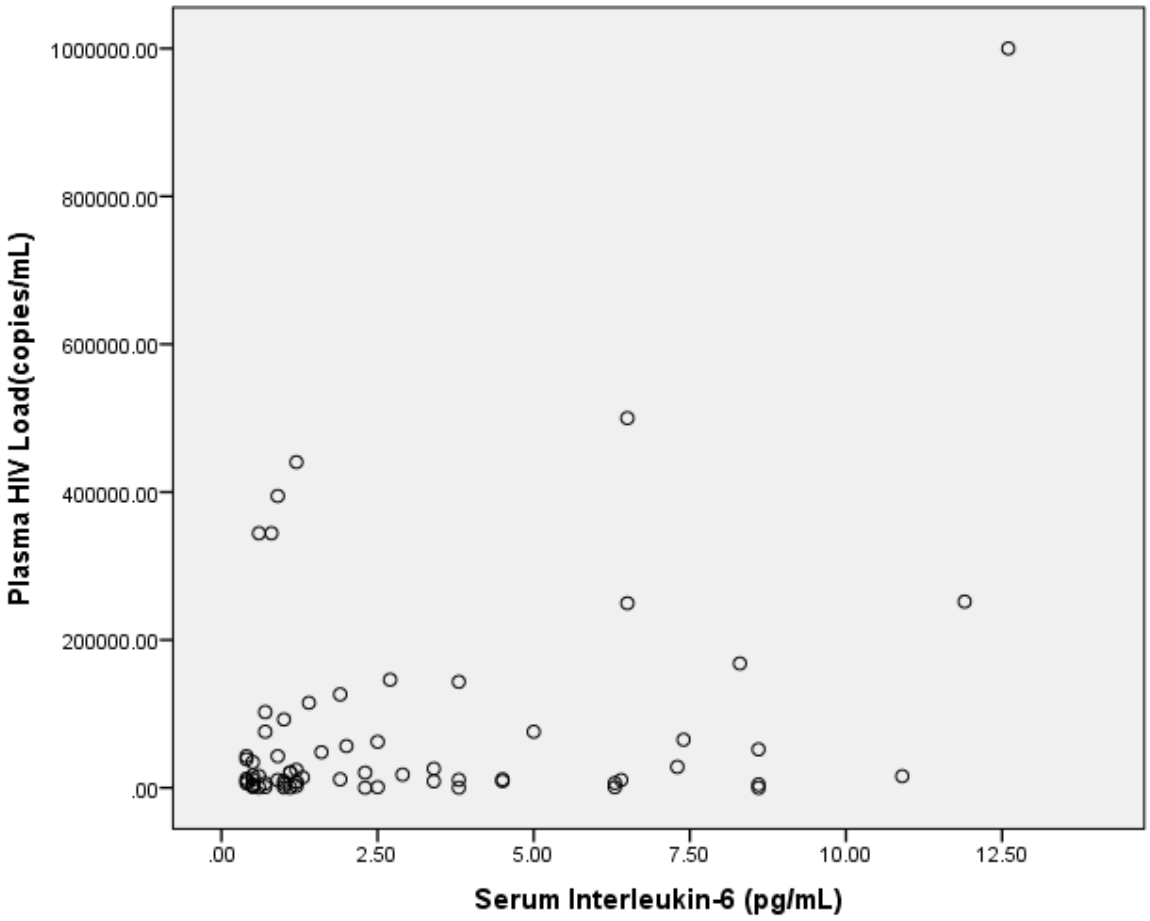


Figure 4.3: Relationship between HIV RNA load and serum interleukin -6 in HIV infected ART-naïve patients at 24 weeks of micronutrient supplementation

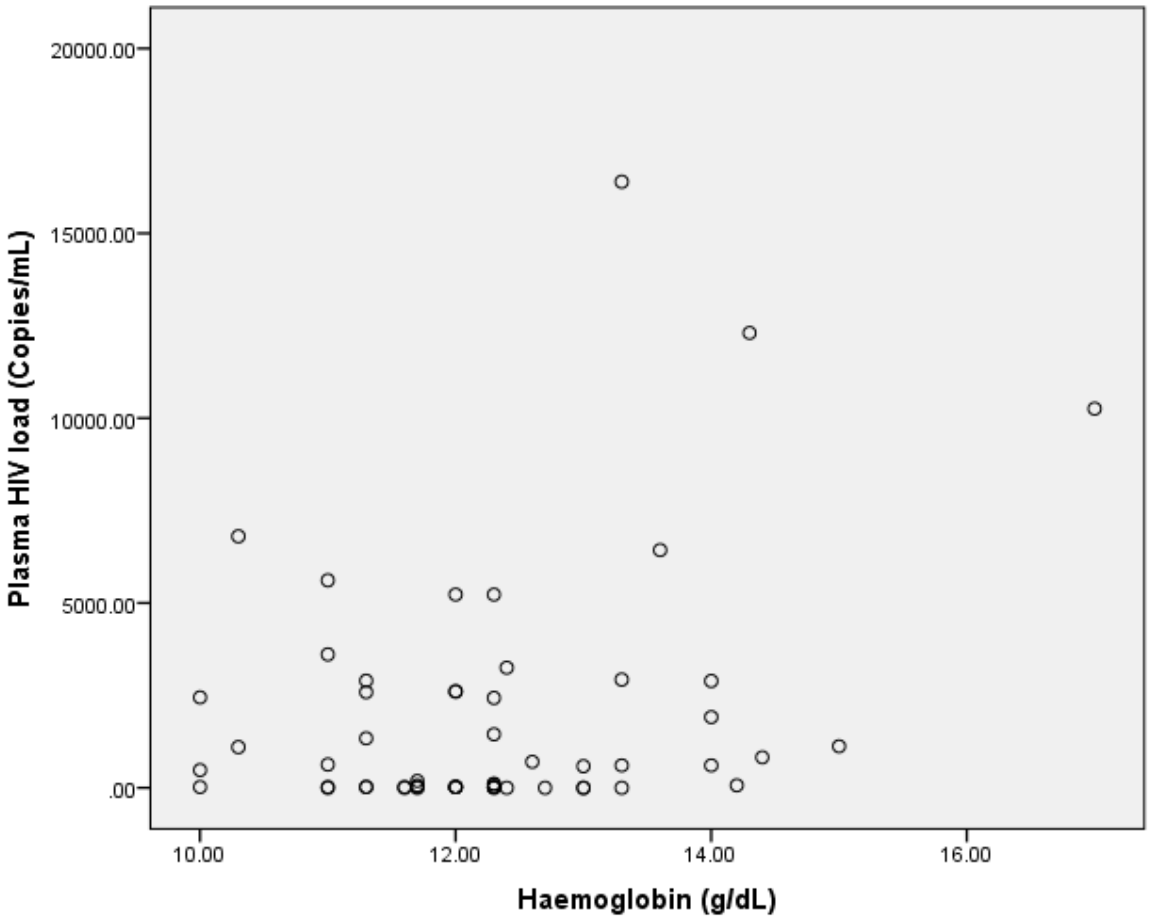


Figure 4.4: Relationship between HIV RNA load and haemoglobinin HIV infected ART-naïve patients at 48 weeks of micronutrient supplementation

CHAPTER FIVE

5.0DISCUSSION

5.1Indices of HIV InfectionProgression among HIV-infected ART-Naïve Patients

5.1.1Socio-demographic characteristics of HIV-infected ART-naïve patients

In this study, females were more than males supporting many previous reports of female dominance in accessing hospital based HIV care, support and treatment service in Nigeria (Obiako *et al.,* 2011; Obiako *et al.,* 2012; NACA, 2015). The female dominance in the HIV treatment response has been attributed to the reluctance of males to access HIV care services due to self-denial and psychological fear of stigmatization and discrimination (Yeldu *et al.,* 2016). Majority of the patients were married, and belonged to young to middle age groups;supporting the findings of Orubuloye*et al.,* (1992) and Adeokun (2006) that the main route of HIV infection in Nigeria is mainly through heterosexual transmission by people of reproductive age groups. All the patients in this study attained formal education and more than 50% at secondary school level. Also, more than 60% of them were employed and 40% earned above the Nigeria minimum wage of 18,000.00 naira ($45.00). These observations are contrary to the NARHS 2012 report,which showed that women, youths, and people of low socioeconomic level were worst affected by the HIV pandemic in Nigeria (NACA, 2012). However, many factors in this research may be responsible for this difference. The research is hospital based as against a community based research. In addition, only patients who came the research sites for care were and met inclusion criteria were recruited into the study.

5.1.2The World Health Organization clinical stage of HIV infected ART-naïve patients

About 73% of the patients were in WHO clinical stage 1 of HIV disease, and were recruited within 4 months of a positive HIV test, while 27% were at WHO clinical stage 2, and had been diagnosed HIV positive for more than two years before enrollment into this study. According to WHO classification of body mass index (Crook, 2012), 25% of the patients were underweight (median BMI of 17.0kg/m^2), 50% were overweight (median BMI of 25.8 kg/m^2) and 25% were either obese (median BMI of ≥ 30.0 kg/m^2)or morbidly obese (median BMI of ≥ 40.0 kg/m^2). These findings are contrary to those of Yeldu *et al.,* (2016), which reported a mean BMI of 24.5±0.6 kg/m^2 among their ART-naïve patients in Sokoto, Nigeria; but support the reports of van der Sander *et al*., (2004) that abnormal BMI, particularly low BMI, is an independent predictor of rapid progression of HIV infection to AIDS and death. However, despite the abnormal BMI, majority of the patients were either asymptomatic or had mild symptoms and signs of HIV disease; and their vital signsand CD4+ cell counts were withinreference range for healthy adults in Nigeria (Oladepo*et al.,* 2009).

5.1.3 The plasma HIV RNA load of HIV infected ART-naïve patients and immunological indices of HIV infection progression

5.1.3.1 *Long term non progressors among the HIV infected ART-naïve patients*

The patients in this study had pVL which ranged from forty (40)copies/mL to above eight million (8,313,012.0) copies/mL. The pVL of 40 copies/mL was found in a 36 year old female patient who was enrolled at 24 months of a positive HIV test with a CD4+ cell count of 1608.0/ µl and without any form of medications. This type of patient is

described as an elite controller (Okulicz*et al.,* 2009), a subgroup of long term non progressors (Sanchez *et al.*,1997). There were three other females who were also enrolled after 24 months of HIV diagnosis with baseline pVL of <2000 copies/ml and CD4+ cell counts of > 700/ µl, and also without any form of medications. These patientsbelong to another subgroup of long- term non progressors (LTNP) called viremic controllers (Okulicz*et al.,* 2009).

5.1.3.2CD4+ cell count

Due to the selective nature of the study, which required that all the enrollees had baseline CD4+ cell counts of above 500.0 /µl, the patients'baseline CD4+ countsof 520.0 to 1552.0 cells/µLwere within the range of 365/µl to 1571/µl for healthy controls reported in Nigeria by Oladepo*et al.*,(2009); yet their median CD4+ cell count of 704.5 cells/µLwas lower than the mean of 927.0±5.0/µl reported by Yeldu *et al.,* (2016) among healthy controls in Sokoto, Nigeria.Also, the progressive decline of CD4+ cell counts among the patients supports the fact that progressive reduction of CD4+ cells is the hallmark of HIV disease; as the key focus of HIV is the competitive annihilation of CD4+ cells through primed apoptosis upon cross-linking of CD4 by gp120 of the virus, and Fas expression on CD4+ cells (Patki*et al.,* 1997; Tateyama*et al.,* 2000). The result of this study also confirmed the well-established inverse relationship between plasma HIV RNA load and CD4+ cell count in our patients (Tateyama*et al.,* 2000; Nixon and Landay, 2010).As a result of this tendency, 13 patients were unable to sustain their CD4+ cell count at above 500.0/ µl for 48 weeks, and had to drop out of the study.

5.1.3. 3Serum interleukin-6 (IL-6)

The baseline serum IL-6 level of 1.9 pg/mLfound among the patients in this study was quite high when compared with the trace quantity (<0.0001 pg/mL) reported by Salter *et al.*,(2013) among healthy adult volunteers in Minnesota, United States of America (USA).Our result corroborates that of Nixon and Landay, (2010) that the levels of circulating IL-6 are directly proportional to HIV RNA load and inversely proportional to CD4+-cell count; thus emphasizing the proinflammatory role of IL-6 in promoting HIV RNA replication (Poli*et al.,* 1990; Arribas*et al.,* 2012). The positive feedback mechanism between HIV-1 replication and IL-6 secretion reported by Douek*et al.,* (2009); and Longo &Fauci(2010) have also been linked to IL-6 elevation in HIV infection.

5.1.3. 4SerumC-reactive protein (CRP)

The baseline CRP level of 29.4 ng/mLin the patients in this study was very high compared to the trace quantity (<0.0003 ng/mL) reported by Salter *et al.*,(2013) among healthy adult volunteers in Minnesota, USA. Pro-inflammatory biomarkers such as CRP, TNF-α, IL-1, D-dimer, fibrinogen, serum amyloid A and amyloid Pare known to be significantly elevated in HIV infection, because HIV is associated with increased expression of cellular markers of T-cell activation and senescence (Lau *et al.,* 2006; Salter *et al.,* 2013). Apart from HIV infection, CRP is also elevated in response to acute injury, other infections, or inflammatory stimuli (Arribas*et al.,* 2012).Therefore CRP is a leading blood marker of systemic inflammation and can only be found in trace amounts in healthy people (Lau *et al.,* 2006; Salter *et al.,* 2013). Elevated CRP levels in HIV

infection have been linked to comorbid diseases such as dyslipidemia, atherosclerosis, stroke and cardiovascular diseases (Feldman *et al.,* 2003; Nixon and Landay, 2010).

5.1.4. The nutritional indicesof HIV infected ART-naïve patients

5.1.4.1 Haemoglobin concentration

The patients' range of baseline haemoglobin concentrations of 7.4 to 15.3g/dL (median=11.0 g/dL), compared to those of healthy controls (range=11.0 to 16.0g/dL,median=12.3g/dL) meant that some of the patients had anaemia. Anaemia has been reported by many workers as a component of malnutrition (Enwonwu, 2006); and an index of HIV infection progression (Awodele*et al.,* 2012; Anyabolu*et al.*, 2014).

5.1.4.2Serum albumin

The wide range of baseline serum albumin of 30 to 44.0g/dL (median of 36.0 g/dL)in the patients when compared with the range of 33.0-48.0g/dL (median of 39.0 g/dL) in the healthy controls; meant that some of the patients had hypoalbuminemia. Hypoalbuminaemia is an important feature of malnutrition (Enwonwu, 2006), and was reported to be anindependent predictor of rapid progression to AIDS and death in both ART-experienced and ART-naïve patients, particularly in resource-limited settings (Piwoz and Preble, 2000).

5.1.4.3Micronutrients (zinc, copper and vitamin B_{12})

The distribution of the patients' baseline serum zinc (range=0.0 to 0.83 ppm), copper (range=-0.8 to 0.05 ppm) and vitamin B_{12}(range=1.0 to 346 ng/L) were significantly lower than those of healthy controls (zinc=0.06 to 0.3 ppm; copper=0.1 to 0.23 ppm;

vitamin B_{12}=111.7 to 1292.0 ng/L) in this study; and from values reported by Zarebavan*et al.,* (2012) in Iranianhealthy controls. The deficiencies of thesemicronutrients has collaborated previous reports that deficiencies of trace elements and vitamins were common among HIV-infected patients (Baum *et al.,* 2000; Jiamton*et al.,* 2003; Fawzie 2005). These deficiencies have been attributed to three main factors: chronic persistent oxidant stress associated with HIV progression and consequent increased demand, utilization and consumption as anti-oxidants (Aquaro*et al.,* 2008; Awodele*et al.,* 2012; Birben*et al.,* 2013;del Valle *et al.,* 2013; Ibeh*et al.,* 2013), and malnutrition (Bowie *et al.,* 2005; Fawzie 2005).

The role of zinc in HIV infection is ambivalent. The HIV-1 virus is a zinc dependent virus because zinc is a component of HIV-1 nucleocapsid proteins and has a strong affinity for binding with HIV-1 trans- activating protein (Tat) necessary for gene expression, multimerization and integration (Baum, *et al.,* 2000). HIV also binds to zinc ions in T-cells in order to induce the production of cytokines such as interleukins IL-1, IL-6 and TNFα and soluble IL-2R which induce HIV replication (Baum, *et al.,* 2000; Prasad, 2007). This may explain why large dosages of zinc induce production of proviral peptides, the basis of new infectious viral particles leading to faster HIV-disease progression (Baum, *et al.,* 2000; Prasad, 2007).On other hand, zinc inhibits NFκβ and TNF-α thereby modulating cytolytic T-cell activity. It stabilizes the thiol groups and phospholipids in biological membranes and protects them against oxidative stress (Fleiger*et al.,* 1995). Zinc deficiency reduces generation of T-cells, and leads to lymphopenia, thymic atrophy, depressed humoral and cell-mediated immunity, and increased frequency and number of new infections (Baum, *et al.,* 2000; Durak, 2014).

Therefore, zinc has both an enhancing and inhibiting activity depending on its concentration in the surrounding tissues.

Superoxide dismutase enzyme, a first line endogenous antioxidant, depends on zinc and copper for its antioxidant activities. The low serum levels of zinc and copper observed in HIV-1 infected patients may partially be explained by their persistent demand and utilizationasantioxidants to counteract the chronic oxidative state of the HIV infection (Prasad, 2007; Oguntibeju*et al.,* 2009).Their deficiencies may also occur in malnutrition (Oguntibeju*et al.,* 2009; Gedle*et al.,* 2015). In addition, the deficiency of copper among the patients in this study may be due to increased demand of the metal for neutrophil mobilization in peripheralblood and T-cell proliferation in HIV infection(Percival, 1998; Elbim*et al.,* 2001). The consequences of its deficiency are: the arrestof granulocytes maturity, neutropenia, reduced superoxide anion formation and phagocytosis (Percival, 1998; Elbim*et al.*,2001; Barasi, 2003).Vitamin B_{12} which was analysed as a prototype of the B vitamins was also deficient among the patients. The deficiency of vitamin B12 in HIV infected persons has been attributed to malnutrition (Guarino*et al.,* 1993; Enwonwu, 2006).

5.1.4.4Lipids

HIV infection is associated with changes in plasma levels of lipoproteins. Data from a Multicenter AIDS Cohort Study (MACS) (Riddler*et al.,* 2003) and another study(Paul *et al.,* 2013) indicated that patients with untreated HIV infection commonly show low levels of total cholesterol, LDL-C, HDL-C and triglycerides.In this study, the median concentrations of the patients' lipids (except triglycerides) were insignificantly lower than those of healthy controls.Although the molecular mechanisms responsible

forchanges in lipid metabolism are not well understood, the process is thought to result from activities of cytokines (TNF-α, IL-1 beta, IL-6 and IFN) and proteins which mediate the acute –phase responseon adipose tissues, monocytes and macrophages and other antigen presenting cells (Rasheed *et al.,* 2008; Renga*et al.,* 2012; Kandi, 2016).

5.2Effect of Micronutrient Supplementation on the Indices of HIV Infection Progression in HIV Infected ART-Naïve Patients

5.2.1 Effectof micronutrient supplementation onsocio-demographic characteristics of HIV infected ART-naïve patients and their retention on care

In this study, more than 60% of the patients were retained on care with micronutrient supplementation for 48 weeks, confirming the postulation that provision of food supplements and other incentives enhanced uptake of pre-ART services, particularly among indigent individuals (Bowie *et al.,* 2005; Govindasamy*et al.,* 2012; Bastard *et al.,* 2013; Govindasamy*et al.,*2014). Sociodemographic factors identified to have influenced the retention of the patients on micronutrientsupplementation at 48 weeks in this study were middle agegroup ($p \leq 0.05$),having an occupation ($p \leq 0.03$) and an income per month ($p \leq 0.04$). About 38% of the patients were aged 41-60 years, 76% were employed, and 49% earned above the minimum wage of 18,000.00 naira (>$45.00) per month. All 22 patients who had no income and 17 out of 24 patients whose incomes were below 18,000.00 naira dropped out of the study before the 12 and 48 weeks respectively. The reasons adduced by the patients were difficulties with transportation to the hospitals for regular clinic appointments and payment for necessary investigations.The above reasons re-affirms the prominent roles of poverty, ignorance and distance from health facilities, and among many others, as causes of the high attrition rate among patients on pre-ART

and ART programmes in Africa and Asia(Govindasamy*et al.,* 2012; Kranzer, *et al.,* 2012; Bastard *et al.,* 2013; Mugglin*et al.,* 2013; CUNY, 2015; WHO/UNICEF/UNAIDS, 2015). Apart from above characteristics, other factors that also led to withdrawal of patients from the study before the 48 weeks, were: decline of CD4+-cell counts to below 500/µL (13 patients), pregnancy (11 patients) and lost to follow-up (9 patients), thus emphasizing the role of diverse factors in the retention of ART-naïve patients on care and support (Govindasamy*et al.,* 2012; Bastard *et al.,* 2013; Mugglin*et al.,* 2013; Govindasamy*et al.,* 2014).

5.2.2 Effect of micronutrient supplementation on the clinicalindices of HIV infected ART-naïve patients

The overall clinical profiles of the patients progressively improved significantly from baseline to 48 weeksof micronutrient supplementation. Their vital signs were stabilized, and there was an increase in their BMI as well as significant reductions in the incidence of opportunistic infections and general morbidity of HIV diseases. The increase in BMI could have resulted from improved appetite, decreased psychological distress and increased emotional stability reported by many of the patients, particularly during the 'Health Talk' component of this research. It could also be the result of reduction or reversal of pro-oxidant hyper-catabolic state of HIV infection progression (Babamento and Kotler, 1997; Macallan*et al*, 1999; Longo and Fauci, 2010). However, the increase in BMI was only beneficial to the 25% of the patients who were underweight (median BMI of 17.0kg/m^2), as the other 75% moved from overweight to obesity (Crook, 2012).

5.2.3 Effect of micronutrient supplementation on plasma HIV RNA loadand immunological indices of HIV infected ART-naïve patients

5.2.3.1 Effect on plasma HIV RNA load

The patients' plasma HIV loads decreased progressively from baseline to 48 weeks when the reduction became significant. The mechanisms by which micronutrients suppress HIV replication are not as explicit as those of antiretroviral drugs (ARVs), which have been well described(Idigbe*et al.,* 2003; Ngondi*et al.,* 2006; Bennett *et al.,* 2012). However, micronutrientshave been shown to inhibit HIV replication through their role as anti-oxidants and cofactors to endogenous antioxidant enzymes such as glutathione (GSH), selenium dependent-glutathione peroxidase, Cu^{2+}/Zn^{2+} dependent SOD, Mn^{2+}dependent SOD, and Fe^{2+}dependent catalase (Pasupathi*et al.,* 2009; Birben*et al.,* 2012). Vitamin C and E act synergistically to counter HIV induced oxidative state and inhibit the replication of HIV-1. Vitamin C is the major water-soluble antioxidant that acts as the first defense against ROS in whole blood and plasma; and helps to regenerate vitamin E during the antioxidant defense process(Niki*et al.,* 1995; Durak 2014); while vitamin E (α-tocopherol) is the most abundant potent lipophilic antioxidant that can alter the membrane integrity and fluidity of HIV-1 and interfere with its ability to bind to CD4+ cell-receptor sites thereby reducing the infectivity of HIV-1 (Harada *et al.,* 2005).Zinc at moderate doses inhibits NFκβ and TNF-αinduced HIV replication by enhancing cytolytic T8-cell and NK cell activities; or by stabilizing biological membranes against oxidative stress (Fleiger*et al.,* 1995); or by through Cu^{2+}/Zn^{2+} dependent SOD deactivation of HIV-1 reverse transcriptase and protease enzymes (Palamara*et al.,* 1996). These inhibitory activities results in a dramatic decrease in both

budding and release of new virus particles from chronically infected macrophages and/or lymphocytes (Palamara*et al.,* 1996).

5.2.3. 2 Effect on CD4+ cell counts

The reduction of plasma HIV RNA load did not result in the complementary increase of CD4+ cell count in this study; rather there was a progressive decline of the patients' CD4+ cell counts from baseline to 48 weeks of micronutrient supplementation. The decline of CD4+ cell counts in HIV infected ART-naïve persons was expected, because continuous annihilation of the CD4+cells, in the absence of ART,is the hallmark of the HIV(Douek*et al.,* 2002). However, in contrast to the above result, Yeldu *et al.*, (2016), reported progressive increase of CD4+ cell counts, from baseline to 48 weeks, among 54 ART-naïve HIV positive patients on micronutrient supplementation in Sokoto, although they did not describe the mechanism by which micronutrient supplementation increased CD4+ cell count in the patients. Some theories suggest that micronutrients such as copper and vitamin A, in the form of retinol or retinoic acid, improve immunity by modulating the growth and function of CD4+ T-cells, B-cells and natural killer cells and their products (Kashou and Agarwal, 2011); while others postulated that the beneficial effect of micronutrients on immune system results from inhibition of programmed apoptosis of CD4+ cells, reversal of oxidative stress and improved functionality of CD4+ T/CD8+ T- cells, rather than the absolute increase in CD4+-cells (Baum *et al.,* 2000; Alimonti*et al.,* 2003; Awodele*et al.,* 2012).This may be the reason multi-micronutrient supplementation produced a 50% reduction in mortality among 481 HIV-positive ART-naïve adults in Thailand at 48 weeks, but produced no effects on the HIV RNA load or CD4 cell count of the patients (Jiamton*et al.*,2003).

5.2.3.3*Effect on interleukin-6*

Micronutrient supplementation reduced serum concentrations of IL-6 significantly at 48 weeks. The constituents of the supplement acted synergistically to reduce this pro-oxidant, pro-inflammatory cytokine. Specifically, vitamin A (retinoic acid) inhibits the production and secretion of IL-6 in a dose-dependent manner by downregulating the expression of IL-6 mRNA in mononuclear and polynuclear immune cells (Oguntibeju*et al.*, 2009; Nixon and Landay, 2010).

5.2.3.4*Effect on C-reactive protein*

Micronutrient supplementation also significantly reduced the serum concentrations of CRP progressively from 24 weeks to 48 weeks respectively. Specifically, the reduction of CRP seem to have occurred through the synergistic anti-inflammatory effects of the trace elements and vitamins on the glutathione enzyme complex (Li-Weber et al., 2002; Biswas et al., 2005; Durak 2014).

5.2.4 Effect of micronutrient supplementation on the nutritional indices of HIV infected ART-naïve patients

5.2.4.1 Effect on haemoglobin, albumin, zinc, copper, and vitamin B_{12}

Each Synovit^{TM} supplement capsule given to the patients in this study is a mixture of essential amino acids, trace elements and vitamins. These moleculesact synergistically to enhance protein synthesis and cellular immunity and reduce pro-oxidant and inflammatory states.The significant increase in the levels of nutritional indices such as haemoglobin, albumin, zinc, copper, and vitamin B_{12}, from baseline to 24 and 48 weeks of micronutrient supplementation, may be an indication that the patients complied with

the medications. The results also corroborate earlier observational studies of the beneficial effects of micronutrients on the quality of life of patients with HIV/AIDS (Allard *et al.,* 1998; de Luis Roman *et al.,* 2001; Jiamton*et al.,* 2003; Bowie *et al.,* 2005).

5.2.4.2 Effect on lipid profile

Micronutrient supplementation produced variable effects on serum lipid concentrations. The first effect was the significant reductions of total cholesterol and HDL-C at 48 weeks. The second effects were initial insignificant reductions of LDL-Cand triglycerides at 24 weeks and a rebound increase of their concentrations to baseline levels at 48 weeks. The variable effects of supplements on lipid levels have been reported in HIV infected patients on ART (particularly protease inhibitors) (Riddler*et al.,* 2003) and in HIV infected ART naïve patients on the anti-lipid drug lovastatin (Montoya*et al.,* 2012). Many theories have been advanced for the complex behaviour of lipids in HIV infection, but the first direct evidence of the molecular mechanisms responsible for the numerous lipid disorders in HIV infection was provided by Rasheed *et al.*,(2008). The result of that study showed that HIV replication alone in human CD4+ cells, without other influences, can stimulate the production of novel cellular enzymes and proteins that enhance synthesis of fatty acids and LDL-C, secretion of triglycerides, alteration of lipid transport and metabolism, and oxidization of lipids (dyslipidaemia); ultimately leading to accumulation of lipids in the blood (hyperlipidaemia) and disproportionate distribution of tissue-associated fats (lipodystrophy) (Rasheed *et al.*,2008).

The short-term effects of the supplements on lipids among the HIV infected patients was postulated to result from the synergistic actions of vitamin E and vitamin C in terminating the chain reactions of lipid peroxidation in the cell membrane by scavenging for the peroxyl radical (Niki*et al.,* 1995; Kashou and Agarwal 2011). The tocopheroxyl radical which is the pro-oxidant form of vitamin E is effectively reduced (regenerated) to the antioxidant form by a network of other antioxidants such as vitamin C, coenzyme Q and glutathione. Apart from prolonging their collective anti-oxidant activities, these cofactors, coenzymes and catalytic enzymes reduce IFN-α induced hepatic de novo fatty acid synthesis, increase peripheral lipoprotein lipase activity and increase clearance of triglycerides in the blood (Allard *et al.,* 1998; Birben et al., 2012; Tang *et al.,* 2002).

The overall long-term effect of the supplements on lipids was potentially pro-atherogenic because of the progressive reduction of HDL-C and the increase of LDL-Cand triglycerides levels to and/or above pre-treatment levels at 48 weeks (Melzi*et al.,* 2010). Increased LDL-C enhance atherosclerosis in HIV infected patients by increasing IFN-α induced hepatic de novo fatty acid synthesis, and decreasing peripheral lipoprotein lipase activity and clearance of triglycerides in the blood (Tang *et al.,* 2002; del Valle *et al.,* 2013; Kandi, 2016). Atherosclerosis has also been linked to the inhibition of ATP binding cassette transporter A1 (ABCA1), an integral transmembrane lipid transporter, by HIV accessory protein Nef. The inhibition of ABCA1 impairs cholesterol efflux from macrophages causing accumulation and internalization of reactive cholesterol species (particularly, LDL-C and apolipoproteinB) in these cells and their transformation into foam cells (Elbim*et al.,* 2001; Lau *et al.,* 2006; Nixon and Landay, 2010; Yvan-Charvet*et al*., 2010).

CHAPTER SIX

6.0CONCLUSIONS,RECOMMENDATIONS, LIMITATIONS AND CONTRIBUTION TO KNOWLEDGE

6.1Conclusions

The result of this study has revealed that HIV infected ART-naïve patients' who were at WHO clinical stage I and II with CD4+ cell counts of ≥520/µL had normal vital signs and few symptoms and signs of HIV infection. Although majority of them were overweight, their nutritional indices were lower than those of healthy volunteers. Their serum C-reactive protein and interleukin-6 were also significantly higher than reference values. Sociodemographic factors that influenced the retention of patients on micronutrient supplementation were middle age, having an occupation and source of income.

Micronutrient supplementation significantly decreased incidence of opportunistic infections, C-reactive protein, interleukin-6, and plasma HIV RNA load, without complementary increase in CD4+ cell counts. Nutritional indices such as haemoglobin, serum zinc, copper, and vitamin B_{12} were significantly increased; while total cholesterol and HDL-C were significantly decreased. The effect on serum albumin, LDL-C and triglycerides were not significant.

The plasma HIV RNA load maintained significant positive correlations with serum interleukin and C-reactive protein and negative correlations with CD4+ cell count, zincandcopper. Significant predictors of plasma HIV RNA load before micronutrient supplementation were serum zinc andinterleukin -6; while serum interleukin-6 and

hemoglobin were the predictors at 24 weeks and 48 weeks of micronutrient supplementation.

In summary, the result of this study has shown that micronutrient supplementation reduced the clinical, nutritional, virological and immunologicalindices of HIV infection progression, and also enhanced retention of the HIV infected ART-naïve patients on the pre-ART care and support programme.

6.2 Recommendations

Since the results of this study have shown some beneficial effects of micronutrient supplementation in HIV infection, it is recommended that

1. The Scientific community should focus on micronutrient supplements with anti-inflammatory, antioxidant, immune boosting and anabolic properties as the therapeutic agents for HIV positive individuals with CD4+ cell counts above 500/µL of whole blood; and/or as adjunctive therapy to individuals on ART.

2. Furtherrandomized clinical trials should be conducted to determine the correct dosages and duration of treatment with micronutrient supplementsas adjunctive therapy in HIV infection.

3. Comparative studies should be undertaken to identify co-factors that may contribute to the progression of HIV infection to AIDS, because a better understanding of the co-factors may assist in the development of diagnostic and therapeutic strategies to, perhaps, alter the course of HIV infection and prevent the onset of AIDS.

6.3Limitations

The limitations of this study can be summarized as:

1. Inability to evaluate other proinflammatory cytokines that stimulate the Th1 response and enhance HIV replication, such as IL-2, IL-10, IL-12, TGF-ß and IFN-γ.

2. Inability to evaluate glutathione enzyme systems complex, the major intracellular hydro-soluble antioxidant agents, involved in the control of the immune response and antioxidant defenses.

3. Inability to evaluate some metabolic biomarkers of oxidative stress such as malondyaldehyde (MDA), and 8-hydroxylguanosine in urine of affected individuals, as the evidence of the role of persistent oxidative stress as a co-factor in progression of HIV infection to AIDS.

6.4Contributions to Knowledge

1.This study has shown that being middle aged, and having an occupation and source of income influenced the retention of WHO clinical stage I/II HIV infected ART-naïvepatients on pre-ART care with micronutrient supplementation for 48 weeks.

2. The study also showed that WHO clinical stage I/II HIV infected ART-naïve patients with none or mild symptoms and signs of the disease and normal CD4+ cell counts

- Had malnutrition i.e.: lower haemoglobin (11.0 g/dL), lower macronutrient [albumin (36.0 g/L) and lipids (TC, 4.6 mmol/L; HDL-C, 1.6mmol/L; LDL-C, 2.4 mmol/L)] and micronutrient [zinc (0.01 ppm), copper (-0.4ppm), vitamin B_{12}(15.0 ng/L)]levels than healthy volunteers[haemoglobin (12.3g/dL; albumin (39.0 g/L) and lipids (TC, 4.7 mmol/L; HDL-C, 1.7mmol/L; LDL-C, 2.6 mmol/L; zinc (0.16 ppm), copper (0.04ppm), vitamin B_{12} (315.0 ng/L)] ($p \leq 0.05$).

- Hadserum C-reactive protein(29.4 ng/mL) andinterleukin-6 (1.9 pg/mL) levels that were significantly higher than reference values (usually CRP <0.001ng/mL and IL-6 <0.001 pg/mL) (*p≤0.05*).

3. The study also showed that micronutrient supplementation to WHO clinical stage I/II HIV infected ART-naïve patientsfor 48 weeks

- Significantly decreased incidence of opportunistic infections (from 18.0% to 5.3%; *p≤0.05)*, plasma HIV RNA load (from 27, 105.0 copies/mL to 585 copies/mL;*p≤0.05*), serum CRP (from 29.4 ng/mL to 25.8 ng/mL; *p≤0.05)*, and IL-6 (1.9 pg/mL to 0.5 pg/mL; *p≤0.05)*,
- Did not lead to an increase in the CD4+ cell counts, but rather significant decrease from 704.5 cells/µL to 595.0 cells/µL (*p≤0.05)*.
- Increased their nutritional status [haemoglobin (from 11.0 g/dL to 12.3 g/dL; *p>0.05*), serum albumin (from 36.0 g/L to 38.0 g/dL; *p>0.05*), zinc (from 0.01 ppm to 0.3 ppm; *p≤0.05*), copper (from -0.4ppm to 0.05 ppm; *p≤0.05*), vitamin B_{12} (from 15.0 ng/L to 30 ng/L; *p≤0.05*).
- Produced variable effects on levels of serum LDL-C and triglycerides, with initial insignificant decreaseat 24 weeks (LDL-C from 2.4 mmol/L to 2.3 mmol/L; *p>0.05)* and triglyceride from 1.1 mmol/L to 1.0 mmol/L; *p>0.05),*and later increaseto levels (LDL-C= 2.6 mmol/L and triglyceride=1.1 mmol/L) similar to those of healthy controls (LDL-C= 2.6 mmol/L and triglyceride=1.1 mmol/L) at 48 weeks (*p>0.05*).

4. The study also showed that the plasma HIVRNA load maintained significant positive correlations withserum IL-6 (rho=0.332, *p=0.00*) and CRP (r=0.250, *p=0.04*) and

negative correlations with CD4+ cell count (rho= -0.152, *p=0.04*); zinc (rho= - 0.349, p=*0.00*); and copper (rho= -0.471, *p=0.00*) respectively.

5. The study showed that significant predictors of HIV infection progression were serum zinc (Beta=0.927, *p=0.00*), and IL-6 (Beta=0.094, *p=0.05*) at baseline, and serum IL-6 (Beta=0.354, *p=0.00*) and hemoglobin (Beta=0.306, *p=0.02*) at 24 weeks and 48 weeks of micronutrient supplementation respectively.

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APPENDICES

Appendix I: MEPIN Award Letter

UNIVERSITY OF IBADAN

MEDICAL EDUCATION PARTNERSHIP INITIATIVE IN NIGERIA (MEPIN)

20th May, 2014

Dr Reginald O. Obiako
Ahmadu Bello University

Dear Dr Reginald,

MEPIN MENTORED RESEARCH AWARD

The Medical Education Partnership Initiative in Nigeria (MEPIN), funded by the National Institute of Health, USA hereby gives a mentored research award in the amount of ₦750,000 (see attached budget) to Dr Reginald O. Obiako of the Department of Pharmacology And Therapeutics, Ahmadu Bello University, Zaria "The effect of micronutrient supplementation on progression of HIV infection in antiretroviral naïve patients." submitted and reviewed for the research training goal of MEPIN. This award is in pursuant of a research training to be mentored by PROF I. abdu-Aguye. Acceptance of this award including the "Terms and Conditions" of NIH funded research projects is acknowledged by the grantee when funds are drawn from the MEPIN program grant account in your University.

Each publication or other document that cites results from NIH grant-supported research must include an acknowledgement of NIH grant support and disclaimer such as "The project described was supported by the Medical Education Partnership Initiative in Nigeria (MEPIN) project funded by Fogarty International Center, the Office of AIDS Research, and the National Human Genome Research Institute of the National Institute of Health, the Health Resources and Services Administration (HRSA) and the Office of the U.S. Global AIDS Coordinator under Award Number R24TW008878. The content is solely the responsibility of the authors and does not necessarily represent the official views of the funding organizations." Award recipients are required to comply with NIH Public Access Policy. This includes submission to PubMed Central (PMC), upon acceptance for publication, an electronic version of a final peer-reviewed, manuscript resulting from research the research supported in whole or in part, with direct cost from National Institutes of Health. The author's final peer-reviewed manuscript is defined as the final version accepted for journal publication, and includes all modification from the publishing peer review process. For additional information, please visit http://publicaccess.nih.gov/

Principal Investigator: Professor David O. Olaleye
Telephone: 08033241317, 08126286262
e-mail: davidoolayele@gmail.com

Contact:
Medical Education Partnership Initiative in Nigeria (MEPIN)
College of Medicine, University of Ibadan, Ibadan, Nigeria.

Award recipients must promote objectivity in research by establishing standards to ensure that the design, conduct and reporting of NIH-funded research are not biased by a conflicting financial interest of an investigator. Investigator is defined as the Principal Investigator and any other person who is responsible for the design, conduct, or reporting of NIH-funded research or proposed research, including the investigator's spouse and dependents children.

Please consult the NIH website http://grants.nih.gov/grants/policy/coi/index.htm for additional information on terms and conditions of NIH funded research.

Please note that the ethical approval from your Institutional Review Board is a pre-requisite and it is mandatory for this project.

If you have any questions about this award, please contact me or mepin2010@gmail.com or your University MEPIN PI.

Congratulations!!!

Yours Sincerely,

Professor D. O. Olaleye
Principal Investigator
MEPIN

Appendix II:The revised World Health Organization (WHO) Clinical Staging ofHIV/AIDS for Adults and Adolescents

Clinical Stage I: asymptomatic, persistent generalized lymphadenopathy& performance Scale 1 (normal activity)

Clinical Stage II: unexplained loss of less than 10% of body weight; recurrentrespiratory infections (sinusitis, tonsillitis, otitis media,pharyngitis); herpes zoster; papular pruritic eruptions; seborrheicdermatitis; fungal nail infections;angular cheilitis& performance scale 2 (symptomatic but normal activity)

Clinical Stage III: unexplained loss of more than 10% of body weight; unexplained diarrhea for more than 1 month; unexplained persistent fever formore than 1 month (>37.6°C, intermittent or constant); persistent oral candidiasis (thrush); pulmonary tuberculosis (current); severe bacterial infections (pneumonia, empyema, pyomyositis, bone or joint infection, meningitis, bacteremia); acute necrotizing ulcerative stomatitis; gingivitis, or periodontitis; unexplained anemia (hemoglobin <8 g/µl); neutropenia (neutrophils <500 cell/µl); thrombocytopenia (platelets <50, 000 cells/µL)&performance scale 3 (bedridden < 50% of the day during the last month).

Clinical Stage IV (AIDS Defining Illnesses): HIV wasting syndrome; HIVencephalopathy; ADC; recurrent bacterial pneumonia (> 2 episodes in 12 months); Pneumocysticjiroveci pneumonia;disseminated herpes simplex infection (orolabial, genital, anorectal) for > 1 month; disseminated tuberculosis; Kaposi sarcoma; non-Hodgkin lymphoma; invasive cervical carcinoma; disseminated candidiasis (esophageal, trachea, lungs or bronchi); cytomegalovirus infection (retinitis orinfection of other organs); Cerebral toxoplasmosis; Cryptococcal meningitis; recurrent nontyphoidal Salmonella; symptomatic HIV-associated nephropathy, or HIV-associated cardiomyopathy; Progressive multifocal leukoencephalopathy; chronic diarrhea due to cryptosporidiosis and isosporiasis; disseminated mycosis (histoplasmosis, coccidioidomycosis, penicilliosis)&performance scale 4 (bedridden > 50% of the day during the last month.

Note: Patient may move from a later stage to an earlier stage if the presenting OI is treated.

Ref: HIV infection, Ed by E Kalabira, M R Kamya, F X Mubiru, N NBakyaita. Makerere Univ. Printery, 2000. 2nd Edition.

Appendix III: The1993 revised CDC HIV classification system and expanded AIDS Surveillancedefinition for adolescents and adults (MMWR 41: RR. 17, December 18, 1992)

	Clinical category		
CD4 cell category	**A**	**B**	**C**
1) ≥ 500 µL	A1	B1	C1
2) 200-499 µL	A2	B2	C2
3) <200 µL	A3	B3	C3

Clinical category A: Asymptomatic, Persistent generalized lymphadenopathy (PGL) with nodes in ≥ 2 extrainguinal sites, at least 1 cm in a diameter for ≥ 3 months, acute (primary) HIV illness.

Clinical category B: Symptomatic, not A or C conditions. Examples include but not limited to: bacillary angiomatosis, candidiasis, vulvovaginal, persistent> 1 month, poorly responsive to treatment. oropharyngealcandiasis, cervical dysplasia, severe or carcinoma in situ, constitutional symptomssuch as fever (38.5^0C) or diarrhea for > 1 month. Above must be attributed to HIV infection or have a clinical course or management complicated by HIV.

Clinical category C: AIDS defining illnesses (stage 4 disease)

Note that categories A3, B3 and C are classified as AIDS.

Appendix IV:Composition of Food Groups recommended in the Balance of Good Health

1. Cereals, roots and tubers: Wheat, oats, barley, rice, maize, millet, rye, yam, cassava, beans, and potatoes together with products made from them. They contain carbohydrates, non-starch polysaccharide (NSP), calcium, iron and vitamin B complex.

2. Fruits and vegetables: Fresh fruits (mango, orange, apple etc); dried fruits (pear, berries etc); fruit juices; and vegetables (tomato, okro, cucumber, onion, lettuce etc). They contain carbohydrates, NSP, vitamins C, E and carotenes, folate, potassium, magnesium, and trace minerals

3. Meat and milk: Carcass meat and meat products; poultry; eggs; fish and fish products, and milk products. They contain protein, vitamin B complex, calcium, iron, phosphorus, zinc, magnesium, long-chain polysaccharide fatty acids (in oily fish), fat-soluble vitamins, and NSP.

4. Fats and fatty foods: Butter, margarine, oils and other fats. They contain fats, essential fatty acids, and fat-soluble vitamins.

5. Sugar and sugary foods: Chocolate and sugar confectionery, soft drinks, ice cream, and biscuits. They contain Carbohydrates, and NSP

Appendix V: Average amount of micro-minerals found in the adult human body

S.No	Trace element	Symbol	Total body content
1	Iron	Fe	4.0 g
2	Zinc	Zn	2.0 g
3	Manganese	Mn	12-20 mg
4	Copper	Cu	80 mg
5	Iodide	I	15-20 mg
6	Chromium	Cr	< 2.0 mg
7	Cobalt	Co	1.5 mg
8	Selenium	Se	3-30 mg

Appendix VI: Certificate from Institutional Health Research Ethical Committee of ABUTH Shika Zaria



HEALTH RESEARCH ETHICS COMMITTEE

AHMADU BELLO UNIVERSITY TEACHING HOSPITAL

SHIKA - ZARIA, NIGERIA.

E-mail: abuth@yahoo.com Website: www.abuth.org

Chairman of Board: Chief. Shuaib Oyedokun Afolabi FNI
Chief Medical Director: Prof. Lawal Khalid, MBBS, FMCS, FWACS, FRCS(ED) FICS
Chairman, Medical Advisory Committee: Prof. Abdullahi Mohammed, MBBS, FWACP, FICS
Director of Administration: Barr. Ishak Bello, LL.B, BL., LL.M, PGDM, AHAN, FCAI

Our Ref: ABUTH/HREC/TRG /36 Date: 7th January, 2014

Your Ref:

ABUTH HREC FULL ETHICAL CLEARANCE CERTIFICATE

Re: ***"The effects of micronutrient supplements on progression of HIV infection in persons yet to start Anti-HIV drugs."***

ABUTH Ethics Committee assigned number:	-	ABUTH/HREC/IO2/2013
Name of the principal investigator:	-	Obiako Onyeadumaraikwe Reginald
Address of the Principal Investigator:	-	Dept. of Medicine, ABUTH, Zaria
Date of receipt of valid application:	-	**2nd January, 2014**
Date of meeting when final determination on ethical approval was made:	-	**8th January, 2014**

This is to inform you that the research described in the submitted protocol, the consent forms, and other participant information materials have been reviewed and ***given full approval by the ABUTH Ethics Committee.***

Please note: this approval dates from 9th **January, 2014 - 9th January, 2015**

No participant recruitment into this research may be conducted outside these dates.

All informed consent forms in this study must carry the ABUTH HREC number assigned to this research and the duration of ABUTH HREC approval of the study.

This HREC expects that you submit your application as well as an annual report for ethical clearance renewal 3 months prior to expiration of study dates. This is to enable you obtain renewal of your approval and avoid interruption of your research.

If there is delay in starting the research, please inform the ABUTH HREC so that starting dates can be adjusted accordingly.

No changes are permitted in the research without prior approval by ABUTH HREC, except in circumstances outlined in national code for Health Research Ethics: http://www.nhrec.net

ABUTH HREC reserves the right to conduct compliance assessment visits to your research site without prior notification.

Prof. A. I. Mamman
Chairman, ABUTH HREC

Appendix VII: Certificate from Institutional Health Research Ethical Committee of Ministry of Health, Kaduna State

MINISTRY OF HEALTH, KADUNA STATE

All Communication to be addressed to:
THE HON. COMMISSIONER
Quoting Reference and Date
Telephone: 234-248048
Website: ttp://www/moh.kd.gov.ng.
Email: info@moh.kd.gov.ng.

Independence Way,
P.M/;B 2014
Kaduna.
Kaduna State, Nigeria.

Health Research Ethical [illegible] State Ministry of Health

MOH/ADM/744/VOL.I/207 11th February, 2015

To.....

Ministry of Health Research Ethical Clearance.

RE: REQUEST FOR PERMISSION TO UNDERTAKE RESEARCH AMONG HIV INFECTED PATIENTS ATTENDING THE HIV CLINIC.

Name of Investigator:-	Dr. OR Obiako
Date of receipt of application:-	3rd February 2015
Date of ethical approval:-	6th February, 2015
Research period:-	March 2015 to March, 2017

You are kindly requested to give researcher maximum cooperation.

However, it is mandatory for researcher to submit his findings to the Ministry please.

F. A. KURAH (MRS)

Secretary Research Ethical Committee

Appendix VIII: Certificate from Institutional Health Research Ethical Committee of St Gerard's Catholic Hospital, Kaduna



ST. GERARD'S CATHOLIC HOSPITAL

P. M. B. 2144 Kaduna State, Nigeria. Telephone 062 - 232749
Website: www.stgerardhospital.org

June 5, 2014

Your Ref: _____ Our Ref: _____ Date: _____ 20____

DR. REGINALD OBIAKO
ABUTH, SHIKA,
ZARIA

Dear Dr. Obiako,

RE: RESEARCH (THESIS) PROPOSAL

This is to inform you that, approval has been granted for you to commence your Research program in this Hospital as requested in your proposal.

Thank you.

ST. GERARD CATH. HOSPITAL DATE 6/6/14

Rev. Sr. Beatrice Danladi
Hospital Administrator

All Correspondence should be Addressed to the Administrator

Appendix IX:Informed Consent Form

Dear Respondent,
I am Reginald Obiako, a Medical Doctor working in Ahmadu Bello University Teaching Hospital Shika. I am also a Postgraduate Student of Ahmadu Bello University Samaru Zaria. I am conducting a research on 'The Effect of Micronutrient Supplementation on some Indices of HIV Infection Progressionamong Persons Yet to Start Anti-HIV Drugs'.

A. THE GOALS OF THIS RESEARCH ARE:
i. To know about your background
ii. To find out some of your health problems
iii. To determine the extent and severity of these health problems
iv. To find out if these health problems can be reduced by nutritional supplements to be given to you by us

B. QUESTIONS AND MEDICAL TESTS
Now I am going to ask you some questions about yourself and how you are feeling about your health, because HIV can affect many areas of your health. Please try to be as honest and as accurate as you can be.As part of our health service,I am going to examine you and give you some tests to do in order to find out how this illness has affected your health.

C. BLOOD TESTS
I will take some blood from you to do tests that will enable me know if the food you are eating and the drug we are giving you are getting to your system, and doing what they are supposed to do.

D. RISKS AND COSTS TO YOU
Taking part in this study will not disturb you too much, except the little time you will spend talking to me, and having the medical examinations and blood tests. You may also feel some pains or see some blood at the place blood will be collected from your arm. You are not to pay for participating in this study, and all procedures carried out on you will be free.

E. BENEFITS TO YOU
The information we will get from this research will assist us in giving you the best possible advice on how to live with this illness.

F. YOUR RIGHTS AND PROTECTION OF YOUR PRIVACY
Your participation in this research is voluntary, and you are free to withdraw from participating at any time. I assure you that all information from you at every stage of this research will be treated in strict confidence and nobody else would know about it except you. If there is anything in this consent form you do not understand or any questions about this research, you should feel free to ask me or call me on GSM 08023735832. Thank you.

CONSENT STATEMENT

I have read (or have been read to) and understand the informed consent form for 'THE EFFECTS OF MICRONUTRIENT SUPPLEMENTS ON PROGRESSION OF HIV INFECTION IN PERSONS YET TO START ANTI-HIV DRUGS'.I have been given the chance to ask questions about this research, and I understand that I can withdraw my consent at any time for any reason. I agree to participate in this research voluntarily and for my blood to be taken for tests relevant to the research.

---------- ----------

Name and signature of Participant Date

Appendix X: Human Vitamin B12 Elisa Kit

i. Microlitre plate: 96-wells coated with 1.0 µg/ml Streptavidin and anti-vitamin B-12 biotinylated purified rabbit IgG conjugate in buffer, blue dye and preservative packaged in an aluminum bag with a drying agent.
ii. vitamin B-12 calibrators (human serum albumin reference) consisting of 6 vials at concentrations of zero (A), 100 (B), 200 (C), 400 (D), 1000 (E) and 2000 (F) in pg/ml or ng/L (by multiplying pg/ml by 0.738).
iii. vitamin B-12 Standard 900ng/L (0.5mL/vial) and Standard Diluent (1.5mL/vial)
iv. vitamin B-12 Enzyme Conjugate: containing 6mL/vial of analog-horseradish peroxides conjugate in a protein-stabilizing matrix.
v. Sample or assay Diluent: 6mL/vial
vi. Wash Solution: 20mL of a 30 fold concentrated saline solution of buffered surfactant with preservatives.
vii. Substrate (colour) Reagents A and B consist of 6mL/vial of stabilized hydrogen peroxide and tetramethylbenzidine (TMB) in buffer respectively.
viii. Stop Solution containing 6mL/bottle of concentrated sulfuric acid (2 N H_2SO_4), as stored at 2-30^0 C.
ix. Stabilizing agent containing 6mL/bottle of dithiothreitol (DTT) solution Releasing agent containing 6mL/bottle of sodium hydroxide (NaOH) and potassium cyanide (KCN).
x. 6mL/bottle of neutralizing buffer that reduces the pH of sample extraction.

Methods of preparing vitamin B12

The human vitamin B_{12} (vitB_{12}) ELISA is based on the principle of a solid phase enzyme-linked immunosorbent assay in which purified human vitB_{12} antibody directed against a distinct antigenic determinant on the sample vitamin B_{12} molecule is used to coat microlitre wells. When combined with an enzyme labeled anti-vitB12conjugate, an antibody-antigen-enzyme-antibody complex is formed. This reaction between the test sample and the two antibodies results in the vitamin B_{12} molecules being sandwiched between the solid phase and enzyme-linked antibodies. After 30 minutes incubation at room temperature, the wells are washed with water to remove unbound labeled antibodies. Addition of Substrate or colour reagents and incubation for 15 minutes at room temperature results in the development of blue colour. The addition of Stop Solution (1N HCl) turns the colour to yellow. The intensity of the yellow colour is measured spectrophometrically at 450 nm, and is directly proportional to the concentration of vitamin B_{12} in the test sample.

Methods and Procedures

i. Sample collection and storage

Blood sample was collected into a serum separator tube (SST). The SST was sealed and blood was allowed to clot for 20 minutes at room temperature before centrifugation at 3000rpm for 5-20 minutes. The serum was immediately removed and stored at ≤ - 20^{0}C (for 4 weeks) and -80^{0}C (for > 4 weeks).

ii. Preparation of Reagents:

a. Wash Solution was diluted to 1000 mL with distilled water in a suitable storage container at room temperature of 20-27 ^{0} C for at least 60 days.

b. 100 µL of Stabilizing agent was added to 3900µL of Releasing agent to make 4000 µL (4mL) of 1: 40 solution.
c. Substrate Solution was formed by mixing Reagents A and B together in equal volumes within 15 minutes of use.
d. Dilution of Standard: This is done by serial dilution of 5 Standard microlitre wells thus:
100 µL of Standard followed by 50 µL of Standard Diluent were added into first well and thoroughly mixed. 100 µL was taken out of first well and added into 2nd well, followed by 50 µL of Standard Diluent, and thoroughly mixed. 100 µL was taken out of 2nd well and added into 3rd well, followed by 50 µL of Standard Diluent, and thoroughly mixed. 100 µL was removed from 3rd well and added into 4th well, followed by 50 µL of Standard Diluent, and thoroughly mixed. 100 µL was removed from 4th well and added into 5th well, followed by 50 µL of Standard Diluent. 100 µL was removed from 5th well and discarded. Different pipette tips were used for each dilution with resultant serial standard density of 600ng/L, 400ng/L, 200ng/L, 100ng/L and 50ng/L respectively.

iii. Sample dilution:
1mL of samples was diluted with 5mL of Assay Diluent to get a 5-fold dilution.

iv. Enzyme-linked immunosorbent assay **(**All samples and reagents were allowed to attain room temperature before use, and samples and standards were assayed in duplicates)
a. 40 µL of sample diluent and 10 µL of sample were added into each well, gently mixed, covered with adhesive strips and incubated at 37^0C for 30 minutes.
b. Excess liquid was aspirated from incubation mixture in each well.
c. Wells were filled with 400 µL of Wash Buffer using a squirt bottle, and excess liquid was completely removed by aspiration. The process was repeated four times for a total of 5 washes. After the last wash, any remaining Wash Buffer was removed by aspiration, decanting, inverting the plate and blotting it against clean absorbent paper towels.
d. 50 µl of Enzyme Conjugate was added into each well, covered with adhesive tape, and incubated for 30 minutes at 37^0C.
e. The wells were washed as in step c above.
f. 50 µl of Substrate Solution was added into each well, and thoroughly mixed. The wells were covered with adhesive tape, and incubated in the dark for 15 minutes at 37^0C.
g. At end of incubation, 50 µL of Stop Solution was added to each well and thoroughly mixed. The colour in the wells changed from BLUE to YELLOW.

v. Calculation of results
a. Optical density (OD) of each well was determined within 15 minutes, using a microplate reader set to 450 nm, corrected at a wavelength of 540nm by subtracting readings at 450nm. The average of the duplicate readings for each sample and Standard was subtracted from the average zero standard OD.
b. A standard curve was constructed using the mean OD values of each standard on the y-axis against the concentration on the x-axis and a best fit curve was drawn through the points on the graph. The unknown values were extrapolated from the standard curve. The Assay range was 28ng/L-800ng/L.

AppendixXI: Quantikine® ELISA Human C-Reactive Protein/CRP Immunoassay ™ kit

a. CRP Microplate: 96 well polystyrene microplate (12 strips of 8 microlitre wells) coated with a mouse monoclonal antibody against CRP
b. CRP Standard: Reference Standard Set (1.0 mL/vial) containing 0, 0.005, 0.010, 0.025, 0.050 and 0.100 mg/L CRP in serum based buffer-BSA solution with preservatives (50ng/vial of recombinant CRP in a buffered protein base with preservatives was also provided as a high standard).
c. hsCRP Assay or Sample Diluent RD1F: 50 mL/vial containing phosphate buffer-BSA solution with preservatives
d. CRP Enzyme Conjugate Reagent: 21 mL/vial containing goat monoclonal anti-CRP conjugated to horseradish peroxidase with preservatives
e. Calibrator Diluent RDSP Concentrate: 21mL/vial of a concentrated buffered protein base with preservatives
f. Colour Reagents (12 ml/bottle): Colour reagent A containing 12mL of stabilized hydrogen peroxide; and Colour reagent B containing 12mL of stabilized chromogen (tetramethylbenzidine)
g. Wash Buffer Concentrate: 21mL/vial containing a 25-fold concentrated solution of buffered surfactant with preservatives.
h. Stop Solution (1 bottle, 11 ml/bottle) containing diluted 1N hydrochloric acid (1N HCl).
i. Microtiter well reader capable of reading absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
j. Absorbance paper and graph paper.

Methods of preparing CRP
The human high sensitive CRP (hsCRP) ELISA is based on the principle of a solid phase enzyme-linked immunosorbent assay (Kindmark, 1972). The assay system utilizes a unique monoclonal antibody directed against a distinct antigenic determinant on the CRP molecule. This mouse monoclonal anti-CRP antibody is used for solid phase immobilization (on the microtiter wells). A goat anti-CRP antibody is in the antibody-enzyme (horseradish peroxidase) conjugate solution. The test sample is allowed to react simultaneously with the two antibodies, resulting in the CRP molecules being sandwiched between the solid phase and enzyme-linked antibodies. After 45-minute incubation at room temperature, the wells are washed with water buffer to remove unbound labeled antibodies. A tetramethylbenzidine (TMB) reagent is added and incubated for 20 minutes, resulting in the development of blue color. The color development is stopped with the addition of 1N HCl changing the color to yellow. The concentration of CRP is directly proportional to the color intensity of the test sample. Absorbance is measured spectrophometrically at 450 nm.

Methods and Procedures
i. Sample collection and storage
Blood sample was collected into a serum separator tube (SST). The SST was sealed and blood was allowed to clot for 30 minutes at room temperature before centrifugation for

15 minutes at 1,000 rpm. The serum was immediately removed and stored at ≤ - 20^0C (for 4 weeks) and -80^0C (for > 4 weeks).

ii. Preparation of Reagent Diluents
a. Calibrator Diluent dilution:
20 mL of Calibrator Diluent RDSP Concentrate was diluted with 80 mL of distilled water to prepare 100 mL of Calibrator Diluent RDSP (1X).
b. CRP Standard dilution:
200 µL of Calibrator Diluent was added into each of 6 polypropylene tubes on the rack. From the Standard bottle containing 50ng/mL of recombinant CRP, 200 µL of Standard Diluent was added into first tube, and mixed thoroughly after which 200 µL was pipetted out and added into second tube, and mixed thoroughly. The 2-fold dilution process was repeated serially up to the sixth tube using different pipette tips for each procedure to yield standard dilutions of 25ng/mL, 12.5ng/mL, 6.25ng/mL, 3.12ng/mL, 1.56ng/mL and 0.78ng/mL respectively. The Calibrator Diluent RDSP (1X) serves as the zero standard (ng/mL). The 50ng/mL serves as the high standard.
c. Substrate Solution:
Colour Reagents A and B were mixed together in equal volumes within 15 minutes of use in a Pyrex tube covered with adhesive strip to protect from light.

iii. Sample dilution:
990 µL of Calibrator Diluent RDSP (1X) was mixed with 10 µL of sample in each polypropylene sample tube to make up to 100 µL dilution.

iv. Enzyme-linked immunosorbent assay **(**All samples and reagents were allowed to attain room temperature before use. Also, all samples and standards were assayed in duplicates)
a. The microwells were secured on the rack and into each well were added 100 µL Assay Diluent RD1F, 50 µl of Standard or sample diluents respectively using separate pipette tips. The wells were covered with adhesive tape and incubated for 2 hours at room temperature.
b. The mixture in each well was decanted and the wells filled with 400 µL of Wash Buffer using a squirt bottle. Excess liquid was completely removed by aspiration and the process was repeated three times for a total of 4 washes. After the last wash, any remaining Wash Buffer was removed by aspiration, decanting, inverting the plate and blotting it against clean absorbent paper towels.
c. 200 µl of CRP Conjugate was added into each well, covered with adhesive tape, and incubated for 2 hours at room temperature.
d. The wells were washed as in step b above.
e. 200 µl of Substrate Solution was added into each well, and thoroughly mixed. The wells were covered with adhesive tape, and incubated for 30 minutes at room temperature.
f. At end of incubation, 50 µL of Stop Solution was added to each well and thoroughly mixed. The colour in the wells changed from BLUE to YELLOW.

v. Calculation of results

a. Optical density (OD) of each well was determined within 30 minutes, using a microplate reader set to 450 nm, corrected at a wavelength of 540nm by subtracting readings at 450nm. The average of the duplicate readings for each sample and Standard was subtracted from the average zero standard OD.

b. A standard curve was constructed using the mean OD values of each standard on the y-axis against the concentration on the x-axis and a best fit curve was drawn through the points on the graph. The unknown values were extrapolated from the standard curve.

AppendixXII: Quantikine®HS ELISA High Sensitivity Human IL-6 Immunoassay ™ kit (HSCC: 30021098)

a. Human highly sensitive IL-6 (hs IL-6) Microplate: 96 well polystyrene microplate (12 strips of 8 wells) coated a monoclonal antibody specific for human IL-6.
b. Human highly sensitive IL-6 (hs IL-6) Standard: Recombinant human IL-6 in a buffered protein base with preservatives; lyophilized.
c. Human highly sensitive IL-6 (hs IL-6) Conjugate: 21 mL/vial of polyclonal antibody specific for human IL-6 conjugated to alkaline phosphatase with preservatives.
d. Assay or sample Diluent RD1-75: 11mL/vial of a buffered animal serum with preservative.
e. Calibrator Diluent RD6-11 Concentrate: 21 mL/vial of a buffered protein base with preservatives.
f. Wash Buffer Concentrate: 100 mL/vial of a 10-fold concentrated solution of buffered surfactant with preservative.
g. Stop Solution: 6mL/vial of 2 N sulfuric acid.
h. Substrate: lyophilised NADPH with stabilizers.
i. Substrate Diluent: 7mL/vial of buffered solution with stabilizers and preservative.
j. Amplifier: Lyophilized amplifier enzymes with stabilizers.
k. Amplifier Diluent: 7mL/vial of buttered solution containing INT-violet with stabilizer and preservative.
l. Plate sealers: adhesive strips.
m. Microplate reader capable of measuring absorbance at 490 nm, with the correction wavelength set at 650 nm or 690 nm.
n. Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ±50 rpm.

Methods of preparing IL-6

The human highly sensitive IL-6 (hsIL-6) immunoassay is based on the principle of a solid phase enzyme-linked immunosorbent assay designed to measure human IL-6 in serum, plasma and urine (Kindmark, 1972). It contains E.coli –expressed recombinant human IL-6 and has been shown to accurately quantitate the recombinant factor. The assay employs a unique monoclonal antibody directed against a distinct antigenic determinant on the IL-6 molecule which has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any IL-6 present is bound by the immobilsed antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for IL-6 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. After an incubation period, an amplifier solution is added to the wells and colour develops in proportion to the amount of IL-6 bound in the initial step. The colour development is stopped and the intensity of the colour is measured.

Methods and Procedures.

i. Sample collection and storage:

Blood sample was collected in a serum separator tube (SST) and allowed to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 rpm. The serum was immediately removed and stored at $\leq$ - 20^0C.

ii. Preparation of Reagent Diluents

a. Calibrator Diluent RD6-11 was used undiluted in serum

b. Standard dilution:

The hsIL-6 Standard was reconstituted with Calibrator Diluent RD6-11 to produce a stock solution of 10 pg/mL. The stock solution was allowed to sit for 15 minutes with gentle agitation prior to making a dilution series in 6 tubes as discussed below: 500 µL of stock solution was pipetted into first tube, thoroughly mixed and 500µL was removed from it and transferred into 2^{nd} tube. The process was repeated serially to the 6^{th} tube to produce 5pg/mL, 2.5pg/mL, 1.25pg/mL,0.625pg/mL,0.313pg/mL and 0.156pg/mL solutions respectively. The reconstituted standard stock served as the high standard (10pg/mL) and Calibrator Diluent served as the zero standard (0pg/mL).

c. Substrate Solution:

The lyophilized Substrate was reconstituted with 6.0 mL of Substrate Diluent and mixed thoroughly at about 10 minutes before use.

d. Wash Buffer solution:

100 mL of Wash Buffer Concentrate was diluted to 1000mL with distilled water.

e. Amplifier Solution:

The lyophilized Amplifier was reconstituted with 6.0 mL of Amplifier Diluent and mixed thoroughly at about 10 minutes before use.

iii. Enzyme-linked immunosorbent assay procedure

All samples and reagents were allowed to attain room temperature before use. Also all samples and standards were assayed in duplicates

a. After the desired numbers of microwells were secured, 100µL of Assay Diluent RD1-75 was added into each well and thoroughly mixed.

b. 100µL of Standard or sample were added into their respective wells, covered with adhesive strip and incubated for 2 hours at room temperature on a horizontal microplate orbital shaker set at 450- 550 rpm.

c. After incubation, each well was filled with 400 µL of Washer Buffer using a squirt bottle, and the liquid was removed by inverting the plate and decanting the contents. Excess liquid was removed by grasping the plate firmly and smartly rapping the plate inverted on a clean absorbent paper towel at least 5 times. The above steps were repeated 5 times for a total of 6 washes. After the last wash, excess Wash Buffer was removed by rapping the inverted plate on a clean absorbent paper towel for at least 10 times.

d. 200µL of hs IL-6 Conjugate was added quickly into each well, covered with a new adhesive strip, and incubated for 2 hours at room temperature on the shaker.

e. The wells were washed as in Step c above.

f. 50µL of Substrate Solution was added to each well, covered with a new adhesive strip, and incubated for 60 minutes at room temperature on the bench top.

g. 50µL of Amplifier Solution was added to each well, covered with a new adhesive strip, and incubated for 30 minutes at room temperature on the bench top. This initiated the colour development.
h. 50µL of Stop Solution was added to each well, and the optical density of each well was read within 30 minutes, using a microplate reader set to 490/492nm, and corrected to 650nm by subtracting the readings at 4490/492nm.

v. Calculation of results
a. Optical density (OD) of each well was determined within 30 minutes, using a microplate reader set to 490/492nm, corrected at a wavelength of 650nm by subtracting readings at 490/492nm. The average of the duplicate readings for each sample and Standard was subtracted from the average zero standard OD.
b. A standard curve was constructed using the mean OD values of each standard on the y-axis against the concentration on the x-axis and a best fit curve was drawn through the points on the graph. The unknown values were extrapolated from the standard curve.

AppendixXIII: Materials forCD4+ cell count using the Partec flow cytometry instrument from CD4 easy count kit. SYSMEX Healthcare /Immunolgy; SysmexPartecGmbH.AmFltgplatz 13.02828. Görlitz. Germany

a. 2000 µl CD4 mAb PE (MEM-241, PE-conjugated monoclonal antibody to human CD4)
b. 100 ml no lyse buffer&Sheath fluid and Cleaning Solution
c. Decontaminating solution, Hypochlorite solution, Eva water and Sodium azide
d. Count check beads green and Tween 20
e. Partec test tubes (Rohren tubes), Micropipettes and pipette tips&Powder-free latex gloves.

Method of preparing CD4+ cell count

1. Principle of the assay.

Patients with HIV/AIDS exhibit T-cell lymphopenia, a loss of $CD4^+$ lymphocytes and a relative increase in the $CD8^+$ subtype and in the $CD3^+CD^-CD8^-$ subtype. The $CD4^+$ is a transmembrane glycoprotein (55 kDA) of the immunoglobin supergene family, present in the "helper/inducer" T-cells, and also expressed on a lower level on monocytes, tissue macrophages and granulocytes. $CD4^+$ is the primary cellular receptor for HIV. A mouse monoclonal IgG1 (MEM-241) antibody recognises and reacts with the human $CD4^+$ antigen to form antibody-antigen complex which is captured at an absorbance of 488nm or 532 nm for blue or green solid state laser. The monocytes, tissue macrophages and granulocytes when stained produce weaker signal intensity than the T-cell lymphocytes. While passing through a flow curette one by one, the cells are individually illuminated by the light spot of the laser. Due to the excitation, the dye molecules emit fluorescence of characteristic colour. This fluorescence light is separated into colour ranges by means of optical filters. The intensity of each colour range is analysed for each cell. Besides fluorescence, the intensity of light scattered by each cell can be measured. Scattered light is measured in forward direction from the light source (forward scatter, FSC) and sideward scatter (sideward scatter, SSC). The scatter intensity is a measure of cell size and morphology. Scatter light can be used to identify a cell before analyzing its fluorescence, but a cell can also be identified by fluorescence before analyzing its scatter properties.

2. Procedure

CD4 cell counts were determined within 4 hours of obtaining the whole blood sample using the CD4 easy count kit (Partec flow cytometry instrument).

i. 20µL of CD4 mAb was added to a Partec test tube
ii. 20µL of whole blood was aspirated from EDTA bottle into the Partec test tube
iii. The contents were gently mixed and incubated for 15 minutes at room temperature, protected from light.
iv. 800µL of no lyser buffer was added.
v. The tube was loaded into COUNTER II (SCRIPT-LOAD CONFIG SCRIPT-CD4) for CD4 absolute measurement.
vi. The machine was 'click start' to start CD4 count and the count continued until it stops automatically.
vii. The gating was adjusted when necessary and the count was noted.

AppendixXIV:Materials for determination of plasma HIV-1 using the AMPLICOR® HIV- 1 MONITOR Test, version 1.5 (Roche Molecular (Diagnostic) Systems, Branchburg, NJ 08876 USA)

a. HIV-1 Monitor Specimen Preparation reagents:
i. HIV-1 Monitor Lysis reagent (Tris-HCl buffer, 68% Guanidine thiocyanate, 3% Dithiothreitol, < 1% Glycogen)
ii. HIV-1 Monitor Quantitation Standard, version 1.5 (Tris-HCl buffer, < 0.001% non-infectious *in vitro* microbial transcribed RNA containing HIV-1 primer binding sequences and a unique probe binding region, < 0.005% synthetic Poly rA RNA, EDTA, Amaranth dye, 0.05% Sodium azide)
iii. HIV-1 Monitor Specimen Diluent (Tris-HCl buffer, < 0.005% synthetic Poly rA RNA, EDTA, 0.05% Sodium azide)

b. HIV-1 Monitor Control reagents:
i. Negative Human Plasma (human plasma, non-reactive by US FDA licensed for antibody to HCV, antibody to HIV-1/2, HIV p24 antigen and HBsAg; No HIV-RNA, HCV RNA or HBV DNA detected by PCR in pooled donor units; No HBV core antibody detected in pooled donor units by an FDA licensed test, 0.1% ProClin ® 300 preservative)
ii. HIV-1 (-) Control [Tris-HCl buffer, < 0.005% synthetic Poly rA RNA, EDTA, 0.05% Sodium azide]
iii. HIV-1 Monitor Low (+) Control [Tris-HCl buffer, < 0.001% non-infectious *in vitro* microbial transcribed RNA containing HIV-1 sequences, < 0.005% synthetic Poly rA RNA, EDTA, 0.05% Sodium azide]
iv.HIV-1 Monitor High (+) Control [Tris-HCl buffer, < 0.005% synthetic Poly rA RNA, EDTA, 0.05% Sodium azide]

c. HIV-1 Monitor Amplification reagents:
i. HIV-1 Monitor Master Mix, version 1.5 [Bicine buffer, Glycerol, <0.01% microbial rTth DNA Polymerase, Potassium acetate, < 0.07% dATP, dGTP, dTTP, dUTP; < 0.001% biotinylated SK145 and SKCC1B primers; < 0.001% AmpErase (microbial uracil-N-glycosylase,) enzyme; 0.05% Sodium azide]
ii. HIV-1 Manganese Solution, version 1.5[<2% Manganese; acetic acid; Amaranth; 0.05% Sodium azide]

d. HIV-1 Monitor Detection reagents:
i. HIV-1 Monitor Microwell Plates [MWP coated with HIV-1- specific DNA probe SK102 (Rows A to F) and QS-specific probe CP35 (Rows G and H); Twelve, B-well strips in one resealable pouch with desiccant
ii. Monitor Denaturation Solution [1.6% Sodium hydroxide, EDTA, Amaranth dye]
iii. Monitor Hybridization Buffer [Sodium phosphate solution; < 25% Sodium thiocyanate, < 0.2% Solubilizer]
iv. Avidin-Horseradish Peroxidase Conjugate [Tris-HCl buffer; < 0.001% Avidin-horseradish peroxidase conjugate; Bovine gamma globulin ; Emulsit 25; 0.1% Phenol; 1% ProClin ® 150 preservative]

v. Substrate A [Citrate solution, 0.01% Hydrogen peroxide; 0.1% ProClin ® 150 preservative]
vi. Substrate B [0.1% 3, 3', 5, 5'- Tetramethylbenzidine (TMB); 40% Dimethylformamide (DMF)

e. Stop reagent (4.9% Sulfuric acid)

f. 10 X Wash Concentrate (< 2% Phosphate buffer, < 9% Sodium chloride, EDTA, < 2% detergent, 0.5% ProClin ® 150 preservative)

g. COBASAmpliPrep and COBAS Taqman machines.
Methods of preparing plasma HIV-1 load

1. Procedure
i. The COBAS AmpliPrep, COBAS Taqman 96/48 was switched on.
ii. The machine was allowed to run for 30 minutes and stabilized, and the reagents were prepared.
iii. Reagent cassettes and consumables were loaded into machine.
iv. Sample and control barcode clips were attached to sample rack.
v. Sample tubes were inserted on sample rack.
vi. 1050µL of samples were swirled on vortex and then dispensed to the corresponding tubes.
vii. 1050µL of controls were swirled on vortex and then dispensed to the corresponding tubes.
viii. An order was created on Amplilink by selecting desired test definition file and assigning controls and patient samples to desired positions.
ix. 'Start the run'
x. Results were reviewed, accepted and printed out.
xi. Used consumables were removed and discarded appropriately.
xii. The COBAS AmpliPrep and COBAS Taqman was switched off.

AppendixXV:Materials forhuman serum zinc (Zn^{2+}) and copper (Cu^{2+}) using Atomic Absorption Spectrophometer (AAS) model number AA240FS (Varian, UK).

a. glassware (Pyrex volumetric flasks, Teflon beakers and watch glass)
b. polyethylene plastic bottles
c. non-ionic detergent solution and tap water and distilled water
d. 10% (v/v) nitric acid and H_2O_2
e. Freshly prepared mixture of concentrated nitric acid and hydrogen peroxide [HNO_3 + H_2O_2] (2:1 v/v).
f. hot plate and oven maintained at 60-80^{0}C

Methods of preparing zinc and copper

1. Pretreatment of sample containers:
All glassware (Pyrex volumetric flasks, Teflon beakers and watch glass) and polyethylene plastic bottles were thoroughly washed with non-ionic detergent solution to remove all dirt, and then rinsed with tap water to remove residues of detergent. They were also thoroughly rinsed with distilled water, and then soaked in 10% (v/v) nitric acid for 48 hours, after which they were rinsed with triply distilled water, and finally dried in the oven at 80^{0}C.
2. Wet acid method of protein digestion:
To each Pyrex volumetric flask was added 0.3 mL of serum sample and 3 mL of freshly prepared mixture of concentrated nitric acid and hydrogen peroxide [HNO_3 + H_2O_2] (2:1 v/v). The mixtures were allowed to stand for 10 minutes, after which the flasks were covered with watch glass and heated on hot plate at 60-70^{0}C for 2 hours. Additional 2mL nitric acid and 100µL of H_2O_2 were added into each flask while heating continued to 100^{0}C until clear digested solutions were obtained. The excess acid mixture was evaporated to semi-dry mass, which was cooled and diluted with 0.1mL nitric acid, and then transferred into 25mL volumetric flask which was filled to the mark using triply distilled water. The digested matrices were stored at 4^{0}C prior to flame atomic absorption spectrophotometry analysis.
3. Instrumentation:
Flame Atomic Absorption Spectrophometer (FAAS) model number AA240FS (Varian, UK) available at Multi-User Chemistry Laboratory, Ahmadu Bello University Zaria was used to measure the concentrations of Zn and Cu in the digested matrices.
4. Validation of extraction and analytic procedures was performed by using
a. Standardized human reference blood containing known quantities (10 ppm each) of Zn and Cu and subjecting them to same procedure as described above.
b. A blank extraction of 10 ppm each of Zn and Cu (without the sample) by subjecting them to same procedure as described above using triply distilled water.
5. Limits of detection:
Limits of detection of Zn^{2+} and Cu^{2+} at absorbance wavelength range of 300 - 400 nm and slit width of 2 were 0.006 and 0.002 respectively.

AppendixXVI: Materials for determining human serum lipids (total cholesterol, triglycerides, high density lipoprotein and low density lipoprotein) using automated Chemistry Analyser (Chemray 120, Kobe-Japan)

a. 1mL of Working Reagent
b. 10 µL of standard solution
c. Calibrator Diluent Concentrate and distilled water
d. Automated colorimetric reader.

Methods of preparing the lipid profiles

1. Specimen collection and storage.

Blood sample was collected after 9-12 hours of fasting into a serum separator tube (SST). The SST was sealed and blood was allowed to clot for 45 minutes at room temperature before centrifugation for 30 minutes at 1,500 x g at 4^0C. The serum was immediately removed and stored at ≤ - 20^0C (for 4 weeks) and -80^0C (for > 4 weeks).

A. Total cholesterol (TCHOL)

i. Reagent composition

a. Cholesterol R1 (50mL) consist of: pipes buffer (50mmol/L, pH 6.90); phenol 24mmol/L);
Sodium cholate (0.5mm/L).

b. Cholesterol R2 (50mL) consist of: cholesterol esterase (> 200 U/L); cholesterol oxidase
(> 250 U/L); peroxidase (> 1000 U/L); 4-aminoantipyrine (0.5mmol/L).

Note: Contents of R1 were dissolved with equal amount of R2 to form a working reagent (W.R) mixture which is stable for 90 days at 2-8^0C

c. Cholesterol Standard Concentration (200mg/dL)

ii. Principles of assay of TCHOL

TCHOL was measured enzymatically in serum in a series of coupled reactions that hydrolyse cholesteryl esters and oxidise the 3-OH group of cholesterol. One of the reaction by-products, H_2O_2 was measured quantitatively in a peroxidase catalysed reaction that produced a colour, measured at absorbance was measured at 500 nm; the colour intensity being proportional to TCHOL concentration.

iii. Procedures for analysis of TCHOL

a. 1mL of Working Reagent each was put into Blank tube, Standard tube and Assay tube.

b. 10 µL of sample was added into the Assay tube, mixed and allowed to stand for 5 minutes at 37^0C or for 10 minutes at room temperature.

c. 10 µL of distilled water was added into the Blank tube, mixed and allowed to stand for 5 minutes at 37^0C or for 10 minutes at room temperature.

d. 10 µL of standard solution was added into the Standard tube, mixed and allowed to stand for 5 minutes at 37^0C or for 10 minutes at room temperature.

e. Colour change in the respective Standard and Assay tubes was recorded at absorbance of 500 nm (480-520) and compared with absorbance of reagent Blank.

The result was calculated thus:

$$\frac{\text{Absorbance of sample (Assay)} \times \text{Standard concentration (200mg/dL)}}{\text{Absorbance of Standard}}$$

B. High Density Lipoprotein Cholesterol (HDL-C)
i. Reagent composition (same as TCHOL)

ii. Principles of assay of HDL-C.
HDL-Cis measured directly in serum. The basic principle of the method is as follows: the apoB containing lipoproteins in the specimen are reacted with a blocking reagent that renders them non-reactive with the enzymatic cholesterol reagent under conditions of the assay. The apoB containing lipoproteins are thus effectively excluded from the assay and only HDL is detected under the assay conditions. The method uses sulfated alpha-cyclodextrin in the presence of Mg^{2+}, which forms complexes with apoB containing lipoproteins, and polyethylene glycol-coupled cholesteryl esterase and cholesterol oxidase for the HDL measurement at absorbance at 600 nm.

iii. Procedures for analysis of HDL-C

These were summarized as illustrated below

Pipette	**Blank tube**	**Calibrator tube**	**Assay tube**
Reagent R1	300 µL	300 µL	300 µL
Calibrator		3 µL	
Specimen (sample)			3 µL

300 µL of reagent (R1) was added to 3 µL of sample in Assay tube, mixed vigorously and allowed to stand for 5 minutes at 37^0C or for 10 minutes at room temperature. A colour change was recorded at absorbance of 600 nm against reagent blank (A1). Then 100 µL of Reagent (R2) was added to the Blank, Calibrator and Assay tubes respectively, mixed vigorously and allowed to stand for 5 minutes at 37^0C or for 10 minutes at room temperature. With manual procedure, the net Absorbance = (A2-0.75nm A1) for Assay and Calibrator were calculated.
Therefore, HDL = $\frac{\text{net Absorbance} \times \text{Calibrator concentration}}{\text{net Absorbance Calibrator}}$
The results were expressed as mg/dL or mmol/L (mg/dL x 0.02586)

C. Triglycerides (TGDS)
i. Reagent composition for TGDs analysis
a. Triglycerides Reagent (100mL) consist of: pipes buffer (5mmol/L, pH 7.00); TOPS (5.3mmol/L); potassium ferrocynate (10mmol/L); magnesium salt (17 mmol/L); 4-aminoantipyrine (0.9 mmol/L); ATP (3.15 mmol/L); lipoprotein lipase (> 1800 U/L);

glycerol kinase (> 450 U/L); glycerol-3-phosphate oxidase (> 3500 U/L); peroxidase (> 450 U/L).
b. Triglyceride Standard Concentration (4mL, 200mg/dL).

ii. Principle of TGD assay.
TGDs were measured enzymatically in serum using a series of coupled reactions in which TGDs were hydrolysed to produce glycerol. Glycerol was then oxidized using glycerol oxidase to yield H_2O_2, one of the by-products, which was then measured quantitatively in a peroxidase catalysed reaction that produced a colour, measured at absorbance of 500 nm, the intensity of colour being directly proportional to concentration of TGD.

iii. Procedure for analysis of TGDs was same as for TCHOL and HDL-C.
D. Low Density Lipoprotein Cholesterol (LDL-C)
LDL-C is calculated from measured values of TCHOL, TGDs and HDL-C according to the relationship: LDL-C = TCHOL – (HDL-C + TGDs/5) (derived from the Friedwald's equation; where TGDs/5 is an estimate of Very low density lipoprotein (VLDL-C)). VLDL-C is one of three major circulating lipoprotein cholesterol fractions.

AppendixXVII: Materials for determining human serum albumin using automated Chemistry Analyser (Chemray 120, Kobe-Japan)

a. Albumin Reagent (75 mmol/L, pH 4.; 0.1M Succinate buffer)
b. Dye Stock/ working solution (0.14g/L, 0.6 M Bromocresol green)
c. Human reference serum albumin (3 g/dL of Albumin Standard)
d. Automated colorimetric reader.

Methods of preparing serum albumin using a colorimetric method as modified by Bartholomew and Delaney (1966); the principle and steps of which are described below:
In an acidic medium, albumin binds with the dye-bromocresol green (BCG) causing a shift in the absorption spectra of the yellow BCG dye. The blue green colour formed is directly proportional to the albumin present when measured at 630 nm (600-650nm or with Red filter).

1. Specimen collection and storage.
Blood sample was collected into a serum separator tube (SST). The SST was sealed and blood was allowed to clot for 45 minutes at room temperature before centrifugation for 30 minutes at 1,500 x g at 4^0C. The serum was immediately removed and stored at ≤ - 20^0C (for 4 weeks) and - 80^0C (for > 4 weeks).

2. Preparation of Reagents.
a. Albumin Reagent consisting of :
i. 0.1M Succinate buffer (75 mmol/L, pH 4.1) was prepared by diluting 11.9 g succinic acid in 990 mL of distilled water, and 10mL of 0.1 M NaOH, to form 1000 mL solution. It was stored at 4^0C until ready to use.
ii. 0.6 M Bromocresol green (0.14g/L) was prepared by dissolving 419 mg Bromocresol green in 10 mL of 0.1 M NaOH, and diluted with 990 mL of distilled water to form 1000 mL of Dye Stock/ working solution.
b. 3 g/dL of Albumin Standard served as human reference serum albumin.

3. Methods and procedures
a. The procedure is as shown below:

Pipette into test tubes	*Blank*	*Standard*	*Test Reagent*
Reagent (mL)	2.5	2.5	2.5
Sample (mL)	-	-	0.01
Standard (mL)	-	0.01	-

b. The content of tubes was mixed well & allowed to stand at room temperature for ten minutes. Absorbance of test and standard was read after ten minutes against reagent blank at 630 nm (600-650 nm or Red filter). The blank reagent (BCG) was used to set instrument to zero.
4. Calculation
Albumin level (gm/dL) = Absorbance of test (OD) x concentration of STANDARD (5g/dL)

Absorbance (OD) of STANDARD

Normal values of albumin= 3-5 g/mL (i.e., 30-50 g/dL)
Many times a ratio of albumin to globulin is considered. For getting the ratio, calculate globulin by using Globulin = serum total protein - serum albumin.

AppendixXVIII: Hemoglobin was measured using the *Sysmex XN* automated Haematology analyzer (Sysmex, Kobe- Japan) available at the ART laboratory at Ahmadu Bello University Teaching Hospital Shika Zaria, using thematerials and procedures of the test outlined below:

a. EDTA vacutainers with venepuncture tube and needle
b. Diluent (cell pack) lyse
c. WBC/HBC lyse reagent (stromatolyser-WH)
d. Detergent (cell clean)
e. *Sysmex XN* Automated Haematology Analyser (Sysmex, Kobe- Japan).

Procedure

The FBC was measured using the *Sysmex XN* automated haematology analyzer (Sysmex, Kobe- Japan) which can process 100 samples of 88µL anticoagulated blood per hour for packed cell volume (PCV), haemoglobin (Hb), total and differential white blood cell count (WBC), total platelet count and mean corpuscular volume (MCV).

Appendix XIX: Letter of Introduction of Constituents of Synovit^{TM} by Synergy Ltd, Lagos

Synergy Healthcare Ltd. RC 606220
Your caring partner.
Obikun Ayo House, Plot 15, Kudirat Abiola Way, Oregun, P. O. Box 13892 Ikeja, Lagos State
Tel: 01 - 896 7965, 893 6876. Email: contact@synergygroup.com. Website: www.synergygroup.com

August 27th, 2013

The Head of Research Team,
Department of Medicine,
Ahmadu Bello University Teaching Hospital,
Shika - Zaria, Kaduna.

ATTN: Dr O.R Obiako
Dear Sir,

INTRODUCING SYNOVIT CAPSULES FOR YOUR RESEARCH PROPOSAL.

Sequel to our agreement to use our Synovit (multivitamin &mineral supplement with Selenium) for your proposed research.

We hereby write to formally introduce the product to you. Below is the composition of Synovit capsules:

CONSTITUENTS	UNITS
Vit.A (as Palmitate)	2000IU
Vit.B1(Thiamine)	3mg
Vit.B2(Riboflavin)	3mg
Vit.B6(Pyridoxine HCL)	1.5mg
Vit.B12	2.5mg
Vit. C	50mg
Vit.E	10mg
Vit.B3(Niacinamide)	20mg
Calcium Panthothenate	10mg
Folic Acid	5mg
Magnesium (as light mag.oxide)	40mg
Zinc(Zinc Sulphate Monohydrate)	20mg
Copper Sulphate Pentahydrate	2mg
Potassium Sulphate	1mg
Choline Bitartrate	10mg
Molybdenum(Sodium Molybdate Dihydrate)	5mg
Manganese(Manganese Sulphate Monohydrate)	2.5mg
Glycine	10mg
L-Lysine(As L-Lysine HCL)	25mg
Ferrous Fumarate	50mg
Selenium(As Sodium Selenate)	100mcg

Aninwike Cyril

Head,Sales &Marketing.



Appendix XX:Comparison between constituents of SynovitTM and the United Nation Food and Agricultural Organization recommended nutrient intake per day (RNI/day)

Constituents of SYNOVIT™	Concentrations in units	RNI/ day
Vitamin A as palmitate	2000 IU	700µg/day (men), 600µg/day (women)
Vitamin B1(thiamine)	3mg	1.2—1.5 mg
Vitamin B2 (riboflavin)	3mg	1.3—1.6 mg
Vitamin B3 (niacinamide)	20 mg	6.6mg/kilocalorie
Vitamin B6 (pyridoxine)	1.5 mg	1.3—2.0 mg
Folate	5mg	200µg/day
Vitamin B12	2.5 mg	5.4 mg/day
Pantothenate as calcium salt	20mg	1.5mg/day
Biotin	-	26-39 mg/day
Vitamin C	50mg	40mg/day
Vitamin E	10mg	4mg (men), 3mg (women)
Zinc as sulphate	20mg	1.0-1.4mg
Copper as sulphate	2 mg	1.2mg
Selenium as sodium	100µg	30-40 µg
Ferrous fumarate	50mg	8.7-58.8mg
Manganese sulphate	2.5mg	-
Potassium sulphate	1mg	-
Magnesium oxide	2.5mg	-
Molybdenum sodium	5mg	-
Choline bitartarate	10 mg	-
L-glycine	10mg	-
L-lysine	25mg	-

Appendix XXI:Proforma

S.No. ---------- ID. No. ---------- Date of enrolment ----------

Tick ∫ on the appropriate response

1. Demographic Characteristics

Participant Name (Surname first) ----------
i. Age ----------
ii. Sex a. male b. female
iii. Religion a. Muslim b. Christian c. other ----------
iv. Marital status a. single b. married c. divorced/separated d. widowed
v. Education a. none b. primary c. secondary d. tertiary
vi. Occupation a. none b. gov't worker c. private worker d. student
e. self-employed f. apprentice g. Arm forces/police
vii. Estimated income per month (in Naira)
a. none b. below 18,000 c. 18,000 -36, 000 d. 37,000 – 50, 000
e. above 50,000

2. Complaints

Any Complaint No [ ] Yes- If Yes, specify below

HIV-related diagnosis a. No b. Yes. If Yes, Specify
---------- ---------- ----------
AIDS- Defining Illness a. No b. Yes. If Yes, Specify
---------- ---------- ----------

3. Physical Examination Findings

i. Height (with shoes/sandals off, in metres) only at first visit----------

ii. Weight [fully clothed, in kg] at all visits/ dates
First visit---------- 12th week ---------- 24th week ----------
36thweek ---------- 48th week ----------

iii. Other relevant findings/ OIs (specify) with dates ----------

---

iv. WHO clinical stage at first visit: a. I b. II

Relevant Laboratory Results

i. Date of Western blot /double ELISA HIV test ----------

ii. CD4+ cell count at HIV Diagnosis---------- Date ----------

iii. CD4+ cell count at other visits
First visit---------- 12th week ---------- 24th week ----------
36thweek ---------- 48th week ----------

CD4+ cell count at exit visit (other than 48 week) ----------
Number of weeks from baseline----------

iv. Viral load at first visit ---------- Date----------
24th week ----------
48th week ----------

v. HGB at first visit ----------

vii. HGB at other visits
First visit---------- 12th week ---------- 24th week ----------
36thweek ---------- 48th week ----------

viii. HGB at exit visit ---------- Date ----------
Number of weeks from baseline----------

viii. Serum albumin at first visit (baseline) Date ----------
ix. Serum albumin at other visits
24th week ----------
48th week ----------

x. Serum albumin at exit visit ---------- Date ----------
Number of weeks from baseline----------

xi. Serum C-reactive protein at first visit (baseline) ---------- Date ----------
xii. Serum C-reactive protein at other visits
24th week ----------
48th week ----------

xiii. Serum IL-6 at first visit (baseline) ---------- Date ----------
xiv. Serum IL-6 at other visits
24th week ----------
48th week ----------

xv. Micronutrient value at first visit (baseline) ---------- Date ----------

Zinc ----------
Copper----------
Vitamin B_{12} ----------
xvi. Micronutrient value at 24th and 48th weeks Date ----------
Zinc ----------
Copper----------
Vitamin B_{12}----------

xix. Date of EXIT from research ----------

Number of weeks from baseline ----------

xx. Reason for EXIT
a. Completed at 48th week
b. Eligible for ART
c. Lost to follow up
d. Voluntary withdrawal
e. pregnancy
f. Death

AppendixXXII: WHO Classification of body mass index and vital signs

A. BMI: Underweight <18.5 kg/m^2; Normal weight 18.5- 24.24.99 kg/m^2;
Overweight 25- 29.99 kg/m^2; Mild obesity 30- 34.99 kg/m^2;
Moderate obesity 35-39.99 kg/m^2; morbid obesity≥40 kg/m^2

B. Blood pressure: Normal <140/<90mmHg; Borderline 140-159/90-94mmHg;
Mild hypertension 140-179/90-104 mmHg; moderate 180/105mmHg;
Severe >180/>105mmHg

C. Temperature: normal axilla 35.8^{0}C-37.7^{0}C; febrile >37.8-40.9^{0}C;
Hyperpyrexia ≥40^{0}C

D. Pulse rates: 60-100 beats/minutes

E. Respiratory rates: 14-18 breaths/minutes