EVALUATION OF SERUM MALONDIALDEHYDE AND VITAMIN E LEVELS AS BIOMARKERS OF OXIDATIVE STRESS IN HIV/TB CO-INFECTED PATIENTS IN KANO, KANO STATE

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OCTOBER, 2014

DECLARATION

I declare that the work in the dissertation entitled EVALUATION OF SERUM MALONDIALDEHYDE AND VITAMIN E LEVELS AS BIOMARKERS OF OXIDATIVE STRESS IN HIV/TB CO-INFECTED PATIENTS IN KANO, KANO STATE was conducted by myself under the supervision of Dr. A. B. Adelaiye, Dr. M.U. Kawu and Prof. A. Abba. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this project was previously presented for another degree or diploma at any university.

AHMED SALISU IBRAHIM — Name

OCTOBER, 2014 — Date

CERTIFICATION

This research work titled "EVALUATION OF SERUM MALONDIALDEHYDE AND VITAMIN E LEVELS AS BIOMARKERS OF OXIDATIVE STRESS IN HIV/TB CO-INFECTED PATIENTS IN KANO, KANO STATE", by Ahmed Salisu Ibrahim meets the regulations governing the award of the Doctor of Philosophy Degree of Ahmadu Bello University Zaria, and is approved for its contribution to scientific knowledge and literary presentation.

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DEDICATION

I have dedicated this research work to my mother Hajiya Hassana Danjuma for the moral upbringing, support, courage and love she has for me.

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ABSTRACT

Both HIV and Tuberculosis infections are associated with lipid peroxidation and antioxidant imbalance. The Pathology of the two diseases produced free radicals which lead to lipid peroxidation indices one of which is Malondialdehyde (MDA) and to disease progression due to CD4 cells apoptosis. The current study was carried out with the objective of determining Serum MDA, Vitamin E, CD4, CD8 and lipid peroxidation index (LPI) in four different subgroups comprising of HIV, Tuberculosis and HIV/TB co-infected patients and the Controls respectively with a view to assessing the interaction between oxidants and antioxidants in the course of HIV/TB co-infection. Lipoperoxides, as Malondialdehyde by thiobarbituric acid reaction and Vitamin E in serum from all the four subgroups were measured spectrophotometrically. Lipid Peroxidation Index (LPI) was determined by obtaining the ratio of MDA and Vitamin E in these cohorts, while CD4 and CD8 cells count was obtained by flowcytometry. A total of 186 [HIV/TB co-infected patients, 44 (23.66%); HIV patients 57 (30.64%); Tuberculosis patients 44 (23.66%) and apparently normal controls 41 (22.04%)] were evaluated in this study. There were 105 men (56.5%) and 81 women (43.5%). The median values were compared among the four subgroups. Results showed the following patients/controls median values respectively for 1. MDA (HIV/TB co-infection = 3.81 umol/l, HIV patients = 4.60 umol/l, Tuberculosis patient = 3.73 umol/l vs 0.55 umol/l for controls), 2. CD4 (HIV/TB co-infection =200.5 cell/mm^3, HIV patients = 356.0 cell/mm^3, Tuberculosis patient = 571.0 cell/mm^3 vs 743.0 cell/mm^3 for controls), 3. Vitamin E (HIV/TB co-infection = 3.85 ug/mL, HIV patients = 7.99 ug/mL, Tuberculosis patient = 8.29 ug/mL vs 18.65 ug/mL for controls) and 4. LPI (HIV/TB co-infection = 0.99, HIV patients = 0.58, Tuberculosis patient = 0.45 vs 0.03for controls). The differences

between patients and controls were statistically significant (p<0, 05). There was a negative correlation between CD4 counts and MDA levels (rho = -0.144, p= 0.350); duration of treatment and MDA level (rho = -0.003, p=0.985) and Vitamin E concentration and CD4 cell counts (rho= - 0,144; p>0, 05) in the HIV/TB co-infection group. The estimated reference limits for MDA (0.025 and 0.975 fractals) were: HIV =1.99 μmol/L - 10.16 μmol/L; TB=1.55 μmol/L - 7.26 μmol/L; HIV/TB co-infection = 4.22 μmol/L - 4.57μmol/L and normal controls = 1.06 μmol/L - 3.29μmol/L lower and upper limits respectively. The higher MDA result from the subjects supports the assertion that HIV/TB co-infection is associated with increased lipid peroxidation and lower antioxidant potential. It can therefore be concluded that co-infection with Tuberculosis exacerbates oxidative stress in HIV patients, while treatment enhances their anti-oxidant status and ameliorates the oxidative stress.

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LIST OF ABBREVIATION

Abs: Antibodies

AIDS: Arquired Immuno Deficiency Syndrome

AKTH: Aminu Kano Teaching Hospital

ANT: adenine nucleotide translocator

ATT: Anti Tuberculosis Theraphy

BCG: Bacillus Calmette Guerin

BMI: Body Mass Index

CAMP: cyclic AMP

CMELIA: Cambodian Early versus Late Introduction of Antiretrovirals

CTGF: connective tissue growth factor

CPT: Co-trimoxazole preventive therapy

Cu: Copper

CVD: Cerebro Vascular Disease

DC-SIGN: DC-specific intercellular-adhesion-molecule-3-grabbingnonintegrin

DR: Death receptors

ELISA: Enzyme Linked Immuno Sorbent Assay

FE^{+2}: Ferous Iron

FE^{+3}: Ferric Iron

FRAP: Feric Reducing Antioxidant Power

GPX: Glutathione Peroxidase

GSH: Glutathione Reductase

GSSG: Glutathione Disulfide

H_2O: Dihydrogen Oxide (Water)

H_2O_2: Hydrogen Peroxide

HAART: Highly Active Anti Retroviral Therapy

HIV: Human Immuno Deficiency Virus

HAD: HIVassociated dementia

HCV: Human Cytomegalos Virus

HSC: Hepatic stellate cells

HT: Height

IDO: Indoleamine 2,3-dioxygenase, tryptophan

IFCC: International Federation of Clinical Chemistry

IHD: Ischaemic Heart Disease

JNK: Jun N-terminal kinase

LAM: lipoarabinomannan

LIP: Lipid Peroxidation Index

MHC: Major Histocompatibilty Complex

MDA: Malondialdehyde

MDM: Monocyte-derived macrophage

MN: Manganase

NAC: N-acetyl-L-cysteine

NADPH: Nucleotide adenine di Phosphate

NO: Nitric Oxide

$NO_{2:}$ Nitirc Dioxide

NOX2: Oxidase-derived superoxide

OxPC: Oxidized phosphatidylcholine

OH: Hydrogyl group

PD: Programmed-Death

PMN: Polymorphonuclear

PI: protease inhibitor

PKC: Protein kinase C

PTB: Pulmonary Tuberculosis

PUFA: Poly Unsaturated Fatty Acids

RNI: Reactive Nitrogen Species

ROS: Reactive Oxygen Species

SAPIT: Starting Antiretrovirals at Three Points in TB Therapy

SOD: Superoxide Dismutase

TAA: Total Antioxidant Capacity

TACE: TNF-alpha converting enzyme

TB: Tuberculosis

TBA: Thiobarbituric Acid

TCR: T cell receptor

Tim-3: T cell immunoglobulin and mucin domain 3

TLRs: Toll-like receptors

Treg: Regulatory T cells

TSLP: Thymic stromal lymphopoietin production

UHMWPE: Ultra High Molecular Weight Polyethylene

VS: Versus

WHO: World Health Organization

WT: weigth

Zn: Zinc

CHAPTER ONE

1.0 INTRODUCTION

Human Immunodeficiency Virus (HIV)/AIDS

Acquired immunodeficiency syndrome (AIDS) is a disease of the human immune system caused by the human immunodeficiency virus (HIV). This condition progressively reduces the effectiveness of the immune system and leaves individuals susceptible to opportunistic infections and tumors (Sepkowitz, 2001).

Scientific evidence has shown that, the Human Immunodeficiency Virus (HIV) is a retrovirus, which is a ribonucleic acid (RNA) virus so designated because of its genome that encodes an unusual enzyme, reverse transcriptase (RT) that enables the virus to make copies of its own genome as RNA in its hosts cells [that is, human T4 helper lymphocytes] (Oguntibeju *et al.,* 2008). The drastic increase in the number of people infected with HIV is not peculiar to a particular racial group, country or community despite the multidimensional efforts which have been made to combat this scourge (Weiss, 1996; Oguntibeju *et al.,* 2007a). It is reported that the virus selectively attacks and depletes T-lymphocyte bearing CD4+ receptors (T-helper cells) causing a predisposition to opportunistic infections and malignancies (Weiss, 1996) thus resulting in Acquired Immunodeficiency Syndrome (AIDS). The cellular receptors to HIV are cells that express the CD4+ T cell receptor (CD4+ T-cells or T4-cells) as well as other white blood cells including monocytes and macrophages. Glial cells in the central nervous system, chromaffin cells in the intestine and Langerhans cells in mucous membranes and skin that express CD4+ T cells receptor can also be infected (Paxon *et al.,* 1996). The possibility that there are other cellular targets apart from CD4+ is proved

by likelihood of neurons that can be infected. This creates the possibility of the presence of co-receptors in addition to CD4+ T cells to mediate fusion between HIV and its target cells (Grossman and Heberman, 1997). Recognition of the CD4+ T-cells by HIV-1 envelope glycoprotein (gp120) as the virus binds to and enters host cells to initiate rapid replication cycles (Oguntibeju *et al.,* 2007b) is the crucial step leading to cytopathic consequences of HIV infection of CD4+ T-cells and is an important factor in the initiation of HIV infection. The shed virions which are immunogenic then stimulate B cells to produce humoral antibodies and plasma cells through lymphoid hyperplasia that ultimately results in decreased number of infected cells as the CD4+ migrate through the germinal cells. The depletion in number of CD4+ T-cells exceeds the formation of new cells and may maintain this phase for many years with resulting general disorganization of the lymph nodes, loss of lymphoid function and integrity. After initial infection of the human host, the pace of immunodeficiency development, susceptibility to infection and malignancies became manifest and are generally associated with the rate of CD4+ decline (Enger *et al.,* 1996). This rate of CD4+ decline varies considerably from person to person and is not constant throughout the stages of HIV infection. Though, the virological and immunological process that take place during the period of rapid fall in the number of CD4+ T-cells are poorly understood, (Koot *et al.,* 1996); however the acceleration of the decline of CD4+ T-cells heralds the progression of the disease that is associated with the increasing rate of HIV-1 replication *in vivo* and declining cell-mediated immune response. Studies have shown that the host immunological alterations due to HIV infection results in progressive development of opportunistic infections and malignancy and is chiefly mediated/induced by deregulation of a cytokine profile production of ROS which also plays a role in the viral replication. In vitro experiments (Schreck *et al.,* 1991) have shown that ROS such as hydrogen peroxide can specifically

activate the nuclear factor kB to induce the expression and replication of HIV-1 in a human T cell line, and addition of antioxidant vitamins blocked activation of nuclear factor kB and inhibited HIV replication (Gagu *et al.,* 1989; Harakeh *et al.,* 1990; Harakeh *et al.,* 1991). Das *et al.,* (1990), stated that excessive production of reactive oxygen species (ROS) such as superoxide anions, OH- radicals, H_2O_2, may be related to an increased activation of Polymorphonuclear leucocytes (PMN) during infection. This is influenced by the pro-oxidant effect of TNFa produced by the activated macrophages during the course of HIV infection and secretion of pro-inflammatory cytokines IL-1, IL-6, and IL-8 (Kedzierska and Crowe, 2001). Gil *et al.,* (2003) reported the presence of substantial oxidative stress in HIV infection which was attributed to the role of viral proteins that increases ROS intracellularly, therefore increasing the apoptotic index and depleting the CD4+ T-lymphocytes. The ROS thus produced can then attack the double bonds in polyunsaturated fatty acids, inducing lipid peroxidation which may result in more oxidative cellular damage to the membrane lipids, proteins or DNA. Chronic oxidative stress experienced by patients infected with HIV leads to a condition in which there is increased consumption of antioxidants (such as Vitamin C, and E , selenium, and carotenoids) as well as micro-nutrients/ trace elements antioxidants (Vijayamalini and Manoharan, 2004). Stephen (2006), therefore, concluded that persistent chronic inflammation such as HIV infection place a long-term strain on antioxidant defenses, impairs immune functions, increases the severity of the disease as well as increases antioxidant requirement by the infected individual. Progression of HIV to AIDS in developed countries after initial infection is about 10 - 12 years for adults in the absence of antiviral therapy. However, some manifest full blown AIDS within 5 years of infection whereas others survive long term (> 10 years) asymptomatic HIV-1 infection without a significant decline in CD4 T cell count which may be attributed to either

infection with genetically defective HIV-1 variants or effective host antiviral immune response where the individual has active cytotoxic T-cell responses against HIV-1 infected cells (Haase, 1999).

Mycobacterium tuberculosis (TB)

Mycobacterium tuberculosis is considered as an etiologic agent of tuberculosis and the unifying feature of the genus is an acid-fast property (Bloom and Murray, 1992). Tuberculosis is a major cause of morbidity worldwide and is spread primarily through airborne transportation by aerosolized droplets during coughing, sneezing or talking especially in poorly ventilated areas (WHO, 1997). *Mycobacteria* are intracellular pathogens that grow and replicate in the host macrophages. It is characterized by unexplained cought persisting for more than three weeks, haemoptasis, pleural pain is not associated with an acute illness, loss of appetite, spontaneous pneu-mothorax, vomiting/dehydration, unexplained tiredness, loss of weight, high remittent or intermittent pyrexia, pleural effusion, severe and continuous diarrhoea in cases of tuberculosis enteritis, drenching sweats during sleep and anaemia (Macleod, 1981).

The primary target for *Mycobaterium Tuberculosis* is the alveolar macrophage which can also be infected with HIV (Landayet *al.,* 1993; Sierra-Mader *et al.,* 1994; Nakata *et al.,* 1995). *Mycobacteria* exacerbate HIV replication in macrophages and lung cells obtained by bronchoalveolar lavage from co-infected individuals (Zhang *et al.,* 1995; Nakata *et al.*, 1997; Orenstein *et al.*, 1997). Also, in vitro studies have demonstrated that *Mycobaterium Tuberculosis* infection can up-regulate both HIV infection and replication within monocyte-derived macrophages (MDMs), increase the efficiency of virus transmission from infected MDMs to T cells, and favor replication of X4 HIV variants by up-regulation of CXCR4 (Mancino *et al.,* 1997). Furthermore, monocytes

from HIV^+ patients display an impaired response to Terminal long receptors (TLR) ligands (Jiang *et al.,* 2005), and viral proteins can interfere with both MDM and DC maturation and function in vitro, including their ability to phagocytose mycobacteria and kill intracellular bacteria (Biggs *et al.,* 1995; Kedzierska *et al.,* 2000; Muthumani *et al.,* 2005; Sacchi *et al.,* 2007).

Mycobacteria are contained within the granuloma, which, by localizing infection and thus potentially preventing spread of the disease between hosts, probably contributes to protection. $CD4^+$ T cells and TNF are important in maintaining granuloma organization. Granuloma formation may fail in individuals with a compromised immune system, and there are several hypotheses about how HIV exacerbates TB pathology through the manipulation of granulomas (Diedrich and Flynn, 2011). Specifically, TB patients with AIDS present a dominant granulocytic infiltrate and necrosis without the typical caseous necrosis seen in non-HIV-infected TB granulomas. This has been associated with the killing of $CD4^+$ cells in the granuloma, probably resulting in a direct disruption of granuloma structure and abolition of the containment of infection. Cavitary lesions are seldom encountered in patients with a CD4 T-lymphocyte count $<200/mm^3$ (Sharma *et al.,* 2005). As a result, while in the majority of adult patients TB is confined preferentially to the lungs, in HIV-infected patients TB can be a systemic disease involving multiple organs that lack well-defined granulomas and instead develop more diffuse lesions leading to all forms of extrapulmonary TB (De-Noronha *et al.,* 2008).

While TNF production in response to *Mycobaterium Tuberculosis* infection is required for control of bacterial growth, TNF is known to activate HIV replication in macrophages (Kedzierska *et al.,* 2003), indicating that the host immune response initiated against one pathogen may promote the replication of another. Thus, both HIV

and *Mycobaterium Tuberculosis* stimulate TNF release from infected cells, and TNF hampers bacterial growth while enhancing HIV replication.

Mycobaterium Tuberculosis survives in DCs and actively down-regulates their pro-inflammatory activity and antigen-presenting function, with concurrent induction of anti-inflammatory cytokines (Jiao *et al.*, 2002). Similarly, HIV can infect and also manipulate DCs and the ensuing T cell functions (Donaghy *et al.*, 2004). In HIV infection, not only is DC-mediated activation of T cells impaired, but the migration of infected DCs can also contribute to pathogen dissemination.

Factors contributing to the resurgence of tuberculosis in developing countries and problems of its control include co-infection with HIV, emergence of multi drug resistance tuberculosis, inadequate treatment, poverty, malnutrition, overcrowding, armed conflict and increasing number of displaced persons. The contribution of Reactive Oxygen Species (ROS) in the bacterial activity of phagocytes has been in the limelight ever since Klebanoff revealed the bactericidal potency of the mycloperoxidase-H O -halide system of human neutrophil granules Klebanoff, (1975). The sensitivity of mycobacteria to the bactericidal potency of phagocytes cells *in vitro* differs from one Mycobacterium species to the other, (Orme and Collins, 1983). A growing literature documents a possible interaction between oxidative stress and pathobiology of mycobacterial infections (Eze *et al*., 1993). Local lung oxidant damage has been implicated to play a role in the pathogenesis of lung mycobacterium infections (Niwa *et al*., 1984), given the combination of direct bacterial lung cell injury and the subsequent massive infiltration of "activated"inflammatory cells that occurs.

In an attempt to kill mycobacterium, host cells namely macrophages, neutrophils and monocytes generate huge amount of reactive oxygen species which contribute to

inflammatory injury to host tissues and may further lead to immunosuppression particularly in those with impaired antioxidant capacity such as HIV infected individuals. Moreover the malnutrition which is commonly present in patients with tuberculosis can add to the impaired antioxidant capacity in these patients (Kauri *et al*., 2005).

HIV/TB co - infection

The emergence of Human Immunodeficiency Virus (HIV) has paved way for the resurgence of *Mycobacterium tuberculosis* infection. While HIV is the most powerful risk factor for the progression of *Mycobacterium tuberculosis* infection to TB disease, TB accelerates the progression of HIV infection to AIDS and shortens the survival of such patients (Yusuph, 2005; Van Altena, 2007). The two are intricately linked to malnutrition, unemployment, poverty, drug abuse and alcoholism and have been referred to as the Cursed Duet (Sharma, 2005).

Human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS), since the time of its description more than three decades ago, has relentlessly spread all around the globe showing no sign of abatement. The sub-Saharan Africa bears the brunt of being the region with the highest burden of both Human Immunodefiency Virus (HIV) infection and of tuberculosis (TB) (Adeiza *et al.*, 2014). In 2004, there were 4.9 million new infections and 3.1 million deaths due to HIV/AIDS (UNAIDS, 2004), mainly in sub-Saharan Africa and South-East Asia. Unfortunately, these are the parts of the world where TB has been flourishing unhindered since ages, forming a deadly synergy (Sharma, 2005). In 2010, about 68% of all people living with HIV resided in sub-Saharan Africa, a region with only 12% of the global population (WHO, 2011). South Africa's HIV epidemic remains the largest in the world with an estimated 5.6

million people living with HIV, while Nigeria continues to have the second largest number of people living with HIV in sub-Saharan Africa (WHO, 2011). Both countries are listed among the 22 high TB burden countries (WHO, 2010). Advent of the HIV/AIDS pandemic has led to a dramatic increase in the number of TB cases worldwide. Globally, 9 percent of all new TB cases (31% in Africa) in adults were attributable to HIV/AIDS. As a result of HIV/AIDS, incidence rates of TB in certain countries have gone up by more than 6 percent per year (Narian, 2002), crippling the already overburdened health care resources. Considering the fact that a third of the world's population are infected with TB, more than a half of which lives in countries ravaged by HIV/AIDS, the gravity of the situation becomes evident (Narian, 2002; Sharma and Mohan, 2004). The full cost of the TB epidemic in sub-Saharan Africa is rarely appreciated (Adeiza *et al.,* 2014). The direct monetary costs of diagnosis and treatment are borne by health services and by patients and their families (Russel, 2004; Sadoh). Added to these are the indirect costs of lost of income and production incurred when TB patients are too sick to work and when young adults-often parents and household heads, die prematurely (Makgoba *et al*., 2006). Beyond these losses, enormous psychological and social costs are associated with TB. These extra costs are less easily quantified, but they are nonetheless real (Adeiza *et al.,* 2014).

TB is a major public-health problem and patients with PTB whose sputum is smear-positive for *M. tuberculosis* form the main source of infection in communities (Adeiza *et al.,* 2014). It is estimated that around two billion people around the world are currently infected with the bacillus but yet only 10% of infected immunocompetent individuals are likely to develop symptomatic TB during their lifetime when compared to 50% in individuals that are immunologically weakened by concurrent HIV infection (Vynnycky and Fine, 2000). The risk of developing the clinical manifestations of the disease is

greatly increased by HIV co-infection (Vynnycky and Fine 2000; Harries and Dye, 2006; Keshinro and Mukadi 2006; Idh *et al.,* 2008). This strong association between HIV and TB in sub-Saharan Africa is responsible for the massive increase in the incidence of TB observed in the region in the last 20 years (Adeiza *et al.,* 2014). This high risk of HIV patients developing TB could be related to the fact that macrophages that are not activated by CD4+ T-lymphocytes are unable to restrict the growth of *M. tuberculosis* (Keshinro and Mukadi 2006).

The WHO global TB control report estimated that 8.8 million incident or new TB cases occurred in the world in 2010 (WHO, 2011). The worldwide TB incidence rates are estimated to have peaked in 2004 and to have decreased at a rate of not less than 1.3% per year since that time. However, the overall worldwide burden continues to rise as a result of the rapid growth of the world population (WHO, 2011). The highest incidence rate was seen in the WHO Asian and African regions with 9 sub-Saharan African countries listed among the 22 high burden TB countries (Adeiza *et al.,* 2014). These countries include Democratic Republic of Congo, Ethiopia, Kenya, Mozambique, Nigeria, South Africa, Uganda, United Republic of Tanzania and Zimbabwe. While 13% of all new infections worldwide were attributable to HIV, in sub-Saharan Africa alone, approximately 39% of all new TB infections were attributable to HIV WHO, (2011).

The interaction between TB and HIV is also characterized by a high mortality rate in individuals co-infected with HIV and *M. tuberculosis* (Adeiza *et al.,* 2014). In 2010, of the estimated 1.40 million deaths from TB, 0.35 million deaths i.e. 25% were attributable to HIV compared to 12% in the year 2000 (WHO, 2011). There were an estimated 440,000 cases of MDR-TB in 2008 (WHO, 2010) and as at July 2010, 58 countries and territories had reported at least one case of extensively drug-resistant TB

(XDR-TB). It is estimated that approximately one third of the 33.2 million people living with HIV or AIDS worldwide are co-infected with TB (Getahun *et al.,* 2010). The relative risk of TB among HIV-infected persons, compared with that among HIV-uninfected persons, ranges between 20- to 37-fold, depending on the stage of the HIV disease (WHO, 2011). The majority of HIV-infected persons do not know their HIV status and there are no global data to quantify the proportion of the population that knows their HIV status (Getahun H *et al.,* 2010).

Reactive Oxygen Species and Reactive Nitrogen Species

Reactive oxygen species / reactive nitrogen species (ROS/ RNS) are constantly being formed in living organisms (Ceconi *et al.,* 2003). In the course of oxygen metabolism, 1 - 5% of all inhaled oxygen becomes ROS (Berk, 2007). Endogenously, ROS are produced from various sources such as mitochondria, activated macrophages and leucocytes, oxidase enzyme (NADPH), cyclo-oxygenase and lipoxygenase (Zalba *et al.,* 2006). Reactive oxygen species have oxidation ability and are classified either as free radicals (superoxide anion $O_2^{\cdot -}$, hydroxyl radical OH^-, nitric oxide NO) or as non-free radicals [hydrogen peroxide H_2O_2, peroxynitrite ONOO-] (Higashi *et al.,* 2006). Previous studies have shown the involvement of ROS in physiological and pathophysiological conditions (Fortuño *et al.,* 2005; Heistad *et al.,* 2009). At low concentrations, ROS are involved in normal cell signaling pathways (smooth muscle and endothelial cell growth, apoptosis and survival) and in the remodeling of vessel walls (Fortuño *et al.,* 2005; Heistad *et al.,* 2009). At high concentrations, ROS are identified as harmful compounds and constitute an important risk factor for the development of many diseases such as cardiovascular diseases (Heistad *et al.,* 2009). The pathophysiology of Cerebro vascular disease (CVD) and Ischemic Heart Disease (IHD) is multifactorial but it has been shown that the underlying pathogenesis is the deposition

of fatty material, mainly LDL cholesterol, on the inner vascular wall of the blood vessels of the heart (Maxwell and Lip, 1997).

Oxidative Stress

Oxidative stress occurs when there is a dysfunction in the overall balance between the production of reactive oxygen and nitrogen species and the antioxidant defense mechanisms (Ceconi *et al.,* 2003). Oxidative stress is believed to play a critical role in the pathophysiology of cardiovascular diseases such as artherosclerosis, stroke and hypertension (Heistad *et al.,* 2009). In the context of oxidative stress in CVD and IHD, the major vascular ROS which is superoxide anion ($O_2^{\cdot -}$) is predominantly generated by the NADPH oxidase enzyme (Fortuño *et al.,* 2005). Superoxide is normally dismutased to hydrogen peroxide (H_2O_2) by a family of superoxide dismutase (intracellular Cu/ Zn SOD, Mn SOD or extracellular Cu/Zn SOD) (Hamilton *et al.,* 2004). Hydrogen peroxide is scavenged into oxygen and water by catalase enzymes or by glutathione peroxidase (GPx) in the presence of reduced glutathione (Hamilton *et al.,* 2004; Zalba *et al.,* 2006). In the pathophysiological process of oxidative stress, excess superoxide has many effects: superoxide combines with NO to form peroxynitrite. Peroxynitrite is a highly toxic oxidant which causes vascular cell damage through oxidation of lipids (lipid peroxidation), proteins (protein nitrosilation) and nucleic acids with superoxides causing vascular dysfunction by removing the protective effects of NO (Heistad *et al.,* 2009); initiates the development of vascular inflammatory state (Hamilton *et al.,* 2004); Facilitates the oxidation of LDL, causing development of artherosclerotic lesions (Zalba *et al.,* 2006); and triggers apoptotic cell death (Ceconi *et al.,* 2003). Accumulating evidence has suggested that oxidative stress, mainly through lipid peroxidation, represents an important risk factor in the development of CVD and IHD (Waterfall *et al.,* 1997; Ceconi *et al.,* 2003). In fact, lipid peroxidation leads to membrane disruption

and release of highly reactive free radicals such as MDA that can severely alter the cellular function (Ceconi *et al.,* 2003).

Activation of Oxygen

Oxygen is essential for energy metabolism and respiration but it has been implicated in many disease and degenerative conditions (Ceconi *et al.,* 2003). Activation of oxygen may occur by two different mechanisms: absorption of sufficient energy to reverse the spin on one of the unpaired electrons and monovalent reduction. Nonactivated oxygen is a bi-radical. It can be activated by either reversing the spin on one of the unpaired electrons to form the singlet state or by reduction. In the monovalent reduction of oxygen, superoxide (O_2-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and finally, water (H_2O) is formed. Superoxide forms the hydroxyl radical (OOH^-) which is a powerful oxidant unit protonated form (Gebicki and Bielski, 1981; Ceconi *et al.,* 2003). Numerous enzymes (peroxidases) use hydrogen peroxide as a substrate in oxidation reactions involving the synthesis of complex organic molecules. Haber and Weiss, (1994) identified the hydroxyl radical as the oxidizing species in the reaction between H_2O_2 and ferrous salts: $Fe^{2+} + H_2O_2 = Fe^{3+} + OH + OH$. Most oxygen is consumed by the cytochrome oxidase enzyme in the mitochondrial electron transport system. Isolated mitochondria produce H_2O_2 and O_2^- in the presence of NADH (Loschen *et al.,* 1974). The various Fe-S-protein and NADH dehydrogenase have also been implicated as possible sites of super-oxide and hydrogen peroxide formation (Waterfall *et al.,* 1997). Various oxidative processes including oxidation hydroxylations, dealkylations, deaminations, dehalogenation and desaturation occur on the smooth endoplasmic reticulum. Mixed function oxygenases that contain a heme moiety add an oxygen atom into an organic substrate using NADPH as the electron donor. The generalized reaction catalyzed by cytochrome P450 is: $RH + NADPH + H^+ + O_2$ ROH +

$NADP^+ + H_3O$. Superoxide is produced by microsomal NADPH dependent electron transport involving cytochrome P450 (Valko *et al.,* 2007).

Compartmentalized enzymes involved in the B-oxidation of fatty acids and glyoxylic acid cycle includes glycolate oxidase, catalase and various peroxidases. Glycolate oxidase produces H_2O_2 in a two-electron transfer from glycolate to oxygen (Lindquist *et al.,* 1991). Xanthine oxidase, urate oxidase and NADH oxidase generate superoxide as a consequence of the oxidation of their substrates (Fridorich, 1970). The xanthine oxidase reaction is often used *in vitro* as a source of superoxide producing one mole of superoxide during the conversion of xanthine to uric acid (Fridorich, 1970). A superoxide generating NADPH oxidase activity has been clearly identified in plasmallema-enriched fractions (Valko *et al.,* 2007). These flavopoteins may produce superoxide by the redox cycling of certain quinones or nitrogenous compounds, while NADPH oxidase reduces Fe^{3+} to Fe^{2+} converting it to a form that can be transported. Dysfunction of NADPH oxidase results in the formation of superoxide radicals (Maxwell and Lip, 1997).

Antioxidants

Maxwell and Lip (1997) reported that a set of scavenging systems called antioxidants has evolved to protect and limit the potential threat of oxidative stress. These include: preventive antioxidant enzymes such as superoxide dismutases (SOD), catalase and glutathione peroxidase; preventive antioxidant proteins such as the iron-binding protein; transferrin, copper-binding proteins; caeruloplasmin and albumin; preventive low molecular weight molecules divided into two groups: water-soluble molecules such as vitamin C, urate, polyphenol (flavonoids) and thiol groups (glutathione); and lipid-soluble antioxidants such as vitamin E (alpha-tocopherol, tocotrienols), ubiquinol-10

(reduced coenzyme Q10) and beta-carotene. Most of these antioxidants are derived from dietary sources (fruits and vegetables including red palm oil).

Tocopherols and Tocotrienols (Vitamin E):

Vitamin E It is a naturally occurring antioxidant, essential for normal reproduction in many animals. It is the collective named for a set of eight related tocopherols and tocotrienols, which are fat-soluble vitamins with antioxidant properties (Herrera and Barbas, 2001; Packer *et al.,* 2001). Of these, α-tocopherol has been most studied as it has the highest bioavailability, with the body preferentially absorbing and metabolising this form (Brigelius-Flohé *et al.,* 1999). It has been claimed that the α-tocopherol form is the most important lipid-soluble antioxidant, and that it protects membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction (Herrera and Barbas, 2001). This removes the free radical intermediates and prevents the propagation reaction from continuing. This reaction produces oxidised α-tocopheroxyl radicals that can be recycled back to the active reduced form through reduction by other antioxidants, such as ascorbate, retinol or ubiquinol (Wang, 1999). This is in line with findings showing that α-tocopherol, but not water-soluble antioxidants, efficiently protects glutathione peroxidase 4 (GPX4)-deficient cells from cell death (Seiler *et al.,* 2008). GPx4 is the only known enzyme that efficiently reduces lipid-hydroperoxides within biological membranes (Seiler *et al.,* 2008).

However, the roles and importance of the various forms of vitamin E are presently unclear, (Brigelius-Flohé *et al.,* 2007 and Atkinson *et al.,* 2008), and it has even been suggested that the most important function of α-tocopherol is as a signaling molecule, and that it has no significant role in antioxidant metabolism (Zingg *et al.,* 2004; Azzi and Angelo, 2007). The functions of the other forms of vitamin E are even less well-

understood, although γ-tocopherol is a nucleophile that may react with electrophilic mutagens, (Brigelius-Flohé *et al.,* 1999), and tocotrienols may be important in protecting neurons from damage (Sen *et al.,* 2006).

Chemistry of Vitamin E

Vitamin E refers to a group of ten lipid-soluble compounds that include both tocopherols and tocotrienols (Brigelius-Flohé and Traber, 1999; Reboul *et al.,* 2006). Alpha-tocopherol, the most biologically active form of vitamin E, is the second-most common form of vitamin E in the diet. This variant can be found most abundantly in wheat, germ oil, sunflower, and sanflower oils (Brigelius-Flohé and Traber, 1999). As a fat-soluble antioxidant, it stops the production of reactive oxygen species formed when fat undergoes oxidation (Herrera and Barbas, 2001; Packer *et al.,* 2001; NHI, 2009). Alpha-Tocopherol is an important lipid-soluble antioxidant and performs its functions as antioxidant in the glutathione peroxidase pathway (Wefers, and Sies, 1988), it protects cell membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction (Herrera and Barbas, 2001; Traber and Atkinson, 2007). This would remove the free radical intermediates and prevent the oxidation reaction from continuing. The oxidized α-tocopheroxyl radicals produced in this process may be recycled back to the active reduced form through reduction by other antioxidants, such as ascorbate, retinol or ubiquinol (Wang and Quinn, 1999). Other forms of vitamin E have their own unique properties; for example, γ-tocopherol is a nucleophile that can react with electrophilic mutagens (Brigelius-Flohé and Traber, 1999).

Absorption, Transport and Storage

Vitamin E is absorbed along with fat in the small intestine. Bile salts are necessary for its absorption. In the liver, it is incorporated into lipoproteins (VLDL and LDL) before been transported. Vitamin E is stored in adipose tissues, liver and smooth muscles.

Biochemical Function of Vitamin E

Most of the functions of vitamin E are related to its antioxidant property. It prevents the nonenzymatic oxidations of various cell components; unsaturated fatty acids by molecular oxygen and free radicals such as superoxide and hydrogen peroxide.

Vitamin E has many biological functions, the antioxidant function being the most important and best known (Brigelius-Flohé and Traber, 1999). Other functions include enzymatic activities, gene expression, and neurological function(s). The most important function of vitamin E has been suggested to be in cell signaling [and it may not have a significant role in antioxidant metabolism] (Azzi, 2007; Zingg and Azzi, 2004).

Other functions include:-

i As an antioxidant, vitamin E acts as a peroxyl radical scavenger, preventing the propagation of free radicals in tissues, by reacting with them to form a tocopheryl radical, which will then be reduced by a hydrogen donor (such as vitamin C) and thus return to its reduced state. As it is fat-soluble, it is incorporated into cell membranes, which protects them from oxidative damage. Vitamin E has also found use as a commercial antioxidant in ultra high molecular weight polyethylene (UHMWPE) used in hip and knee replacements, to help resist oxidation (UHMWPE , 2009).

ii As an enzymatic activity regulator, for instance, protein kinase C (PKC), which plays a role in smooth muscle growth, can be inhibited by α-tocopherol. α-Tocopherol has a stimulatory effect on the dephosphorylation enzyme, protein phosphatase 2A, which in turn, cleaves phosphate groups from PKC, leading to its deactivation, bringing the smooth muscle growth to a halt (Schneider, 2005).

iii Vitamin E also has an effect on gene expression. Macrophages rich in cholesterol are found in the atherogenetic tissue. Scavenger receptor CD36 is a class B scavenger receptor found to be up-regulated by oxidized low density lipoprotein (LDL) and binds it (Devaraj *et al.,* 2001). Treatment with α-tocopherol was found to downregulate the expression of the CD36 scavenger receptor gene and the scavenger receptor class A (SR-A) (Devaraj *et al.,* 2001) and modulates expression of the connective tissue growth factor (*CTGF*) (Azzi and Stocker, 2000; Villacorta *et al.,* 2003). The *CTGF* gene, when expressed, is responsible for the repair of wounds and regeneration of the extracellular tissue lost or damaged during atherosclerosis (Villacorta *et al.,* 2003).

iv Vitamin E also plays a role in neurological functions (Muller, 2010), and inhibition of platelet aggregation (Dowd and Zheng, 1995; Brigelius-Flohé and Davies, 2007; Atkinson *et al.,* 2008).

v Vitamin E also protects lipids and prevents the oxidation of polyunsaturated fatty acids (Whitney and Sharon, 2011).

So far, most human supplementation studies about vitamin E have used only α-tocopherol. This can affect levels of other forms of vitamin E, e.g. reducing serum γ- and δ-tocopherol concentrations. Moreover, a 2007 clinical study involving α-tocopherol

concluded supplementation did not reduce the risk of major cardiovascular events in middle-aged and older men (Sesso *et al.,* 2008).

Recommended Dietary Allowance (RDA)

Vitamin E requirement increases with increased intake of Poly Unsaturated Fatty Acids (PUFAs). A daily consumption of about 10mg of tocopherol for man and 8mg for woman is recommended. Regular consumption of more than 1,000 mg (1,500 IU) of tocopherols per day (NIH, 2000) may be expected to cause Hypervitaminosis E, with an associated risk of vitamin K deficiency and consequently of bleeding problems.

Deficiency Symptoms

Vitamin E deficiency can cause: spinocerebellar ataxia(Traber and Atkinson, 2007), myopathies (Brigelius-Flohé, 1999), peripheral neuropathy, ataxia, skeletal myopathy, retinopathy, impairment of the immune response (NIH, 2009; Kwodley, 1999) and red blood cell destruction (Whitney and Sharon, 2011).

1.1 Background of the study

The molecular mechanisms of increased HIV replication in patients with HIV-TB are increasingly being understood. An oxidative stress occurs in HIV infection even at the early stages of disease (Repeto, 1996; Weissman, 1996). This pro-oxidant state is the result of an imbalance between the generation of reactive oxygen and nitrogen species (ROS) and the antioxidant system, which is responsible for scavenging these oxidants and ensuring that they avoid interacting with cellular targets. ROS and antioxidants are essential parts of the basic biology and physiology (Halliwell, 2006). They were considered as products of normal physiological processes, involved for instance in the transport of electrons in the mitochondrion and also serve as signaling molecules (Valko *et al.,* 2007). Several metabolic pathways release ROS following polymorphonuclear

activation during respiratory burst leading to the release of hypohalite ions such as superoxide anion radical, hydrogen peroxide, hydroxyl radical, singlet oxygen, nitric oxide, and peroxynitrite, including the activation of immune competent and phagocytic cells (Schwarz, 1996). The ROS productions are particularly increased in the activated phagocytes and neutrophils representing one part of the microbial activity. ROS are capable also in neutralizing the ever-changing virulence of micro-organisms. The ability of immune system to sterilise a site of infection, by rapid production of ROS keeps organism alive. Activation of the immune system may produce a significant local or systemic increase of ROS generation. An excessive amount of these molecules could leads to oxidative modification and, therefore, dysfunction of proteins, nucleic acids, carbohydrates and lipids (Block *et al*., 2002). The extent of damage that this stress could cause depends on the condition of the antioxidant defense system at the site of inflammation. Proteins modified by ROS exhibit altered structures, undergo spontaneous fragmentation, manifest increased proteolytic susceptibility, and loss of function. Oxidative protein damage has been found to be high in different parts of the body and to be increased during aging, neurodegenerative disorders and HIV infection (Roberts *et al.,* 2010). Phospholipids of biological membranes are highly susceptible to oxidation by ROS and also are involved in the process known as lipid peroxidation. This is a free radical mediated pathway and its final products are used as an index of irreversible damage of cell membrane phospholipids (Roberts *et al.,* 2010). Oxidative modification to DNA and its consequences such as gene mutation and DNA deletions have long been implicated in the pathogenesis of a variety of human disorders associated with mitochondrial dysfunction and aging (Devasagayam *et al.,* 2004). Oxidative damage to DNA can be variable, producing structural injury such as strand breaks, protein/DNA cross-links, and/or modification of base pairs. Hydroxyl radicals

can also react with carbohydrates by randomly abstracting a hydrogen atom from one of the carbon atoms, producing a carbon- centered radical. This leads to chain breaks in important molecules like hyaluronic acid in a process involving intermediates such as peroxyl radicals. In the synovial fluid surrounding joints, an accumulation and activation of neutrophils during inflammation produces significant amounts of oxyradicals. This phenomenon apparently accounts for a significant decrease in the synovial fluid of affected joints.

In addition, excessive production of ROS occurs by a pro-oxidant effect of TNF-α produced by activated macrophages (Peterhans, 1987). Reactive oxygen species (ROS) activate the nuclear transcription factor NF-kB, which is obligatory for HIV replication (Nabel and Baltimore, 1987; Lenardo and Baltimore 1989). Persistent HIV-1infection of macrophages results in increased levels of NF-κB in the nucleus secondary to increased inhibitors (IκBα, IκBβ, and IκBε) degradation, a mechanism postulated to regulate viral persistence. Then, as the synthesis at the transcriptional level proceeds, replication is negatively controlled by Bcl-2 to reach a balance characterized by low virus production and higher Bcl-2 and TRX levels resulting in low but sustained viral production compatible with cell survival.

1.2 Justification

As a result of HIV/AIDS, incidence rates of TB in certain countries has increased by more than 6 percent per year (Corbett *et al.,* 2003), crippling the already overburden health care resources. Also, TB is the leading cause of morbidity and mortality in patients with HIV/AIDS (Raviglione, 1992). The direct and indirect costs of illness due to TB and HIV are enormous, estimated to be more than 30 percent of the annual household income in developing countries with catastrophic impact on the economy of the developing world (Russell, 2004). Despite support from donor agencies, substantial

number of HIV/TB co-infected patients remained undiagnosed in many settings. Follow up tests using CD4 kits is erratic especially in resource constrained areas. False negative results of sputum smears, atypical radiographic findings in the advanced diseased condition affect rational clinical decision leading to excessive morbidity and mortality**.** Both HIV and Tuberculosis have bi-directional relationship and are associated with oxidative stress (Sharma, 2005). It is widely reported that oxidative stress, representing an imbalance between production and elimination of chemically reactive species, such as reactive oxygen species (ROS) [Halliwell, 1994], is involved in HIV infection (Schreck *et al.,* 1991). Oxidative stress also has been shown to be associated with TB infection through activation of phagocytes by mycobacteria which may further contribute to immunosuppression (Jack *et al.*, 1994; Grimble, 1994). Furthermore, an earlier report (Macallan, 1999) has shown high levels of oxidative stress in HIV-TB co-infected patients. A variety of enzymatic (superoxide dismutase, catalase, glutathione peroxidase) and nonenzymatic (ascorbate) antioxidants present in human serum become insufficient to circumvent HIV-1 replication secondary to cellular ROS production by a pro-oxidant effect of inflammatory cytokines and/or a polymorphonuclear leukocyte activation (Perl and Banki, 2000). Friis-Moller *et al*., (2007) have shown that HIV-infected patients are in oxidative imbalance early in the disease and both serum and tissue anti-oxidants levels are low and peroxidation products elevated. High serum levels of malondialdehyde (MDA), reduced serum glutathione (GSH), and decreased superoxide dismutase (SOD) activities are normally found and both HIV and TB infections also result in considerably reduced vitamins E and C concentrations (Bogden *et al.,* 1990). There is therefore the need to access an oxidative stress parameter like Malondialdehyde (MDA) and compare its values with that of serum vitamin E in order

to tract the progression of HIV/TB co-infection and also generate a reference value to be used as an adjunct diagnostic step in a resource constrained environment.

1.3 Aims/Objectives

1.3.1 Aim

To assess the magnitude of oxidative stress, by assessing the level of oxidants and antioxidants systems in HIV/TB co-infected patients in Kano State, Nigeria.

1.3.2 Objectives

1. To determine the level of Malondialdehyde (MDA) as a marker of oxidative stress in HIV-TB co-infected patients
2. To find out if there is correlation or not between MDA level and the progression of HIV-TB co-infection
3. To compare the MDA level with CD4 and CD8 levels using WHO classification to allow for the development of a reference value to be used in the rural settings.
4. To assess the effect of treatment in relation to the stages of HIV/TB co-infection
5. To obtain the profile of serum vitamin E among people with HIV/TB co-infection to allow for comparison with MDA values obtained from this study

1.4 Hypothesis

1. The level of Malondialdehyde (MDA) will not increase in HIV/TB Co-infected patients.
2. The level of Malondialdehyde (MDA) will not increase with progression of HIV/TB severity.
3. The level of MDA will not increase with decreasing CD_4^+ level
4. Serum Vitamin E level will not decrease with progression of HIV/TB co-infection

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 HIV/TB co - infection

Tuberculosis (TB) and HIV co-infections place an immense burden on health care systems and pose particular diagnostic and therapeutic challenges (Pawlowski *et al.*, 2012). Infection with HIV is the most powerful known risk factor predisposing for *Mycobacterium tuberculosis* infection and progression to active disease, which increases the risk of latent TB reactivation 20-fold. TB is also the most common cause of AIDS-related death. Thus, *M. tuberculosis* and HIV act in synergy, accelerating the decline of immunological functions and leading to subsequent death if untreated. The mechanisms behind the breakdown of the immune defense of the co-infected individual are not well known (Pawlowski *et al.*, 2012).

Tuberculosis (TB) and human immunodeficiency virus/Acquired immune deficiency syndrome (HIV/AIDS) constitute the main burden of infectious disease in resource-limited countries. *Mycobacterium tuberculosis*–HIV co-infections pose particular diagnostic and therapeutic challenges and exert immense pressure on health care systems in African and Asian countries with large populations of co-infected individuals (Pawlowski *et al.*, 2012).

In the individual host the two pathogens, *M. tuberculosis* and HIV, potentiate one another, accelerating the deterioration of immunological functions and resulting in premature death if untreated. Some 14 million individuals worldwide are estimated to be dually infected (Getahun *et al.*, 2010). TB is the largest single cause of death in the setting of AIDS (Corbett *et al.*, 2003), accounting for about 26% of AIDS-related deaths

(Getahun *et al.,* 2010), 99% of which occur in developing countries (Collins *et al.,* 2002).

Both TB and HIV have profound effects on the immune system, as they are capable of disarming the host's immune responses through mechanisms that are not fully understood. HIV co-infection is the most powerful known risk factor for progression of *M. tuberculosis* infection to active disease, increasing the risk of latent TB reactivation 20-fold (Selwyn *et al.,* 1989; Getahun *et al.,* 2010). Likewise, TB has been reported to exacerbate HIV infection (Modjarrad *et al.,* 2010). Various lines of evidence indicate that inborn errors of immunity, as well as genetic polymorphisms, have an impact on susceptibility to TB and HIV (Moller and Hoal, 2010).

2.2 Aspects of Immune Response to HIV Infection

HIV-1, which most commonly infects via the genital mucosa, persists as a chronic infection even though the virus elicits strong innate and adaptive, including cellular and humoral, immunity. Explanations for this may be linked to virus genomic integration and subsequent cellular latency, as well as an extreme genetic variability, which translates into constant immune escape. HIV-specific $CD8^+$ lymphocytes play a key role in the initial reduction of viremia during acute infection, but become increasingly dysfunctional and exhausted under conditions of chronic antigen persistence (Hess *et al.,* 2004; Streeck *et al.,* 2008). Virus-neutralizing antibodies are also elicited but are frequently accompanied by immune escape, and even if some individuals develop cross-neutralizing antibodies, it is debatable whether they play a role in the control of the virus (Euler *et al.,* 2010).

The hallmark of HIV infection is the depletion of $CD4^+$ T cells. Interestingly, during the primary HIV infection, the cells that are preferentially depleted are the effector memory $CD4^+$ T cells in the gut mucosa (Brenchley and Douek, 2008). These immunopathogenic features, together with the systemic and chronic state of immune activation, including accelerated T cell turnover, are thought to contribute to progression of HIV disease (Moir *et al.,* 2011). Thus, constant antigenic stimulation is characterized by a dysfunctional T cell population displaying loss of functional potential, i.e., cytokine production and cytotoxic activity, and proliferative ability in response to antigen stimulation. In addition, the loss of immune balance between Th17 and regulatory T cells (Treg) during HIV disease progression has recently been implicated in permeabilization of gut integrity and the pathogenesis of HIV (Kanwar *et al.,* 2010).

Microbial translocation caused by gut permeability has also been suggested to contribute to systemic immune activation observed during chronic HIV infection (Brenchley *et al.,* 2006). Hyper-responsiveness of plasmacytoid DCs during the primary infection, which results in excessive type-1 IFN production and the following chronic activation of these cells, may additionally contribute to systemic immune activation and HIV-1 disease progression (Fitzgerald-Bocarsly and Jacobs, 2010).

$CD8^+$ T cells have been implicated in the control of chronic HIV replication as suggested by studies on simian immunodeficiency virus (SIV) viremia in non-human primates after in vivo $CD8^+$ T cell depletion (Schmitz *et al.,* 1999). In addition, there are rare individuals who control HIV-1 replication to undetectable levels, i.e., elite controllers. This phenotype is strongly associated with some MHC class I alleles (Miura *et al.,* 2009) and with the presence of HIV-specific $CD8^+$ T cells showing superior

cytotoxic capacity to kill HIV-infected targets (Migueles *et al.,* 2008; Hersperger *et al.,* 2010).

Programmed-Death 1 (PD-1) and T cell immunoglobulin and mucin domain 3 (Tim-3) are two examples of markers of T cell exhaustion in HIV-1^+ patients caused by constant antigenic stimulation (Day *et al.,* 2006; Jones *et al.,* 2008). Both molecules are involved in the down-regulation of host immune responses and play a role in maintaining T cell tolerance. A recent finding is that Tim-3 was up-regulated on virus-specific $CD8^+$ T cells in patients with chronic progressive HIV infection (Jones *et al.,* 2008); another recent publication reports that Tim-3 was up-regulated on antigen-specific $CD8^+$ T cells in patients with active TB (Wang *et al.,* 2011), indicating that similar inhibitory receptor/ligand interactions play a role in modulating host immunity to both HIV and *M. tuberculosis* infections in humans.

2.3 TB Reactivation by HIV

It is generally thought that one-third of the world's population is latently infected with *M. tuberculosis* (Dye *et al.,* 1999), although the data supporting this notion may be questioned. Also, the rate of progression from infection to disease varies greatly. Approximately 10% of *M. tuberculosis*–infected individuals are thought to develop overt clinical disease (Selwyn *et al.,* 1989) and about half of them develop disease more than two years after infection; these cases are commonly named “reactivation” or post-primary TB (Lillebaek *et al.,* 2003). Thus, the lifetime risk of developing active TB in immunocompetent adults is estimated to be 5%–10% during their lifetime, but in HIV-positive individuals this risk is increased to 5%–15% annually (Aaron *et al.,* 2004).

The depletion of $CD4^+$ T cells, which is a main feature of AIDS, is certainly an

important contributor to the increased risk of reactivation of latent TB and susceptibility to new *M. tuberculosis* infection. There is also some evidence that $CD8^+$ T cells play a role in the control of latent TB (van Pinxteren *et al.,* 2000; Lewinsohn *et al.,* 2003; Lewinsohn *et al.,* 2007; Chen *et al.,* 2009). Other mechanisms reported to facilitate *M. tuberculosis* infection and disease in individuals with HIV are up-regulation of *M. tuberculosis* entry receptors on macrophages (Rosas-Taraco *et al.,* 2006), HIV manipulation of macrophage bactericidal pathways (Spear *et al.,* 1990), deregulated chemotaxis and a tipped Th1/Th2 balance (Havlir and Barnes, 1999). It has also been shown that HIV impairs tumor necrosis factor (TNF)-mediated macrophage apoptotic response to *M. tuberculosis* and thus facilitates bacterial survival (Patel *et al.,* 2007).

In the latent phase of TB, the bacteria are not completely eradicated despite a seemingly robust Th1 immune response. A failure or an alteration of the quality or levels of the protective adaptive immune responses or of the cross-talk with innate immune responses leads to reactivation of infection. Several immune mechanisms, such as increased levels of $FoxP3^+$ Treg cells (Roberts *et al.,* 2007), increased production of IL-27 (Pearl *et al.,* 2004), TGF-β (Bonecini-Almeida et al., 2004; Carow *et al.,* 2011), PGE-2 (Hirsch *et al.,* 1996), SOCS1, or the decoy receptor D6 (Di Liberto *et al.,* 2008), or diminished levels of IFN-γ, TNF, and polyfunctional specific T cells, are believed to play a role in such reactivation. Many of these factors, such as SOCS1 or IL-27, down-regulate the IFN-γ/IL-12 axis, thereby impairing bacterial control, while others, such as the D6 decoy receptor, are mainly anti-inflammatory, but may indirectly inhibit efficient bacterial clearance. Some of these mechanisms may also underlie HIV-infected patients' increased susceptibility to active TB.

Granulomas are organized cellular structures that constitute TB's pathologic hallmark. Mycobacteria are contained within the granuloma, which, by localizing infection and thus potentially preventing spread of the disease between hosts, probably contributes to protection. $CD4^+$ T cells and TNF are important in maintaining granuloma organization. Granuloma formation may fail in individuals with a compromised immune system, and there are several hypotheses about how HIV exacerbates TB pathology through the manipulation of granulomas (Diedrich and Flynn, 2011). Specifically, TB patients with AIDS present a dominant granulocytic infiltrate and necrosis without the typical caseous necrosis seen in non-HIV-infected TB granulomas. This has been associated with the killing of $CD4^+$ cells in the granuloma, probably resulting in a direct disruption of granuloma structure and abolition of the containment of infection. Cavitary lesions are seldom encountered in patients with a CD4 T-lymphocyte count $<200/mm^3$ (Sharma *et al.,* 2005). As a result, while in the majority of adult patients TB is confined preferentially to the lungs, in HIV-infected patients TB can be a systemic disease involving multiple organs that lack well-defined granulomas and instead develop more diffuse lesions leading to all forms of extrapulmonary TB (De Noronha *et al.,* 2008).

In macaques, SIV induces distortions in pro-inflammatory and anti-inflammatory T cell responses within the granuloma that may have significant effects on reactivation of latent TB. Reduction of T cell numbers also occurred within lung granulomas of monkeys co-infected with SIV compared with monkeys exclusively infected with TB (Diedrich *et al.,* 2010). It is important to note that besides the known increased risk of disseminated disease in adults with HIV, there is a growing recognition from prevalence surveys of subclinically active TB infection in co-infected individuals (Mtei *et al.,* 2005).

2.4 Aspects of Immune Response to *M. tuberculosis* Infection

M. tuberculosis infects the host mainly through inhalation of aerosolized bacilli; alveolar macrophages are the primary target cells for this intracellular pathogen. Detection of *M. tuberculosis* by innate cells recognizing pathogen-associated molecular patterns, via toll-like receptors (TLRs) and nucleotide-binding oligomerization domain receptors, initiates a local inflammatory response and results in increased numbers of macrophages and dendritic cells (DCs) in infected lung tissue and draining pulmonary lymph nodes. Following activation by cytokines and innate receptor agonists, infected macrophages elicit direct bactericidal effector functions, such as reactive oxygen or nitrogen intermediates (Walker *et al.,* 1981; MacMicking *et al.,* 1997), or expression of small GTPases that can regulate endosomal trafficking (MacMicking *et al.,* 2003). DCs can phagocytose the bacteria in lung tissue, migrate to draining lymph nodes, and initiate the adaptive immune response by priming naïve T lymphocytes (Chackerian *et al.,* 2002).

Cell-mediated immunity is essential for control of *M. tuberculosis* infection; activation of both $CD4^+$ and $CD8^+$ T cells is seen in active TB in humans, as well as in mice after experimental infection (Cooper, 2009). $CD4^+$ T lymphocytes of T helper cell type 1(Th1) are thought to be most critical (North and Jung, 2004). Also, there is experimental evidence that $CD8^+$ T cells (Flynn *et al.,* 1992; Feng *et al.,* 1999), as well as unconventional T cells such as CD1-restricted cells recognizing mycobacterial lipids (Ladel *et al.,* 1995), contribute to optimal control of the disease. T cells recruited to the infected lung are thought to control infection by producing interferon gamma (IFN-γ) in response to mycobacterial antigens presented by macrophages (Orme *et al.,* 1993; Lazarevic *et al.,* 2002). In turn, IFN-γ activates macrophages to kill the intracellular bacteria through reactive nitrogen and oxygen intermediates (Chan and Flynn, 2004), and by inducing phagolysosome formation (Chackerian *et al.,* 2002). However, these

mechanisms might even be present in susceptible hosts, in which the infection progresses to disease. The full knowledge of the constituents of an effective protective immune response to TB is still incomplete.

In the *M. tuberculosis*–infected host there is also a robust humoral response, with a wide spectrum of antibodies (Abs) of different specificities and isotypes; although secondary to the cellular immune responses in terms of protection, B cells as well as certain antibodies responses have been shown to be capable of playing an important role in protective immunity to TB (Kallenius *et al.,* 2007).

2.5 Exacerbation of HIV Infection by *M. tuberculosis* Infection

The incidence and mortality rates for new AIDS-defining opportunistic infections have been shown to be higher if individuals with HIV are co-infected with TB (Pape *et al.,* 1993; Whalen *et al.,* 1995). Despite these epidemiological data supporting the notion that *M. tuberculosis* infection has a negative impact on the immune response to HIV and on progression to AIDS, research on possible mechanisms is scarce. The function of many immune cells, including macrophages and DCs, is modulated by both HIV and *M. tuberculosis*. Increased replication of the virus was demonstrated locally, at sites of *M. tuberculosis* infection in the lung (Nakata *et al.,* 1997), and within activated cells, including lymphocytes and $CD14^{+}$ macrophages, of the pleural space (Lawn *et al.,* 2001) of co-infected patients. *M. tuberculosis* has been reported to up-regulate HIV-1 replication in chronically or acutely infected T cells or macrophages (Shattock *et al.,* 1993; Zhang *et al.,* 1995), as well as ex vivo in alveolar macrophages and lymphocytes from patients with HIV (Toossi *et al.,* 1997; Goletti *et al.,* 1998). These in vitro/ex vivo findings are also reflected in vivo where elevated plasma viral loads have been detected in HIV-infected individuals with concomitant active TB disease (Goletti *et al.,* 1996).

The primary target for *M. tuberculosis*, the alveolar macrophage, can also be infected with HIV (Landay *et al.,* 1993; Sierra-Madero *et al.,* 1994; Nakata *et al.,* 1995). Mycobacteria exacerbate HIV replication in macrophages and lung cells obtained by bronchoalveolar lavage from co-infected individuals (Zhang *et al.,* 1995; Nakata *et al.,* 1997; Orenstein *et al.,* 1997). Also, in vitro studies have demonstrated that *M. tuberculosis* infection can up-regulate both HIV infection and replication within monocyte-derived macrophages (MDMs), increase the efficiency of virus transmission from infected MDMs to T cells, and favor replication of X4 HIV variants by up-regulation of CXCR4 (Mancino *et al.,* 1997). Furthermore, monocytes from HIV^+ patients display an impaired response to TLR ligands (Jiang *et al.,* 2005), and viral proteins can interfere with both MDM and DC maturation and function in vitro, including their ability to phagocytose mycobacteria and kill intracellular bacteria (Biggs *et al.,* 1995; Kedzierska *et al.,* 2000; Muthumani *et al.,* 2005; Sacchi *et al.,* 2007). SOCS1, which is stimulated by infection with *M. tuberculosis* (Rosas-Taraco *et al.,* 2006), has been shown to facilitate the late replication pathways of HIV infection (Ryo *et al,* 2008) and mediate viral evasion of type I IFN anti-viral signalling (Fenner *et al.,* 2006).

While TNF production in response to *M. tuberculosis* infection is required for control of bacterial growth, TNF is known to activate HIV replication in macrophages (Kedzierska *et al.,* 2003), indicating that the host immune response initiated against one pathogen may promote the replication of another. Thus, both HIV and *M. tuberculosis* stimulate TNF release from infected cells, and TNF hampers bacterial growth while enhancing HIV replication.

M. tuberculosis survives in DCs and actively down-regulates their pro-inflammatory activity and antigen-presenting function, with concurrent induction of anti-inflammatory cytokines (Jiao *et al.,* 2002). Similarly, HIV can infect and also manipulate DCs and the ensuing T cell functions (Donaghy *et al.,* 2004). In HIV infection, not only is DC-mediated activation of T cells impaired, but the migration of infected DCs can also contribute to pathogen dissemination.

The DC-expressed C-type lectin receptor DC-SIGN (DC-specific intercellular-adhesion-molecule-3-grabbingnonintegrin) has been suggested to facilitate transmission and immune escape of both *M. tuberculosis* and HIV (van Kooyk *et al.,* 2003). HIV attaches to DC-SIGN through interaction with the viral envelope glycoprotein gp120, and this interaction is thought to contribute to efficient spread and transmission of the virus to $CD4^+$ T cells in *trans* (Geijtenbeek *et al.,* 2000; Kwon *et al.,* 2002). *M. tuberculosis* has been reported to target DC-SIGN by a mechanism that is distinct from that of HIV, leading to inhibition of pro-inflammatory IL-12 and TNF production and induction of IL-10 by DCs (Geijtenbeek *et al.,* 2003) and, hence, down-regulation of protective immune responses.

It has been suggested that TB patients have a microenvironment that facilitates HIV infection by; *i*) increasing the expression of co-receptors CXCR4 and CCR5 regulated by *M. tuberculosis* products, *ii*) increasing pro-inflammatory cytokines, especially TNF; and *iii*) down-regulation of CCL5 (Rosas-Taraco *et al.,* 2006). It has also been shown that *M. tuberculosis* and its cell wall component, lipoarabinomannan (LAM) (Briken *et al.,* 2004), may activate replication of HIV in provirus-carrying cells by inducing TNF and IL-6 production through the NF-κB pathway, which in turn triggers transcriptional

activation of the long terminal repeat (LTR) promoter and supports replication of HIV (Collins *et al.,* 2002).

2.6 Animal Models to Study *M. tuberculosis* and HIV Co-Infection

One of the most important challenges in studies of co-infection is to identify appropriate animal models, since HIV does not cause disease in rodents or even non-human primates. Thus, while mice are ideal models to study immune response to infection and vaccination due to the large diversity of tools and knowledge about their immune system, conventional mice are not susceptible to HIV infection owing to the restricted specificity of the virus for the human cell. To circumvent this limitation, two complementary mouse models were recently generated. Using these models, the most relevant features of *M. tuberculosis* and HIV infections can be reproduced in mice (e.g., typical TB granuloma formation; virus replication in splenic lymphocytes, peritoneal macrophages, and brain; immune exhaustion and/or chronic immune activation; and susceptibility to systemic, vaginal, and rectal HIV infection). In one, the "humanized mouse", human hematopoietic progenitor cells ($CD34^+$) from human cord blood are used to reconstitute the immune system of immunodeficient mice (Traggiai *et al.,* 2004). A variation of this model incorporates the engraftment of a fetal human thymus fragment, which gains activity as a functional human thymus, allowing a more proper positive and negative T cell selection than the original model (Gorantla *et al.,* 2010). Features such as $CD4^+$ cell depletion, prolonged viremia, and co-receptor-mediated tropism were observed during HIV infection of humanized mice (Baenziger *et al.,* 2006; Sun *et al.,* 2007; Zhang *et al.,* 2007;Denton *et al.,* 2010; Gorantla *et al.,* 2010). These mice show transplanted human cells in mucosal surfaces and could thereby be infected by the intravaginal and intrarectal routes (Joseph *et al.,* 2010). The model has been used to evaluate new approaches to the prevention or treatment of HIV infection,

including human-neutralizing antibodies (Joseph *et al.,* 2010), prophylactic usage of antiretrovirals (Denton *et al.,* 2010), and T cell–specific siRNA delivery (Kumar *et al.,* 2008). Inoculation of humanized mice with mycobacteria enhances the CD4/CD8 cell ratio, the differentiation of $CD4^+$ cells into memory/effector types, and the translocation of IFN-γ-secreting T cells into the lung. Of particular importance is that lungs and livers from infected mice show typical features of mycobacterial granuloma. On the negative side, the adaptive immune responses, especially the specific IgG levels in response to immunization or infection, are low.

The other mouse model used to study *M. tuberculosis*/HIV interaction, the "EcoHIV" model, makes use of a modified HIV-1 strain, in which the coding region of gp120 has been replaced by that of gp80 from ecotropic murine leukemia virus that is able to infect the immune cells of conventional mice (Potash *et al.,* 2005). The resulting chimeric virus construct, EcoHIV, productively infects immunocompetent mice. Replicating virus is detected in splenic lymphocytes, peritoneal macrophages, and the brain of mice. The chimeric virus also elicits an immune response directed against viral proteins, and stimulates mouse genes similar to those stimulated by HIV in humans. This murine model of HIV infection has proven useful in vaccine challenge studies and for preclinical evaluation of antiretroviral drugs (Hadas *et al.,* 2007; Saini *et al.,* 2007; Roshorm e*t al.*, 2009).

HIV transgenic mice incorporating the entire viral genome have also been used to study the effect of *M. tuberculosis* infection on the induction of HIV gene expression (Scanga *et al.*, 2007). In this model, viral gene expression was activated by *M. tuberculosis* and suppressed after anti-mycobacterial chemotherapy (Scanga *et al.,* 2007).

Although non-human primates are resistant to infection by HIV, they can be infected by SIV, a retrovirus causing immunodeficiency similar to AIDS in Asian macaques. Thus, SIV infection in macaques has been used as a model for AIDS. Macaques also develop TB that is very similar to TB in humans, and can develop cavitary lung disease and necrotic lesions. They also can maintain TB latency for years, and only a small proportion of latently infected macaques develop reactivation (Diedrich *et al.,* 2011). Macaques infected with SIV can develop persistent *Mycobacterium bovis* bacillus Calmette Guerin (BCG) (Shen *et al.,* 2002) and *M. tuberculosis* co-infection (Safi *et al.,* 2003; Diedrich *et al.,* 2010). Co-infection with SIV and BCG accelerated progression to AIDS (Zhou *et al.,* 1999) and coincided with a severe depletion of $CD4^+$ T cells, loss of BCG-specific T cell responses, and reactivation of the clinically latent BCG infection into a TB-like disease (Shen *et al.,* 2004). *M. tuberculosis* reactivation in SIV-infected macaques is associated with early peripheral T cell depletion and not virus load (Diedrich *et al.,* 2010).

2.7 General effects of ROS in immune response

ROS have a critical role in several physiological events such as regulation of redox-dependent signaling cascades, by acting as cofactors for hormones production (Drummond *et al.,* 2011), intracellular signaling post- T-cell activation (Kiessling *et al.,* 2010), antigen cross-presentation (Mantegazza *et al.,* 2008), autophagy (Lam *et al.,* 2010), both apoptotic and necrotic cell death pathways (Saito *et al.,* 2006; Morgan *et al.,* 2010) and chemotaxis (Urao *et al.,* 2008), by increasing CCR5, and CXCR4 expression (Saccani *et al.,* 2000;.Li *et al.,* 2009), major determinants of HIV interaction with mononuclear phagocytes and T lymphocytes.

ROS can also orchestrate Th2 responses, by inducing lipid oxidization which trigger

thymic stromal lymphopoietin (TSLP) production by epithelial cells, a cytokine known to be involved in Th2 differentiation, which suppresses the production of Th1 molecules such as IL-12 and CD40 by DCs, in lymph node; and induce DCderived chemokine CCL7, which mediates basophils recruitment (Tang *et al.,* 2010). Interestingly, NOX2 oxidase-derived superoxide from macrophages is essential for Treg generation, contributing to control T cell-mediated inflammation (Kraaij *et al.,* 2010). Besides, NOX2 deficiency affects both FoxP3 and RORγt expression in CD4+ T cells, which is traduced as increased Th17 cells and diminished Treg development in a ROS-dependent and T-cell–intrinsic manner (Lee *et al.,* 2011).

On the other hand, excessive ROS production by an overactive NADPH oxidase system, both in phagocytic and non-phagocytic cells, may set in motion a vicious cycle of radical and non-radical oxidant generation in various cellular compartments, which disrupts redox circuits that are normally controlled by thiol-dependent antioxidant defenses, and induces a state of oxidative stress. In fact, many adverse effects of ROS are attributable to the oxidation of important signaling proteins, including kinases and phosphatases, and activation of the pro-inflammatory redox-dependent transcription factor NF-κB; this leads to the expression of adhesion molecules, leukocytes proliferation and migration (Jones *et al.,* 2008; Drummond *et al.,* 2011). ROS may also oxidize and activate matrix metalloproteinases, responsible for tissue remodeling. ROS may initiate the assembly of multiprotein signaling complexes known as inflammasomes, which activate caspase-1, leading to the processing and secretion of the pro-inflammatory cytokines interleukin-1β (IL-1β) and IL-18 (Martinon, 2010).

Several studies suggest that ROS affect the intrinsic apoptotic pathway in neuronal cells, with mitochondria being the major source and primary target (Sacktor *et al.,* 2004). ROS may oxidize mitochondrial pores that lead to cytochrome c release and

caspase-9 activation due to the disruption of the mitochondrial membrane potential (Cai and Jones, 1999; Simon *et al.,* 2000). ROS generation change the intracellular redox status as well, with subsequent effects on specific kinases, phosphatases, and transcription factors, increasing cell susceptibility to apoptotic stimuli (Cimino *et al.,* 1997; Gardai *et al.,* 2002; Alvarado-Kristensson *et al.,* 2004). ROS are also involved in T lymphocytes cell death process (Kiessling *et al.,* 2010), hence, inhibition of superoxide generation upon T-cell receptor engagement, rescue from activation induced cell death (Norell *et al.,* 2009). Sustained Junction N-terminal kinase (JNK) activation is ROS dependent in T cells and, during T cell neglect induced death increased levels of ROS has been detected (Purushothaman *et al.,* 2009).

Thus, ROS are important signaling molecules that regulate many signal-transduction pathways and play critical roles in cell survival, death, and immune defenses (Huang *et al.,* 2011), involving them in many different diseases including cancer, neurological disorders, among many others (Lovell *et al.,* 2007; Ziech *et al.,* 2011).

2.8 ROS and HIV-1 infection

An increased oxidative stress condition has been repeatedly described in chronically HIV-1-infected patients, based on: elevated extracellular and intracellular ROS levels (Dobmeyer *et al.,* 1997; Elbim *et al.,* 1999; Munoz *et al.,* 1999; Elbim *et al.,* 2001; Sánchez-Pozo *et al.,* 2003; Olivetta *et al.,* 2005; Wang *et al.,* 2010; Masanetz *et al.,* 2011), systemic reduction in glutathione (GSH) and thioredoxin concentrations (Sahaf *et al.,* 2005), disturbance of mitochondrial membrane potential (Perl *et al.,* 2000), and changes in expression and activation status of cell death receptors (Zhu *et al.,* 2011), which may lead to host cell death (Salmen *et al.,* 2007; Wang *et al.,* 2010). An increased HIV-1 replication, induced by ROS, has been observed in reservoir cells (Yang *et al.,* 2009). This effect is mediated by H_2O_2-induced LTR activation during

middle stage of infection, where a great deal of oxidative stress occurred (Pyo *et al.,* 2008).

It has been demonstrated that HAART partially restore the antioxidant capacity by suppressing HIV (De Martino *et al.,* 2001), and it has been suggested that antioxidant therapy in combination with HAART could protect the blood brain barrier from oxidative stress-induced damage, could be considered as a viable therapeutic option for patients with HIVassociated dementia (HAD) (Banerjee *et al.,* 2010). However protease inhibitors (PIs) for HIV, enhance ROS production in several types of cells including macrophages, vascular smooth muscle cells, umbilical vein endothelial cells, adipocytes, and pancreatic β-cells, and contributed to intestinal barrier integrity disruption (Wu *et al.,* 2010) suggesting that ROS greatly contribute to HIV PIs-induced side effects (Lei *et al.,* 2010).

2.9 Effects of HIV induced ROS on immunity

There is no doubt about the crucial role played by ROS in the immunopathogenesis of HIV infection. HIV induced ROS have been connected to decreased immune cell proliferation, loss of immune function (Sánchez-Pozo *et al.,* 2003; Gülow *et al.,* 2005; Yang *et al.,* 2009), followed by a cellular dysfunction and cell death, loss of memory T cell response (Cummins and Badley, 2010), T helper imbalance, related to premature Treg response (Amarnath *et al.,* 2007), which are highly susceptible to HIV infection (Antons *et al.,* 2008), premature ageing of immune system because of a direct and quantitative shortening of telomere (Pollicita *et al.,* 2009), altogether disturbing the adaptative immune response.

ROS has been connected to several critical signaling pathways during HIV Infection. Thus, ROS induce HIV LTR activation by early NF-κB activation, by inducing both IkB degradation and covalent modification of p65, CBP/p300-induced hyperacetylation

as well as phosphorylation of p65 (Pyo *et al.,* 2008), these events may explain the explosive increase in viral replication after the middle stage of infection (Buhl *et al.,* 1990) and, can promote HIV replication in macrophages (Aquaro *et al.,* 2007). Cross link of death receptors such as DR5, induced production of ROS and subsequent apoptosis in HIV-1 infected monocyte-derived macrophage, associated with JNK phosphorylation (Zhu *et al.,* 2011), a kinase known to be involved in the apoptotic signaling pathway initiated by stress or toxic stimuli (Saeki *et al.,* 2002). Besides, HIV infection facilitates TRAIL-induced cell death in monocyte-derived macrophage (MDM) by down regulating the TRAIL decoy receptors and intracellular c-FLIP, dependent of ROS generation and subsequent JNK phosphorylation (Zhu *et al.,* 2011).

Excessive ROS production is also explained by polymorphonuclear chronic activation during HIV infection (Elbim *et al.,* 2001) or, through a pro-oxidant effect of TNF-α, as released by activated macrophages, which may be accompanied by a concomitant deficient antioxidant defense system (Jahoor *et al.,* 1999); altogether increasing the susceptibility to cell death. Indeed, oxidative stress is the common mediator of programmed cell death in HIV/AIDS on this subpopulation (Salmen *et al.,* 2007). Previous results show that neutrophils from HIV infected patients have increased basal levels of superoxide production (Elbim *et al.,* 1994), particularly before AIDS is established, which is associated to their dysfunction (Elbim *et al.,* 2001). In fact, apoptosis could be responsible for HIV-related neutropenia (Pitrak, 1999), which could occur either spontaneously or triggered via death receptors such as Fas/ FasL (Pitrak *et al.,* 1996; Salmen *et al.,* 2004). Spontaneous cell death during HIV infection is at least partially mediated by ROS, because it can be significantly reduced in the presence of oxygen radical scavengers (Salmen *et al.,* 2007).

2.10 Effects of HIV induced ROS on different cells

Oxidative stress not only affects HIV infected human CD4 T lymphocytes, macrophages, dendritic cells and neutrophils, human hepatocytes has been documented to be affected as well during the disease. HIV virions and its envelope gp120 protein, induce ROS production within hepatocytes, hepatic stellate cells (HSC) and other immune cells through its interaction with CCR5 or CXCR4 chemokine receptors (Rotman *et al.,* 2009; Bruno *et al.,* 2010), and during HIV/HCV co-infection, leads to accelerated hepatic fibrosis development, higher rates of liver failure and death, compared with patients with HCV only (Macías *et al.,* 2009). Thus, HIV regulates hepatic fibrosis progression through the generation of ROS, in a NF-κB -dependent fashion, and a subsequent increment of profibrogenic genes (Lin *et al.,* 2011). Oxidative stress has been recently involved in endothelial cell dysfunction, vascular injury, and pulmonary arterial hypertension, during HIV infection (Mermis *et al.,* 2011). A unifying mechanism of HIV-related ROS effects is currently unknown, despite the intensive efforts unraveling the immunopathogenesis of the disease; which may make difficult to design the appropriate therapeutic approach capable of controlling oxidative stress.

2.11 ROS and mucosal integrity during HIV infection

A prominent role of the intestinal mucosal integrity has been postulated has been connected with chronic immune activation during HIV infection. Gut mucosa is a compartment where the interchange between HIV and the host's immune system, takes center stage (Brenchley *et al.,* 2004; Mehandru *et al.,* 2004). Within 1–2 weeks, infection becomes systemic, with extensive viral replication and CD4+ T-cell depletion in the intestinal lamina propria (Guadalupe *et al.,* 2003; Mehandru *et al.,* 2004; *Li et al.,* 2009; Shacklett *et al.,* 2010), because of a direct infection or apoptosis of bystander

cells (Haase, 2010); associated to enteropathy (Kotler *et al.*, 1984) induced by proinflammatory molecules, and cytotoxic effects that provoke intestinal epithelial apoptosis (Li *et al.,* 2008), and immune activation. The T regulatory response that has been described as premature in this region, because the immunosuppressive effects of cellular immune response precede clearance or control of viral replication (Estes *et al.,* 2006; Haase, 2010).

ROS play an important role in epithelial intestinal cells injury, contributing to gut mucosal barrier dysfunction. The ROS-induced gut mucosal injury implies loss of epithelial integrity between the cellular tight junctions and enteric bacterial translocation, which can be attenuated by radical scavenger (Mukojima *et al.,* 2009). Oxidative stress is known to exist in intestinal epithelium, and activate TACE (TNF-alpha converting enzyme), a pleiotropic metalloprotease also known as ADAM17, which is required for TNF-α production (Forsyth *et al.,* 2007).

Current evidence also indicates that Th17 cells are even more profoundly depleted than CD4+CCR5+ T cells in the intestinal mucosa of HIV- infected individuals (Brenchley *et al.,* CD4+CCR5+ T cells in the intestinal mucosa of HIV- infected individuals (Brenchley *et al.,* 2008). Loss of Th17 cells (Lackner *et al.,* 2009), is associated with loss of the integrity of the gut mucosal barrier, allowing microbial translocation products from the gut, associated to increased plasma levels of lipopolysaccharide (LPS) and soluble CD14 (sCD14), in the absence of overt bacteremia, which correlates with systemic immune activation (Brenchley *et al.,* 2006; Douek *et al.,* 2009) and mortality prediction in HIV infection (Sandler *et al.,* 2011). NOX2 deficiency or ROS depletion significantly promotes development of effector Th subsets such as Th17 cells, and suppressed development of natural and inducible Treg cells. ROS is essential for immune homeostasis by controlling Th17/Treg balance. Increased generation of ROS

attenuated Th17 cell differentiation and its related immune response (Won *et al.,* 2010). It is likely that disruption of the precise fine-tuning between generation and elimination of ROS may cause inflammation in multiple tissues in a T-cell-dependent manner (Lee *et al.,* 2011).

2.12 Effect of HIV proteins on ROS production

Several mechanisms have been proposed to explain how HIV could modulate ROS generation: 1) by inactivation of the GDP-bound form of RhoA and activation of p190 RhoGAP-A protein (.Fausther-Bovendo *et al.,* 2010). A previous report showed that Rac-dependent ROS production, leads to downregulation of RhoA through oxidative inactivation of low molecular-weight protein tyrosine phosphatase, and the subsequent activation of p190 RhoGAP-A (Nimnual *et al.,* 2003); 2) by phosphorylation of p47phox (Wang *et al.,* 2010) and association with p22-phox (Salmen *et al.,* 2010) inducing a direct activation of the NADPH oxidase complex, 3) by activation of p66ShcA, a protein involved in phosphatidylinositol 3-kinase/Akt/PKB signaling module, a pathway which is upstream of NADPH (Husain *et al.,* 2009), 4) by activating the PI3K/ Akt pathway, turning on the NF-κB Transcription factor (Zhang *et al.,* 2011) and inducing p42/44 MAPK activation (Wu *et al.,* 2007).

Several HIV proteins (structural and regulatory) have been shown to modulate ROS production (Vilhardt *et al.,* 2002; Wu *et al.,* 2007; Olivetta *et al.,* 2009; Deshmane *et al.,* 2009; Agrawala *et al.,* 2010). Thus, HIV regulatory protein Tat has pro-oxidant function, which could induce long terminal repeat region (LTR) transactivation, and this effect is prevented by NADPH oxidase inhibitors (Zhang *et al.,* 2011). Furthermore, Tat can reduce SOD synthesis (Westendorp *et al.,* 1995; Song *et al.,* 2007) and intracellular glutathione (GSH) levels (Zhang *et al.,* 2009). Exposure of microglia and astrocytes to

HIV-1 Tat leads to ROS generation, which activates signal transduction processes leading to expression of proinflammatory cytokines, as well as adhesion molecules on endothelial cells, microglia, and astrocytes (Pu *et al.,* 2003; D'Aversa *et al.,* 2004; Song *et al.,* 2007). On the other hand, inhibition of NADPH oxidase significantly attenuates inflammatory mediators (TNF-α, IL-6, CXCL10, IFN-γ and MCP-1) in microglia, astrocytes and macrophages (Turchan-Cholewo *et al.,* 2009; Williams *et al.,* 2010).

HIV-1 Nef is another regulatory protein that also has pro-oxidant properties. The effect of Nef on ROS activation has been previously demonstrated in different cells (Vilhardt *et al.,* 2002; Olivetta *et al.,* 2009). Nef has been detected in brain, where it associates with astrogliosis and recruitment and activation of monocytes/macrophages (Mordelet *et al.,* 2004). HIV-1 Nef increases oxidative stress in primary human astrocytes (Acheampong *et al.,* 2009) and led to their rapid cell death (Masanetz *et al.,* 2011). Furthermore, ROS-induced astrocyte death is thought to play a role in the occurrence of HAD (Robb and Connor, 19998; Feeney *et al.,* 2008).

Several pathways could explain Nef modulating effects on ROS: Nef may induce superoxide production by activating PAK (p21- activated kinase) in a Cdc42/Rac dependent manner (Vilhardt *et al.,* 2002). Through the interaction with Hck (hemopoietic cell kinase), Nef may induce phosphorylation and membrane translocation of p47-phox, a mechanism that could explain activation of superoxide, bypassing the typical pathway stimulated by pro inflammatory cytokine GM-CSF (Olivetta *et al.,* 2009). Additionally, through its association with p22-phox (Salmen *et al.,* 2010), Nef could directly affect NADPH-activity, a possibility that requires further investigation.

A hypothetical predictive model of protein-protein association, between p22-phox and Nef, showing low values of free energy between the molecules (-1117.6). In particular, residues VRGE (126-129) from p22-phox (gray) corresponding to an intracellular

portion of the protein and residues RRQDI (105-109) from Nef, demonstrated the highest probability of interaction.

Vpr an HIV protein that contribute to HIV-1 pathogenesis has been proven to increase ROS and HIF-1(a biomarker of oxidative stress) expression in human microglial cells, promoting mitochondrial dysfunction as well as oxidative stress (Roc *et al.,* 2007; Deshmane *et al.,* 2009). Oxidized phosphatidylcholine (OxPC), formed from phospholipids in response to oxidative stress, were identified in atherosclerotic lesions (Imai *et al.,* 2008) to be induced by rVpr, in a ROS-dependent manner, because it is reverted by the addition of N-acetyl-L-cysteine (NAC), a ROS scavenger molecule (Hoshino *et al.,* 2010). In this model, ROS are likely generated as a result of mitochondrial dysfunction, since Vpr binds ANT (adenine nucleotide translocator), a member of the permeability transition pore complex (Jacotot *et al.,* 2000), and disrupts the MMP (Kitayama *et al.,* 2008). Additionally, HIV- 1 gp120-induced neurotoxicity use ROS as signaling and effector of oxidant damage. Antioxidant gene blocks gp120-induced proapoptotic signaling in vitro and protects cell viability in vitro and in vivo, therefore specifically hypersensitive to gp120-induced apoptosis, signaling for which involves ROS intermediates (Agrawala *et al.,* 2010).

2.13 The triad: Immune chronic activation, Oxidative stress and HIV infection

A continue loss of CD4+ T lymphocytes, immune response dysfunction and systemic Immune system exhaustion (Said *et al.,* 2010) associated with constitutively activation of lymphocyte populations (Boassoand Shearer, 2008) and expression of programmed death-1 (PD-1) (Grossman *et al.,* 2002), has been associated with impaired immune reconstitution in patients on ART (Hunt *et al.,* 2003).

A number of factors have been identified for sustained chronic immune activation, which are both directly or indirectly related to HIV replication, they include: the innate

and adaptive immune responses against HIV, the translocation of bacterial products as a consequence of compromised integrity of the mucosal barrier, and the potential bystander stimulation of lymphocytes and macrophages by HIV gene products (Roederer *et al.,* 1995; Douek *et al.,* 2009). Such immune activation is manifested in many ways including: increased T-cell turnover (Hellerstein *et al.,* 1999; Douek *et al.,* 2009), increased frequencies of T-cells with activated phenotype (Freguja *et al.,* 2011), polyclonal B-cell activation (Lane *et al.,* 1983), increased serum levels of proinflammatory cytokines and chemokines (Douek *et al.,* 2009), enhanced oxidative stress (Mollace *et al.,* 2001; Toborek *et al.,* 2003) and premature development of regulatory T cells (Tregs) (Freguja *et al.,* 2011). Immune exhaustion is an aberrant component of the immune chronic activation during HIV-1 infection and is associated with ongoing virus replication (Sachdeva *et al.,* 2010), and elevated intracellular cyclic AMP (cAMP), which inhibits T cell activation capability (Aandahl *et al.,* 1998). In T cells, cAMP triggers a protein kinase A-Csk-Lck inhibitory pathway that inhibits proximal T cell receptor (TCR) signaling events (Vang *et al.,* 2001). This mechanism may also be involved in the inhibitory function of Tregs (Mahic *et al.,* 2006). Therefore, anti inflammatory therapy has been suggested to download the immune activation in order to improve T cell-dependent functions in vivo (Pettersen *et al.,* 2011).

Chronic immune activation of innate immune cell is also evident during HIV infection. Hence, pDCs are highly susceptible to HIV-induced activation due to its interaction with the cellular receptor CD4, and the subsequent production of type I interferon. Also, pDCs during HIV infection show high levels of the chemokine receptor CCR7 (Fonteneau *et al.,* 2004), indoleamine 2,3-dioxygenase, tryptophan depletion and the subsequent suppressive effects by contributing with Treg generation. Increased levels of IDO and tryptophan depletion is mediated in part by TLR activation, (Boasso and

Shearer, 2008) and by oxidative stress, which creates a niacin "sink" effect that depletes both niacin and tryptophan (Taylor, 2010). These events are associated with inhibition of HIV-induced CD4 T cell proliferative responses in vitro (Boasso *et al.,* 2007), and increment of regulatory T cells (Treg) in lymphoid tissues, where IDO is overexpressed (Boasso and Shearer, 2008). The dysregulation of innate immunity could contribute both to the numerical depletion of CD4 T cells and to the progressive loss of functional responsiveness of lymphocytes.

2.14 ROS and Tuberculosis (TB)

Increased reactive oxygen species (ROS) has been reported in patients with TB. High endogenously produced ROS in activated phagocytes of TB patients that escape to its surroundings can damage tissue or cellular DNA as well as impair immune function (Madebo *et al.,* 2003). It has been shown that the bactericidal potency of the myeloperoxidase-H_2O_2-halide system of neutrophilic granules demonstrates the bactericidal activities of the phagocytes that invariably produce increased ROS and reactive nitrogen intermediates (RNI) during phagocytic respiratory burst and that lower antioxidant potential as shown by significant reduction of enzymatic antioxidants (superoxide dismutase, catalase and glutathione) and non-enzymatic antioxidants as well as high MDA concentrations suggesting increased generation of ROS due to lipid peroxidation (Reddy *et al.,* 2004). Di Massio *et al.,* (1991), reported significantly reduced vitamin C and α- tocopherol that are integral components of antioxidants, which when present in sufficient quantity may act synergistically to protect cells from oxidative stress induced damage in TB patients. Several factors such as inadequate nutrient, malnutrition, nutrient malabsorption, low food intake, inadequate nutrient release from the liver, acute infections including other than HIV may be the cause of low or impaired antioxidant capacity in TB patient (Das *et al.,* 1990).

Presentation of the disease is variable as regards the pathology as well as infections in a variety of tissues such as the meninges, lymph nodes and tissue of the spine; where response to antibiotic medication/treatment to clear the bacilli from tissues, partial reversal of the granulomatous process and subsequent clearance from the sputum may be found in clinical cases (Jargirda and Zagzag, 1996). The progression and nature of disease may be affected by factors such as conditions that impact on the host immune system like poorly controlled diabetes mellitus, renal failure, chemotherapy, malnutrition or intrinsic host susceptibility (Madebo *et al.,* 2003). Host susceptibility has been known to affect endogenous re-activation and exogenous re-infection of the bacilli (Madebo *et al.,* 2003).

2.15 Malondialdehyde (MDA)

Lipid peroxidation in serum or plasma have been measured by a variety of chemical tests and the most frequently used test is the measurement of malonaldehyde (MDA) by thiobarbituric acid (TBA) reaction (Plaser and Cushman, 1966). Lipid peroxidation is an indicator of oxidative stress in cells and tissues. Oxidative stress and over production of inflammatory cytokines has also been shown to increase disease progression of HIV/AIDS patients. This may result from poor nutrition; due mostly to diets deficient in antioxidants (Moseson *et al.,* 1989), psychological stress (Burack *et al.,* 1993), chemical toxicity from dye and environment and microbial activity from multiple or concurrent infections (Vittinghoff *et al.,* 2001). The lipid peroxidation index malondialdehyde (MDA) is used as a measure of oxidative stress in cells. Lipid peroxidation being a free radical reaction occurs when hydroxyl radicals possibly oxygen reacts with the unsaturated lipids of bio-membranes resulting in the generation of lipid peroxide radical (ROO^{-}), lipid hydroperoxide (ROOH) and fragmentation

products such as Malondialdehyde (MDA) (Aust and Svingen, 1982; Uchida, 2000). To infect cells, the membrane enveloped HIV transfers its genome both across the viral and cellular membranes, causing them to fuse and provide portal of entry (Chan and Kim, 1998); thus, membrane lipids are required for HIV-entry (Chan and Kim, 1998). Peroxidation of lipids can have numerous effects including, increased membrane rigidity, decreased activity of membrane bound enzymes, altered activity of membrane receptors (sodium pumps), altered permeability (increased permissibility), formation of hydrophobic centers which, may approach the external phase, alteration of protein structure, mutagenicity (resulting from DNA/RNA damage or bonding), and inhibition of growth and protein synthesis. This is due to the high nucleophilic properties, which enable them to react with electrophilic sites such as amino and thiol groups (Chan and Kim, 1998). Reactive oxygen species (ROS, hydroxyl radicals (HO^-), superoxide anions (O_2^-), nitric oxide (NO) and hydrogen peroxide (H_2O_2), are constantly generated in aerobic organism in response to both external and internal stimuli (Lee *et al.,* 1997).

Increased levels of lipid peroxidation products such as MDA have been reported in a variety of animal diseases like *Dicrocoelium dendriticum* infection in sheep (Simsek *et al.,* 2006) and kidney diseases in dogs (Kargin and Fidanci, 2001). Brain injury is reported to be caused by superoxide radical and hydrogen peroxide (Kotos and Wel, 1986). Distomatosis (*Fasciola hepatica, Fasciola gigantica* and *Dicroceolium dendriticum* infections) in sheep causes production of reactive oxygen species and lipid peroxidation by significant increase in liver MDA (Deger *et al.,* 2008).

2.16 Treatment of HIV/TB Co-infection

The availability of highly active antiretroviral therapy (HAART) has greatly improved the outcome of HIV/AIDS, in terms of prevention of opportunistic infections as well as mortality (Palella, 1998). Specifically, benefit in terms of prevention of TB has been

demonstrated in South Africa (Badri *et al*, 2002) and outcome of patients with HIV/TB co-infection has improved over the years, attributable to improvements in antiretroviral and antituberculosis treatment (Leonard *et al.*, 2002). Thus understandably, both antituberculosis treatment and HAART are indispensable in the management of patients with HIV/TB co-infection.

The decision regarding when to start HAART during TB treatment needs to balance several considerations. The two most important variables in the decision are the incidence and mortality associated with TB-IRIS (Tuberculosi-Immune Reconstitution Inflammatory Syndrome) and the excess mortality associated with delaying HAART and the development of other opportunistic infections. Data are now emerging from controlled clinical trials of the optimum time to start HAART in such patients (Piggot & Karakousis, 2011). These studies have shown that irrespective of the CD4+ cell count, deferral of HAART to the end of treatment for TB is associated with high mortality risk and that mortality was reduced by 34% in patients in the Cambodian Early versus Late Introduction of Antiretrovirals (CAMELIA) trial with very low CD4+ cell counts (median 25cells/μL) who are started on ART within the first 2 weeks of treatment rather than after 2 months (Blanc *et al.*, 2010). The Starting Antiretrovirals at Three Points in TB Therapy (SAPIT) trial in South Africa observed 56% lower mortality rates among patients who started HAART during TB treatment, compared with patients who waited until Anti Tuberculosis Treatment (ATT) had been completed (Abdool Kareem *et al.*, 2010). Reasons to delay the initiation of HAART until after 2 months of ATT include drug interactions between rifampicin and Non Nucleoside Reverse Transciptase Inhibitors (NNRTIs), cumulative drug toxic effects (especially hepatotoxicity), pill burden and Immune Reconstitution Inflammatory Syndrome (IRIS). The current WHO guidelines recommend that all HIV-infected individuals with active TB (regardless of

CD4+ cell count) receive HAART as soon as TB treatment is tolerated, generally within 2-8 weeks (WHO, 2010). In all HIV-positive TB patients, co-trimoxazole preventive therapy (CPT) should be initiated as soon as possible and given throughout TB treatment irrespective of CD4+ T-cell count (WHO, 2010) but barriers to implementing CPT still exist in TB/HIV programs (Date *et al.,* 2010): CPT substantially reduces hospitalization and mortality in HIV-positive TB patients (Wiktor *et al.,* 1999; Harries *et al.,* 2011). The exact mode of activity is not clear but co-trimoxazole is known to prevent *Pneumocystis jirovecii* pneumonia and malaria and is likely to have an impact on a range of other bacterial infections in HIV-positive TB patients. Co-infected patients should be given co-trimoxazole 960 mg daily or thrice weekly while ATT is administered.

Access to potent antiretroviral therapy (ART) decreases morbidity and mortality due to HIV infection and AIDS (Walensky, 2006), and has made HIV infection a manageable chronic illness. Unfortunately, many patients who are prescribed chronic ART, experience drug toxicities that include metabolic, hepatic, neurological and cardiovascular complications (Morse and Kovacs, 2006).

Highly active anti-retroviral therapy (HAART) may increase chemically reactive species in circulation, possibly by producing more oxidized metabolites deriving from the interaction between ROS and infected-cell biomolecules (Martín *et al.,* 2001 and Hulgan *et al.,* 2003). This is supported by several biochemical mechanisms, such as mitochondrial interference, following treatment with HAART-NRTI [nucleoside reverse transcriptase inhibitors] (Hulgan *et al.,* 2003), and activation of the P450 hepatic system by HAART, which are protease inhibitors [PI] (Martín *et al.,* 2001). A putative mechanism of these toxicities includes inhibition of mitochondrial DNA polymerase-γ, with resultant mitochondrial DNA depletion and tissue-specific mitochondrial

dysfunction (Brinkman *et al.*, 1999). Other metabolic toxicities such as dyslipidemia and insulin resistance appear to be predominately associated with protease inhibitor (PI) exposure, likely through effects on lipid metabolism and cellular glucose uptake, (Morse and Kovacs, 2006). Although specific mechanisms of ART-associated toxicities are largely unknown, it has been shown that all of these processes may be mediated by free radical-induced cellular damage, also referred to as oxidant stress (Day and Lewis, 2004). Montine *et al.*, (2004); Shishehbor *et al*., (2006) independently described oxidative stress as an underlying pathogenic mechanism during various human disease conditions including cardiovascular and neurodegenerative diseases.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Background information of study area:

Established in 1988, the Aminu Kano Teaching Hospital began offering clinical services in 1994 at a temporary site in the Murtala Mohammed Specialist Hospital, Kano and later moved to its present, permanent site in 1997. Situated in Kano, the largest commercial center of northern Nigeria, with over 9 million people, this 700-bed hospital receives patients from Kano, and from the neighboring states of Jigawa, Katsina, Kaduna, Bauchi, and Zamfara. The majority of patients are indigenous Hausa-Fulani, although the Ibo and Yoruba ethnic groups are also well represented. Most patients are farmers, traders, businessmen, or civil servants. Five days a week, the hospital operates a multidisciplinary, specialist clinic for HIV/AIDS patients, which is located at the Prof. S. S. Wali Virology Centre. Examinations and antiretroviral drugs are provided free to all patients under USA President Emergency Program For Aids Relief (PEPFAR). Similarly, a DOTS (Directly Observed Treatment, Short-Course) clinic has been established within the same complex; it provides free antituberculosis treatment to all TB patients under Kano State Tuberculosis and Leprosy Control Program. The present study population comprised HIV/AIDS and Tuberculosis patients attending the Aminu Kano Teaching Hospital HIV/AIDS specialist clinic from April to September 2012.

3.2 Study Population

The study population comprised of four different groups as follow:

1. HIV-TB co-infected patients attending HIV/TB clinics
2. HIV patient attending the HIV clinic
3. TB patients attending the TB outpatient clinic
4. Apparently normal people that served as controls

3.3 Study design:

This is a cross sectional study which took place at Prof. S. S. Wali Virology Centre within Aminu Kano Teaching Hospital (AKTH)/Bayero University, Kano, Nigeria.

3.4 Sample Size Determination

A random sampling method was used to select the patients and the sample size was determined using the following formula:

$$n = Z^2pq/d^2$$ (Lwanga S, 1991)

Where, n = Minimum sample size required,

Z = the standard normal deviate corresponding to 5% level of significance. The value obtained from the normal distribution table is 1.96,

p = Prevalence of TB/HIV Co-infection in Kano State = 10.5% (Iliyasu, 2009).

q = Complementary probability to p = 1- p

d=precision of the estimate (5%=0.05)

$$n = \frac{(1.96)^2 \text{ X } (0.105) \text{ X } (1\text{-}0.105)}{(0.05)^2}$$

$$= 145$$

Adding 5% attrition, the sample size = 152

This number was divided into three groups; 50 subjects representing each sub-group of HIV, Tuberculosis and HIV/TB co-infection. Additional 50 controls were included in the study to allow for better comparison. The apparently normal controls were the staff in the two clinics and students from Community Health Officers' (CHO) class recruited

during the study period. A brief history was taken to rule out Diabetes and Hypertension or drug intake in the subjects, but for the control group in addition to these diseases, Tuberculosis infection was also ruled out. Thereafter, blood was collected for ELISA to exclude the presence of HIV infection and their sputum tested for the presence of Acid Fast Bacilli. All the controls tested negative for the ELISA Kits antibodies.

3.5 Inclusion Criteria

Inclusion criteria for both patients and control subjects include: Those between 20 - 70 years old; Patients with diagnosis of Tuberculosis by standard methods; Patients diagnosed as HIV seropositive by standard method; Patients diagnosed as HIV/TB co – infected by standard methods.

3.6 Exclusion Criteria

These include; presence of pregnancy, serological evidence of hepatitis B or C, diabetes or hypertension, smoking cigarette, or involved in alcohol consumption. Patients with complications such as renal, endocrine or hepatic diseases were also excluded from the study.

3.7 Data Collection:-

The data collection was made with the use of a structured questionnaire (Appendix I). The questionnaire composed of four different parts; (i) Sociodemographic characteristics, which include: age, sex, marital status and occupation of the cohorts; (ii) Anthropometric data, which consist of weight, height and Body Mass Indesx; (iii) Clinical history, consisting of the presenting complain, type of medication, associated comobidities, social history involving smoking and alcohol consumption and (iv) Laboratory data, which include CD4, CD8 counts, serum Malondialdehyde and vitamin E.

3.7.1 Collection of Socio-demograpic information

The Doctors and chief Matron of the two clinics screened the patients to be included in the study. The lower cadre staff helped in directing the patients from the various clinics to the focal area where the study was conducted. They also collected and arranged the files of the patients and also supply the basic laboratory forms to be filled off for the patients. Information about socio-demographic characteristics of the cohorts were obtained by Junior Community Health Extension Workers (JCHEW) in the two clinics and entered in to the questionnaires. The cohorts were later directed to the Phlebotomist who collected their blood for CD4, CD8, MDA and vitamin E analysis. Trained Scientists analysed the CD4 and CD8 cells using flowcytomery machine.

3.7.2 Collection of Anthropometric Data:-

i The Weight in Kilogram of the cohorts was taken by the JCHEW staff, the cohorts were made to remove their shoes and with light clothing, asked to stand erect on the weighing machiene; bathroom scale (Beexon, Hungry). One measurement was taken on each subject and the value obtained approximated to the next whole number.

ii The height in Centimeter was also taken by the same staff with patients on bare feet using standard measuring meter rule. The values in centimeters were converted in to their corresponding meter values before entered into the questionaires.

iii The Body Mass Index (BMI) calculated from the measured height and weight using the formula; $BMI = Wt/Ht^2$. Where, Wt stands for Weight in kilogram, while Ht stands for Height in meter.

3.7.3 Determination of Blood pressure:-

This was taken using the manual Accoson mercury sphygmomanometer by the researcher or the supporting staff. The subjects were made to sit down and allowed to rest for a minute or two to ensure they were calm before the measurement was taken. Brief description of the process involved was given to each subject before the reading was taken. After that subjects were made to pull up their clothes to expose the upper arm region where the cuff will be tied on. The cuff was then tied around the mid upper arm region of the left arm and its bulb inflated until the radial pulse was no longer felt. Thereafter, the pressure was slowly deflated. The first sound heard (Koroftko) was taken as the systolic blood pressure while the level of disappearance of the last sound when it was muffled was considered as the diastolic blood pressure (Ganong, 2012). Single readings were normally taken unless where there was doubt, then a second or even third reading was taken before the value obtained was documented.

3.7.4 Blood Sample collection:-

Using aseptic techniques, 10milliliter cube ($10ml^3$) of Peripheral blood was taken from each participant through the cubital veins by the Phlebotomist after an overnight fasting from 8.00pm to 8.00am. First, tourniquet was applied tightly around the mid upper arm region of the hand where the blood was to be taken. Methylated sprit was then applied on the site of blood collection thereafter a hypodermic syring needle was inserted in to one of the prominent veins so as to draw the blood until the 10milliliter volume was reached. The blood was then transferred into two different specimen bottles; one plain and the other containing Ethylene Diamine Tetra Acetic Acid (EDTA). The whole blood was placed into plain bottles for the CD4 and CD8 counts immediately after collection. The blood transferred into EDTA bottles was then centrifuged at 3000 rpm for 15minutes to obtain the serum for MDA and vitamin E

estimation. The serum in sample bottles was wrapped with aluminum foil and kept at -80^{0}C before MDA and vitamin E analysis.

3.7.5 Determination of CD4 and CD8 lymphocytes counts

CD4 and CD8 count was determined by Flowcytometry using the T4-T8 Easy count kit (Cyflow SL – 3, USA). Into a Rhoren test tube, 20µl of CD4 PE mAb was added, then 20 µl of well mixed EDTA whole blood collected within 6 hours was mixed and incubated in the dark for 15 minutes at room temperature. Later, 800 µl of CD4 & CD8 buffer each were added into separate containers and mixed thoroughly. The count estimation was carried out using the Cyflow meter. The CD4 values obtained were further classified in to four different subgroups according to WHO classification.

3.7.6 Determination of Serum Malondialdehyde (MDA)

Serum malondialdehyde was measured using the method of Ohakawa (1979). The principle is based on Lipid peroxidation which generates peroxide intermediates which upon cleavage releases MDA, a product which reacts with Thiobarbitutic Acid (TBA). The product of the reaction is colored complex which absorbs light at 532nm.

The method involves putting 0.20 ml of plasma in a test tube containing 3 ml of glacial acetic acid. Later 3ml of 1% TBA in 2% NAOH was added. The mixture was placed in a boiling water for 15minutes, allowed to cool and the absorbance of the pink colored product read at 532nm. Calibration curve was constructed using malondialdehyde tetrabutylammonium salt which was obtained from Sigma (St Lious, USA).

3.7.7 Determination of serum α-tocopherol

This was estimated using Fabianek (1968) method. A volume of 0.5 ml of serum with an equal volume of purified absolute ethanol was added in to a test tube for protein precipitation. Then 0.5 ml of xylen was added and shaken vigorously for 30 seconds and

later centrifuged for 10 minutes at 3000 rpm. After the centrifugation, the upper xylene layer which contains the extracted tocopherol was collected with a dropper and transferred in to a cuvet. To a 0.1ml of BA (Bathophenanthroline), 0.2 ml of serum xylene was added and mixed. Then 0.1ml of ferric chloride and 0.1ml of orthophosphoric acid was added to the mixture and mixed thoroughly. The absorbance was determined at 536 nm. The blank solution was prepared with 0.2 ml of xylene alone.

3.8 Limitations of the Study

1. Security situation in Kano State that spanned up to two years delayed the early completion of the work.
2. Some reagents being photo labile, got destroyed before utilizing them, hence they were not use for the intended purpose of SOD and Glutathione estimation.

3.9 Data Analysis

The data was analyzed using simple percentages, range, mean and standard deviation with Minitab version 6 and SSPS statistical software package version 16. Microsoft Word and Excel were used for the tabulations. Kruskal Walis test was used to compare median values between the four subgroups and Mann Whitney test was performed to compare variation in median values between the co-infection and normal control groups. Correlation analysis was performed using Spearman's test. The level of significance was set at $p<0.05$.

3.10 Consent for the Study

Ethical approval from AKTH Ethical and Scientific Committee (Appendix II) was sought for. In addition, written/Oral consent of the patients was obtained prior to the commencement of the study.

CHAPTER FOUR

4.0 RESULTS

A total of 186 [HIV/TB co-infection, 44 (23.66%); HIV patients 57 (30.64%); Tuberculosis patients 44 (23.66%) and apparently normal controls 41 (22.04%)] were evaluated in this study. There were 105 men (56.5%) and 81 women (43.5%). Except for the Tuberculosis patients who have the median age of 31 years, all the other three subgroups have the median age of 36 years ($P>0.05$). Fifty eight percent (58%) were married, 33.87% divorced, while widowed and singled constituted 3.76% each. Majority of the cohorts; 39.78% had secondary school education followed by 30.11% with tertiary education, 5.38% non-formal education and 4.84% primary education. Morethan 37% were self employed, 33.33% civil servants and 29.57% were unemployed as of the time of this study.

Table 4.1: Socio-demographic characteristics of subjects and controls

Characteristics	HIV/TB co-infection	HIV	TB	Controls
Age (yr)	36	36	31	36
Sex				
Males	24	22	26	33
Females	20	35	18	08
Total	44	57	44	41
Marital status				
Single	4	1	2	0
Married	22	39	18	30
Widowed	3	4	0	0
Divorced	15	13	24	11
Total	44	57	44	41
Educational Level				
Non-formal	13	14	9	1
Primary	3	4	2	0
Secondary	14	29	14	17
Tertiary	14	0	19	23
Total	44	57	44	41
Occupation				
Unemployed	13	20	21	1
Civil servant	9	12	8	33
Others	22	25	15	7
Total	44	57	44	41

Table 4.2: Anthropometric and Hematological variables among the four groups

Variables	Controls (n= M33, F8)	HIV (n= 22M, F35)	TB (n= M26, F18)	Co-infection (n=M24, F20)
Weight (kg)	75.00[2]	68.00[2]	52.50[2]	55.00[2]
Height (m)	1.65[3]	1.64[3]	1.67[3]	1.70[3]
BMI (kg/m^2)	25.85[4]	24.68[4]	19.35[4]	19.88[4]
MAC (cm)	28.10[5]	27.20[5]	23.15[5]	24.65[5]
CD4	743.0[6]	356.0[6]	571.0[6]	200.5[6]
CD8	369.0[7]	621.0[7]	331.5[7]	563.0[7]
CD4/CD8 ratio	1.86[8]	0.50[8]	1.92[8]	0.39[8]
MDA (umol/l)	0.55[9]	4.60[9]	3.73[9]	3.81[9]
Vitamin E (ug/mL)	18.65[10]	7.99[10]	8.29[10]	3.85[10]

TB, pulmonary tuberculosis; BMI, Body Mass Index; MAC, mid- arm circumference; MDA, Malondialdehyde

Kruskal Walis test with associated P values;

1. H = 3.53, P=0.317[1]
2. H = 33.08, P=0.0001[2]
3. H = 3.11, p=0.375[3]
4. H = 26.20, P=0.0001[4]
5. H = 28.24, p=0.0001[5]
6. H = 89.03, P=0.0001[6]
7. H = 40.25, P=0.0001[7]
8. H = 114.20, p=0.0001[8]
9. H = 40.33, P=0.0001[9]
10. H = 146.6, P = 0.000[10]

In Table 4.2 above, the BMI was found to be significantly ($p<0.05$) lower in patients with Tuberculosis (19.35 kg/m^2), HIV/TB co-infection (19.88 kg/m^2), HIV (24.68

kg/m^2) when compared to normal controls (25.85 kg/m^2). CD4 count was significantly $p<0.05$ lower in HIV/TB co-infection group (200.5 cells/mm^3), followed by HIV group with (356.0 cells/mm^3), then Tuberculosis with (571.0 cells/mm^3) when compared with normal controls (743.0 cells/mm^3). Conversly, the MDA value was the least in the normal control group; 0.55 umol/l and highest in the HIV group; 4.60 umol/l, while HIV/TB co-infection had 3.81 umol/l. The vitamin E value was found to be high; 18.65 ug/mL in the normal control group, while Tuberculosis and HIV patients had 8.29 ug/mL and 7.99 ug/mL respectively. The least value recorded was in the HIV/TB co-infection group; 3.85 ug/mL.

Table 4.3: Distribution of median serum Malondialdehyde values by Age in Normal Controls

Age group	Number (%)	Median	Range
21-30	13 (31.71%)	0.55	0.45 – 6.60
31-40	16 (39.02%)	0.52	0.344 -6.838
41-50	10 (24.39%)	0.45	0.241- 6.564
51-60	1 (2.44%)	3.88	3.88 - 3.88
61-70	1 (2.44%)	1.55	1.55 -1.55

Table 4.3 shows the distribution of serum Malondialdehyde values by age among the normal Controls. There is reduction in the median MDA values from the age group 21 – 30 to 41 – 50, while, absolute values represent both the age groups of 51 – 60 and 60 – 70.

Table 4.4: Distribution of median serum Malondialdehyde values by Age in HIV Patients

Age group	Number (%)	Median	Range
21-30	9 (15.79)	4.78	2.89 – 7.32
31-40	26 (45.61)	4.88	0.83 – 8.73
41-50	18 (31.57)	5.02	2.06 – 7.97
51-60	3 (5.26)	4.40	3.54 – 5.19
61-70	1 (1.74)	2.58	2.58 – 2.58

The median MDA values show an increase from the ages 21 – 30 up to 41 – 50 years, thereafter the values declined up to the age of 61 – 70.

Table 4.5: Distribution of median serum Malondialdehyde values by Age in Tuberculosis Patients

Age group	Number (%)	Median	Range
10 – 19	4 (9.09)	3.60	0.38 – 4.54
20 – 29	13 (29.54)	3.51	0.34 – 7.42
30 – 39	14 (31.81)	4.07	2.65 – 7.29
40 – 49	5 (11.36)	3.37	2.68 – 4.47
50 – 59	5 (11.36)	4.64	1.51 – 7.29
60- 69	2 (4.54)	4.74	3.30 – 6.19
70 - 70	1 (2.27)	4.19	4.19 – 4.19

No clear pattern in MDA values was produced among the age groupings in the Tuberculosis Patients.

Table 4.6: Distribution of median serum Malondialdehyde values by Age in HIV/TB co-infected patients

Age grouping	Number (%)	Median	Range
20-29	8 (18.18%)	4.26	1.65 - 20.96
30-39	21 (47.73%)	3.99	2.47 - 71.06
40-49	11 (25.00%)	4.60	1.79 - 71.51
50-59	3 (6.82%)	2.4	2.1 - 39.4
60+	1 (2.27%)	2.58	2.58 - 2.58

No clear pattern in MDA values was produced among the age groupings in HIV/TB co-infected Patients

Table 4.7: Median values of CD4 and serum Malondialdehyde values in HIV/TB co-infected patients according to WHO clinic staging

CD4 Count	WHO staging	Number	CD4	MDA
>600	WHO stage I	2	703.5	5.70
400 - 599	WHO stage II	4	470.0	3.71
200 - 399	WHO stage III	17	285.0	3.44
<200	WHO stage IV	21	135.0	3.85

There is a significant decline ($p<0.05$) in the CD4 median values as the disease progresses from stage I to IV. The MDA values progressively declined from stage I to III, thereafter, a slight increase was noticed from stage III to IV.

Table 4.8 Mann-Whitney test for the HIV/TB Co-infection and Controls

Diagnosis	**Variables**						
	No.	**MDA**	**Vit. E**	**CD4**	**CD8**	**CD4/CD8**	**MDA**
Co-infection	44	3.81[1]	3.85[2]	200.5[3]	563[4]	0.39[5]	3.81[6]
Controls	41	0.55[1]	18.65[2]	743[3]	369[4]	1.86[5]	0.55[6]

1. W = 2454.5, P = 0.0001
2. W = 990.0, P = 0.0001
3. W = 1043.5, P = 0.0001
4. W = 2296.0, P = 0.0004
5. W = 1007.0, P = 0.0001
6. W = 1398.0, P = 0.0001

In Table 4.8 above, the vitamin E level, CD4 cell count, CD4/CD8 ratio and BMI values are significantly higher ($p<0.05$) in normal controls than the in HIV/TB co-infected subjects. Conversly, values of MDA, and CD8 cells are significantly ($p<0.05$) higher in co-infection compared to the normal controls.

To generate reliable reference intervals for Serum Malondialdehyde (S-MDA), in both co-infection and control groups, the data was log-transformed and normalized to the base of 0.975. The reference interval was calculated as recommended by International Federation of Clinical Chemistry (IFCC) with REFVAL 3.42 after log transformation of the data. The estimated reference limits for MDA (0.025 and 0.975 fractals) were: HIV =1.99 μmol/L - 10.16 μmol/L; TB=1.55 μmol/L - 7.26 μmol/L; HIV/TB co-infection = 4.22 μmol/L - 4.57μmol/L and normal Controls = 1.06 μmol/L - 3.29μmol/L lower and upper limits respectively

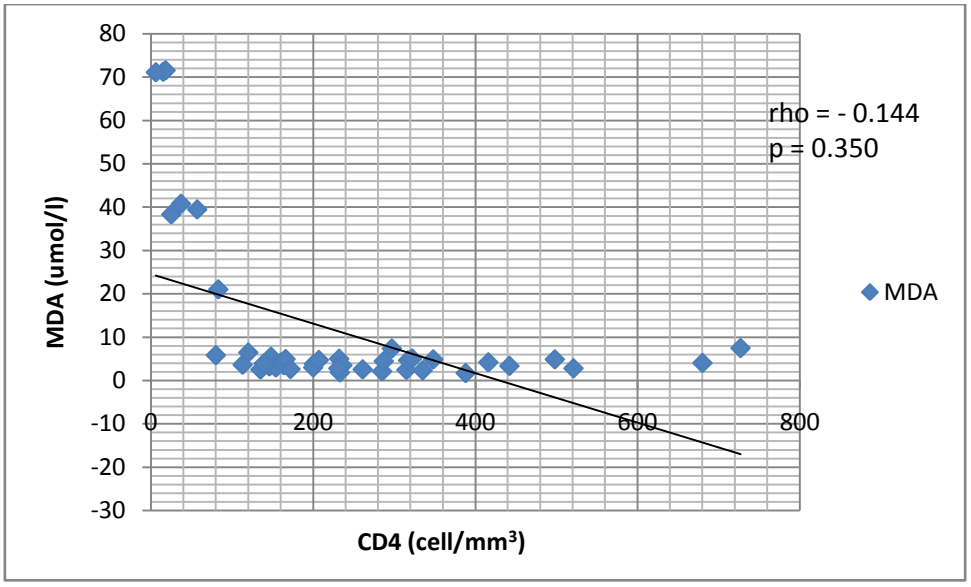


Figure 1: Correlation between Malondialdehyde and CD4 count in HIV/TB co-infected Patients

Figure 1, shows the Spearman's Correlation Coefficient between MDA and CD4 cell count. There was no significant relationship (rho = -0.144; p=0.350) between CD4 counts and MDA levels in HIV/TB co-infected patients.

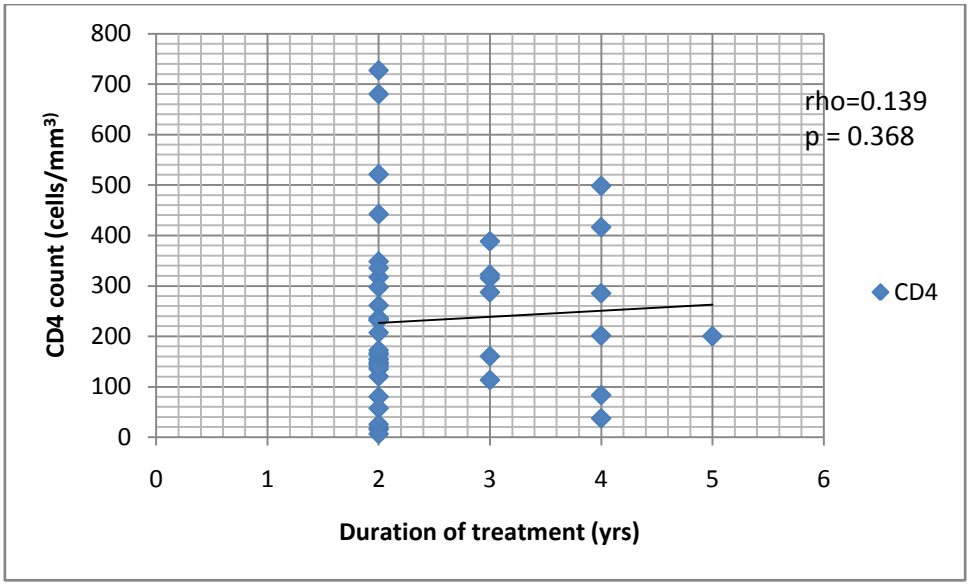


Figure 2: Correlation between CD4 count and Duration of treatment in HIV/TB co-infected Patients

Figure 2, shows the correlation between CD4 count and duration of treatment. There was no significant relationship (rho = 0.139, p 0.368) between the duration of treatment and CD4 counts in HIV/TB co-infected Patients

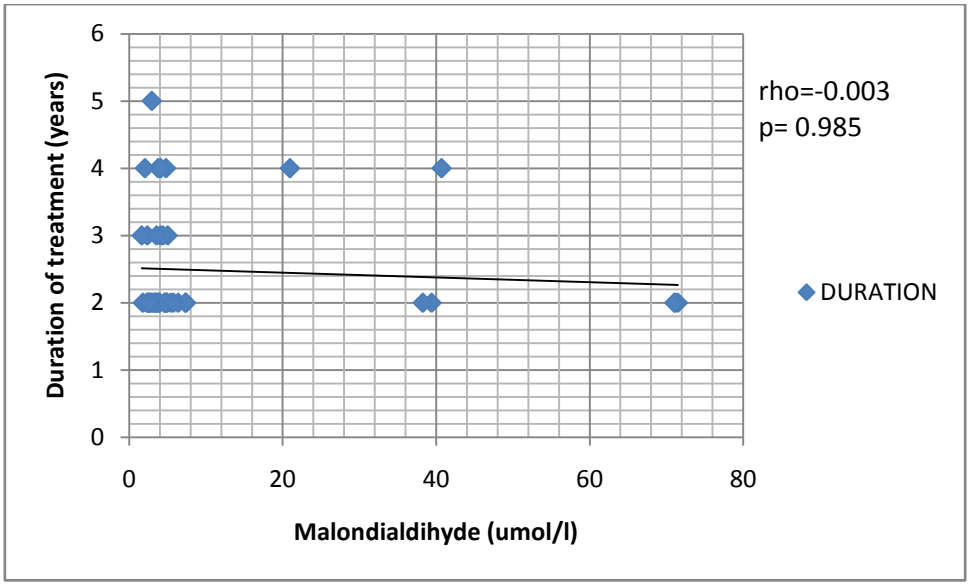


Figure 3: Correlation between Duration of treatment and Malondialdehyde in HIV/TB co-infected Patients

Figure 3, shows a Spearman's Correlation Coefficient between duration of treatment and MDA values. There was no significant relationship (rho = -0.003; p=0.985) between duration of treatment and MDA level in HIV/TB co-infected Patients.

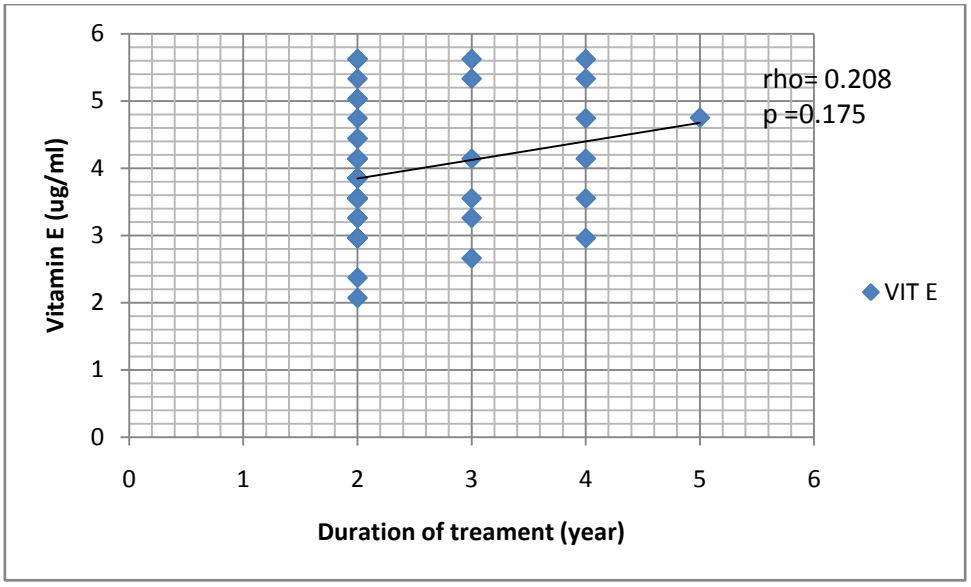


Figure 4: Correlation between vitamin E and Duration of treatment in HIV/TB co-infected Patients

Figure 4: shows the Spearman's Correlation Coefficient between vitamin E and duration of treatment. There is an insignificant rho = 0.208, p = 0.175 positive relationship, indicating an increase in the anti oxidant level as the duration of treatment increases.

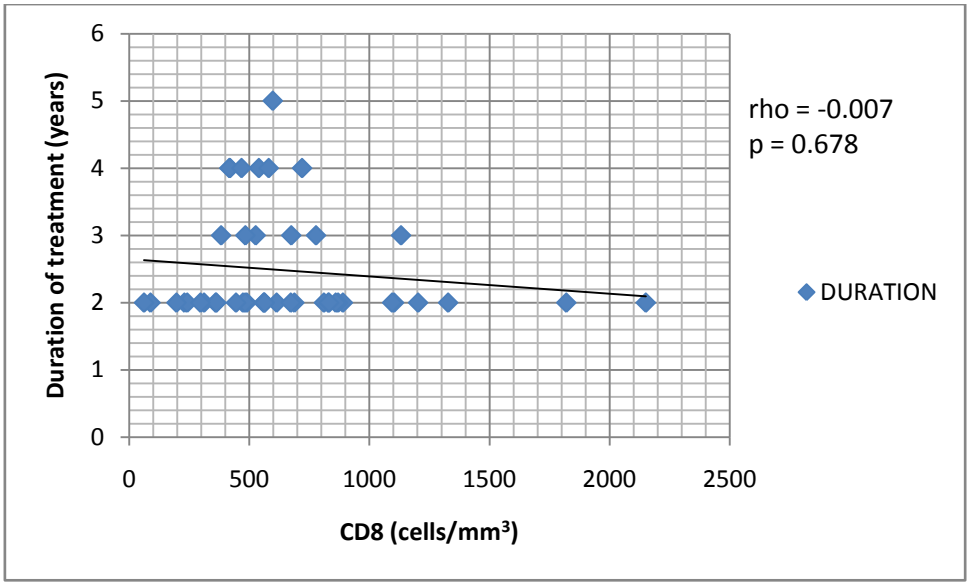


Figure 5: Correlation between Duration of treatment and CD8 counts in HIV/TB co-infected Patients

Figure 5: shows the Spearman's Correlation Coefficient between duration of treatment and CD8 count. There is an insignificant rho = -0.007, p = 0.678 negative relationship between the duration of treatment and CD8 cell counts.

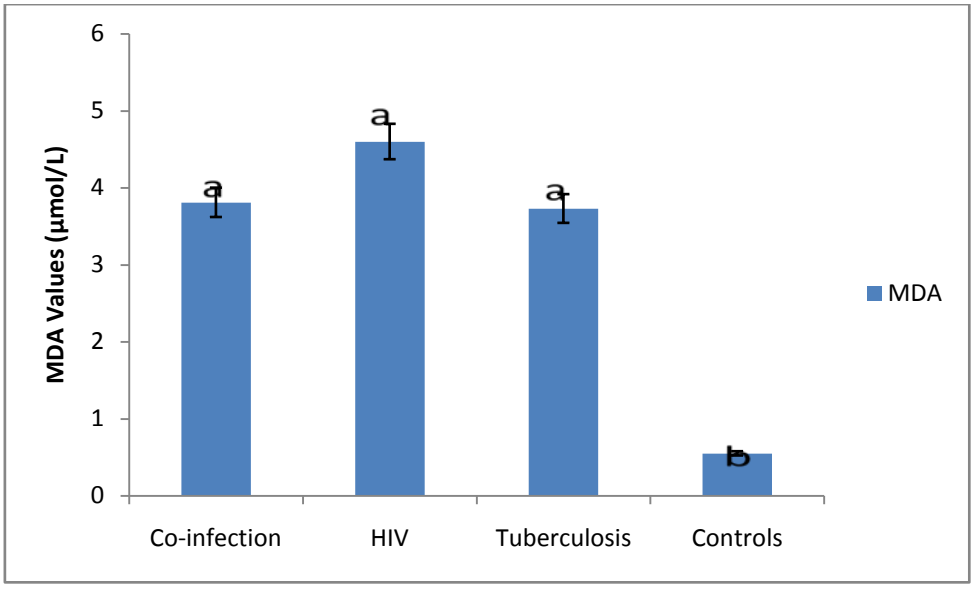


Figure 4.6: Bar chart showing median MDA values among the four subgroups

a and b = Values with different letters are statistically different ($p<0.05$)

Malondialdehyde (MDA) is one of the most frequently used indicators of lipid peroxidation. Figure 4.6, showed that the median MDA values are statistically significant $p<0.05$ with higher median value observed in HIV patients (4.60 umol/l) followed by HIV/TB co-infection (3.81 umol/l) then Tuberculosis (3.73 umol/l) and finally (0.55 umol/l) for the normal controls.

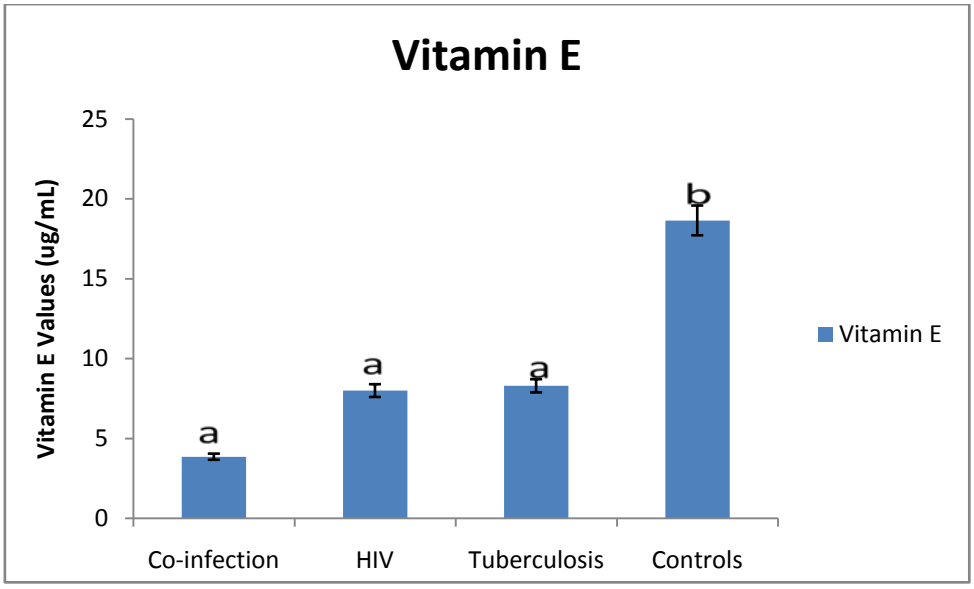


Figure 4.7: Bar chart showing median Vitamin E values among the four groups

a and b = Values with different letters are statistically different ($p<0.05$)

When compared among the four sub- groups, there was a significant ($p<0.05$) difference with higher value observed in the normal controls 18.65 ug/mL, followed by Tuberculosis; 8.29 ug/mL, then HIV patients; 7.99 ug/mL and the least value was seen in the co-infection group; 3.85 ug/mL.

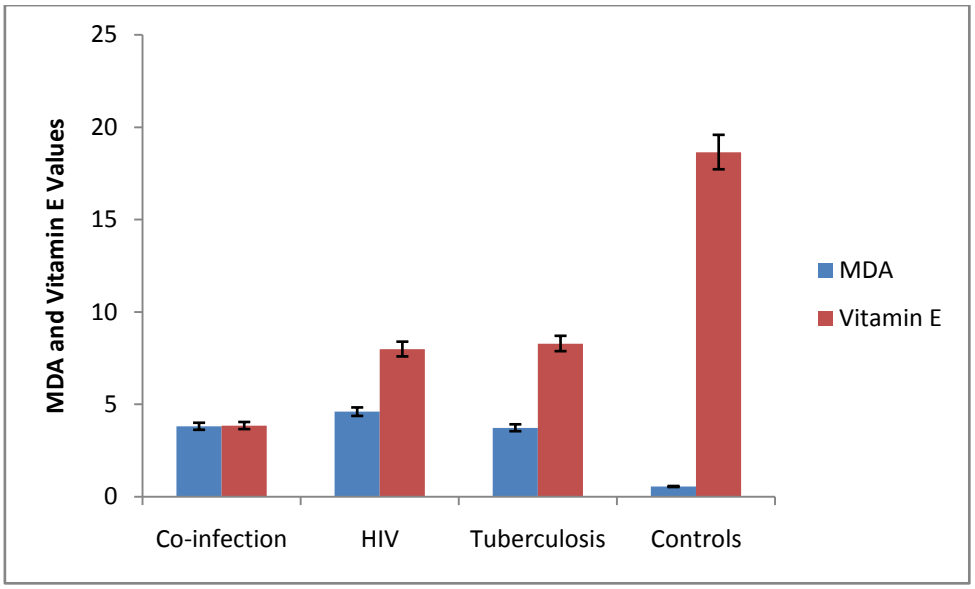


Figure 4.8: Bar chart comparing median values of MDA and Vitamin E among the four groups

The values are similar in the HIV/TB co-infection subgroup, but widely differed in the remaining three subgroups with Vitamin E much higher compared to MDA especially in the normal Controls.

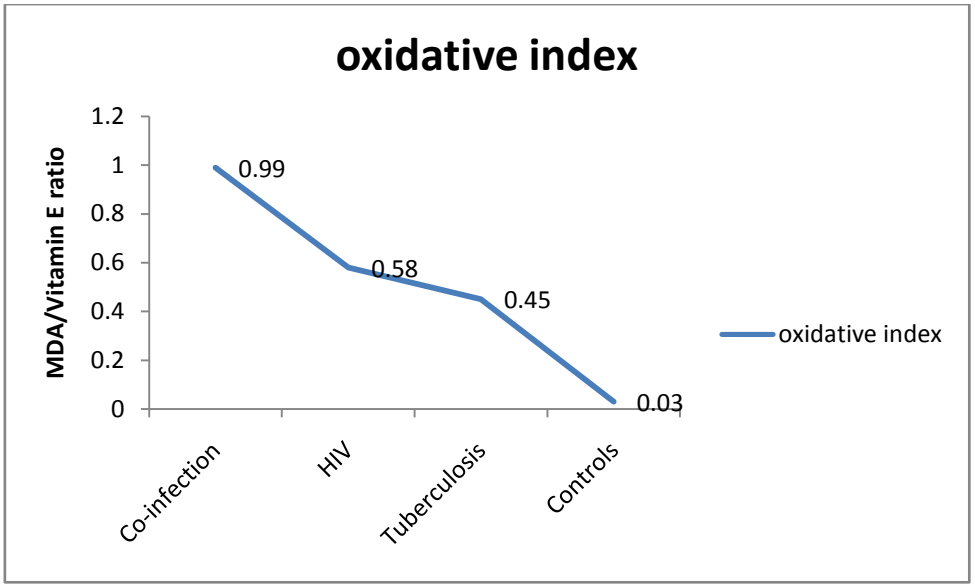


Figure 4.9: Line diagram showing Lipid Peroxidative Indexes among the four groups

The Lipid Peroxidation Index was shown in Figure 4.9. This was determined by dividing the values of MDA with that of Vitamin E. A significant ($p<0.05$) difference was observed with higher value obsevered in the co-infection group (0.99), followed by HIV patients (0.58), then Tuberculosis (0.45) and the last in normal controls (0.03). This means that the oxidative index is 33 times in HIV/TB co-infection, 19.3 times in HIV patients and 15 times in Tuberculosis patients than in the normal controls.

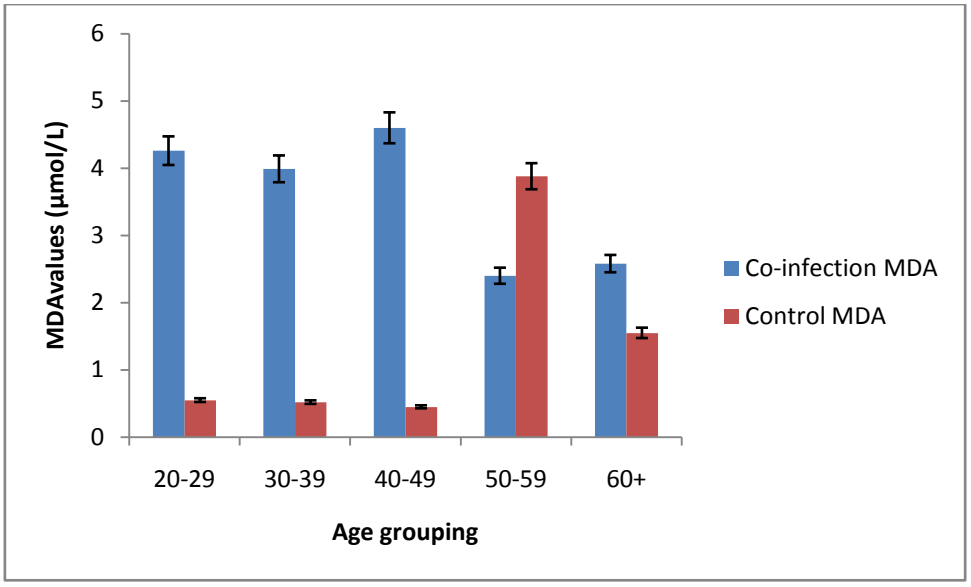


Figure 4.10: Bar chart showing the MDA values among age groupings between HIV/TB Co – infection and the normal Controls

Figure 4.10, shows the MDA values among age groupings between HIV/TB Co – infection and the normal Controls. There is no clear pattern established in both Subjects and Controls.

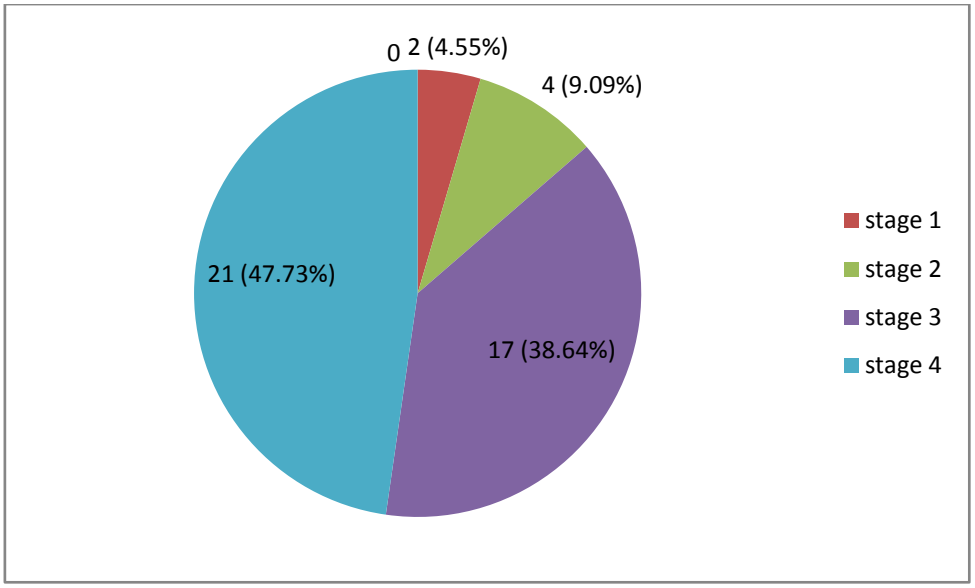


Figure 4.11: Pie chart showing Percentage distribution of the Co – infected Subjects according to Clinical Staging of the Disease

Figure 4.11, indicates that majority of the subjects are in the WHO stage IV of the disease; 21 (47.73%), meaning that majority of the clients presented late to the clinic. This predisposes them to the development of full blown AIDS and other opportunistic infections.

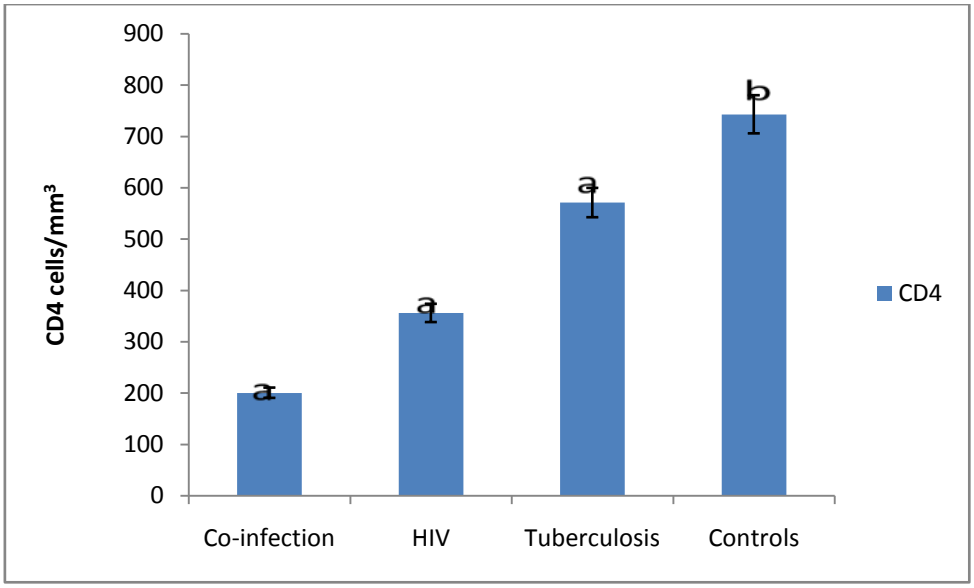


Figure 4.12: Bar Chart showing the median values of CD4 counts among the four subgroups

a and b = Values with different letters are statistically different ($p<0.05$)

Figure 4.12, shows the median values of CD4 counts among the four subgroups. The HIV/TB co-infection has the lowest ($p>0.05$) CD4 counts compared to the remaining three subgroups.

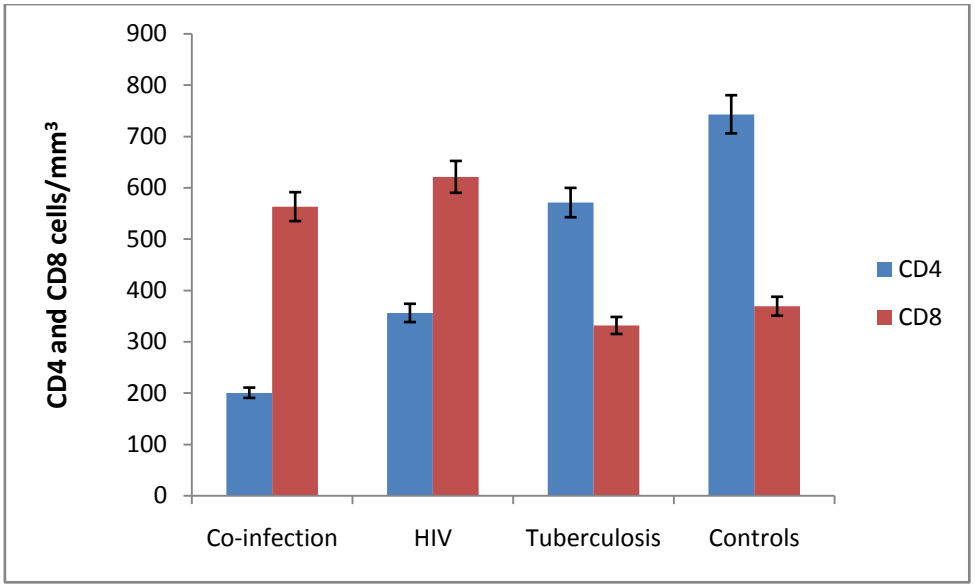


Figure 4.13: Bar Chart showing median values of CD4 and CD8 among the four subgroups

Figure 4.13, shows the median values of CD4 and CD8 among the four subgroups. The CD4 cells showed a linear increase in value from HIV/TB co-infection subgroup to the normal controls subgroup. CD8 on the other end appeared to be higher in Co-infection and HIV subgroups, while values in Tuberculosis and normal Controls remained low.

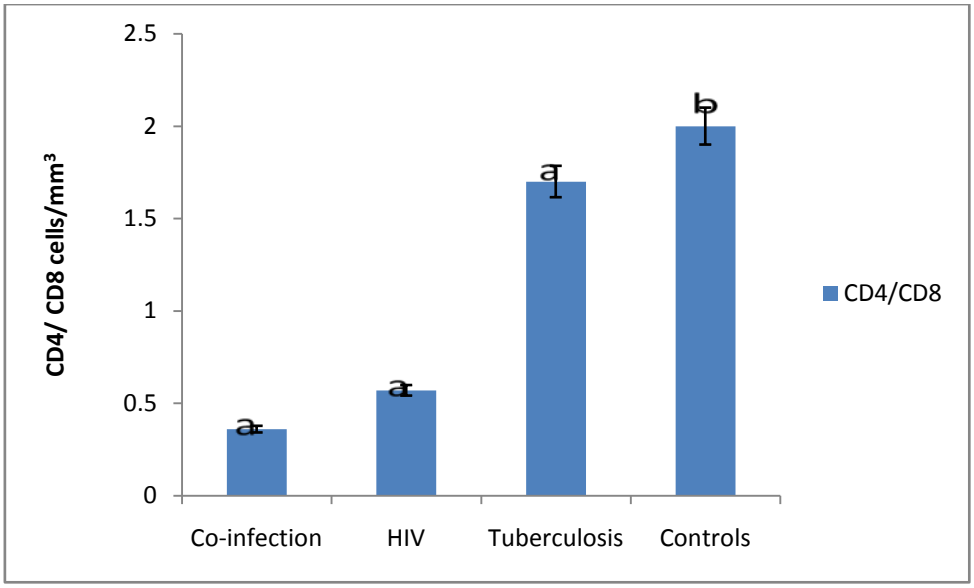


Figure 4.14: Bar Chart showing CD4/CD8 ratio among the four subgroups

a and b = Values with different letters are statistically different ($p<0.05$)

Figure 4.14, shows CD4/CD8 ratio among the four subgroups. It is (1.86) in control group, (1.92) in HIV group, with least value recorded in the HIV/TB co-infection group (0.39), followed by HIV infection group (0.05). There is significant ($p<0.05$) difference between the four different groups.

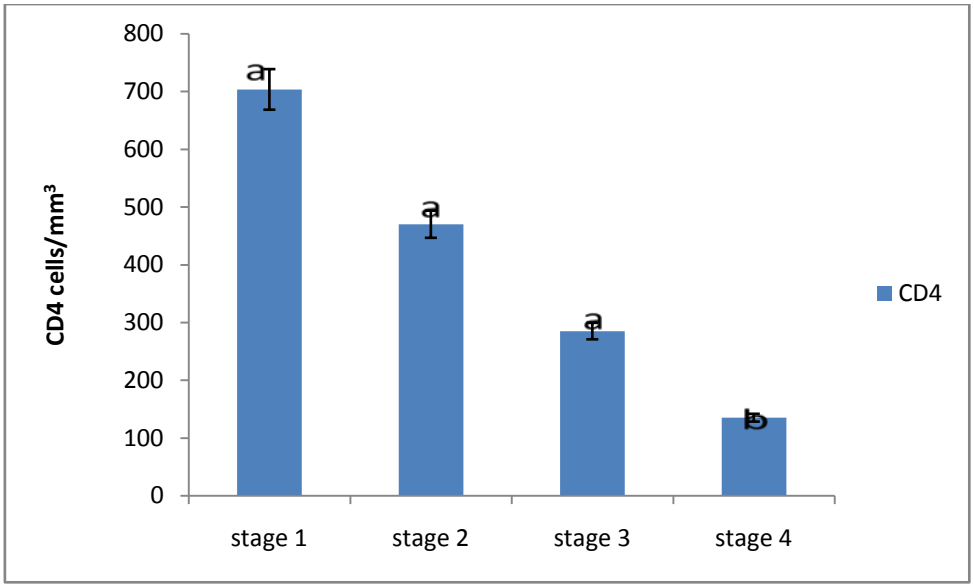


Figure 4.15: Bar Chart showing CD4 counts in relation to WHO Clinical staging of HIV/TB Co-infection

a and b = Values with different letters are statistically different ($p<0.05$)

Figure 4.15, shows CD4 counts in relation to WHO Clinical staging of HIV/TB Co-infection subgroup. There was progressive decline in the CD4 count as the disease progresses from WHO stage I to IV respectively.

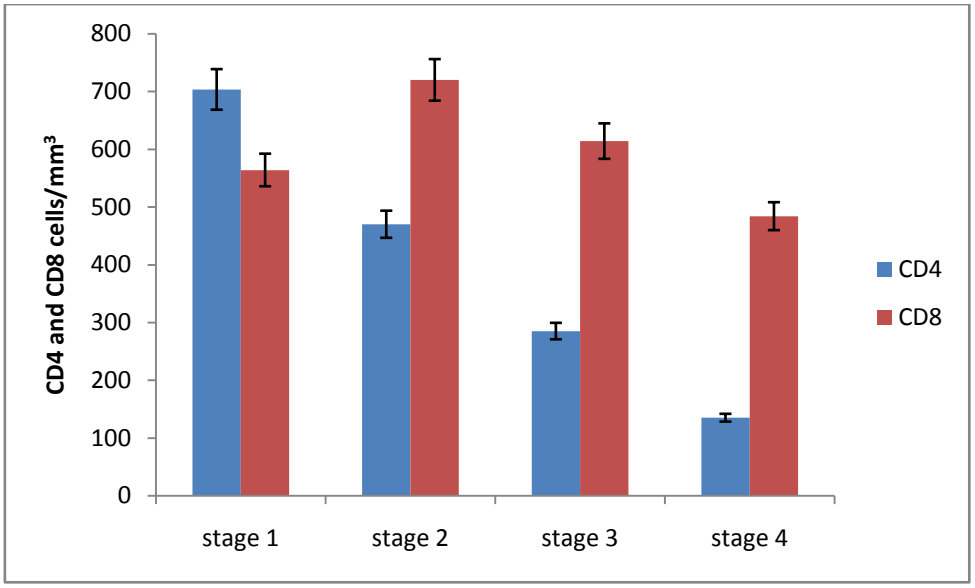


Figure 4.16: Bar Chart showing variation of CD4 and CD8 counts in relation to WHO Clinical staging of HIV/TB Co-infection

Figure 4.16, shows variation of CD4 and CD8 counts in relation to WHO Clinical staging of HIV/TB Co-infection. The CD4 cells progressively declined from stage I to IV, while CD8 remained well above 480 cells in all the stages of the disease.

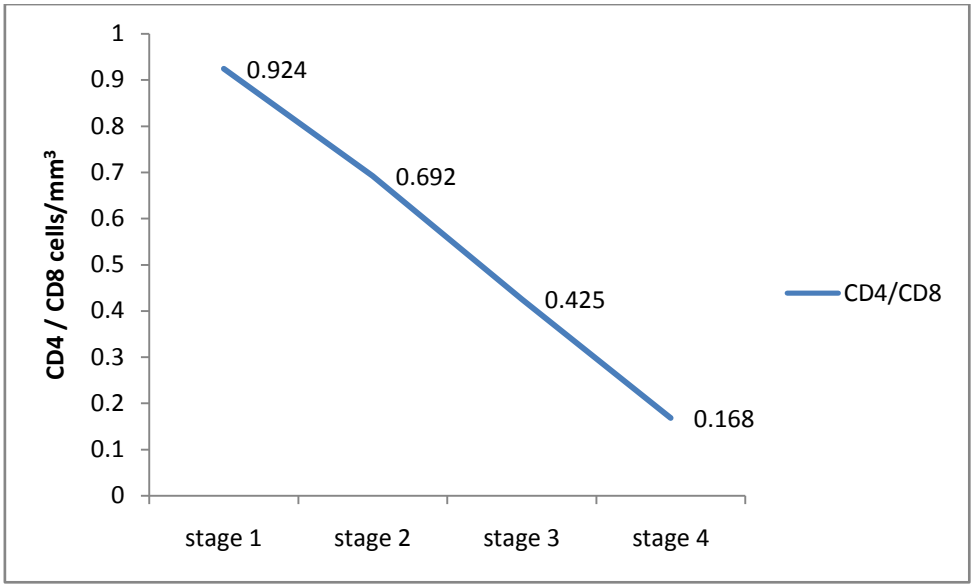


Figure 4.17: A line diagram showing variation in MDA values in relation to WHO Clinical staging of HIV/TB Co-infection

Figure 4.17, shows the individual values of CD4/CD8 cell ratio among the WHO clinical staging of HIV/TB Co-infection. The values are; 0.924, 0.692, 0.425 and 0.168 from stage I to IV respectively. This shows a decline in the CD4/CD8 ratio as the disease progresses from stage I to IV.

CHAPTER FIVE

DISCUSSION

Much research has been conducted in the areas of HIV-TB co-infection, but there are few studies in the literature report on oxidative stress marker; MDA, comparing its value in relation to single infection, co-infection and normal controls and also comparing the oxidative stress indices in these categories of diseases. The present study was developed to generate data in these areas.

The median MDA values among the four subgroups showed a significant difference, with higher value seen in HIV patients (4.6 umol/l), followed by HIV/TB co-infection (3.81 umol/l), then Tuberculosis patients (3.73 umol/l) and (0.55 umol/l) for the normal controls. This finding supports earlier study (Teto *et al.,* 2013) that documented higher MDA values in HIV patients, compared to controls. Studies have shown that humans infected with HIV are under chronic immune activation characterized by increased generation of ROS and perturbations of the antioxidant defence system (Gil *et al*., 2003; Pasupthi *et al.,* 2009; Coaccioli, *et al.,* 2010; Awodele *et al.,* 2012). Oxidative stress (OS) has been detected in many tissues of HIV infected individuals using different biochemical's markers and diverse biotechniques (Gil *et al.,* 2013).

Reports have shown that even in the earliest stages of HIV infection, a deleterious reductive-oxidative (redox) imbalance may occur (Osuji *et al.,* 2013), this means that increased reactive oxygen intermediates are generated at the same time that stores of naturally occurring reducing agents are depleted (Osuji *et al.,* 2013). The possible result is the uncontrolled presence of oxygen-containing molecules which may overwhelm the antioxidant capacity and cause damage to cell membranes, protein and nucleic acids and

also cause alterations in the intra and inter- cellular environment (Carole and Sterrit, 1994; Osuji *et al.,* 2013).

The higher serum MDA concentrations in the three infected subgroups as against the normal controls in this study is believed to be due to the effects of free radicals on polyunsaturated lipids which induce oxidative stress, producing deleterious effects such as cellular apoptosis. This may also be the main cause of CD4 cell depletion during HIV infection, as observed by other workers (Lopez *et al.,* 1996).

In HIV-infected patients, increased oxidative stress has been implicated in increased HIV transcription through the activation of nuclear factor B (NF-B) (Kumar *et al.,* 1998). NF-B is bound to factor I B in the cytoplasm in its inactive form, but various factors, such as TNF-α and reactive oxygen species (ROS) can cause the release of NF-kB from factor I kB; and NF-kB translocates to the nucleus and binds to DNA (Mingyan *et al.,* 2009). NF-kB is involved in the transcription of HIV-1. Thus, ROS may potentially be involved in the pathogenesis of HIV infection through direct effects on cells and through interactions with NF-kB and activation of HIV replication (Isreal and Gougerot- Pocidalo, 1997).

Studies have demonstrated that viral Tat protein liberated by HIV-1-infected cells interferes with calcium homeostasis, activates caspases and induces mitochondrial generation and accumulation of ROS (Gil *et al.,* 2004). All these are important events in the apoptotic cascade of several cell types. CD4 cell subset depletion in HIV/AIDS patients is the most dramatic effect of apoptosis mediated by redox abnormalities.

The higher oxidative index observed in this study in the HIV/TB co-infected as compared to controls and other infected groups may be due to possible interaction

between oxidative stress and pathobiology of mycobacterial infections (Eze *et al*., 1993). Mycobacteria are intracellular pathogens that grow and replicate in the host macrophages (Osuji *et al.,* 2013). In an attempt to kill mycobacterium, host cells namely macrophages, neutrophils and monocytes generate huge amount of reactive oxygen species which contribute to inflammatory injury to host tissues and may further lead to immunosuppression particularly in those with impaired antioxidant capacity such as HIV infected individuals (Osuji *et al.,* 2013). Moreover the malnutrition which is commonly present in patients with tuberculosis can add to the impaired antioxidant capacity in these patients (Kauri *et al*., 2005). Therefore TB and HIV infections lead to continuous production of free radicals that may cause a resultant low level of antioxidants (Wiid *et al*., 2004).

Furthermore, the role of oxidative stress in the pathogenesis of TB suggests lower antioxidant capacity and higher free radical levels in TB patients than in control subjects (Samuel *et al.,* 2013). Significantly increased MDA in TB patients than in non-TB individuals has been previously reported by some authors (Tesfaye *et al.,* 2003; Reddy *et al.,* 2004; Reddy *et al.,* 2009). Samuel *et al.,* (2013) reported that; increased production of reactive oxygen species / reactive nitrogen intermediates (RNI) secondary to phagocyte respiratory burst occurs in pulmonary TB. Evidence suggest that increased circulating levels of free radical activity are found in pathogenesis of active pulmonary TB and hence play a role in resultant fibrosis (Ramesh *et al.,* 2011). Free radicals have been implicated in the development of lung fibrosis which may be a long term sequel of pulmonary tuberculosis (Jack, 1994). Free radicals attack the cell membrane causing tissue damage and wasting disease in pulmonary TB patients. In the same vein,

Mycobacterium can induce reactive oxygen species production by activating phagocytes (Reddy *et al.,* 2004).

Individually, HIV and TB infections lead to oxidative stress, it will be assumed that the combined effects of these infections will produce more stress in the system, leading to higher MDA value than the individual diseases as has been observed by previous workers (Awodele *et al.,* 2012). The relatively lower MDA value recorded in the HIV/TB co-infection as opposed to HIV infection alone in this study is contrary to the findings of a previous study of Awodele *et al.,* (2012). Differences in the nutritional status of the affected patients may be responsible for the variation between the findings observed in the present study.

The fact that, both HIV and TB infections alone produce high MDA value in the present study, support the role of oxidative stress in the pathogenesis of these diseases. The correlation between high MDA concentration and low concentrations of the anti-oxidant in this study suggests increased utilization of vitamin E by ROS as documented in earlier study (Gil *et al.,* 2013). HIV-infected individuals have been reported to show decreased antioxidant concentrations, disruption in glutathione metabolism, and enhanced spontaneous generation of ROS (Tejasvinis *et al.,* 2012). Apart from the HIV/TB induced oxidative stress, malnutrition may have influenced the anti-oxidant status of the subjects used in this study as reported in a similar study by (Hemilia, 1994). The present study shows a significant ($p<0.05$) decrease in the level of vitamin E in the HIV/TB co-infected patients (3.85ug/mL), HIV patients (7.99 ug/mL) and Tuberculosis patients (8.29 ug/mL) compared to the normal controls (18.65ug/mL). In many studies, concentration of vitamin E was found to be significantly lower in tuberculosis patients than healthy controls (Reddy *et al.,* 2004). Others have found significantly low levels of

vitamin E, vitamin A, total antioxidant, Zn and beta-carotene in pulmonary tuberculosis compared to normal controls (Plit *et al.*, 1998; Madebo *et al.*, 200; Wiid *et al.*, 2004). Several factors such as low food intake, nutrient malabsorption and inadequate nutrient release from the liver, acute infections and inadequate availability of carrier molecules may influence circulating antioxidant concentrations in these patients (Siez and, Stahl 1995; Rock *et al.*, 1996; Das *et al.*, 1996).

Vitamin E, a potent chain breaking lipid soluble antioxidant, reacts with lipid peroxyl radicals eventually terminating the peroxidation chain reaction and thereby reducing oxidative damage. While in this study, only vitamin E was assayed and compared with MDA in order to assess the individual antioxidant role in HIV and Tuberculosis patients with or without accompanying co-infection, (Suresh *et al.*, 2009) studied the total anti oxidant capacity (TAC) in HIV patients and compared the value with serum MDA level. A significantly lower serum TAC concentration in the HIV-1 patients compared to controls documented reflects a lower total antioxidant capacity. Negative correlation between MDA and TAC suggests an increased oxidative stress in HIV-1 infected patients. This antioxidant deficiency in HIV-1 seropositive populations was believed to be due to depletion of antioxidant molecules when they are consumed in the process of protecting cells against ROS induced oxidative damage in a magnitude that is related to advancement of the disease to AIDS (Suresh *et al.*, 2009). A weakened antioxidant defense system, in turn, could lead to further enhancement in lipid peroxidation (Ogunro *et al.*, 2005; Valko *et al.*, 2007). It is clear that whether a single antioxidant is assessed, as seen in this study or the total anti oxidant capacity is assessed as presented by (Suresh *et al.*, 2009), both procedures revealed a reduction in the antioxidant potential in patients with HIV and TB with or without accompanying co-infection. Certainly, total antioxidant assessment gives an overall picture of the interplay between oxidants and

TAC both (enzymatic and non enzymatic). However, one will prefer, an assessment of a single antioxidant especially in a resource constrain environment, like the location of this study. Ultimately, the choice of which antioxidant to assess will depend on the aim of the study, availability of facilities and equipment and financial status of the individual undertaking the research.

This study did not show any age – related influence on MDA reference intervals. In contrast, a relationship between lipid peroxidation and aging has been reported in rat testes and liver (Mihara M *et al.,* 1980). Similarly, workers have reported the accumulation of lipofuscin in testes of aging mice, (Miquel J *et al.,* 1978). A report of age – related reference intervals for serum MDA by (Kharb *et al.,* 2003) agrees with findings of (Miquel J *et al.,* 1978; Mihara M *et al.,* 1980). The higher MDA values in the three subgroups as compared to normal controls cannot be linked to age related changes as observed in other studies, but to be due to chronic nature of these diseases, with continued stimulation of the immunocompetent cells like macrophages, lymphocytes and neutrophils. These activated immune cells produced superoxide radicals and hypohalide ions through NADPH and myeloperoxidases (Block *et al.,* 2002). The radicals produced attack components of the cell membranes, there by breaking lipids and producing MDA as a bye product (Block *et al.,* 2002). The extent of damage that this stress could cause depends on the condition of the antioxidant defense system at the site of inflammation (Gil *et al.,* 2013). In addition, all the infected patients are on medication with anti retroviral or anti tuberculosis drugs or both. The pro-oxidant side effects of these drugs may add to the MDA already generated by the primary infection in these patients.

Phospholipids of biological membranes are highly susceptible to oxidation by ROS and also are involved in the process known as lipid peroxidation (Gil *et al.,* 2013). This is a free radical mediated pathway and its final products are used as an index of irreversible damage of cell membrane phospholipids as reported by (Block *et al*., 2002; Roberts *et al*., 2010). The 33 – fold higher oxidative stress index observed in HIV/TB co-infected, 19.3 in HIV only, and 15 – fold in TB infected as compared to controls signifies an additive effect of oxidation on the HIV/TB co-infected group. Suresh *et al.,* **(**2009) reported a 76-fold increase in LPI in HIV patients compared to the controls, which is in agreement with previous studies (Rabaud *et al.,* 1997). It has also been established that HIV-1 uses available antioxidants for its replication and this phenomenon adds to the chronic inflammatory process that speeds up CD4 cells apoptosis and disease progression (Fawzi, 2004). The LPI in this study was found to be below the level documented in previous studies (Suresh *et al.,* 2009). The variation may be due to difference in analytical techniques or anthropological response to the HIV virus.

The median CD4 cell count for the four subgroups was compared using Kruskal Walis test. A significant $P<0.01$ statistical difference was found with HIV/TB co-infection having the value of 200.5 cell/mm^3 followed by HIV patients with 356.0 cell/mm^3, Tuberculosis patients with 571.0 cell/mm^3 and normal controls with 743.0 cell/mm^3. The lower CD4 cell counts observed in this study are in agreement with an earlier report by Awodele *et al.,* 2012 who documented a decrease in the level of CD4 counts in HIV-TB co-infection, HIV patients under treatment, and the HIV naive group when compared to the HIV seronegative normal human volunteers. They also documented a significant decrease in the levels of the CD4 counts in HIV naïve patients compared with those of the HIV and HIV-TB co-infected patients on HAART. In the present study, all the cohorts were on HAART; therefore comparison was made between the

three subgroups and the normal controls. The observed decline in the levels of CD4 counts in these subgroups compared to the normal controls is an indication of the level of depletion of the host immune system that is often associated with HIV infection.

In HIV/TB co-infection, CD4 cells are essential for the containment of *M. tuberculosis* and the long-term survival of infected mice, which was demonstrated by a significant decrease in survival time and an increase in the bacterial burden in major histocompatibility complex class II and CD4 mice (Caruso., *et al.,* 1999). HIV and Simian Influenza Virus (SIV) cause substantial reductions in peripheral, mucosal, and gut CD4 cells shortly after infection by preferentially infecting activated CD4 cells (Schneider *et al.,* 1995; Veazey *et al.,* 2000; Mattapallil *et al.,* 2005; Centlivre *et al.,* 2007) and resting memory CD4 cells (Brenchley *et al.,* 2004; Li *et al.,* 2005). Studies have determined that SIV, and presumably HIV, kills up to 60% of the gut CD4 cells within the first 10 days of infection, with an 80% reduction of these cells by 2 weeks post - infection (Mattapallil *et al.,* 2005; Centlivre *et al.,* 2007). The affected cells are mostly effector memory T cells (Veazey *et al.,* 2000; Li *et al.,* 2005), which are abundant at these sites. Because HIV depletes these cells within the periphery, gut, and mucosal tissue (Schneider *et al.,* 1995; Veazey *et al.,* 2001), it has been hypothesized that HIV-induced depletion of CD4 cells in granulomas patients with Tuberculosis infection leads to a direct disruption of the containment of *M. tuberculosis* infection (Law *et al.,* 1996; Lawn *et al.,* 2002). It therefore appears that the attack on CD4 cells by HIV infection is much severe in the HIV/TB co-infection than in HIV infection alone as depicted in this study, with co-infection having CD4 of 200.5 cell/mm^3 as compared to HIV patients with 356.0 cell/mm^3.

The peripheral CD4 cell count is a standard measure of disease progression in HIV-infected individuals, and this has been reported by many studies on HIV/TB co-infection (Hertoghe *et al.,* 2000; Breen *et al.,* 2006; Geldmacher *et al.,* 2008; Diedrich *et al.,* 2010). The values observed in this study supported previous studies on the level of CD4 counts in relation to disease progression on HIV/TB co-infection. In the current study, WHO stage I CD4 count was 703.5cell/mm^3; 470cell/mm^3 in stage II; 285 cell/mm^3 in stage III and 135 cell/mm^3 in stage IV respectively. The decline in the CD4 count values with progression of the disease, predisposes the patients to full blown AIDS and subsequent development of opportunistic infections including Tuberculosis for patients in stage IV as their CD4 count is far less than 200 cell/mm^3. It has been documented that HIV positive individuals are more susceptible to TB than HIV negative people regardless of their peripheral CD4 cell counts (Selwyn *et al.,* 1992), although susceptibility increases with decreasing peripheral CD4 cell counts. HIV positive individuals with <200 CD4 cell/mm^3 blood are more susceptible to TB than HIV positive individuals with >500 CD4 cell/mm^3 blood, regardless of antiretroviral therapy (Lawn *et al.,* 2009). Similarly, an acute and transient decrease in peripheral CD4 T cells after SIVmac251 inoculation in monkeys with latent TB significantly correlated with time to development of reactivation TB (Diedrich *et al.,* 2010). The reduction in CD4 T cell counts was not confined to the periphery, as co-infected monkeys with reactivated latent TB within 17 weeks of SIVmac251 inoculation had a trend toward fewer BAL fluid CD4 T cells than monkeys with reactivated latent TB after 26 weeks. A lower frequency of BAL fluid CD4 T cells has also been noted in HIV positive individuals with TB than in HIV negative individuals with TB (Law *et al.,* 1996; Breen *et al.,* 2006; Kalsdorf *et al.,* 2009) and also in HIV positive individuals without TB who live in areas

with high TB incidence rates than in HIV negative individuals in the same community (Kalsdorf *et al.,* 2009).

Even though, only hematological analysis was conducted in this study, histological analysis conducted by other workers have also provided the data on CD4 counts in granulomas in AIDS patients with Tuberculosis (Shen, *et al.,* 1988). This test can also be carried out to monitor HIV disease progression in patient with or without accompanying Tuberculosis infection. Lymph node biopsy specimens from patients with AIDS and tuberculous adenitis had fewer CD4 cells than individuals without AIDS and tuberculous adenitis. In the absence of CD4 cells within the granulomas, CD8 cells were distributed throughout the granuloma without being confined to the periphery, as is normally observed. This suggests that CD4 cells help maintain the architecture and integrity of the granuloma during co-infection. The reduction in CD4 cell counts within granulomas of AIDS patients is not surprising because one characteristic of AIDS patients is having <200 CD4 - T cell/mm^3 blood and likely reflects a long duration of CD4 - T cell depletion. Another study demonstrated that similar numbers of granulomas were observed in HIV positive and HIV negative patients with pleural TB (Heyderman *et al.,* 1998). No difference in the number of bacilli assessed by staining of culture-positive tissues was observed between the two groups. However, individuals with <100 CD4 cell/mm^3 blood were more likely to have acid-fast bacilli within biopsied granulomas than individuals with >100 CD4 cell/mm^3. A threshold CD4 - T cell count may be needed to prevent bacterial growth in co-infected individuals. Taken together, these studies demonstrate that the peripheral decrease in T- cells correlates with a decrease within the granuloma during AIDS. If this correlation also occurs throughout the course of HIV infection, this may also explain why co-infected individuals with fewer CD cells are more prone to developing active TB (Lawn *et al.,* 2009). However,

many co-infected people present with TB well before the development of AIDS, so these granulomas may not represent what is occurring in most co-infected people (Lawn *et al.,* 2009). Significantly fewer CD4 and CD8 cells were observed within lung granulomas of co-infected monkeys than in granulomas from those with active TB alone (Diedrich *et al.,* 2010). The decrease in lung T- cell numbers was independent of peripheral CD4 cell counts, which means that HIV may selectively kill T- cells directly involved in maintaining granulomas (i.e., activated T- cells at the site) prior to loss of peripheral T- cells and signs of AIDS.

Taken together, these studies demonstrate that CD4 (and possibly CD8) T- cells play a very important role in preventing the development of TB in co-infected individuals. However, more studies are needed to confirm that T- cell depletion is occurring within granulomas. Future studies may also provide a correlation of peripheral, airway, and granuloma T- cell counts, which may be used as a biomarker of disease progression.

The CD8 cells are however found to be high with a statistical significant P<0.01value observed in the HIV patients, 621.0 cell/mm^3 followed by, HIV/TB co-infection, 563.0 cell/mm^3, then normal controls, 369.0 cell/mm^3 with least value observed in the Tuberculosis patients, 331.5 cell/mm^3. CD8 cells being cytotoxic in function are believed to be activated following HIV infection in order to carry out cell mediated immune response against the HIV virus. The lower values observed in normal controls and Tuberculosis patients indicate their quiescence state in the later two subgroups. The CD4/CD8 cells ratio was also calculated to access their dual roles in both HIV and Tuberculosis patients with or without co-infection. A statistically significant $p<0.01$ difference was observed, with HIV/TB co-infection having the least value of 0.39,

followed by HIV patients with 0.50, Tuberculosis with 1.92 and normal controls having 1.86.

The comparison of CD4 median values in HIV/TB co-infection was made with regard to WHO clinical staging of HIV disease. A significantly higher value was observed in WHO clinical stage I, 703.5cell/mm^3; followed by stage II, 470.0 cell/mm^3; stage III, 285.0 cell/mm^3 and the least was observed in stage IV, 135.0 cell/mm^3.

Memory CD8 T cells are an important component of protective immunity against viral infections. The CD8 median values were also compared using a similar pattern of WHO CD4 cell classification. The CD8 cells showed an increase in value from stage I to II (564 cell/mm^3 to 720 cell/mm^3), thereafter, the cell counts decrease from stage II down to stage IV (720 cell/mm^3 to 614 cell/mm^3 in stage III and 484 cell/mm^3 in stage IV respectively). The CD8 response observed in this study agrees with documented findings in the literature taht indicated that a CD8 cell response to a viral infection undergoes an expansion phase, culminating in the generation of effector CD8 cells and viral clearance. The expansion phase is followed by a death phase, when 90 to 95% of the effector T cells die. The surviving 5 to 10% of the effector CD8 cell pool further differentiates and generates a memory T-cell population that is maintained for long time in the absence of antigen (John and Rafi, 2004). Memory CD8 cell generation is linear and progressive. Antigenic stimulation causes naïve CD8 cells to proliferate and acquire effector functions. The effector T- cells that survive the death phase further differentiate, giving rise to memory T- cells that continue to differentiate in the absence of antigen and acquire the ability to persist in the absence of antigen via homeostatic turnover (John and Rafi, 2004). In this study, it is observed that CD8 cells remained high above

480cell/mm^3 in all the four Clinical stages of the disease. This may suggest some degree of immunocompetence to the HIV virus in these subjects.

An inverse relationship (rho = -0.144, p >0.05) between the CD4 counts and MDA levels was observed in this study indicating an increase in MDA level as the disease progresses from WHO stage I to IV. The high serum MDA concentrations reported here are probably the consequences of the effects of free radicals on polyunsaturated lipids which induce oxidative stress, deleterious destructive effects such as cell apoptosis, leading to CD4 cell depletion during HIV infection. This agrees with findings of earlier workers in this regard (Georges *et al.,* 2003).

The positive but weak relationship (rho = 0.139, p>0.05) between the duration of treatment and CD4 counts observed in this study signifies an increase in CD4 count as the duration of treatment increases. This agrees with the observation of earlier studies which suggest that access to Highly active antiretroviral therapy (HAART) decreases morbidity and mortality due to HIV infection and AIDS (Walensky *et al.,* 2006), and has made HIV infection a manageable chronic illness. Unfortunately, many patients who are on chronic HAART experience drug toxicities that include metabolic, hepatic (De Clercq, 2009), neurological and cardiovascular complications (Morse and Kovacs 2006). Even though, drugs toxicities were not assessed in this work, it is pertinent to note that, highly active anti-retroviral therapy (HAART) may increase chemically reactive species in circulation, possibly by producing more oxidized metabolites derived from the interaction between ROS and infected-cell biomolecules (Martín *et al.,* 2001; Hulgan *et al.,* 2003). This is supported by several biochemical mechanisms, such as mitochondrial interference, following treatment with HAART-NRTI [nucleoside reverse transcriptase inhibitors] (Hulgan *et al.,* 2003); activation of the P450 hepatic system by HAART, which are protease inhibitors (PI) (Martín *et al.,* 2001). A putative mechanism of these

toxicities includes inhibition of mitochondrial DNA polymerase-γ, with resultant mitochondrial DNA depletion and tissue-specific mitochondrial dysfunction (Brinkman K, 1999; Feng *et al*., 2001). Other metabolic toxicities such as dyslipidemia and insulin resistance appear to be predominately associated with protease inhibitor (PI) exposure, likely through effects on lipid metabolism and cellular glucose uptake, respectively (Feng *et al*., 2001; Morse and Kovacs 2006).

Although specific mechanisms of HAART-associated toxicities are largely unknown, these processes may be mediated at a fundamental level by free radical-induced cellular damage, also referred to as oxidant stress (Day and Lewis 2004). It is therefore possible that the high MDA values recorded in HIV, TB and the HIV/TB co-infection was exacerbated by the drug treatment in addition to the respiratory burst that produce the free radicals in the process of attacking the microorganisms. This should be born in mind by physicians attending to HIV patients and effort to include antioxidants supplements may help to reduce the secondary effect of HAART on these patients. The inverse relationship (rho = -0.003, $p>0.985$) between duration of treatment and MDA level, showing a decline in MDA level as the duration of treatment increases points to effectiveness of the therapy.

The positive but weak relationship (rho = 0.208; $p>0.05$) between duration of treatment and Vitamin E level indicates an increase in the antioxidant level as the duration of treatment increases. In this study, no any antioxidant therapy was given to the cohorts; the Vitamin E assayed is the one present in the staple foods of the patients. The low levels recorded in the three subgroups compared to the normal controls may suggest that it was used up by the oxidants generated during the disease processes. Whether addition of oral Vitamin E in addition to the one present in staple will reduce oxidative damage in the body or not should be addressed by future studies. Clearly antioxidants are

recognized as potential biomolecules that can influence disease process if ROS caused or significantly contributed to the progression of the disease (Edeas, 2009; Edeas *et al.,* 2010; Gil *et al*., 2005). Presently, there are conflicting reports on the role of antioxidant therapy in many disease conditions including the aging process. Some studies have shown that antioxidant therapy have no effect and can even increase mortality (Heart Protection Study Collaborative Group 2002; Miller *et al.,* 2005). Proponents of the theory claim that, this phenomenon can be explained by hormesis: The addition of antioxidants can lead to a decrease of normal biological response to free radicals and therefore lead to a more sensitive environment to oxidation. Evidences support the view that increased ROS formation is usually a consequence of tissue damage by a disease or toxin and sometimes it contributes significantly, more often it does not and, in some cases ROS may be exerting a protective role, e.g. by down-regulating inflammation (Gil *et al.,* 2013). Under such circumstances, an administered antioxidant reaching the site of increased ROS formation would at best marginally affect secondary tissue damage and could even be deleterious (Gil *et al.,* 2013). Sometimes pro-oxidants might be more beneficial as suggested (Ma, 2010 and (Milazzo *et al.,* 2010). Indeed, flavonoids may act just as much as pro-oxidants in the gastrointestinal tract and in cell culture systems as they do as antioxidants (Gil *et al.,* 2013).

Furthermore, a recent study tracking the eating habits of 478,000 Europeans suggests that consuming lots of fruits and vegetables has little if any effect on preventing cancer (Boffetta *et al.,* 2010). While vitamin E supplementation was initially hoped to have a positive effect on health, research has not supported this hope (Haber, 2006). Vitamin E does not decrease mortality in adults, even at large doses (Abner, 2011), and high-dosage supplementation may slightly increase it (Miller *et al.,* 2005; Bjelakovic *et al.,* 2008). It does not improve blood sugar control in an unselected group of people with

diabetes mellitus (Abner, 2011) or decrease the risk of stroke (Bin, 2011). Studies on its role in age-related macular degeneration are ongoing as, though it is of a combination of dietary antioxidants used to treat the condition, it may increase the risk (Olson, 2011). The concept of hormesis believes that oxidative stress may promote life expectancy of lower animals like *Caenorhabditis elegans* by inducing a secondary response to initially increased levels of reactive oxygen species (Schulz *et al.*, 2007). This observation was purely on a hypothetical basis (Tapia PC (2006/2005(epub)). In mammals, the question of the net effect of reactive oxygen species on aging is even less clear (Sohal *et al.*, 2002). A 2012 Cochrane Review examined the potential effectiveness of antioxidant vitamin supplementation in preventing and slowing the progression of age-related cataract. The included studies involved supplementation of vitamin E, along with β-carotene and vitamin C, either dosed independently or in combination, and compared to the placebo. The systematic review showed that vitamin E supplementation had no protective effect on reducing the risk of cataract, cataract extraction, progression of cataract, and slowing the loss of visual acuity (Mathew, 2012). Epidemiological findings (Bjelakovic *et al.*, 2007) support the process of mitohormesis in humans, and even suggest that the intake of exogenous antioxidants may increase disease prevalence in humans, according to the theory, because they prevent the stimulation of the organism's natural response to the oxidant compounds which not only neutralizes them but provides other benefits as well. Therefore, future studies should explore the process of mitohormesis in human subjects to help improve the management of chronic diseases associated with oxidative stress.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary

Both HIV and Tuberculosis infections are associated with lipid peroxidation and antioxidant imbalance. Pathology of the two produced free radicals which lead to lipid peroxidation indices, one of which is Malondialdehyde (MDA) and to disease progression due to CD4 cells apoptosis. The current study was carried out with the objective of determining Serum MDA, Vitamin E, CD4, CD8 and lipid peroxidation index (LPI) in four different subgroups comprising of HIV, Tuberculosis and HIV/TB co-infected patients and the Controls with a view to assessing the interaction between oxidants and antioxidants in the course of HIV/TB co-infection. Lipoperoxides, as Malondialdehyde by thiobarbituric acid reaction and Vitamin E in serum from all the four subgroups were measured spectrophotometrically. Lipid Peroxidation Index (LPI) was determined by obtaining the ratio of MDA and Vitamin E in these cohorts, while CD4 and CD8 cells count were obtained by flowcytometry. The higher MDA result from the subjects supports the assertion that HIV/TB co-infection is associated with increased lipid peroxidation and lower antioxidant potential.

Contribution to knowledge in this study includes the following:

(i) Findings from this study support a link between oxidative stress, HIV and tuberculosis infections.

(ii) It also shows that both Tuberculosis and HIV/TB co –infection infections are associated with lower BMI values:

   a. Normal controls = 25.85 kg/m^2

b. HIV = 24.68 kg/m^2

c. TB = 19.35 kg/m^2

d. Co – infection = 19.88 kg/m^2

(iii) The CD4 count was found to be lowest in the HIV/TB co – infection subgroup:

a. Normal controls = 743.0 cell/mm^3

b. HIV = 356.0 cell/mm^3

c. TB = 571.0 cell/mm^3

d. Co – infection = 200.5 cell/mm^3

(iv) The CD8 count among the four subgroups were:

a. Normal controls = 369.0 cell/mm^3

b. HIV = 621.0 cell/mm^3

c. TB = 331.5 cell/mm^3

d. Co – infection = 563.0 cell/mm^3

(v) The CD8 count was however found to remain high at all stages of the HIV/TB co – infection disease:

a. Stage I = 564 cell/mm^3

b. Stage II = 720 cell/mm^3

c. Stage III = 614 cell/mm^3

d. Stage IV = 484 cell/mm^3

(vi) Reference values for MDA in Kano State for both Subjects and Controls have been established as follows:

- Normal Controls = 1.062 and 3.29 μmol/L, lower and upper limits respectively

- HIV =1.99 and 10.16 μmol/L, lower and upper limits respectively
- TB=1.55 and 7.26 μmol/L, lower and upper limits respectively
- HIV/TB Co – infection = 4.22 and 4.57 μmol/L, lower and upper limits respectively

(vii) Lipid peroxidation indices between HIV/TB co-infection and normal controls have also been established as follows:

- Controls = 0.03
- HIV = 0.58 (19.3 times that of normal controls)
- TB = 0.45 (15 times that of normal controls)
- HIV/TB Co-infection = 0.99 (33 times that of normal controls)

6.2 Conclusion

The higher MDA result from the subjects supports the assertion that HIV/TB co-infection is associated with increased lipid peroxidation and lower antioxidant potential. It is therefore concluded that co-infection with Tuberculosis exacerbates oxidative stress in HIV patients, while treatment enhances their anti-oxidant status and ameliorates the oxidative stress.

6.3 Recommendation

(i) The use of the ration of CD4/CD8 should be considered as one of the prognostic criteria in assessing the progression of HIV/TB co – infection. The higher the ratio, the better the prognosis.

(ii) It is recommended that routine tests to ascertain level of antioxidants should form part of the protocols for the management of HIV/TB co – infection.

(iii) Malondialdehyde and CD4 tests should always be compared in the assessment of immune response in relation to oxidative stress in patients with HIV/TB co –infection

(iv) It is further recommended that Lipid peroxidation Index (LIP) be calculated with the help of a formular developed in this study as LPI = MDA/Vit E, where MDA = Malondialdehyde, Vit E = Vitamin E to assess oxidative stress level in a Laboratory setting.

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