BIOCHEMICAL MARKERS OF OXIDATIVE STRESS AS INDICES OF HIV/AIDS PROGRESSION

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by

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JUNE , 2009

DECLARATION

I thereby declare that this submission is my own work towards the PhD and that, to the best of my knowledge, it contains no material previously published by another person nor material which has been accepted for award of any other degree of the University, except where due acknowledgement has been made in the text.

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ABBREVIATIONS

+LR	-	Positive Likelihood Ratio
+PV	-	Positive Predictive Value
$^1O_2$	–	Singlet oxygen
3,5-DHBS- 3,5	-	dichloro-2-hydroxybenzene
4-AAP	-	4-aminoantipyrine
AIDS	-	Acquired Immune Deficiency Syndrome
ANOVA	-	Analysis of Variance
ART	-	Antiretroviral Therapy
AUC	-	Area under the Curve
AZT	-	3′-azido-3′-deoxythymidine
B2-M	-	Beta 2-Microglobulin
BD	-	Becton Dickinson
BRH	-	Bolgatanga Regional Hospital
CAT	-	Catalase
CD4	-	Cluster of Differentiation
CDC	-	Centre for Disease Control
CE	-	Cholesterol Esterase
cGMP	-	Cyclic Guanosine Monophosphate
CHRPE	-	Committee on Human Research, Publication and Ethics
CI	-	Confidence Interval
Cl^-	-	Chloride
CO	-	Cholesterol Oxidase
CRH	-	Central Regional Hospital
CSW	-	Commercial Sex Workers

DIYTC	-	Diethyldithiocarbamate
DNA	-	Deoxyribonucleic Acid
DNPH	-	Dinitrophenylhydrazine
ECF	-	Extracellular Fluid
EIA	-	Enzyme Immunosorbent Assay
ELISA	-	Enzyme Linked Immunosorbent Assay
FDCs	-	Follicular Dendritic Cells
FIV	-	Feline Immunodeficiency Virus
FRAP	-	Ferric Reducing Ability of Plasma
G3P	-	Glycerol-3-Phosphate
GALT	-	Gut-associated Lymphoid Tissue
GHS	-	Glutathione
Gp	-	Glycoprotein
GPx	-	Glutathione Peroxidase
GSSH	-	Glutathione
H_2O_2	–	Hydrogen Peroxide
HAART	-	Highly Active Antiretroviral Therapy
Hb	-	Haemoglobin
HCT	-	Haematocrit
HDL	-	High Density Lipoprotein
HIV	-	Human Immunodeficiency Virus
IFN-α	-	Interferon-alpha
IL	-	Interleukin
IL-1	-	Interleukin-1
ISE	-	Ion Selective Electrode
K^+	-	Potassium
KNUST	-	Kwame Nkrumah University of Science and

		Technology
LDL	-	Low Density Lipoprotein
LED	-	Light Emitting Diode
-LR	-	Negative Likelihood Ratio
LTR	-	Long Terminal Repeat
MCH	-	Mean Cell Haemoglobin
MCHC	-	Mean Cell Haemoglobin Concentration
MCV	-	Mean Cell Volume
MDA	-	Malondialdehyde
Na^+	-	Sodium
NAC	-	N-acetyl cysteine
NaCl	-	Sodium Chloride
NACP	-	National AIDS Control Programme
NAD	-	Nicotinamide Adenine Diphosphate
Nef	-	Negative factor
NFkB	-	Nuclear Factor kappa B
NK	-	Natural Killer
NO	-	Nitric Oxide
NSI	-	Non- Syncytia-forming Variant
O_3^-	-	Ozone
OH^-	-	Hydroxyl Radical
p	-	Level of Significance
PGE2	-	Prostaglandin E2
PLT	-	Platelets
PLWHAs	-	People Living with HIV/AIDS
POD	-	Peroxidase
PUFA	-	Polyunsaturated Fatty Acid

-PV	-	Negative Predictive Value
R	-	Coefficient of Variation
RBC	-	Red Blood Cell
Rev	-	Regulator of Expression of Viral Proteins
RNA	-	Ribonucleic Acid
RNS	-	Reactive Nitrogen Species
ROC	-	Receiver Operator Characteristic
ROS	-	Reactive Oxygen Species
SIV	-	Simian immunodeficiency virus
SOD	-	Superoxide dismutase
STD	-	Sexually transmitted disease
TBA	-	Thiobarbituric Acid
TCA	-	Trichloroacetic Acid
TH1	-	T helper 1
TH2	-	T helper 2
USAID	-	United States International Development Agency
XOD	-	Xanthine Oxidase

ABSTRACT

Reactive Oxygen Species (ROS) has been implicated in the pathogenesis and the progression of HIV infection. This study was aimed at investigating the levels of oxidative stress and their probable relationship as markers of HIV disease progression in HIV positive patients in two established HIV/ART centres in Ghana. In all two hundred and twenty eight (228) confirmed People Living with HIV/AIDS (PLWHAs) were included in the study. The subjects were recruited from the Central Regional Hospital (CRH) and Bolgatanga Regional Hospital (BRH). One hundred and forty-three (143; 62.72%) PLWHAs were recruited from CRH and they comprised of eighty one (81; 56.64%) males and sixty two (62; 43.36%) females whilst eighty five (85; 37.28%) PLWHAs were recruited from BRH comprising of forty three (43; 50.59%) males and forty two (42; 49.41%) females. These two hospitals were chosen to give a fair representation of PLWHAs in Ghana. Another 100 sex- and age-matched healthy, HIV-seronegative individuals were studied in parallel as controls. Ethical clearance was obtained from Committee on Human Research, Publications and Ethics (CHRPE), School of Medical Sciences, Kwame Nkrumah University of Science & Technology (KNUST), Kumasi. All subjects gave informed consent to take part in the study after verbal and written explanation of the methods and risks involved had been given. Venous blood samples were taken and assayed for the haematological parameters (haemoglobin (Hb), haematocrit (HCT) and total white blood cell count (WBC), biochemical assays (total Serum Protein, Serum Albumin, Triglycerides, Total Cholesterol, HDL-cholesterol and LDL-cholesterol and markers of oxidative stress (Serum Malondialdehyde (MDA), Ferric Reducing Ability of Plasma (FRAP) and serum Vitamin C and E, Superoxide Dismutase (SOD) Glutathione Peroxidase (GPx). The subjects with the mean age of 36.9±10.9 years which was not significantly different from that of the control group of 39.4±13.4 years. The PLWHAs subjects were grouped per the criteria of the Center for Disease Control (CDC) as: CD4 count (1) ≥ 500 mm^{-3}; (2) 200 – 499 mm^{-3}; and (3) < 200 mm^{-3}. Forty three (43; 18.86%) patients (38.7±10.94 years) had a $CD4^+$ count ≥ 500 mm^{-3}, sixty three (63; 27.63%) patients (37.4±9.54 years) had a $CD4^+$ count between 200 – 499 mm^{-3} and one hundred and twenty two (122; 53.51%) patients (36.7±10.93 years) had a $CD4^+$ count < 200 mm^{-3}. Markers of oxidative stress revealed significant differences between the patients and control subjects. Malondialdehyde ($p<0.001$) in the patients was markedly increased as compared to the control group. This suggests increased lipid peroxidation in HIV infected individuals and this increased with the progression of the infection. Ferric reducing ability of Plasma ($p<0.0001$), Glutathione peroxidase ($p<0.0001$), Superoxide Dismutase ($p<0.0001$) were decreased in the patients compared to the control group indicating an increase in free radicals product. Vitamin C ($p<0.0001$) and E ($p<0.0001$) were reduced in the patients compared to the control group suggesting a decrease in the antioxidant level as the HIV infection progressed in the patients. Results from the haematological assay revealed a significant decrease in the mean blood haemoglobin levels of the HIV positive patients compared to the control subjects. The significant positive

correlation (p<0.0001) between Hb and CD4 count highlights its usefulness in the progression of HIV infection. The haematocrit result pattern showed a consistency with the Hb. There was a significant positive correlation between HCT and CD4 counts (p<0.0001). No significant difference was observed in the total white blood cell count (WBC) of HIV positive patients and the control group (p=0.1830). Apart from serum total Protein (p<0.0001) and Triglycerides (p<0.001), which showed significant increase as compared to the control group, serum albumin (p=0.0106), Total cholesterol (p<0.0001), HDL-cholesterol (p<0.0001) and LDL cholesterol (p<0.0001) showed a significant decrease as compared to the control group. From the correlation analysis, serum Albumin(R=0.41), Total cholesterol (R=0.67), HDL-cholesterol (R=0.27) and LDL cholesterol (R=0.27) showed a positive significant correlation in relation to the CD4 count. However serum total Protein (R=-0.36), Potassium (R=-0.67) and Triglycerides (R=-0.27) did show a negative correlation in relation to the CD4 count. Altogether the findings of this study have revealed that oxidative stress increases as HIV infection progressed. We propose from the study that the interaction of the mechanism underlying oxidative stress and HIV progression and subsequent apoptosis is a receptor-mediated process. During the early phase of HIV infection, generation of ROS has been known to activate HIV replication in vivo through the activation of a factor that binds to a DNA-binding protein, NF-kappa B which is a known activator of HIV replication. This leads to increase in the disturbance in the antioxidant system leading to an increase in ROS production with concomitant biochemical and haematological derangement as observed in the study. Therefore an early intervention with antioxidant supplements in the early phase of HIV infection is likely to reduce HIV progression and improve the immune function.

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1.1 GENERAL INTRODUCTION

1.1.1 History of the Human Immunodeficiency Virus (HIV)

The human immunodeficiency virus (HIV) was unknown until the early 1980's but since that time has infected millions of persons in a worldwide pandemic. The result of HIV infection is relentless destruction of the immune system leading to onset of the acquired immunodeficiency syndrome (AIDS). The AIDS epidemic has already resulted in the deaths of over half its victims. All HIV-infected persons are at risk for illness and death from opportunistic infections and neoplastic complications as a consequence of the inevitable manifestations of AIDS (Moss and Bacchetti, 1989).

There have been many theories as to the origin of HIV. Robert Gallo (1984) speculated that HIV crossed species to humans from an African green monkey. Although this idea has since been discredited, it is still widely believed that HIV came into the human population from one or more non-human primate species. HIV-2, for instance, has an almost exact counterpart in a virus of the sooty mangabey, a type of African monkey (Bosch *et al.*, 1989).

1.1.2 Phases of Evolution of HIV

Despite considerable evidence to the contrary, a small, but well-known group of scientists argue that HIV does not cause AIDS. They contend that AIDS is brought about by lifestyle factors such as drug use, inadequate nutrition, and contraction of multiple STDs. The AIDS pandemic has evolved over time, with four main phases of evolution. In the initial phase, HIV emerged from endemic rural areas to spread among urban populations at an accelerating rate. In the second phase dissemination occurred and involved definable risk groups. Behaviors in these risk

groups, including sexual promiscuity and injection drug use, led to the third phase of escalation which occurred through the 1980's. A fourth phase of stabilization has occurred in some regions such as Western Europe, North America, and Australia, where control measures appear to be having a positive effect. However, some regions such as Central Africa and Asia continued to experience escalation of the pandemic through the 1990's (Quinn, 1996).

1.1.3 Consequences of the HIV Epidemic

The scope of the AIDS pandemic has already led to serious consequences, not only for health care systems of countries unable to cope with many AIDS victims, but also for the national economies of those countries because of the loss of young to middle aged who are economically most productive. Worldwide, about half the victims of AIDS are women and a consequence of this is perinatal infection resulting in a significant number of children born with HIV infection (Quinn, 1996).

Costs for detection, diagnosis and management are considerable when effective therapies for persons with complications of HIV infection are instituted to prolong survival. Though the pharmacologic therapies exist for prolonging the lives of persons infected with HIV, such therapies are expensive and out-of-reach for most persons worldwide. The years of useful life lost by the predominantly younger population infected by HIV also has a serious economic impact (Whiteside, 2001).

A proper understanding of AIDS issues, including the nature of HIV and its means of spread, should precede decisions regarding allocation of health care resources and control measures (Gottlieb, 2001).

Prevention strategies for HIV will require ongoing education, despite a general public perception, particularly among young persons, that AIDS is a peripheral threat that does not call for changes in lifestyle. The battle against AIDS will

require political alliances that allow prevention strategies to be implemented across national borders. The reservoir of infected persons is so large, global human interaction so broad, and costs of AIDS so high that everyone on earth is affected in some way by the AIDS pandemic (Lert, 2000).

1.2 HIV/AIDS STATISTICS

1.2.1 HIV/AIDS Worldwide

The estimated number of persons living with HIV/AIDS worldwide in 2007 was 33.2 million [30.6-36.1 million], a reduction of 16% compared with the estimate published in 2006 (39.5 million [34.7-47.1 million]). The estimated number of deaths due to AIDS in 2007 was 2.1 million [1.9-2.4 million] worldwide, of which 76% occurred in sub-Saharan Africa. Declines in the past two years are partly attributable to the scaling up of antiretroviral treatment services. Sub-Saharan Africa remains the most affected region in the global AIDS epidemic. More than two thirds (68%) of all people who are HIV-positive, live in this region where more than three quarters (76%) of all AIDS deaths in 2007 occurred. It is estimated that 1.7 million [1.4 million-2.4 million] people were newly infected with HIV in 2007, bringing to 22.5 million [20.9 million-24.3 million] the total number of people living with the virus. Unlike other regions, the majority of people living with HIV in sub-Saharan Africa (61%) are women. Global HIV incidence likely peaked in the late 1990s at over 3 million new infections per year, and was estimated to be 2.5 million [1.8–4.1 million] new infections in 2007 of which over two thirds (68%) occurred in sub-Saharan Africa. This reduction in HIV incidence likely reflects natural trends in the epidemic as well as the positive impact of prevention programmes which predictably resulted in behavioural change in different contexts (USAIDS, 2007).

Table 1.1 Global summary of the AIDS epidemic December 2007

Number of People living with HIV in 2007	
Total	33.2 million [30.6 – 36.1 million]
Adults	30.8 million [28.2 – 33.6 million]
Women	15.4 million [13.9 – 16.6 million]
Children under 15 years	2.5 million [2.2 – 2.6 million]
People newly infected with HIV in 2007	
Total	2.5 million [1.8 – 4.1 million]
Adults	2.1 million [1.4 – 3.6 million]
Children under 15 years	420 000 [350 000 – 540 000]
AIDS deaths in 2007	
Total	2.1 million [1.9 – 2.4 million]
Adults	1.7 million [1.6 – 2.1 million]
Children under 15 years	330 000 [310 000 – 380 000]

The ranges around the estimates in this table define the boundaries within which the actual numbers lie, based on the best available information. Source: USAID, 2007.

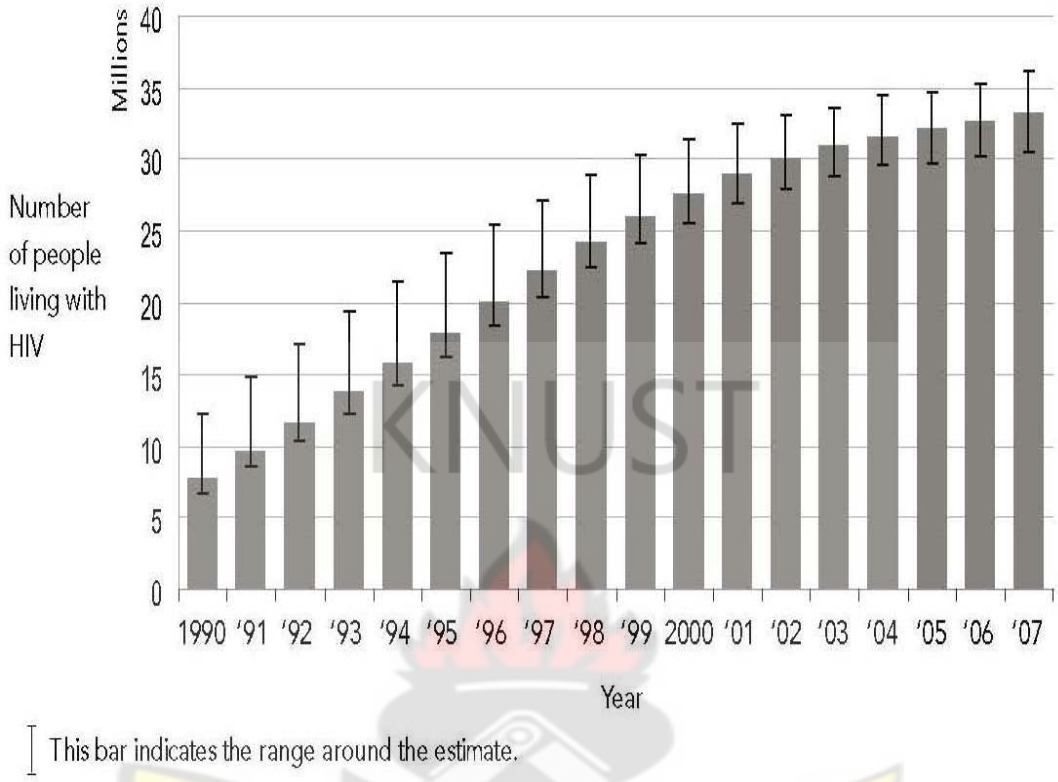


Figure 1.1 Estimated number of people living with HIV globally, 1990–2007

Source: USAIDS, 2007.

1.2.2 HIVAIDS infection in Ghana

The first reported cases of 42 Human Immune-Deficiency Virus / Acquired Immune Deficiency Syndrome (HIV/AIDS) in Ghana were recorded in 1986, mainly among women who had travelled outside the country. By the end of December 1999, a cumulative total of 37,298 cases had been recorded. Nearly 90% of the cumulative AIDS cases from 1986-1999 were between 15-49 years of age, with 63% of all reported HIV/AIDS cases being females. The female-to-male HIV/AIDS infection ratio is, however, gradually attaining parity, changing from 6:1 in 1987 to approximately 2:1 in 1998. The peak ages for infection are 25-29 years for females and 30-34 years for males (HIV Sentinel Survey Report, 2007; NACP, 2007).

The national prevalence rate of HIV has risen from 2.6% in 1994 to 4.6% according to the 1998 sentinel surveillance report. HIV Sero-prevalence among Sexually-Transmitted Disease (STD) patients and blood donors is recorded to be 17% and 4%, respectively. Among Commercial Sex Workers (CSW) in Accra and Kumasi, it has been found that 75.8% and 82%, respectively, are HIV positive. The National AIDS Control Programme (NACP) projected the average national prevalence rate to increase to 6.4% by 2004, 8.2% by 2009 and 9.5% by the year 2014 if the trend at that time continued. Heterosexual transmission of HIV accounts for 75-80% of all HIV/AIDS infection. Vertical transmission (from mother to child) accounts for 15% while transmission through blood and blood products accounts for 5%. HIV I and II are both present. The former is the most commonly found, with the subtypes A and D identified as the most predominant The prevalence rate in Ghana dropped from 2.22 per cent in 2006 to 1.9 per cent in 2007. According to the 2007 HIV Sentinel Survey and National HIV Prevalence, AIDS Estimates Reports released, an estimated 264,481 people are living with HIV/AIDS. Out of this number, 110,666 are males and 153, 815 are females (NACP, 2007).

1.2.3 Regional Distribution of HIV/AIDS in Ghana

The distribution of HIV/AIDS in Ghana is higher in densely populated areas. Higher numbers of cases occur in the Southern regions of the country particularly in densely populated regional capitals like Kumasi, Koforidua and Accra. Prevalence of HIV/AIDS is also very high in mining towns like Obuasi and Tarkwa as well as in border towns(NACP, 2007).

1.2.4 The Socio-Economic Factor

Even though poverty levels have been estimated to have dropped from 36% in 1987/88 to 29.4% in 1998/99, there are regional as well as rural-urban variations. Poverty and other economic pressures on individuals in the country constitute major factors in the spread of HIV/AIDS (NACP, 2007).

1.2.5 Causes of high rate of HIV infection in Ghana

Ghana has a diverse ethnic and cultural composition that is reflected by different cultural practices and sometimes by different political orientations. A common feature of the traditional system is the strong communal and family support system such as the extended family system. In recent times, particularly with urbanisation and the consequent rural-urban migration, these systems are breaking down resulting in the development of nuclear families. The price being paid for this is the inadequate social and family support for PLWHA and people affected by HIV/AIDS. Other socio-cultural factors such as stigma and denial make the care and support for PLWHA and those affected by HIV/AIDS a daunting challenge. Polygamy, sexual attitudes and belief systems which underlie gender inequalities, make it difficult for women to negotiate on issues about sex, reproduction and condom use(NACP, 2007).

1.3 CLASSIFICATION OF HUMAN IMMUNODEFICIENCY VIRUS (HIV)

Human immunodeficiency virus (HIV) and its subtypes are retroviruses, and they are the etiologic agents of AIDS. Human retroviruses were unknown until the 1980's, though animal retroviruses such as feline leukemia virus had been detected previously. HIV belongs to a large family of ribonucleic acid (RNA) lentiviruses that are characterized by association with diseases of immunosuppression or central nervous system involvement and with long incubation periods following infection before manifestations of illness become apparent (Fauci, 1993; Levy, 1993).

1.3.1 Family Retroviridae

HIV is a special type of retrovirus containing RNA. There are three families of retroviruses: oncoviruses (causing cancer), lentiviruses (slow viruses, of which HIV is one), and foamy viruses or spumaviruses (about which much less is known). There are also retroviral infections of animals, e.g., SIV (simian immunodeficiency virus) infects nonhuman primates, FIV (feline immunodeficiency virus) affects cats, and visna virus infects sheep (UNAIDS Report on the global HIV/AIDS epidemic June 2000).

1.3.2 Types of HIV

There are two main forms of HIV: HIV-1 and HIV-2. HIV-1 was discovered by Luc Montagnier and his associates at the Institute Pasteur in Paris in 1983. HIV-2 was first identified among patients in Cameroon in 1985. HIV-2 is more similar to SIV (Simian Immunodeficiency Virus) than is HIV-1 and it is much less virulent (UNAIDS Report on the global HIV/AIDS epidemic June 2000).

1.3.2.1 HIV 1

Molecular epidemiologic data suggest that HIV type 1, the most common subtype of HIV that infects humans, has been derived from the simian immunodeficiency virus, called SIVcpz, of the Pan troglodytes troglodytes subspecies of chimpanzee. The lentivirus strain SIVcpz is highly homologous with HIV-1, and another form of simian immunodeficiency virus found in sooty mangabeys (SIVsm) has similarities as well. There is molecular epidemiologic evidence for at least seven cross-species transmissions of SIV to humans occurring in the first half of the 20th century, probably through exposures to primate blood (Hahn *et al.*, 2000; Korber *et al.*, 2000).

Table 1.2 Distribution of Subgroups of HIV 1

Group	
• Subtype A	West and Central Africa
• Subtype B	South America (including Brazil and Argentina), United States, Europe, Thailand, Russia
• Subtype C	India, Sudan, Southern and Eastern Africa
• Subtype D	East and Central Africa
• Subtype E	Thailand, Philippines, China, Central Africa
• Subtype F	Brazil, Argentina, Eastern Europe, Central Africa
• Subtype G	Western and Eastern Africa, Central Europe
• Subtype H	Central Africa
• Subtype J	Central America
• Subtype K	Democratic Republic of Congo, Cameroon
• **N**	Cameroon
• **O**	West Africa

1.3.2.2 HIV-2

The numerous strains of HIV-1 isolated from various geographic regions of the world are all immunologically similar and differ only slightly in their DNA sequences. A second retrovirus designated HIV-2 has been isolated from a number of patients with AIDS, first in West African countries and subsequently in Western Europe, the United States, and elsewhere. Most cases have appeared in West Africa and have appeared only sporadically in other parts of the world (De Cock *et al.*, 1993). HIV-2 is believed to have been present in Africa as early as the 1960's. (Miyazaki, 1995). HIV-2, which has greater homology to simian immunodeficiency virus (SIV) than to HIV-1, appears to have become established in human populations as a zoonotic infection from the primate reservoir of sooty mangabeys *(Cercocebus atys*) (Hahn *et al.*, 2000).

1.3.2.3 Subtypes of HIV 2

The subtypes of HIV-2 have been designated from A through F. There is up to a 25% difference in genetic homology among these subtypes. All six subtypes can be detected by enzyme immunoassay (EIA) and Western blot assays for HIV-2 similar to those for HIV-1. Infection with HIV-2 eventually leads to AIDS. Persons can be co-infected with HIV-1 and HIV-2 (De Cock *et al.*, 1993; Gao *et al.*, 1999).

1.3.3 Mode of Transmission of HIV 2

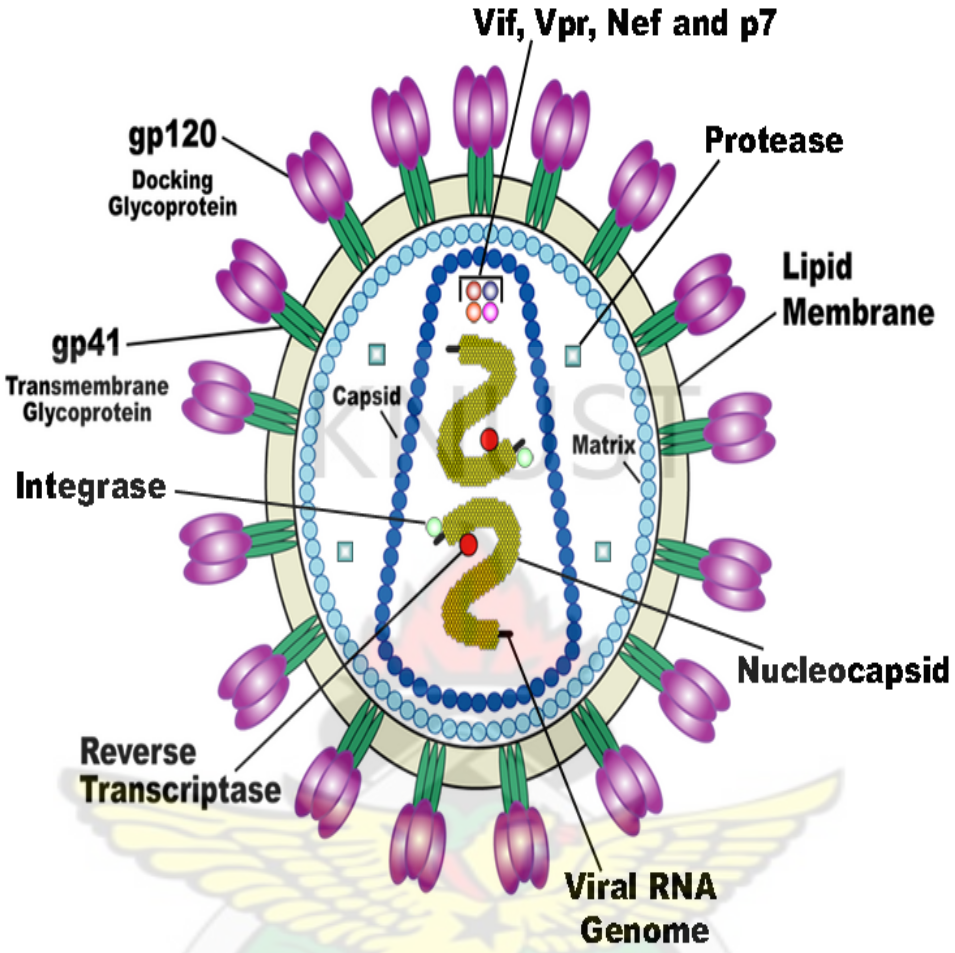
HIV-2 is spread in a manner similar to HIV-1, though the high-risk groups are commercial sex workers and persons with other sexually transmitted diseases. The peak age of persons infected with HIV-2 appears to be higher than that of HIV-1, but there appears to be no sex difference in rates of infection (Miyazaki, 1995).

HIV-2 appears to utilize the same cellular mechanisms for infection as HIV-1, including the use of CD4 receptors and chemokine coreceptors (Murdoch and Finn, 2000).

1.4 THE STRUCTURE OF HIV

It is roughly circular in shape, although the pliability of the viral envelope may change the spherical shape to oval or even to a somewhat irregular outline (Schoub, 2000).

The mature virus consists of a bar-shaped electron dense core containing the viral genome: two short strands of ribonucleic acid (RNA) about 9200 nucleotide bases long--along with the enzymes reverse transcriptase, protease, ribonuclease, and integrase, all encased in an outer lipid envelope with 72 surface projections containing an antigen, gp120, that aids in the binding of the virus to the target cells with CD4 receptors. By electron microscopy, the plasma membrane of an infected CD4+ lymphocyte exhibits budding virus particles approximately 90 to 100 nanometers in diameter (Greene, 1993; Ferguson *et al.*, 2002).



Source: US National Institute of Health (2005)

Figure 1.2 A diagrammatic representation of HIV

1.4.1 Retroviral Genome of HIV

1.4.1.1 Structural Genes

HIV is composed of three genes, which code for the inner core proteins, the envelope proteins and the virus enzyme. These genes are named respectively, the gag gene (group antigen, as the antigenicity of this protein is the same throughout this group of viruses), the env gene (envelope), and thirdly the pol gene (polymerase, referring to the reverse transcriptase which functions as a polymerase (Schoub, 2000).

The gag gene codes for the production of the dense cylindrical core protein (p24, a nucleoid shell protein with a molecular weight of 24,000; and several internal proteins, p7, p15, p17 and p55). The gag protein has the ability to direct the formation of virus-like particles when all other major genes (pol and env) are absent. It is only when the gag gene is non-functional that the retroviruses (HIV) lose their capacity to bud out of the host cell (Wills and Craven, 1991).

The pol gene codes for protease (p10), the virus-associated polymerase that is active in two forms, p51 or p66, and the endonuclease (intergrase, p31) enzymes. The polymerase is made up of two subunits, alpha and beta. The alpha subunit has three functions: (1) to make DNA from RNA; (2) to digest the RNA from RNA: DNA hybrids and (3) to make DNA from DNA. The beta subunit is non-enzymatic (Dimmock and Primrose, 1983).

The env gene codes for two major envelope glycoprotein (gp120, located on external spikes of HIV and gp41, the transmembrane protein that attaches gp120 to the surface of HIV) that become embedded throughout the host membrane, which ultimately becomes the envelope that surrounds the virus as it 'buds' out (Stine, 1993).

1.4.1.2 Regulatory Genes

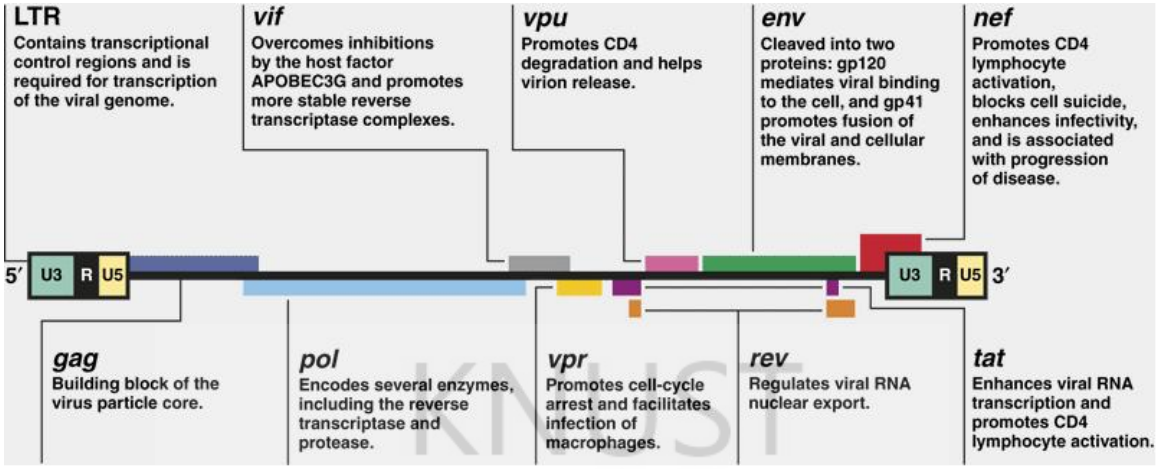
The regulatory genes can be divided into three groups- positive regulatory genes, negative regulatory genes and regulatory genes whose precise functions still need to be determined.

1.4.1.3 Positive Regulatory Genes

There are four positive regulatory genes that produce regulatory proteins which promote the expression of other genes and which positively regulate the formation of viral particles by promoting the production of proteins and the assembly of the components of the virus. The first gene is *tat* gene (transactivator of transcription) which occurs as two separate segments of nucleic acid and produces a protein which attaches itself to a specific site in the long-terminal repeat (LTR) and thereby stimulates the expression of the three structural genes by promoting the transcription of the messenger RNA for the manufacturing of their proteins. The second regulatory gene is the *rev* gene (regulator of expression of viral proteins), which functions as a promoter of the export of viral RNA from the nucleus of the cell. The third gene, *vif* (virus infectivity factor) influences the infectivity of the virus particles and also the release of infectious virus from cells. The fourth gene, vpu (viral protein u) is known to enhance the production of virus particles by promoting release of infectious virus from cells (Schoub, 2000) .

1.4.1.4 Negative Regulatory Genes

The negative regulator gene, *nef*, (*ne*gative *f*actor), produces proteins which act on a section of the long terminal repeat (LTR) called NRE (negative regulatory element) which in turn sends a message down regulating viral replication by inhibiting the production of structural proteins. The virus for efficient replication needs it. Two further regulatory genes, *vpr* (*v*iral *p*rotein *r*) and *vpt,* (*v*iral *p*rotein *t*) have been described although their function still needs to be elucidated (Schoub, 2000).



Source: www.gladstone.ucsf.edu

Figure 1.3 The viral genome for HIV-1

1.4.2 Replication of HIV

Retroviruses are unable to replicate outside of living host cells and do not contain deoxyribonucleic acid (DNA). The pathogenesis of HIV infection is a function of the virus life cycle, host cellular environment, and quantity of viruses in the infected individual. After entering the body, the viral particle is attracted to a cell with the appropriate CD4 receptor molecules where it attaches by fusion to a susceptible cell membrane or by endocytosis and then enters the cell. The probability of infection is a function of both the number of infective HIV virions in the body fluid which contacts the host as well as the number of cells available at the site of contact that have appropriate CD4 receptors (Ferguson *et al.*, 2002).

1.4.3 Route of Entry of HIV into the Body

HIV infection can occur through oropharyngeal, cervical, vaginal and gastrointestinal mucosal surfaces, even in the absence of mucosal disruption. Routes of HIV entry into mucosal lamina propria include M cells, dendritic cells, and epithelial cells. Dendritic cells can bind to gp120 through a C type lectin, suggesting that dendritic cells that squeeze between "tight" epithelium may

capture HIV-1 and deliver it to underlying T cells, resulting in dissemination to lymphoid organs. HIV can cross a tight epithelial barrier by transcytosis during contact between HIV-infected cells and the apical surface of an epithelial cell (Belyakov and Berzofsky, 2004).

1.4.4 HIV Attachment and Penetration

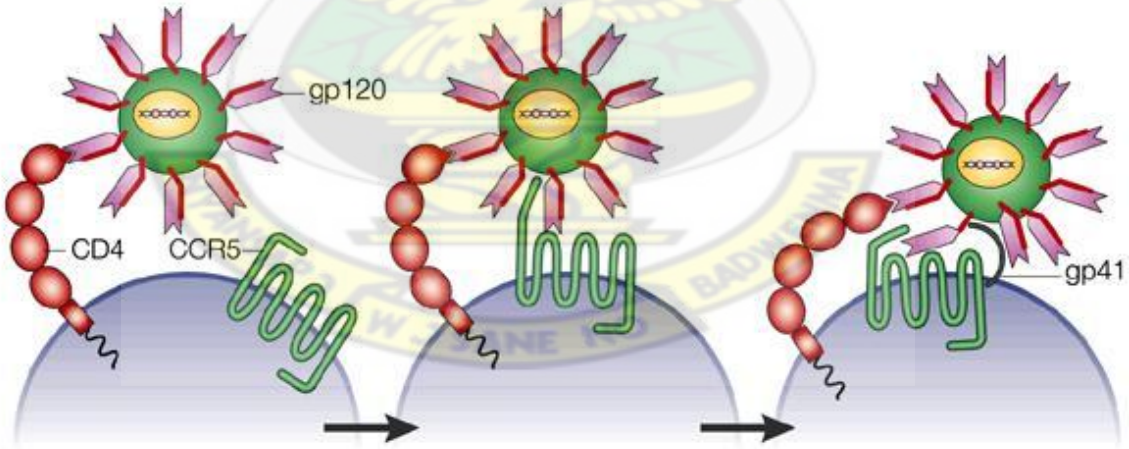
1.4.4.1 CD4 Cells as a Receptor

HIV primarily infects cells that have CD4 cell-surface receptor molecules, using the receptors to gain entry. Many cell types share common epitopes with this protein, though CD4 lymphocytes play a crucial role. Cells with CD4 receptors susceptible to HIV infection may include cells of the mononuclear phagocyte system, principally blood monocytes and tissue macrophages, T lymphocytes, B lymphocytes, natural killer (NK) lymphocytes, dendritic cells (Langerhans cells of epithelia and follicular dendritic cells in lymph nodes), hematopoietic stem cells, endothelial cells, microglial cells in brain, and gastrointestinal epithelial cells. The galactosylceramide receptors on cells within the brain and bowel may play a role as well (Levy, 1996).

1.4.4.2 **Chemokine Receptor as HIV Coreceptors**

The first report suggesting a possible role of chemokines and their receptors in HIV pathogenesis was published in the late 1980s, when Walker and co-workers showed that HIV infection was inhibited by soluble factors, produced by CD8+ lymphocytes (Walker and Levy, 1989). The identification of CXCR4 (initially known as Fusin (Feng *et al.*, 1996) as a coreceptor for HIV strains adapted to infect transformed T-cell lines (T-tropic strains), its relation with chemokine receptors family and the observation that β- chemokines inhibited the infection by HIV strains able to infect primary macrophages (M-tropic), lead to the identification of the β-chemokine receptor CCR5 as a second coreceptor, mediating M-tropic strains entry (Alkhatib *et al.*, 1996; Deng *et al.*, 1996). Nowadays, nineteen of these seven-

transmembrane domain G-protein-coupled receptors (GPCRs) have been thus considered, *in vitro*, as coreceptors for HIV-1, HIV-2 and Simian Immunodeficiency Virus (SIV): CCR1, CCR2b, CCR3, CCR4, CCR5, CCR8, CCR9, CXCR2, CXCR4, CXCR5, CXCR6, CX3CR1, GPR1, GPR15, APJ, ChemR23, RDC1, BLTR and US28 (Dimitrov *et al.*, 1999; Simmons *et al.*, 2000). Interestingly, despite the extensive range of molecules that potentially could act as viral coreceptors, CCR5 and CXCR4 are the major coreceptors for HIV-1 and they seem to be of major importance in HIV-1 pathogenesis (Zhang *et al.*, 1998; Simmons *et al.*, 2000). CCR5-dependent (R5) strains are predominant during early stages of HIV infection and only in approximately 40% of infected humans, a viral population arises that can use CXCR4 in addition to (R5/X4 strains), or sometimes instead of CCR5 (X4 strains) (Berger *et al.*, 1998; Berger *et al.*, 1999).



Source: Nature Immunology (2002)

Figure 1.4 A diagrammatic representation of the relationship of the chemokine receptor to the CD4 receptor and the HIV

1.4.5 Uncoating of HIV

1.4.5.1 Assembly of Virion

Cellular enzymes now remove the capsid, releasing the RNA and two enzymes, which are collectively known as transcriptase. One of the enzymes, DNA polymerase forms a single-stranded copy of DNA, using the viral RNA as a template. The second enzyme ribonuclease then destroys the original RNA. During this period the DNA polymerase synthesizes a complementary DNA strand, using the first DNA strand as a template. This activity results in a double-stranded DNA molecule and the loss of the viral RNA. Once the double-stranded DNA molecule has been formed, it migrates to the nucleus of the T-lymphocyte. Here a viral enzyme named integrase incorporates the DNA molecule into the cell's 46 chromosomes. At this stage the host carries the genetic information of HIV as a provirus in the T-lymphocyte (Alcamo, 1993).

1.4.5.2 Infection of Additional Cells

Cells with CD4 receptors at the site of HIV entry become infected and viral replication begins within them. The infected cells can then release virions by surface budding, or infected cells can undergo lysis with release of new HIV virions which can then infect additional cells. Some of the HIV virions are carried via the lymphatics to regional lymph nodes (Pantaleo and Fauci, 1996).

After initial entry of HIV into host cells and the establishment of infection, HIV virions released from infected cells may then enter the systemic circulation and be carried to widespread sites within the body. Cells of the mononuclear phagocyte system, including those in lymph nodes, spleen, liver, and bone marrow can then become infected with HIV. Besides lymph nodes, the gut associated lymphoid tissue in gastrointestinal submucosa provides a substantial reservoir for HIV. Primary HIV infection is followed by a burst of viremia in which virus is easily detected in peripheral blood in mononuclear cells and plasma. In the period of

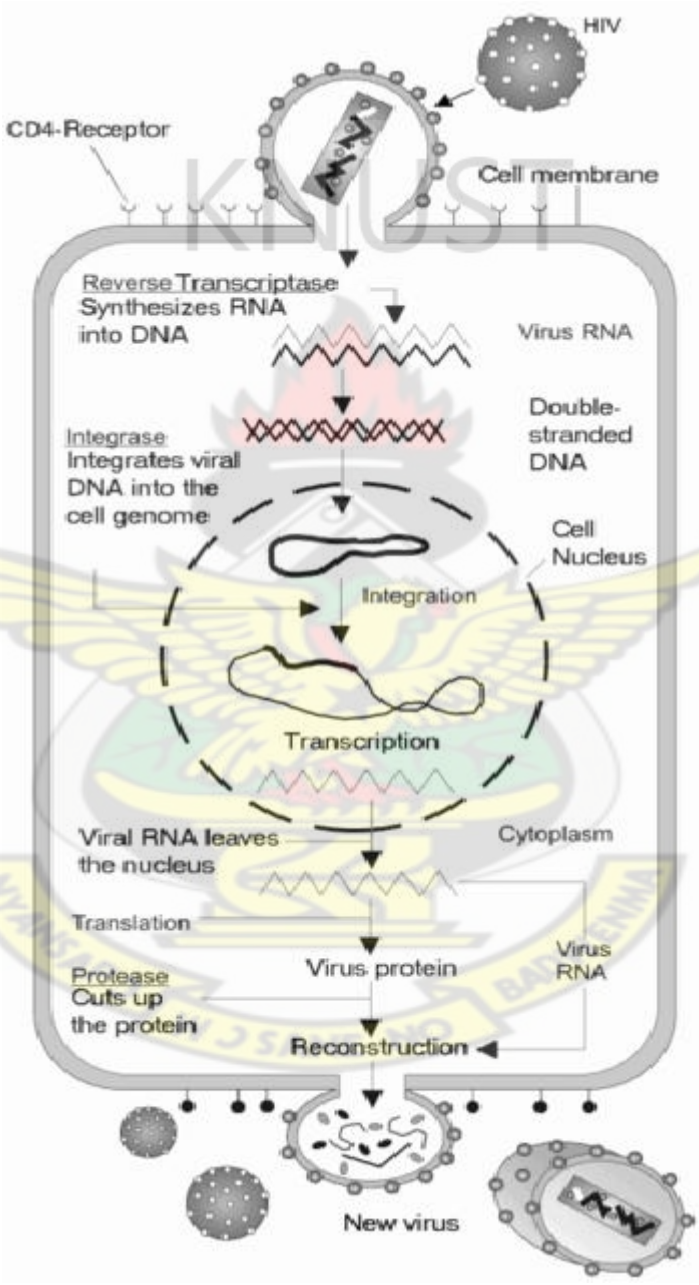
clinical latency of HIV infection, there is little detectable virus in peripheral blood, but viral replication actively continues in lymphoid tissues (Pantaleo and Fauci, 1996).

Once the HIV proviral DNA is within the infected cell's genome, it cannot be eliminated or destroyed except by destroying the cell itself. The HIV provirus then directs its replication by infected host cells. This replication may at first occur within inflammatory cells at the site of infection or within peripheral blood mononuclear cells (CD4 lymphocytes and monocytes) but then the major site of replication quickly shifts to lymphoid tissues of the body (lymph nodes and gastrointestinal tract). The initial burst of viral replication that ensues infection is followed by replication at a lower level, which accounts for the clinically apparent latency of infection. However, viral replication is stimulated by a variety of cytokines such as interleukins and tumor necrosis factor which activate CD4 lymphocytes and make them more susceptible to HIV infection (Pantaleo and Fauci, 1996).

1.4.6 HIV Activation and Release

Activation of viral synthesis leads to release of new infective particles from the host cell surface by budding. Budding virions utilize host cell membrane to help form the outer virion envelope. A protease enzyme encoded by the *pol* gene of HIV cleaves the large precursor proteins in the budding virion necessary for production of infectious particles. Replication may also cause cell lysis with release of additional infective viral particles. Host cell death may be mediated via several diverse mechanisms: direct viral cytopathic effects, fusion to multinucleated giant cells (syncytia formation), cytotoxic immune response by other lymphocytes (CD8+ cytotoxic T- lymphocytes), autoimmune mechanisms, disruptive interaction of HIV envelope proteins with the cell membrane, immune clearance from alteration of antigenicity of the host cell, activation of apoptosis (programmed cell

death), or toxic accumulation of viral DNA, RNA, or proteins (Levy, 1996; Pantaleo and Fauci, 1996).



Source: GFDL (2006)

Figure 1.5 Steps in HIV Replication

1.5 RATE OF PRODUCTION OF HIV IN AN INFECTED PERSON

The magnitude of HIV-1 production in infected persons is enormous. The numbers of "productively infected cells" (those cells with 20 or more copies of HIV-1 RNA) are quite high. When primary HIV-1 infection occurs, most of the productively infected cells are CD4 lymphocytes, accounting for about 80% of all infected cells at the site(s) of mucosal inoculation and 90% of infected cells in lymphoid tissues. However, follicular dendritic cells (FDCs) within the lymphoid tissues provide the greatest reservoir in well-established HIV-1 infections, particularly throughout the clinically latent period before the onset of AIDS, harboring an estimated 10^{11} copies of HIV-1 RNA. The pool of 10^7 to 10^8 productively infected CD4 cells within the body, averaging 50 - 100 copies per cell, gradually diminishes over time, eventually leading to immune failure and the onset of AIDS. The total virion production per day in an infected person averages greater than 10^9 to 10^{10} copies. Additional reservoirs of HIV-infected cells may be present in the central nervous system, lung, and liver (Haase, 1999).

1.6 HOST RESPONSE TO HIV INFECTION

Subsets of the CD4+ lymphocyte population are important in determining the host response to infection. The subset known as TH1 (T helper 1) is responsible for directing a cytotoxic CD8+ T-lymphocyte response, but the TH2 (T helper 2) subset of CD4+ and CD8+ T- lymphocytes diminishes the cytotoxic lymphocyte response while increasing antibody production. Persons infected with HIV who have a dominant TH1 response tend to survive longer. The switch from a TH1 to a TH2 response has been suggested as a factor in the development of AIDS. Production of interleukin-5 (IL-5) and interferon-gamma (IFN-gamma) by CD4+ and CD8+ T-lymphocytes expressing CD30 is associated with promotion of B-lymphocyte immunoglobulin production (Graziosi *et al.*, 1994; Pizzolo *et al.*, 1994).

The imbalance in the TH response to a predominantly TH2 response is mediated by HIV proteins gp120 and Tat, which trigger the release of cytokines necessary for a TH2 response. These HIV proteins stimulate mast cells and basophils. The Tat protein upregulates chemokine receptor CCR3 on mast cells and basophils, rendering them susceptible to infection by CCR3 tropic HIV. Increased serum IgE levels suggest that a TH2 response has occurred and predict a poorer prognosis (Marone *et al.*, 2001).

1.7 TRANSMISSION OF HIV

Considerable epidemiologic and clinical work has been performed to understand the transmission of HIV from one person to another. As in past epidemics, the spread of AIDS is facilitated by human travel. Modern means of travel by jet aircraft readily available to many people provide an easy route for the spread of AIDS from one location or population to another. Besides, regional integration and closer cooperation among states have eased the travel of subjects across international borders (Levy *et al.*, 2005).

However, unlike most infections in past epidemics, AIDS is distinguished by a very long latent period before the development of any visible signs of infection in affected persons. The average HIV-infected person may have an initial acute self-limited illness, may take up to several weeks to become seropositive, and then may live up to 8 or 10 years, on average, before development of the clinical signs and symptoms of AIDS. In virtually all past infectious disease epidemics, infected persons were soon easily recognized so that measures could be taken to prevent the spread of disease. But persons infected with HIV cannot be recognized by appearance alone, are not prompted to seek medical attention, and are often unaware that they may be spreading the infection (Levy *et al.*, 2005).

1.7.1 Reservoir of HIV

The transmission of HIV is a function both of where the virus appears in the body and how it is shed. HIV can be present in a variety of body fluids and secretions. The presence of HIV in genital secretions and in blood, and to a lesser extent breast milk, is significant for spread of HIV. However, the appearance of HIV in saliva, urine, tears, and sweat is of no major clinical or social importance, as transmission of HIV through these fluids does not routinely occur, primarily because of the low concentration of HIV in these fluids (Shepard *et al.*, 2000).

Though infectious particles of HIV are frequent in cerebrospinal fluid, contact with this fluid in daily life is extremely rare (Yeung *et al.*, 1993).

1.7.2 Mode of Transmission of HIV

The most important feature of HIV is the means of spread. Unlike most epidemics of infectious diseases wherein much of a population is at risk, HIV infects definable population subgroups ("risk groups"). This happens because HIV is primarily a sexually transmissible disease. Homosexual, bisexual, and heterosexual transmission all can occur. Although sexual intercourse between males has remained the greatest risk for transmission in developed nations of Western Europe and the United States, heterosexual transmission is increasing in those regions but still remains less common than in Africa, Asia, or parts of the Caribbean (Hader *et al.*, 2001; Morison, 2001).

Transmission of HIV can occur from male to male, male to female, and female to male. Female to female transmission remains extremely rare, though women with same-sex contact are also often bisexual and have additional risk factors for HIV infection (Rich *et al.*, 1993; Bevier *et al.*, 1995).

Worldwide, heterosexual transmission accounts for the majority of cases of HIV infection. The important factors that promote heterosexual transmission include:

- More sexual partners
- Frequent change of sexual partners
- Unprotected sexual intercourse (lack of barrier precautions)
- Presence of additional sexually transmitted diseases
- Lack of male circumcision
- Social vulnerability of women and young persons
- Economic and political instability of the community

(Lamptey, 2002).

1.7.3 Rate of HIV Transmission

There are three major variables that explain the sexual transmission of HIV:

(1) Transmission efficiency,

(2) Number of sexual partners, and

(3) Seroprevalence (numbers of infected individuals in a population).

HIV transmission through sexual exchange of semen or vaginal fluids is much less efficient than transmission of either gonorrhea or hepatitis B virus (Miller *et al.*, 1993). Usually, multiple sexual exposures are necessary to increase the likelihood for transmission of HIV from infected persons. It is estimated that gonorrhea may be transmitted in 22 to 25% of sexual encounters involving an infected individual, hepatitis B virus in 20 to 30% of encounters, and hepatitis C in 2% of sexual encounters, while HIV transmission occurs much less often--approximately 0.3% per sexual contact with an HIV-infected person. However, some persons have

become HIV-infected after a single sexual contact, while other persons have remained uninfected after hundreds of contacts (Peterman and Curran, 1986; Miller *et al.*, 1993). The rate of sexual transmission of HIV may depend upon the number of viral particles in genital secretions. The number of CD4 cells per μL of seminal fluid ranges from 10^2 to 10^3, while the number of virions can range from undetectable to over 10^6. The numbers of virions in the female genital tract is generally lower. Transmission can occur both cell-to-cell as well as from cell- free fluid (Gupta *et al.*, 1997; Iversen *et al.*, 1998). Thus, the transmission rate is two to three times higher from infected males to females than from infected females to males, without other cofactors (Vernazza *et al.*, 1999).

1.7.4 Other Sources of HIV Infection

Cocaine can increase the transmission rate for HIV with oral sex. This increase in infectivity can be due either to the greater numbers of oral sores with inflammatory cells containing HIV in the infected person or to the increased numbers of inflammatory cells with CD4 receptors in the contact person waiting to become infected, from the loss of an intact epithelial barrier (Faruque *et al.*, 1996).

Even though HIV has been found in a variety of body fluids such as saliva, urine, feces, and tears, non-sexual transmission of HIV by these body fluids is improbable (Gershon and Vlahov, 1990). There is no evidence for HIV transmission by the aerosol route (Beltrami *et al.*, 2000).

1.7.5 Parenteral Infection of HIV

HIV has another important secondary means of spread through blood or blood products. Parenteral exposure to blood and blood products is the most highly efficient method of HIV transmission--close to 90% (Perkins *et al.*, 1987).

1.7.6 Mother-To-Child-Transmission (MTCT)

HIV infection can also be acquired as a congenital infection perinatally or in infancy. Mothers with HIV infection can pass the virus to their babies transplacentally, at the time of delivery through the birth canal, or through breast milk. In the absence of breast-feeding, intrauterine transmission accounts for 25 to 40% of infections, while 60 to 75% occur during labor and delivery (Perkins *et al.*, 1987).

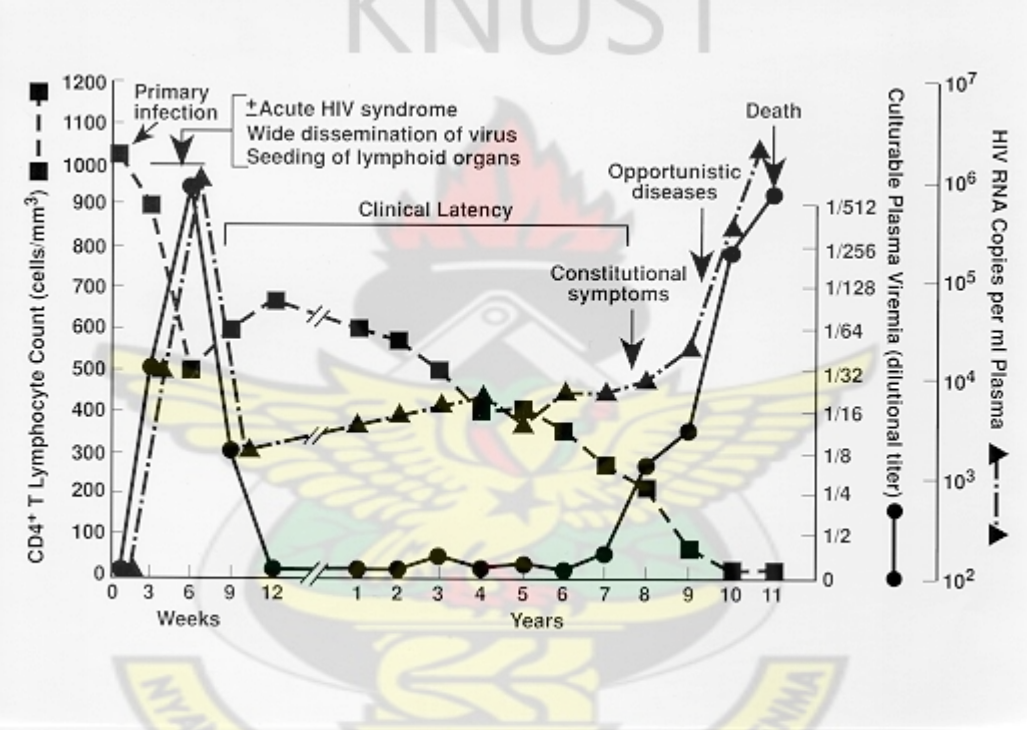


Figure 1.6 Typical course of HIV infection

1.8 PATTERNS FOR HUMAN IMMUNODEFICIENCY VIRUS INFECTION

Worldwide, three patterns of HIV infection have been identified. In pattern 1, affecting primarily urban areas of the Americas and western Europe, the majority of HIV infections occur in males having sexual intercourse with other males (homosexual and bisexual males), followed by infections in intravenous drug users. Fewer cases are observed among heterosexuals. Pattern 2 occurs in those

areas in which HIV has been present longer and the number of HIV-infected persons in the population is greater. Men and women are affected equally, and heterosexual intercourse is the major means of transmission for HIV. These areas include sub-Saharan Africa and parts of the Caribbean where HIV infection occurs throughout the heterosexual population, and congenital AIDS is a significant problem. Pattern 3 occurs in areas of the world in which HIV has been introduced only recently, defined risk groups have not emerged, and only sporadic cases are reported (Adjorlolo-Johnson *et al.*, 1994).

1.8.1 Acute Phase of HIV Infection

In acute HIV infection, the peripheral blood may demonstrate lymphopenia and/or thrombocytopenia. However, atypical lymphocytes are absent. Although the CD4 cells are decreasing, the levels may initially remain in the normal range, but depletion continues. Simultaneously, there is an increase in cytotoxic CD8 lymphocytes that continues as symptoms subside and viremia decreases (Kahn and Walker, 1998).

During this acute phase of HIV infection, there is active viral replication, particularly in CD4 lymphocytes, and a marked HIV viremia. This peripheral blood viremia is at least as high as 50,000 copies/mL and often in the range of 1,000,000 to 10,000,000 copies/mL of HIV-1 RNA. High titers of cytopathic HIV are detectable in the blood so that the p24 antigen test is usually (but not always) positive, while HIV antibody tests (such as enzyme immunoassay) are often negative in the first three weeks. The viremia is greater in persons whose primary HIV infection is symptomatic (Quinn, 1997; Kahn and Walker, 1998).

1.8.2 Viremic Phase of HIV Infection

During this viremic phase, HIV disseminates throughout the body to lymphoid tissues and other organs such as brain. There are alterations in peripheral blood mononuclear cells marked by a decline in CD4+ lymphocytes. Persons acutely

infected with HIV are highly infectious as a consequence of the high levels of HIV, both in blood as well as in genital secretions. Over half of all HIV infections may be transmitted during this period (Quinn, 1997).

Generally, within 3 weeks to 3 months following initial infection with HIV, the immune response is accompanied by a simultaneous decline in HIV viremia. Both humoral and cell mediated immune responses play a role. The CD4 lymphocytes rebound in number after primary HIV infection, but not to pre-infection levels. Seroconversion with detectable HIV antibody by laboratory testing such as enzyme immunoassay accompanies this immune response, sometimes in as little as a week, but more often in two to four weeks (Levy, 1996; Pantaleo and Fauci, 1996).

1.8.3 Latent Phase of HIV Infection

The HIV infection then becomes clinically "latent." During this phase, there is little or no viral replication detectable in peripheral blood mononuclear cells and little or no culturable virus in peripheral blood. The CD4 lymphocyte count remains moderately decreased. However, the immune response to HIV is insufficient to prevent continued viral replication within lymphoid tissues. Though lymph nodes may remain unenlarged and their architecture is maintained, active viral replication continues (Tenner-Racz *et al.*, 1998). Tests for HIV antibody will remain positive during this time but p24 antigen tests are usually negative. There is no evidence to suggest that seroreversion, or loss of antibody, occurs in HIV-infected persons (Roy *et al.*, 1993).

1.8.4 Clinical Aids Stage of HIV Infection

The stage of clinical AIDS that is reached years after initial infection is marked by the appearance of one or more of the typical opportunistic infections or neoplasms diagnostic of AIDS by definitional criteria. The progression to clinical AIDS is also marked by the appearance of syncytia-forming (SI) variants of HIV in about half of HIV-infected patients. These SI viral variants, derived from non-syncytia-forming

(NSI) variants, have greater CD4+ cell tropism and are associated with more rapid CD4+ cell decline. The SI variants typically arise in association with a peripheral blood CD4 lymphocyte count between 400 and 500/μL, prior to the onset of clinical AIDS. However, appearance of the SI phenotype of HIV is a marker for progression to AIDS that is independent of CD4+ cell counts (Ghose *et al.*, 2001).

1.9 PROGRESSION OF HIV INFECTION

The development of signs and symptoms of AIDS typically parallels laboratory testing for CD4 lymphocytes. A decrease in the total CD4 lymphocyte count below 500/μL presages the development of clinical AIDS, and a drop below 200/μL not only defines AIDS, but also indicates a high probability for the development of AIDS-related opportunistic infections and/or neoplasms. The risk for death from HIV infection above the 200/μL CD4 level is low (Phillips *et al.*, 1992; Gallant *et al.*, 1994; Bozzette *et al.*, 1995).

Other laboratory findings which indicate progression to AIDS include HIV p24 antigen positivity, increased serum beta2-microglobulin (B2-M), elevated serum IgA, or increased neopterin levels in serum, cerebrospinal fluid, or urine. The p24 antigen is a highly specific predictor of progression, but only about 60% of HIV-infected persons develop p24 antigenemia prior to onset of clinical AIDS (Tsoukas and Bernard, 1994).

The best laboratory measure for determination of the progression of AIDS is the level of HIV-1 RNA in peripheral blood. The predictive value of HIV-1 RNA levels is independent of the CD4 lymphocyte count and of age in adults. During the acute phase of HIV infection prior to any immune response, plasma levels of HIV-1 RNA typically exceed 10,000 copies/μL. There is a sex difference indicated by levels of viral load. The initial viral load following HIV infection is 50,766 copies/mL in males and 15,103 copies/mL in females (Sterling *et al.*, 2001).

These levels of HIV-1 RNA generally drop, but fluctuate for a period of 6 to 9 months. After this time, a "set point" is reached for the level of HIV-1 RNA that remains relatively constant during the latent phase of HIV infection. Factors influencing this set point include the strain of HIV-1, host anti-HIV response, and the number of cells, including CD4 lymphocytes and macrophages, available for infection. The initial viremia may be higher, and the set point may not be reached until after infancy in cases of congenital HIV infection (Sterling *et al.*, 2001).

The set point levels of HIV-1 RNA correlate with the time to development of AIDS. The set point can range from <50 to 1,000,000 copies/mL. Persons with a higher set point tend to lose CD4 cells more rapidly and progress to AIDS more quickly. Levels of HIV-1 RNA can remain at a steady state for months to years, but usually fall with time. Levels in any individual person may vary with time and even change rapidly, though a variation of <0.7 $\log_{10}$ copies/mL is typical, but an upward progression is an ominous sign of probable progression to AIDS. Less than half of persons with low levels (<4500 copies/mL) of HIV-1 RNA have progressed to AIDS at 10 years following seroconversion, and those with levels <200 copies/mL do not appear to progress at all. Conversely, persons with >100,000 copies/mL are 10 times more likely to progress to AIDS in 5 years. For persons in the top quartile (>36,270 copies/mL) the median time to development of AIDS is 3.5 years (Sterling *et al.*, 2001).

1.10 THE ROLE OF ANTIRETROVIRAL THERAPY IN HIV/AIDS MANAGEMENT

There is currently no vaccine for HIV infection. One of the ways that an HIV/AIDS patient is managed is the use of antiretroviral therapy (ART). The primary goal of ART for HIV infection is the suppression of the viral replication. When the drugs are given in combination, HIV replication and immune deterioration can be delayed and the survival and quality of life improved (Furtado *et al.*, 1999).

Strategies for the treatment of HIV infection began changing with the recognition that viral replication occurs during the years preceding the development of clinical disease. HIV infects cells with $CD4^+$ receptors which are able to fuse with *gp 120* in the viral coat causing entry into the cells. Cells with this receptor in the body are predominantly T-lymphocytes which play a major role in cellular immunity in response to invasion of the body by foreign substances. Between the time of initial infection and the development of the clinical disease, the T lymphocytes $CD4^+$ count progressively declines as a result of their destruction by the HIV after they have been used as site for viral replication. This results in the lesser number of T-lymphocytes production with a corresponding progressive suppression of the immune system (Furtado *et al.*, 1999).

1.10.1 Classes of Antiretroviral drugs

There are different classes of antiretroviral drugs that act at different stages of the HIV life-cycle.

1.10.2 Nucleotide and Nucleoside Reverse Transcriptase Inhibitors (NRTIs)

Drugs in this class inhibit reverse transcription by being incorporated into the newly synthesized viral DNA and preventing its further elongation. Examples of drugs in this class are zidovudine (AZT), stavudine (d4T), lamivudine (3TC), emtricitabine (FTC), didanosine (ddl), abacavir (ABC) and tenofovir (TDF)(Weller and Williams, 2001).

1.10.3 Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)

Drugs in this class inhibit reverse transcriptase directly by binding to the enzyme and interfering with its function. Examples include nevirapine (NVP) and efavirenz (EFV)(Weller and Williams, 2001).

1.10.4 Protease Inhibitors (PIs)

Protease inhibitors (PIs) target viral assembly by inhibiting the activity of protease which is an enzyme used by HIV to cleave nascent proteins for final assembly of

new virons. Examples are saquinavir (SQV), indinavir (IDV) and nelfinavir (NFV)(Weller and Williams, 2001).

1.10.5 Integrase Inhibitors

Drugs in this class inhibit the enzyme integrase, which is responsible for integration of viral DNA into the DNA of the infected cell. An example is raltegravir (de Soultrait *et al.*, 2002).

1.10.6 Entry Inhibitors of Fusion Inhibitors

These drugs interfere with binding, fusion and entry of HIV-1 to the host cell by blocking one of several targets. Maraviroc and enfuvirtide are the two currently available agents in this class (Kilby *et al.*, 1998).

1.10.7 Maturation Inhibitors

These drugs inhibit the last step in gag processing in which the viral capsid polyprotein is cleaved, thereby blocking the conversion of the polyprotein into the mature capsid protein (p24). Because these viral particles have a defective core, the virions released consist mainly of non-infectious particles. There are no drugs in this class currently available, though two are under investigation, bevirimat (Panacos Pharmaceuticals) and Vivecon (Weller and Williams, 2001).

1.11 HISTORIC BACKGROUND OF FREE RADICALS

The presence of free radicals in biological materials was discovered less than six (6) decades ago (Commoner *et al.*, 1954). Soon thereafter, Denham Harman hypothesized that oxygen radicals may be formed as by-products of enzymic reactions in vivo. In 1956, he described free radicals as a Pandora's box of evils that may account for gross cellular damage, mutagenesis, cancer, and, last but not least, the degenerative process of biological aging (Harman, 1956).

The science of free radicals in living organisms entered a second era after McCord and Fridovich (1969), discovered the enzyme superoxide dismutase (SOD) and, finally, convinced most colleagues that free radicals are important in biology. Numerous researchers have since been inspired to investigate oxidative damage inflicted by radicals upon DNA, proteins, lipids, and other components of the cell (Beckman and Ames, 1998).

A third era began with the first reports describing advantageous biological effects of free radicals. (Mittal and Murad, 1977) provided suggestive evidence that the superoxide anion through its derivative, the hydroxyl radical, stimulates the activation of guanylate cyclase and formation of the "second messenger" cGMP. There is now a large body of evidence showing that living organisms have not only adapted to an unfriendly coexistence with free radicals but have, in fact, developed mechanisms for the advantageous use of free radicals. Important physiological functions that involve free radicals or their derivatives include the following: regulation of vascular tone, sensing of oxygen tension and regulation of functions that are controlled by oxygen concentration, enhancement of signal transduction from various membrane receptors including the antigen receptor of lymphocytes, and oxidative stress responses that ensure the maintenance of redox homeostasis (Droge, 2002).

1.11.1 Oxidants, Free Radicals and Reactive Oxygen Species (ROS)

Oxidants may be broadly defined as endogenous or exogenous substances, which have the capacity to bring about the oxidation of target molecules, either directly by abstraction of electrons, or indirectly through the production of highly reactive intermediate chemical entities. Free radicals represent a class of such entities whose reactivity derives from the presence of unpaired electrons in their atomic structure, but which are capable of independent existence for very brief intervals of time (Cui *et al.*, 2004).

ROS is a collective term that includes all reactive forms of oxygen, including both the radical and non radical species that participate in the initiation and/or propagation of radical chain reactions. Consequent to their reactivity, accumulation of ROS beyond the immediate needs of the cell may affect cellular structure and functional integrity, by bringing about oxidative degradation of critical molecules, such as DNA, proteins, and lipids (Ames *et al.*, 1993; Mitra *et al.*, 2002; Cui *et al.*, 2004; Schaller, 2005).

1.11.1.1 Endogeneous Sources and Effects of Oxidants

Basically there appear to be four endogenous sources which account for most of the oxidants produced by cells.

i. As a consequence of normal aerobic respiration, mitochondria consume 0_2, reducing it by sequential steps to produce H_20. Inevitable byproducts of this process, are O_2, H_20_2, and -OH (Chance *et al.*, 1979).

ii. Phagocytic cells destroy bacteria or virus infected cells with an oxidative burst of nitric oxide (NO), O_2^-, H_20_2, and $OC1^-$. Chronic infection by viruses, bacteria, or parasites results in a chronic phagocytic activity and consequent chronic inflammation, which is a major risk factor for cancer. Chronic infections are particularly prevalent in third-world countries (Kasai *et al.*, 1989).

iii. Peroxisomes, which are organelles responsible for degrading fatty acids and other molecules, produce H_20_2 as a by-product, which is then degraded by catalase. Evidence suggests that, under certain conditions, some of the peroxide escapes degradation, resulting in its release into other compartments of the cell and in increased oxidative DNA damage (Kasai *et al.*, 1989).

iv. Cytochrome P450 enzymes in animals constitute one of the primary defense systems against natural toxic chemicals from plants, the major source of dietary

toxins. The induction of these enzymes prevents acute toxic effects from foreign chemicals but also results in oxidant by-products that damage DNA (Kasai *et al.*, 1989).

1.11.1.2 Exogeneous Sources and Effects of Oxidants

Three exogenous sources may significantly increase the large endogenous oxidant load.

i. The oxides of nitrogen (NO.) in cigarette smoke (about 1000 ppm) cause oxidation of macromolecules (Kiyosawa *et al.*, 1990) and deplete antioxidant levels (Schectman *et al.*, 1991). This is likely to contribute significantly to the pathology of smoking. Smoking is a risk factor for heart disease as well as a wide variety of cancers in addition to lung cancer (Kneller *et al.*, 1992).

ii. Iron (and copper) salts promote the generation of oxidizing radicals from peroxides. Men who absorb significantly more than normal amounts of dietary iron due to a genetic defect (hemochromatosis disease) are at an increased risk for both cancer and heart disease. It has therefore been argued that too much dietary copper or iron, particularly heme iron (which is high in meat), is a risk factor for cardiovascular disease and cancer in normal men (Sullivan, 1989).

iii. Normal diets contain plant food with large amounts of natural phenolic compounds, such as chlorogenic and caffeic acid, that may generate oxidants by redox cycling (Gold *et al.*, 1992).

1.11.2 TYPES OF ROS

The most common ROS include: Ozone (O_3), singlet oxygen ($^1O_2$), the superoxide anion (O2), hydrogen peroxide (H_2O_2), the hydroxyl radical (OH^-), and Peroxyl radicals.

1.11.2.1 Singlet oxygen

Singlet oxygen ($^1O_2$), which is largely involved in photochemical reactions, is very reactive, although it does not contain unpaired electrons and therefore is not a free radical. It is formed in vivo by enzymatic activation of oxygen, for example, through lipooxygenase activity during prostaglandin biosynthesis (Cadenas and Sies, 1984). It can also be produced by physicochemical reactions, such as energy transfer due to type II photosensitization (Sies, 1993), thermal decomposition of endoperoxides and dioxetanes (Di Mascio *et al.*, 1989), reaction of ozone with human body fluids and interaction between hydrogen peroxide and peroxynitrite. Singlet oxygen is a very reactive ROS and induces various genotoxic, carcinogenic, and mutagenic effects through its action on polyunsaturated fatty acids (PUFAs) and DNA (Di Mascio *et al.*, 1994).

1.11.2.2 Superoxide anion (O_2^-)

The superoxide is an anionic radical formed by the reduction of molecular oxygen through the acceptance of a single electron. The hydroperoxyl radical, which is unstable at physiological pH, dissociates to superoxide. In vivo, it is mainly produced by the electron transport chains in the mitochondria and microsomes through electron leakage—a phenomenon that increases with an increase in oxygen utilization (McCord and Omar, 1993). Superoxide radicals are also formed by metal ion-dependent oxidation of epinephrine and norepinephrine, and by the action of enzymes such as tryptophane hydroxylase, indoleamine dioxygenase, and xanthine oxygenase. Activated phagocytes also possess metabolic pathways for the production of superoxide radicals in response to bacterial infection (Curnutte and Babior, 1987).

1.11.2.3 Hydrogen peroxide

Hydrogen peroxide and superoxide may undergo further transformations in the presence of transition metals (particularly iron and copper) (Halliwell and Gutteridge, 1990) to give rise to the highly reactive hydroxyl radicals, by the Haber–Weiss or Fenton reactions. This property, combined with the membrane permeability of hydrogen peroxide, gives superoxide and hydrogen peroxide the ability to affect the integrity of distant molecules within the cell (Halliwell and Gutteridge, 1989; Cochrane, 1991).

1.11.2.4 Hydroxyl radical

The hydroxyl radical can be produced experimentally by various procedures, including exposure to radiation (von Sonntag, 1988), or by decomposition of peroxynitrite (Beckman *et al*., 1994). Because of its low half-life the direct action of the hydroxyl radical is confined to regions immediately in the vicinity of its formation. However, being the most aggressive member of the ROS family, it can bring about extensive damage to different types of molecules, including proteins, nucleic acids, and lipids. In DNA, the OH can induce several effects including base and sugar modifications, cross-linking between bases, cross-linking between DNA and protein, strand breaks, and formation of adducts (Cochrane, 1991). The action of hydroxyl radicals on proteins leads to extensive protein–protein cross-linking (Stadtman, 1992). This may be further aggravated in metalloproteins and metalloenzymes by transition metals, which act as foyers for the formation of hydroxyl radicals from their precursors, thus resulting in site-specific destruction of the critical regions of the molecule. The most extensive studies on the oxidative properties of OH radicals have been carried out on membrane lipids in which the PUFAs are particularly vulnerable to oxidation. The peroxidation of PUFAs by

hydroxyl radicals constitutes one of the most severe attacks on cellular integrity (Niki *et al.*, 1993; Gutteridge, 1995).

1.11.2.5 Peroxyl radicals

Peroxyl radicals are, however, believed to be produced primarily during lipid peroxidation (Halliwell and Gutteridge, 1989), which is initiated by abstraction of a hydrogen atom from unsaturated lipids. Although lipid peroxidation has been found to play a useful role in some biological processes, peroxidation of membrane PUFAs may adversely affect many functionally important parameters, such as membrane fluidity, permeability, electrical potential, and controlled transport of metabolites across the membrane (Halliwell and Gutteridge, 1989).

1.11.3 Lipid Peroxidation

The oxidation of lipids by ROS generally consists of three steps as discussed below (Niki *et al.*, 1993).

1.11.3.1 Initiation step

The free radical attacks a methylene group in the polyunsaturated fatty acids (PUFAs), leading to a rearrangement of the double bonds to the conjugated diene form, and simultaneously producing a carbon-centered alkyl radical. The alkyl radical reacts with molecular oxygen to give rise to a peroxyl radical (Niki *et al.*, 1993).

1.11.3.2 Propagation step

The peroxyl radical, in its turn, starts a self-perpetuating chain reaction in which most of the membrane lipids are converted to a variety of hydroperoxides and cyclic peroxides. The hydroperoxides can be further degraded to hydrocarbons, alcohols, ether, epoxides, and aldehydes. Of these products, malondialdehyde and 4-hydroxynonenal have the additional ability to inactivate phospholipids, proteins,

and DNA by bringing about cross-linking between these molecules (Esterbrauer *et al.*, 1990).

1.11.3.3 Termination step

The chain reaction is stopped by interactions between the radicals themselves, or between the radicals and antioxidants, giving rise to nonradical products or unreactive radicals (Esterbrauer *et al.*, 1990).

1.11.4 Antioxidants

Halliwell & Gutteridge (1989) have defined antioxidants as substances that are able, at relatively low concentrations, to compete with other oxidizable substrates and, thus, to significantly delay or inhibit the oxidation of these substrates. This definition includes the enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase, as well as nonenzymic compounds such as -tocopherol (vitamin E), beta-carotene, ascorbate (vitamin C), and glutathione. They may do so by removing or lowering the local concentrations of one or more of the participants in this reaction, such as oxygen, ROS, or metal ions, which catalyze oxidation ($Fe^{3+,}$ Cu^{2+}, etc.), or by interfering with the chain reaction that spreads oxidation to neighboring molecules. They may also act by enhancing the endogenous antioxidant defenses of the cell. Thus, antioxidants may intervene at any of the three major steps: initiation, propagation, or termination of the oxidative process (Cui *et al.*, 2004).

1.11.4.1 Mode of action of Antioxidants

Critical structures in the cell are protected not only by the availability of several types of antioxidants, but also by other mechanisms, such as the repair or elimination of damaged molecules by appropriate enzymes. Antioxidants may be classified according to their chemical nature and mode of function:

1.11.4.2 Enzyme antioxidants

They act on specific ROS after they are formed and degrade them to less harmful products. Examples include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). SODs convert the superoxide radical to hydrogen peroxide, which is not a free radical by itself, but is a precursor of the highly reactive hydroxyl radical. The SODs, catalase, and GPx constitute the major intracellular enzymic antioxidants, while the extracellular antioxidants are mainly of the preventive and scavenging types (Cui *et al.*, 2004).

1.11.4.3 Preventive antioxidants

They act by binding to and sequestering oxidation promoters and transition metal ions, such as iron and copper, which contain unpaired electrons and strongly accelerate free radical formation. Examples of preventive antioxidants include transferrin and lactoferrin (which bind ferric ions), ceruloplasmin (which binds Cu, catalyzes the oxidation of ferrous ions to ferric due to its ferroxidase activity, and increases the binding of iron to transferrin), haptoglobins (which bind hemoglobin), hemopexin (which binds heme), and albumin (which binds copper and heme) (Cui *et al.*, 2004).

1.11.4.4 Scavenging or chain-breaking antioxidants

They act by presenting themselves for oxidation at an early stage in the free radical chain reaction and giving rise to low energy products that are unable to propagate the chain further. Lipid-soluble and water-soluble scavengers act in cellular environments that are either hydrophobic or hydrophilic, respectively. The major lipid-soluble scavengers are vitamin E (α-tocopherol), β-carotene, and coenzyme Q (CoQ) (Murthy, 2001), while ascorbic acid, various thiols, uric acid, and bilirubin function in the aqueous milieu (Frei *et al.*, 1988).

1.11.5 Vitamin E (alpha Tocopherol)

The recommended dietary allowance for vitamin E is 10 mg for men and 8 mg for women. Vitamin E is very safe compared to other fat soluble vitamins. Few side effects have been reported even at high doses as 3200 mg daily. Vitamin E is most widely known for its biological function as a chain breaking lipid soluble antioxidant. Cell culture studies suggest that when cell growth is inhibited by lipid peroxidation, α-tocopherol may re-stimulate cell growth and proliferation by removing the inhibitor. Vitamin E is necessary for neurological function and for the prevention of certain genetic disorders, red blood cell breakdown and other disorders in premature infants and adults (Meydani, 1995).

One mechanism by which vitamin E may contribute to the reduction in the risk of oxidative stress is through the antioxidant protection of LDL-Cholesterol. The susceptibility of LDL particles to oxidation appears to be important in the pathogenesis of oxidative stress. Oral supplementation with Vitamin E is known to increase LDL resistance to oxidation. Vitamin C increases the resistance of LDL to oxidation by recycling vitamin E and other phenolic antioxidants in lipoprotein particles (Hannigan, 1994). Low intake of Vitamin A, β-carotene, Vitamin C and E have been associated with abnormally low immune responses and greater risk of cancer. Extreme vitamin C deficiency is associated with depressed phagocytic activity and vitamin E deficiency reduces antibody T-cell function if accompanied by deficiency of other antioxidants, particularly selenium (Hannigan, 1994).

1.11.5.1 Antioxidant activity of Vitamin E

Vitamin E is well accepted as nature's most effective lipid soluble, chain breaking, antioxidant, protecting cell membranes from peroxidative damage. Vitamin E is well accepted as the first line of defense against lipid peroxidation, protecting polyunsaturated fatty acids contained in cellular and sub-cellular membranes through its free radical quenching activity in bio-membranes at early stages of free

radical attack (Packer, 1991). Tocopherols are lipid soluble, sterically hindered phenols that react more rapidly with peroxyl radicals than to polyunsaturated fatty acids. Thus vitamin E is the major chain breaking antioxidant. In vivo α-tocopherol is the major lipid soluble antioxidant in the plasma (Kayden, 1993). The phospholipids of mitochondria, endoplasmic reticulum and plasma membranes possess affinities for α-tocopherol and the vitamin appears to concentrate at these sites. The tocopherol act as antioxidants breaking free radical chain reactions as a result of their ability to transfer phenolic hydrogen to a peroxy free radical of a peroxidised polyunsaturated fatty acid as shown below.

$$ROO^- + ToCOH \rightarrow ROOH + ToCO$$

$$ROO^- + ToCO \rightarrow ROOH + Non\ free\ radical\ product$$

The phenoxy free radical formed then reacts with a further peroxy free radical. Thus tocopherol does not readily engage in reversible oxidations and the side chains are oxidized to the non free radical products shown (Allard *et al.*, 1998b).

Another mechanism by which vitamin E enhances immune functions is through its antioxidant properties. Rapidly proliferating cells of the immune system are highly susceptible to peroxidative damage by free radicals, peroxides and superoxides (Tengerdy, 1990). Vitamin E acts as a free radical scavenger and prevents the lipid peroxidation of cell membranes. Vitamin E also modulates the production of prostaglandin E2 (PGE2) in the body. Elevated levels of PGE2 decrease the production of IL-2, a cytokine critical for the growth and differentiation of T and B lymphocytes, and increases the differentiation of PGE2-receptor-bearing T-cells into T-suppressor cells. PGE2 also inhibits activation of natural killer cells, which are a major source of IFN-g, another important cytokine in the host defence system. It is believed that downregulation of PGE2 by vitamin E serves to increase the

production of IL-2 and IFN-g, thereby stimulating the immune system. Other proposed actions of vitamin E on the immune system are through its direct interaction with macrophages, causing upregulation of IL-1 and IL-2. Vitamin E is also believed to decrease levels of TNF-a through its antioxidant properties. Thus, in addition to its role as a free-radical scavenger, the impact of vitamin E on the regulation of various cytokines in the immune process is believed to play a major role in inhibiting HIV-1 replication (Odeleye and Watson, 1991).

1.11.6 VITAMIN C

Vitamin C is ascorbic vitamin. Although scurvy was first described during the crusades, and commonly plagued early explorers and voyages, the specific relationship between scurvy, citrus fruits and ascorbic acid was not established until the 20th century. English sailors have been nick named "limeys' since the days when ships were required to carry citrus fruits (lemons) as scurvy preventive(Benzie, 1999).

1.11.6.1 Antioxidant Property of Vitamin C

Vitamin C is water soluble and is present in its deproportionated state under most physiological conditions. It is considered to be the most important antioxidant in extracellular fluids and has many cellular activities of an antioxidant nature. Vitamin C has been shown to efficiently scavenge superoxide, hydrogen peroxide, hypochlorite, the hydroxyl radical, peroxyradicals and O_2. In studies with human plasma lipids it was shown that ascorbic acid was far more effective in inhibiting lipid peroxidation initiated by a peroxy radical initiator, than other plasma components, such as protein thiols, urate, bilirubin and alpha tocopherol (Frei *et al.*, 1989).

Thus by efficiently trapping peroxyl radicals in the aqueous phase before they can initiate lipid peroxidation, ascorbic acid can protect biomembranes against peroxidation damage. Ascorbic acid can also act to protect membranes against peroxidation by enhancing the activity of tocopherol, the chief lipid soluble, chain breaking antioxidant. With in vitro studies it was shown that ascorbic acid reduces the tocopheroxy radical and thereby restores the radical scavenging of tocopherol (Packer *et al.*, 1979).

Tappel *et al* (1962) has suggested that the two vitamins act synergistically, vitamin E acting as the primary antioxidant and the resulting vitamin E radical then reacting with vitamin C to regenerate vitamin E. The recycling of vitamin E at the expense of vitamin C may account in part for the fact that clinically overt vitamin E deficiency has not been demonstrated. More over in certain conditions vitamin C radical itself can be enzymatically reduced back to vitamin C by an NADH dependent system (Huang *et al.*, 2002).

1.11.7 Vitamins and HIV infection

Vitamins A and E are both important for optimal functioning of the immune system (Beisel, 1996). Deficiencies of these vitamins have been found to impair immune responses in a variety of studies (Semba, 1994). There is ample evidence from both human and animal studies that supplementation with vitamins A and/or E enhances immunity and resistance to various infectious diseases (Odeleye and Watson, 1991). Previous studies have found significant proportions of HIV-1-seropositive individuals with low serum levels of either or both vitamins (Semba, 1994). Low serum levels or low intakes of vitamin A in HIV-1 infection have been associated with lower CD4+ cell counts (Semba and Tang, 1999), increased maternal-fetal transmission (Semba, 1994), increased mortality from AIDS or infections and increased risk of progression to AIDS (Tang *et al.*, 1993).

Low serum vitamin E levels in HIV-1-infected individuals have been correlated with a higher degree of lipid peroxidation (Malvy *et al.*, 1994).

Supplementation with vitamins A and E has been shown to improve immune parameters in several AIDS models (Shor-Posner *et al.*, 1995). There is also evidence that vitamin A supplementation in children with HIV-1 may decrease diarrhoeal symptoms (Coutsoudis *et al.*, 1995).

1.11.8 Oxidative stress

Formation of highly reactive oxygen-containing molecules is a normal consequence of a variety of essential biochemical reactions. Therefore, there is the need to maintain a critical balance between free radical generation and antioxidant defense. Oxidative stress occurs when this balance between pro-oxidants and antioxidants is potentially upset. Thus oxidative stress is essentially an imbalance between the production of various reactive species and the ability of the organism's natural protective mechanisms to cope with these reactive compounds and prevent adverse effects. Because of their reactivity, accumulation of ROS beyond the immediate needs of the cell may affect cellular structure and functional integrity, by bringing about oxidative degradation of critical molecules, such as DNA, proteins, and lipids (Ames *et al.*, 1993; Mitra *et al.*, 2002; Cui *et al.*, 2004; Schaller, 2005).

Acute oxidative stress as well as chronic oxidative stress have been implicated in a large number of human degenerative diseases affecting a wide variety of physiological functions, such as atherosclerosis, diabetes, ischemia/reperfusion (I/R) injury, inflammatory diseases (rheumatoid arthritis, inflammatory bowel disease, and pancreatitis), cancer, neurological diseases, hypertension, ocular diseases (cataract, senile muscular degeneration, and retrolental fibroplasia), pulmonary diseases, and hematological diseases (Halliwell and Gutteridge, 1989).

Even aging and age-related loss of physiological fitness have been attributed to the chronic effects of ROS on various biological macromolecules (Droge, 2003).

1.11.8.1 Disturbance in oxidant-antioxidant balance

Living cells and tissues have several mechanisms for reestablishing the original redox state after a temporary exposure to increased ROS or RNS concentrations. Cells or tissues are in a stable state if the rates of ROS production and scavenging capacity are essentially constant and in balance. Redox signaling requires that this balance is disturbed, either by an increase in ROS concentrations or a decrease in the activity of one or more antioxidant systems. In higher organisms, such an oxidative event may be induced in a regulated fashion by the activation of endogenous ROS-generating systems. However, similar responses may be induced by oxidative stress conditions generated by environmental factors. If the initial increase in ROS is relatively small, the antioxidative response may be sufficient to compensate for the increase in ROS and to reset the original balance between ROS production and ROS scavenging capacity. Under certain conditions, however, ROS production is increased more strongly and persistently, and the antioxidative response may not be sufficient to reset the system to the original level of redox homeostasis. In such cases, the system may still reach equilibrium, but the resulting quasi-stable state may now be associated with higher ROS concentrations. This leads to what is referred as oxidative stress (Droge, 2002).

1.11.9 Human Immunodeficiency Virus and Oxidative Stress

Humans infected with human immunodeficiency virus (HIV) have been shown to be under chronic oxidative stress. Perturbations of the antioxidant defense system in HIV-infected humans include changes in ascorbic acid, tocopherol, carotenoids, selenium, superoxide dismutase and glutathione. In addition, elevated levels of hydroperoxides and malondialdehyde are found in plasma of HIV-infected individuals. In vitro, manipulations that result in enhanced oxidative stress increase the replication of HIV, possibly via activation of nuclear factor kappa B

(NF-ķB), a transcription factor that stimulates the replication of HIV and of certain cytokines, among them tumor necrosis factor-ά (TNF-ά) (Droge *et al.*, 1994).

Concentrations of intracellular GSH in the peripheral blood mononuclear cells and lymphocytes of asymptomatic HIV-seropositive patients were found to be somewhat below those of healthy controls (Buhl *et al.*, 1989).

How increased oxidant production is initially triggered in HIV-infected humans has not been determined to date. Possibilities include stimulatory effects of gp125 (Pietraforte *et al.*, 1994) and Tat, the viral-transactivating protein secreted from virus-infected cells (Westendorp *et al.*, 1995).

1.11.9.1 Mechanism of Oxidative Stress in HIV Infection

Oxidative stress is a known activator of HIV replication in vitro through the activation of a factor that binds to a DNA-binding protein, NF-kappa B. HIV responds to transcriptional stimuli similar to those leading to the induction of a series of cellular genes during T cell activation. The pivotal event is the activation of NF-kB transcription factor (Kopp and Ghosh, 1995).

Activation of this factor was shown to be one of the main requirements in the early stages of viral replication in T lymphocytes. The event necessary to efficiently trigger NF-kB-induced viral transcription is recognition of a specific antigen (Hazan *et al.*, 1990)

NF-kB is composed of two subunits (p50 and p65) and is retained in an inactive form in the cytoplasm by interaction with inhibitory molecules designated IkBs (Baeuerle and Henkel, 1994).

Under various stimuli mediated through a number of cell surface molecules, such as receptors to antigens or to cytokines (Kopp and Ghosh, 1995), this factor is

translocated into the nucleus. There, it interacts with two specific cis-acting sequences present within the long terminal repeat sequence (LTR) of HIV-1 (Dornand and Gerber, 1989).

Recombinant TNF generates reactive oxygen species (ROS) through neutrophils at concentrations as low as 0.5 ng/ml. This effect can be neutralized by specific antibodies (Larrick and Wright, 1990).

TNF-ά is produced by activated macrophages and may contribute directly to disease progression by activation of HIV replication through the action of NF-ķB. TNF-ά levels are elevated in the serum of patients with AIDS and TNF-ά can use oxidative stress as a second messenger (Lahdevirta *et al.*, 1988).

Oxidative stress may also play an important role in the genesis of cellular DNA damage and, in this context, may be related to HIV-associated malignancies and disease progression.

1.11.9.2 Oxidative Stress Stimulates HIV Replication in-vitro

HIV gene expression can be activated in vitro by oxidative stress. H_20_2 can induce the expression of HIV in human T cell lines by activating transcription of NF-ķB. NF-ķB is inactive in the cytosol when complexed to a second regulatory molecule termed IķB (Brown *et al.*, 1995). Activation with a variety of oxidative stimuli resulted in dissociation of the complex between NF-ķB and IķB and subsequent translocation of NF-ķB to the nucleus. NF-ķB binding activity in the nucleus can be modulated by oxidoreduction in vitro. Activation of cells by a variety of inflammatory stimuli, including mitogens, cytokines, and oxidative stress, induces phosphorylation and degradation of the IķB proteins (Brown *et al.*, 1995; DiDonato *et al.*, 1995) and releases active NF-ķB heterodimers, which translocate to the nucleus to stimulate transcription of responsive genes. Whereas homodimers of p50 appear to be the predominant form of NF-KB in resting cells, activated cells

contain high levels of p50/p65 and related heterodimers, as well as homodimers of p65 (Ganchi *et al.*, 1993).

Transient expression assays reveal that the p65 subunit of NF-KB is responsible primarily for transcriptional activation by NFKB (Schmitz and Baeuerle, 1991; Perkins *et al.*, 1992), and a potent *trans-activation* domain was mapped to a carboxyl-terminal region of p65. Among the various well-characterized targets for activation by NF-KB is the enhancer for the human immunodeficiency virus (HIV-1). The HIV-1 enhancer contains two tandem binding sites for NF-KB that are recognized efficiently by at least four different Rel-related protein heterodimers as well as by homodimers of either p50 or p65 (Liou and Baltimore, 1993).

Several different NF-KB subunit combinations have been shown to activate HIV-1 transcription strongly in vivo and in vitro (Fujita *et al.*, 1992), and various studies have reported that the NF-KB-binding sites (Ross *et al.*, 1991) as well as the NF-KB proteins (Qian *et al.*, 1994) are important for virus replication in peripheral blood.

NF-kB nuclear binding activity by oxidative stress is specific and occurs at low concentrations (Stein *et al.*, 1989) . A variety of antioxidants, including GSH, GSH ester, pentoxyphylline, desferrioxamine and and N-acetylcysteine, can block activation of NF-kB (Dworkin, 1994).

1.11.9.3 Evidence for in-vivo Oxidative Stress in HIV Infection

Direct measurement of oxidative stress under clinical conditions is difficult. However, indirect evidence indicates that HIV infection is associated with increased ROS production and increased consumption of antioxidants. Concentrations of intracellular GSH in the peripheral blood mononuclear cells and lymphocytes of asymptomatic HIV-seropositive patients were found to be somewhat below those of healthy controls. However, intracellular GSH levels were profoundly depressed in patients with AIDS and AIDS-related complex.

Zidovudine treatment of patients with AIDS produced a rise in measurable GSH levels (Buhl *et al.*, 1989).

One mechanism of GSH action is through removal of intracellular H_20_2 by providing a substrate for GSH peroxidase, the major H_2O_2 removing enzyme. It may be significant that this enzyme is selenium dependent and that selenium levels are decreased in HIV-infected patients (Dworkin *et al.*, 1986).

In addition, HIV infected patients often have lower concentrations of acid soluble thiol, an important marker of antioxidant activity in the blood (Eck *et al.*, 1989).

HIV-infected patients commonly excrete higher than average quantities of malondialdehyde into their urine, reflecting increased levels of lipid peroxidation.

It was shown that rhesus monkeys that were acutely infected with the simian immunodeficiency virus had lower plasma thiol levels than uninfected animals within 2 weeks of infection. These early metabolic changes coincided with a rise in levels of urinary neopterin, a nonspecific marker of macrophage activation (Sonnerborg *et al.*, 1988).

Oxidative stress and hydroxyl radical formation can lead to increased lipid peroxidation and to modifications in both membrane fluidity and receptor conformation (Carson *et al.*, 1986).

In the absence of a proper antioxidant system, the DNA repair capacity of the cell may be altered and lymphocytes may be killed or suffer from impaired function. Although oxidative bursts of small magnitude are associated with lymphocyte activation and are regulated by intracellular GSH, depletion of the latter is associated with immunosuppression and down-regulation of IL-2 receptors (Roederer *et al.*, 1990b).

1.11.9.4 Oxidative Stress and DNA Damage

Certain malignancies of lymphoid origin are dramatically elevated in patients with AIDS (Karp and Broder, 1991). Failure to repair DNA strand breaks is more common in lymphocytes that are depleted of catalase and/or GSH than in other cell type under the same circumstances (Ameisen and Capron, 1991). Lymphocytes are more susceptible to DNA damage than are macrophages, because the latter have a more potent antioxidant system. The role of chronic antigenic stimulation in DNA damage and carcinogenesis in HIV-infected persons is potentially intriguing, because DNA repair capacity is related to levels of intracellular antioxidant activity (Schraufstatter *et al.*, 1988). CD4 cell death in HIV-infected individuals is thought by some investigators to result from apoptosis, i.e., programmed cell death, characterized by specific patterns of DNA fragmentation (Ameisen and Capron, 1991; Arends and Wyllie, 1991). Apoptosis can be activated by the endonuclease system, which itself can be activated by oxidative stress (Larrick and Wright, 1990). TNF-a is a potent inducer of apoptosis (Dealtry *et al.*, 1987; Larrick and Wright, 1990). Oxidative stress, secondary to chronic macrophage activation, could thus be a factor in the events leading to CD4 cell depletion. In addition, 8-hydroxyguanosine is often induced in DNA damaged cells. This nucleoside binds to DNA with mutagenic consequences, another potential factor in the development of malignancies (Floyd, 1990).

1.11.10 Apoptosis induction by ROS

It has been shown that hydrogen peroxide is able to induce apoptosis, which is prevented by catalase (Pierce *et al.*, 1991). Besides catalase, high intracellular glutathione levels oppose the apoptosis triggered by ROS. Since then many authors have shown that ROS can induce apoptosis in many different cell systems. For instance H_2O_2 induced apoptosis in neutrophils, which can be prevented by catalase (Kasahara *et al.*, 1997). Catalase also prevented spontaneous neutrophil apoptosis, suggesting that the generation ofH_2O_2 might be one important trigger mechanism responsible for the short life-span of mature neutrophils. In addition,

augmented intracellular glutathione levels prevented Fas receptor-mediated apoptosis in these cells (Watson *et al.*, 1997). Similar studies have been performed in eosinophils (Wedi *et al.*, 1999). The apoptosis- inducing effects were blocked by glutathione and *N*-acetylcysteine. These two antioxidants were also able to block spontaneous and Fas receptor-mediated eosinophil apoptosis (Wedi *et al.*, 1999). Induction of apoptosis using low concentrations and inhibition of apoptosis by higher concentrations was also observed by using phenylarsine oxide in both neutrophils and eosinophils (Yousefi *et al.*, 1994). Taken together, there is growing evidence that ROS are important for the induction of apoptosis in inflammatory but also other cells. The functional importance of ROS generation for the activation of death mechanisms has been demonstrated by inhibitor studies. There are numerous examples of the inhibition of apoptosis through antioxidative drugs or enzymes (Simon *et al.*, 2000).

1.11.11 Role of ROS in triggering death receptor-mediated apoptosis

Receptors of the tumor necrosis factor (TNF)/nerve growth factor (NGF) family display pleiotropic functions, ranging from cell growth and differentiation to cell death (Ashkenazi and Dixit, 1998; Wallach *et al.*, 1999). A subset of this family has been shown to induce apoptosis in several systems and termed death receptors. In addition to the common cystein-rich motifs found in the extracellular domain of all members of the TNF/NGF receptors family, these receptors contain a common sequence in their cytoplasmic domain termed death domain that is sufficient and necessary to induce death via caspase dependent mechanisms (Ashkenazi and Dixit, 1998; Wallach *et al.*, 1999). However, other mechanisms such as ROS generation appear to be equally important. For instance, the antioxidant *N*-acetylcysteine blocked TNF-induced apoptosis in some systems (Cossarizza *et al.*, 1995; Talley *et al.*, 1995) suggesting a functional role of ROS during this process. ROS appear to be mitochondria derived (Schulze-Osthoff *et al.*, 1992; Schulze-Osthoff *et al.*, 1993) and responsible for later mitochondrial events leading to full activation of the caspase cascade. Besides actions on mitochondria, ROS may also

activate sphingomyelinase generating ceramide,(Liu *et al.*, 1998) an intracellular mediator of apoptosis in granulocytes (Hebestreit *et al.*, 1998). Strikingly, TNF induced ROS can also activate anti-apoptotic pathways, at least in some systems (Wong *et al.*, 1989), due to the activation of the transcription factor NF-·B. The anti-apoptotic genes expressed via this pathway are not clear at the moment, but may involve superoxide dismutase (Wong *et al.*, 1989) and, perhaps, catalase. Activation of the Fas receptor (Krammer, 1999) which also belongs to the TNF/NGF receptor family, results in a signal transduction pathway that most likely uses ROS. This assumption is based on the fact that antioxidants blocked Fas receptor-mediated apoptosis (Chiba *et al.*, 1996; Um *et al.*, 1996). Similarly to the TNF system, Fas receptor-induced ROS may generate ceramide (Hebestreit *et al.*, 1998). Moreover, besides induction of apoptosis, ROS have also been shown to be involved in induction of both Fas receptor (Delneste *et al.*, 1996) and Fas ligand (Hug *et al.*, 1997; Bauer *et al.*, 1998) genes.

1.11.12 AIDS and Apoptosis

Infection of CD4+ T cell cultures with HIV is associated with a cytopathic effect of the virus, manifested by ballooning of cells and formation of syncytia leading to cell death by apoptosis of both infected and non-infected cells. CD4+ T cell destruction can be mediated directly by HIV replication as a consequence of viral gene expression, such as gp120-gp41, or indirectly through priming of uninfected cells to apoptosis. Expression of the viral envelope gp120-gp41 complex in infected cells mediates onset of apoptosis of both infected and non-infected cells. Thus chronically HIV-infected cells can serve as effector cells to induce apoptosis in uninfected target CD4+ T cells. Peripheral T lymphocytes from HIV-infected subjects are prematurely primed for apoptosis. This means that those cells are obviously more sensitive to the induction of apoptosis in response to various stimuli than T lymphocytes from uninfected controls. Interestingly, not only T cells of the CD4 subset but also of the CD8 subset are primed for apoptosis. It also was

observed that not only T cells but all blood mononuclear cells, including B cells, T cells, NK cells, granulocytes and monocytes, show increased sensitivity to apoptosis. The central paradox of HIV pathogenesis is that the viral burden, either free or cellular, seems too low to deplete the CD4+ population by direct killing. The observation that an important fraction of T cells are prematurely primed for apoptosis in HIV-infected subjects prompted the hypothesis that some indirect mechanisms are responsible for inappropriate cell death and significantly contribute to CD4+ T cell depletion as well as to CD8+ destruction in AIDS. Indeed, it has been observed that apoptotic T cells in lymph nodes of HIV-infected individuals contained many apoptotic but uninfected bystander cells whereas infected cells were not found to be apoptotic (Gougeon, 2000).

1.11.13 THERAPEUTIC USE OF ANTIOXIDANTS IN HIV INFECTION

1.11.13.1 In vitro

Antioxidant drugs have been demonstrated to have anti-HIV activity in chronically infected monocytes stimulated in tissue culture by phorbol myristate acetate or TNF-a (Kalebic *et al.*, 1991). Antioxidants such as glutathione, glutathione ester, N-acetylcysteine (Kalebic *et al.*, 1991) pentoxyphylline, desferrioxamine (Baruchel *et al.*, 1991), vitamin C, and 2-L-oxothiazolidine have also been shown to have some anti- HIV activity in cultured lymphocyte lines, although the mechanism of action is unclear. The degree of inhibition observed in these studies with regard to production of progeny virus and antigens was shown to have several orders of magnitude below that obtained with nucleoside antagonists of viral reverse transcriptase, such as 3'-azido-3'-deoxythymidine (AZT). For this reason, the antioxidants should not be considered as direct antiretroviral agents but rather as potentially immunomodulating drugs that achieve their effect through indirect mechanisms, possibly through inactivation of

triggers of HIV gene expression, such as NF-kB [(Yamauchi *et al.*, 1990). Diethyldithiocarbamate (DIYTC), which has a GSH peroxidase-like activity, has not shown any in vitro antiviral activity, despite reasonable results in human clinical trials on HIV-infected subjects. In animals, DIYTC has been shown to increase glutathione levels in a variety of tissues (Baruchel and Wainberg, 1992).

1.11.13.2 In vivo

The antioxidant drug that has been extensively studied in clinical trials is Diethyldithiocarbamate (DIYTC). Its efficacy has been demonstrated in two randomized placebo-controlled studies (Lang *et al.*, 2006) and one randomized non-placebo controlled study. In the largest of these trials, 387 patients were randomized to receive either DIYTC or placebo. A significant (56%) reduction in rate of progression to new opportunistic infection was reported in AIDS patients receiving DIYTC as compared to placebo (*p*=0.03).

In a randomized, 8-week double-blind, placebo-controlled trial followed by optional open-label drug for up to 24 weeks in which 81 HIV subjects with no active opportunistic infections were given oral N-acetylcysteine (NAC), whole blood GSH levels significantly increased from 0.88nM to 0.98nM representing 89% over the uninfected controls. Thus NAC offers useful adjunct therapy to increase protection against oxidative stress and improve immune system function. These findings suggest that NAC therapy could be used in other clinical situations in which GSH deficiency or oxidative stress plays a role in the disease pathology (De Rosa *et al.*, 2000).

Caution should probably be exercised before using antioxidant drugs in therapy of HIV-associated disease. An important prerequisite is that good pharmacological monitoring of oxidative stress be enacted as an important part of such trials, along with studies of viral burden in the patients under investigation. Furthermore, whereas low levels of reactive oxygen species might have beneficial biological

effects, it is also likely that excessive antioxidant protection could be deleterious. (Roederer *et al.*, 1990a).

1.12 STATEMENT OF PROBLEM

Evidence supports the premise that a pro-oxidant condition exists in seropositive patients, a result of an overbalance in production of reactive oxygen forms combined with a multilevel deficiency in nutritional and metabolic sources of antioxidants. Apoptosis (a programmed cell death) is recognized as a possible pathway of immune cell loss in patients with HIV infection and AIDS. The cascade of events that results from oxidative stress is markedly similar to that which can initiate apoptosis and includes oxidation of cellular membranes, alteration of metabolic pathways, disruption of electron transport systems, depletion of cellular ATP production, loss of Ca^{2+} homeostasis, endonuclease activation and DNA/chromatin fragmentation (Papadopulos-Eleopulos *et al.*, 1992).

Reduced levels of antioxidants have been found in patients with HIV infection. This raises the question whether only antiretroviral therapy might be beneficial in patients with HIV infection.

The capacity to effectively manage the food and nutrition implications of ART is a critical factor in the success of antiretroviral therapy in resource-limited settings. Failure to address drug-food interactions can reduce drug efficacy, lead to poor adherence to drug regimens, aggravate side effects, or damage the nutritional status of PLWHA. Increased access to ART in developing countries must be accompanied by measures to identify and enable feasible dietary responses to the drugs interactions with food and nutrition. Policies, strategies, and programs involving ART should include mechanisms that provide information and guidance on drug-food interactions and that enable appropriate management of these interactions, especially in food insecure contexts (WHO, 2003).

Clinical trials have identified in detail the vitamin and mineral needs of HIV-positive persons and those with AIDS. These studies suggest the need for increasing the intake of the following micronutrients as supplementation for the prevention and treatment of AIDS: vitamin A and carotenoids, vitamin C, vitamin E, selenium, n-acetyl cisteine, l-glutamine, zinc, copper, manganese, alphalipoic acid, coenzyme Q10, flavonoids or vitamin P, and B-complex vitamins (Dworkin et al, 1986).

There are currently increasing numbers of scientific publications demonstrating that oxidizing stress is an absolute requisite for both testing positive on the tests for HIV and developing the clinical manifestations of AIDS (Fuchs et al, 1991) Moreover, there have been international meetings on the role of oxygen free radicals in HIV/AIDS.

1.13 STUDY HYPOTHESIS

HIV infection sets in motion a variety of cellular derangements including Reactive Oxidative Stress (ROS) which in the absence of antioxidants accelerate immune cell death leading to apoptosis and progression of HIV replication and likely Acquired Immunodeficiency Syndrome (AIDS).

1.14 RATIONALE OF STUDY

Although Highly Active Antiretroviral Therapy (HAART) has resulted in dramatic changes in disease course, long term therapy is limited by development of resistance, toxicity and cost element. The role of nutrients and sound nutrition practices have been well documented in supplementation studies with glutamine, micronutrients, branched chain amino acids, whey proteins etc. As these help strengthen the immune system and reduce the adverse effects of infection related oxidative stress while lengthening survival time (Bijlsma, 1997).

Currently there is a strong scientific opinion that more than 90 percent of diseases are caused by oxidative stress due to active oxygen, other free radicals and lipid peroxides. Active oxygen, though vital to our body, causes considerable damage to the essential components of human body and disturbs the physiologically important functions of proteins, lipids, enzymes and DNA bearing the genetic code. This leads to disorder in our body, which in turn leads to development of diseases and accelerating aging (Bandaru *et al.*, 2007).

It is now generally accepted that a central pathologic feature of HIV disease involves oxidative stress, leading to programmed cell death (apoptosis) and depletion of CD4 cells. Evidence of increased oxidation reactions, depletion of the antioxidant defense system, and increased levels of oxygen radicals have been demonstrated in the blood and tissues of HIV-infected individuals. Elevated levels of hydroperoxides, malondialdehyde and deficiencies of the critical antioxidant enzymes manganese, superoxide dismutase, glutathione peroxidase, thioredoxin, and catalase have been demonstrated in plasma, lung lining, erythrocytes, and lymphocytes in HIV infected persons (Cutler and Jotwani, 2006).

In Ghana, management of PLWHAs is focused on the administration of antiretroviral drugs but research suggests that since the beginning of the AIDS epidemic, free radicals and, specifically, oxidizing agents have been implicated in the pathogenesis of this new syndrome. We envisage that such a finding will augment and perhaps improve the care and management of PLWHAs by reducing the oxidative stress associated with HIV infection.

1.15 AIM AND OBJECTIVES OF RESEARCH

The main aim of this study was to conduct investigations to determine the levels of oxidative stress and their probable relationship as markers of HIV disease progression in PLWHAs

The objectives of the research included:

- To conduct laboratory investigations to review some markers of oxidative stress in PLWHAs to that of age-matched HIV seronegative individuals.

- To assess relevant Hematological parameters in the progression of HIV infection of PLWHAs to that of age-matched HIV seronegative individuals so as to ascertain the trend of these parameters as the HIV infection progresses.

- To assess relevant Biochemical parameters in the progression of HIV infection of PLWHAs to that of age-matched HIV seronegative individuals so as to ascertain the trend of these parameters as the HIV infection progresses.

- To assess the best predictor(s) of markers of oxidative stress in HIV/AIDS progression.

Chapter 2

MATERIALS AND METHODS

2.1 SUBJECTS

Two hundred and twenty eight (228) confirmed People Living with HIV/AIDS (PLWHAs) (36.9±10.9 years) were recruited into the study. The subjects were selected from the Central Regional Hospital (CRH) and Bolgatanga Regional Hospital (BRH). One hundred and forty-three (143) PLWHAs (38.8±6.5 years) were recruited from CRH and they comprised of eighty one (81) males and sixty two (62) females whilst eighty five (85) PLWHAs (37.2±6.7 years) were recruited from BRH comprising of forty three (43) males and forty two (42) females. The mean age of the subjects group was 37.10±0.84 years. One hundred (100) healthy individuals who were HIV-seronegative with similar age and sex distribution were used as controls. The mean age of the control group was 38.76±1.73 years. Table 2.1 shows the summary of the demographic characteristics of the subjects and Control groups.

Inclusion criteria for the study required patients to be HIV positive and not taking multivitamins/mineral supplements for at least 3 months prior to the taking of the blood samples. Patients had to be clinically stable with no active opportunistic infections. Exclusion criteria were as follows: smoking, initiation of antioxidant vitamin therapy before study, diabetes, kidney or liver dysfunction (Bonithon-Kopp *et al.*, 1997).

PLWHAs were placed in three (3) groups according to the Centers for Disease Control and Prevention Criteria (CDC) classification system that emphasizes the importance of CD4+ T lymphocyte testing in clinical management of HIV-infected persons. The system is based on three ranges of CD4 counts (1) ≥500 mm^{-3}; (2) 200–499 mm^{-3}; and (3) <200 mm^{-3} . of the total subjects recruited, 18.9% had CD4

counts ≥500 mm^{-3}, 27.2% had CD4 counts between 200–499 mm^{-3} and 53.9% had CD4 counts <200 mm^{-3}.

Control subjects were one hundred (100) sex- and age-matched healthy (39.4±13.4 years), HIV-seronegative individuals. All subjects gave informed consent to take part in the study after verbal and written explanation of the methods and risks involved.

This study was approved by the Committee on Human Research, Publications and Ethics (CHRPE), School of Medical Sciences, Kwame Nkrumah University of Science & Technology (KNUST), Kumasi. All patients enrolling in the study completed a written informed consent form in accordance with the Helsinki Declaration.

Table 2.1 Demographic Characteristics of the Subjects and Controls. The values are expressed as mean ± SEM

Parameter	Control	Total Subjects	CD4>500	CD4 200-500	CD4<200
Age, (years)	38.76± 1.73	37.10±0.84	38.72±2.58	37.43±1.57	36.68±1.08
Males, (n)	72	157	22	47	88
Females, (n)	28	71	21	16	34
CD4 count, (mm^{-3})	1045.45±77.53	226.0±21.17***	823.1±60.75	310.70±14.44***	84.30±77.53***
HIV serotype 1, (n)	-	138	22	34	82
HIV serotype 2, (n)	-	1	-	-	1
HIV serotype 1 and 2,(n)	-	89	20	27	42

2.2 SAMPLE COLLECTION

Blood samples were collected from the ante cubital vein. Rubber tourniquet was applied for less than one minute and the site to be punctured cleaned with 70% methylated spirit. 8ml to 10ml of blood was taken. 2ml of the blood was placed in anticoagulated sequestrene bottles-EDTA for haematological analysis and some of the oxidative stress markers analysed in the study. The samples were then mixed by hand to prevent clotting by turning the EDTA tubes up and down and then transferred onto mechanical mixer to dissolve the anticoagulant.

The rest of the blood samples were placed in plain tubes and allowed to clot. After the blood had clotted it was placed in a centrifuge and spun at 3000 x g for 10 minutes to obtain the sera. The sera obtained were immediately used for the determination biochemical assay and some of the markers of oxidative stress analysed in the study.

2.3 IMMUNOLOGICAL ASSAY

The immunological markers that were determined were absolute cell counts of CD4 and CD3 T lymphocytes in unlysed whole blood. The CD4 and CD3 T lymphocytes counts were determined using the Becton Dickinson (BD) FACSCount system (Becton, Dickinson and Company, Califonia, USA).

2.3.1 BD FACS Count System

The BD FACSCount system is a compact, self-contained system, incorporating instrument, reagents, and controls, for automatic counting of CD4, CD8, and CD3 T lymphocytes. The BD FACS Count system uses whole blood, eliminating lyse and wash steps. A unique software algorithm automatically identifies the lymphocyte populations of interest. The BD FACS Count system precisely measures absolute numbers of CD4-positive T lymphocytes, the cellular parameter most closely associated with HIV/AIDS disease progression and therapy decisions. It gives consistent, accurate results even with low CD4 counts. In

addition, absolute counts of CD3- and CD8-positive T lymphocytes, and the helper/suppressor ratio (CD4-positive: CD8-positive T lymphocytes) are measured. The BD FACS Count system offers a complete T lymphocyte panel to monitor immune status.

2.3.2 CD4 and CD3 T Lymphocytes Quantification

The BD FASCount system used flow cytometry for the quantification of CD4 and CD3 T Lymphocytes. Flow cytometry uses the principles of light scattering, light excitation, and emission of fluorochrome molecules to generate specific multi-parameter data from particles and cells in the size range of 0.5um to 40um diameter. Flow cytometry is the recognized gold standard for CD4 testing, and is used to stage HIV/AIDS, guide treatment decisions for HIV-infected persons, and evaluate effectiveness of therapy.

2.4 MARKERS OF OXIDATIVE STRESS

2.4.1 Malondialdehyde (MDA) Determination

MDA levels were determined by the MDA- Thiobarbituric acid (TBA) test which is the colorimetric reaction of MDA and TBA in acid solution. TBA reacted with MDA, a secondary product from lipid peroxidation, which generated an adduct of red colour, which was detected spectrophotometrically. This method is a fast, sensitive, and low-cost method that can be used to indicate the extent of lipid peroxidation in a variety of systems (Shlafer and Shepard, 1984).

The method used for this assay was based on that of (Kamal *et al.*, 1989). Serum (0.5 ml) was added to 2.5 ml of 20% trichloroacetic acid (TCA) and then 1 ml of 0.67% TBA. The mixture was boiled at 100^{o}C for 30 minutes. After cooling, the sample was extracted with 4 ml n-butanol and centrifuged at 3000 rpm for 10 min. The absorbances of supernatant were measured at 535 nm and the results were

expressed as µmol/l, using the extinction coefficient of 1.56 x 10^5l/mmol cm. The concentrations were calculated as follows:

$$C = \frac{A}{\epsilon l}$$

Where C= concentration

A= absorbance

ϵ= extinction coefficient

l= light path

2.4.2 The Ferric Reducing/Antioxidant Power (FRAP) Assay

Serum total antioxidant status was evaluated using ferric reducing antioxidant power (FRAP) assay The FRAP assay used antioxidants as reductants in a redox-linked colorimetric method. In this assay, at low pH a ferric-tripyridyltriazine (Fe 3++-TPTZ) complex was reduced to the ferrous form, which was blue colored and monitored by measuring the change in absorption at 593 nm. Acetate buffer (300 mmol/l, pH 3.6); 10 mmol/l 2,4,6-tri-pyridyl-s-triazine (TPTZ) in 40 mmol/l HCl and 20 mmol/l $FeCl_3$_$6H_2O$ in the ratio of 10:1:1 giving the working FRAP reagent. FRAP reagent (750µl) was mixed with 25µl serum or standard in a test tube. After exactly 10 min at 25^{o}C, the absorbance at 593 nm was read against reagent blank. Fe (II) standards were used. The change in absorbance was directly proportional to the reducing power of the electron-donating antioxidants present in the serum. The absorbance change was translated into a FRAP value (in µmol/lit) by relating the change of absorbance at 593 nm of test sample to that of a standard solution of known FRAP value. The concentrations in mmol/lwere calculated as follows:

$$\boldsymbol{C\,(t) = \frac{A\,(t)}{A\,(s)}\,x\,C\,(s)}$$

Where C (t) = Concentration of test

A (t) = Absorbance of test

A (s) = Absorbance of standard

C (s) = concentration of standard

2.4.3 Vitamin C (ascorbic acid)

Vitamin C in the serum was determined by micro technique using Dinitrophenylhydrazine (DNPH) by (Natelson, 1961). Ascorbic acid in serum was oxidized by Cu (II) to form dehydroascobic acid, which reacted with acidic 2,4 ditrophenylhydrazine to form a red dishydrazone, which was measured at 520nm. Reagents that were used for the assay included:

- Ascorbic acid standard- 100mg of ascorbic acid plus 1ml of glacial acetic acid and this was diluted to 100ml of distilled water.
- 10% Trichloroacetic acid (TCA) - 10grams of TCA and was diluted with 100ml of distilled water.
- Dinitrophenylhydrazine reagent- 2grams of DNPH was added to 0.25grams of thiourea and 0.03grams of $CuSO_4.5H_2O$ and this was made to 100ml with 9M H_2SO_4. 50ml of concentrated H_2SO_4 was further added and 150ml of distilled water was added.
- 65% H_2SO_4 -70ml of concentrated H_2SO_4 was added to 30ml of distilled water.

The sample was prepared by rapidly adding 1.6ml of 10% TCA to 0.4ml of serum. This was mixed well and allowed to stand for 5 minutes at room temperature. The

mixture was then centrifuged at 1006 x g. The aliquot (1ml) was transferred to a test tube.

	Standard	Test	Blank
Sample	-	1ml	-
Standard	1ml	-	-
TCA			1ml
DNPH reagent	0.4ml	0.4ml	0.4ml

This was stoppered and incubated at 37°C for three hours. It was then chilled in an ice bath and 1.6ml of cold 65% H_2SO_4 was added to it. This was then allowed to stand at 25°C for 30 minutes. The standard and the test were then read against the blank at 520nm. The concentration of the test was calculated as follows:

$$\boldsymbol{C\,(t) = \frac{A\,(t)}{A\,(s)}\; x\; C\,(s)}$$

Where C (t) = Concentration of test

A (t) = Absorbance of test

A (s) = Absorbance of standard

C (s) = concentration of standard

2.4.4 Vitamin E (Tocopherol)

Serum tocopherol was measured by the method described by (Baker H and Frank O, 1969). Serum tocopherol was measured by the reduction of ferric to ferrous ions which then formed a red complex with alpha, alpha dipyridyl. Tocopherol was then extracted in xylene followed by the addition of ferric chloride. The mixture was then read at 520 nm. Reagents used in the assay included:

- Absolute alcohol
- Xylene
- Alpha, alpha dipyridyl- 1.20 g/l in n-propanol
- Ferric chloride- 1.20 g $FeCl_3.6H_2O$ in ethanol.
- Standard solution- 10 mg/l DL-alpha-tocopherol in ethanol.

	Standard	Test	Blank
Serum	-	1.5ml	-
Standard	1.5ml	-	-
Distilled water	1.5ml	-	1.5ml
Ethanol	-	1.5ml	1.5ml
Xylene	1.5ml	1.5ml	1.5ml

The test samples and the standard were then centrifuged and 1.0 ml of the xylene layer transferred into clean test tubes. 1.0ml of dipyridyl reagent was then added to each tube followed by 0.33 ml of ferric chloride. The absorbance was then read at 520 nm. The concentration of the test was calculated as follows:

$$\boldsymbol{C\,(t) = \frac{A\,(t)}{A\,(s)}\,x\,C\,(s)}$$

Where C (t) = Concentration of test

A (t) = Absorbance of test

A (s) = Absorbance of standard

C (s) = concentration of standard

2.4.5 Glutathione Peroxidase (GPx)

The glutathione peroxidase (GP_X) was determined using the RANDOX RANSEL® reagent. Glutathione peroxidase (GPx) is a selenium dependent enzyme. There is a correlation between GPx and selenium status. GPx levels provide a more accurate reflection of selenium status than measuring the levels of selenium directly by atomic absorption, because concentrations of the enzyme are less affected by sudden dietary changes. The method used was based on that of (Valentine, 1967). GPx catalysed the oxidation of glutathione (GHS) by cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH the oxidized glutathione (GSSG) was immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance was then measured at 340nm.

$$2GSH + ROOH \xrightarrow{GPX} ROH + GSSG + H_2O$$

$$GSSG + NADPH + H^+ \xrightarrow{GR} NADP^+ + 2GSH$$

The sample was prepared by diluting 0.05ml of anticoagulated whole blood with 1.0ml of the diluting reagent. This was incubated for five minutes and 1.0ml of haemoglobin reagent was added. This was mixed well and assayed with 20 minutes of adding the haemoglobin reagent.

	Diluted sample (ml)	**Reagent blank (ml)**
Diluted sample	0.02	-
Distilled water	-	0.02
Glutathione reagent	1.0	1.0
Cumene reagent	0.04	0.04

The test tubes were mixed and the initial absorbance of the sample and reagent blank was read after one minute and the timer was started simultaneously. The

absorbances were read again after one and two minutes. The absorbance of the reagent blank was subtracted from that of the sample. The glutathione peroxidase concentration was calculated as follows:

$$\boldsymbol{glutathione\ peroxidase\ concentration\ (U/l)} = \mathbf{8412}\ \boldsymbol{X\ change\ of\ absorbance\ per\ minute}$$

2.4.6 Superoxide Dismutase (SOD)

The superoxide dismutase was determined using the RANDOX RANSOD® reagent. The method used employed xanthine and xanthine oxidase (XOD) to generate superoxide radicals which reacted with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. The superoxide dismutase activity was then measured by the degree of inhibition of the reaction.

$$\boldsymbol{xanthine \xrightarrow{XOD} uric\ acid + O_2}$$

$$\boldsymbol{INT \xrightarrow{O_2} formazan\ dye}$$

$$\boldsymbol{O_2 + O_2 + 2H^+ \xrightarrow{SOD} O_2 + H_2O_2}$$

The samples were prepared by centrifuging 0.5ml of whole blood for 10 minutes at 3000rpm and the plasma was aspirated. The erythrocytes were then washed four times in 3.0ml of the NaCl solution by centrifuging at 3000rpm for 10 minutes for each wash. 2.0 ml of cold distilled water was then added to the washed erythrocytes and the lysate was left to stand at +4^{o}C for 15 minutes. The lysate was then diluted with 25 fold of the phosphate buffer. Serial dilutions of the standard were also prepared with distilled water.

	Sample diluent (ml)	Standards (ml)	Diluted sample (ml)
Diluted sample	-	-	0.05
Standard		0.05	-
Phosphate buffer	0.05	-	-
Mixed substrate	1.7	1.7	1.7
Mix well			
Xanthine oxidase	0.25	0.25	0.25

The test tubes were mixed well and the initial absorbance A_1 read after 30 seconds. The final absorbance A_2 was read after 3 minutes. The SOD concentration was calculated as follows:

$$\frac{\mathbf{A2 - A1}}{\mathbf{3}} = \boldsymbol{change\ in\ absorbance\ of\ standard\ or\ sample}$$

All standard and sample rates were converted to percentages of the sample diluents rate and subtracted from 100% to give the percentage inhibition.

$$\mathbf{100} - \frac{\boldsymbol{A\ (standard\ or\ diluted\ sample\ x\ 100)}}{\boldsymbol{A\ (sample\ diluent)}} = \%\ \boldsymbol{inhibition}$$

The percentage inhibition for each standard was plotted against Log_{10} of the standard concentration. The units of SOD of the diluted samples were then obtained from the standard curve.

$$\boldsymbol{SOD\ units\ per\ ml\ of\ whole\ blood} = \boldsymbol{SOD\ units\ per\ ml\ from\ standard\ curve\ x\ dilution\ factor}$$

2.5 HAEMATOLOGICAL ASSAY

Various haematological parameters including white blood cell count (WBC), haemoglobin concentration (HGB), haematocrit (HCT), Mean Cell Volume (MCV), Mean Cell Haemoglobin (MCH) and Mean Cell Haemoglobin Concentration (MCHC) were determined by automated blood analyzer (CELL-DYN 1800, Abbott Laboratories Diagnostics Division, USA).

2.5.1 The CELL-DYN (R) 1800

The CELL-DYN (R) 1800 is an automated, multiparameter haematology analyzer designed for in vitro diagnostic use in the clinical laboratory. It is menu-driven, controlled by a microprocessor, and comes in an Open System model.

2.5.2 Mode of Operation of the CELL-DYN (R) 1800

2.5.2.1 Aspiration

The CELL-DYN (R) 1800 aspirated approximately 30 µL (microliters) of whole blood from an open collection tube that has been held under the Sample Aspiration Probe, and transferred the sample to the Pre-Mixing Cup.

2.5.2.2 Dilution

A 7.5-milliliter (mL) volume of diluent was added to the Pre-Mixing Cup to attain a dilution ratio of 1:251. The diluted sample was then divided into two. One hundred microlitres of the 1:251 µL sample dilution was aspirated and mixed with an additional 5 mL of diluent in the RBC/PLT mixing chamber and this gave a dilution ratio of 1:12801 µL. A specimen of the 1:12801 µL dilution was analyzed and this generated results for the red blood cell and platelet parameters.

The remainder of the 1:251 µL sample dilution was then mixed with 1.0 mL of lyse reagent in the WBC Mixing Chamber. The lyse reagent ruptured the membrane of each red blood cell and this caused the cytoplasm and hemoglobin to be quickly released.

2.5.2.3 Cell Measurement

The CELL-DYN (R) 1800 used two independent measurement methods which were Electrical Impedance Method for determining WBC, RBC, and PLT data and Modified Methemoglobin Method for determining HGB.

2.5.2.4 Electrical Impedance Measurement

This method was based on the measurement of changes in electrical resistance created by a particle suspended in a conductive diluent as it passed through an aperture of known dimensions.

2.5.3 White Blood Cell Count (WBC) Determination

Electrical impedance was used to count the White Blood Cells (WBC) as they pass through the aperture of the von Behrens WBC transducer. As each cell was drawn through the aperture, a change in electrical resistance occurred and this generated an equivalent voltage pulse. The number of pulses sensed during each cycle corresponded to the number of white cells counted. The amplitude of each pulse was essentially proportional to the cell volume.

2.5.3.1 WBC Measurement Process

The 1:251 WBC/HGB dilution was delivered to the WBC mixing chamber where it was bubble-mixed with 1.0 mL of lyse reagent. A metered volume of the lysed sample was drawn through the aperture into the Counting Chamber by vacuum. The WBCs were then counted by impedance.

2.5.4 Haemoglobin concentration (Hb) Determination

A modified Methemoglobin method was used for the colorimetric determination of hemoglobin. A portion of the lysed, diluted sample from the WBC Mixing Chamber was used for Hb measurement. A low-energy Light-Emitting Diode (LED) was used as the light source. The LED shone through the Hb flow cell and a 540 nm narrow-bandwidth filter onto a photo detector. The Hb concentration was directly proportional to the absorbance of the sample.

2.5.5 MCV, MCH and MCHC Determination

The CELL-DYN 1800 determined the mean cell volume (MCV) from the RBC size-distribution data. Hematocrit (HCT) results were calculated from the RBC count and the MCV value as follows:

$$MCV = \frac{HCT\ x\ 10}{RBC}$$

Mean Cell Hemoglobin (MCH) and Mean Cell Hemoglobin Concentration (MCHC) values were calculated automatically whenever appropriate parameters were measured, for example, red blood cell count (RBC), hematocrit (HCT), and hemoglobin (HGB). Results for the Mean Cell Hemoglobin (MCH) were calculated from the HGB and RBC count as follows:

$$MCH = \frac{Hb}{RBC}\ x\ 10$$

Results for the Mean Cell Hemoglobin Concentration (MCHC) were calculated from the HGB and RBC count as follows:

$$MCHC = \frac{Hb}{HCT}\ x\ 100$$

2.6 BIOCHEMICAL ASSAYS

Various biochemical assays including total Protein, albumin, total cholesterol, triglycerides, High Density Lipoprotein (HDL)-Cholesterol and Low Density Lipoprotein (LDL)-Cholesterol were analysed using the ATAC® 8000 Random Access Chemistry System (Elan Diagnostic Systems, USA).

2.6.1 Total Protein

The method used for this assay was based on a colour reaction of protein molecules with cupric ions and this was known as the Biuret colour reaction. This procedure was first described by (Riegler, 1914). This procedure was later modified by (Weichselbaum, 1946) and (Gornall *et al.*, 1948) where sodium potassium tatrate was introduced to stabilise the cupric ions in the alkaline reagent.

$$\boldsymbol{Protein + Cu^{2+} \xrightarrow{Alkaline} Colour\ complex}$$

Protein in the serum formed a blue coloured complex when it reacted with cupric ions in the alkaline solution. The intensity of the violet colour was directly proportional to the amount of protein present in the serum and this was measured at 540nm.

2.6.2 Albumin

The method used for this assay was based on that of (Doumas *et al.*, 1971) where at a controlled pH, bromocresol green formed a coloured complex with albumin. The intensity of the colour at 630nm was directly proportional to the albumin in the serum.

2.6.3 Cholesterol

This method for the measurement of total cholesterol (Allain *et al.*, 1974) in serum involves the use of three enzymes: cholesterol esterase (CE), cholesterol oxidase (CO) and peroxidase (POD). In the absence of the former the mixture of ethyl-N-propyl-m-anisidine (ADPS) and 4-aminoantipyrine (4-AA) are condensed by hydrogen peroxide to form a quinoneimine dye proportional to the concentration of cholesterol in the sample. The intensity of the red colour produced is directly proportional to the total cholesterol in the sample when read at 500 nm.

$$\boldsymbol{Cholesterol\ Esters \xrightarrow{CE} Cholesterol + Fatty\ Acids}$$

$$\boldsymbol{Cholesterol + O_2 \overset{CO}{\rightarrow} Cholest-4-en-3-one + H_2O_2}$$

$$\boldsymbol{4-AA + ADPS + H_2O_2 \overset{POD}{\longrightarrow} Quinoneimine\,(red\,dye) + 4H_2O}$$

2.6.4 Triglycerides

The present method uses a modified Trinder (Barham and Trinder, 1972) colour reaction to produce a fast, linear, endpoint reaction(Fossati and Prencipe, 1982). Triglycerides in the sample are hydrolyzed by lipase to glycerol and fatty acids. The glycerol is then phosphorylated by ATP to glycerol-3-phosphate (G3P) and ADP in a reaction catalyzed by glycerol kinase. G3P is then converted to dihydroxyacetone phosphate (DAP) and hydrogen peroxide by glycerophosphate oxidase (GPO). The hydrogen peroxide then reacts with 4-aminoantipyrine (4-AAP) and 3, 5-dichloro-2-hydroxybenzen (3,5-DHBS) in a reaction catalyzed by peroxidase to yield a red coloured quinoneimine dye. The intensity of the colour produced is directly proportional to the concentration of triglycerides in the sample.

$$\boldsymbol{Triglycerides + H_2O \xrightarrow{Lipase} Glycerol + Fatty\ Acids}$$

$$\boldsymbol{Glycerol + ATP \xrightarrow{Glycerol\ kinase} G3P + ADP}$$

$$\boldsymbol{G3P + O_2 \xrightarrow{Glycerolphosphate\ oxidase} DAP + H_2O_2}$$

$$\boldsymbol{H_2O_2 + 4AAP + 3,5-DHBS \xrightarrow{Peroxidase} Quinoneimine\,(red\,dye) + 2H_2O}$$

2.6.5 HDL-Cholesterol

The method employed herein is in a two reagent format. The first reagent contains anti human β-lipoprotein antibody which bind to lipoproteins (LDL, VLDL and

chylomicrons) other than HDL. The second reagent contains enzymes which then selectively react with the cholesterol present in the HDL particles. Consequently only HDL cholesterol is subject to cholesterol measurement. The intensity of the red colour produced is directly proportional to the HDL-cholesterol in the sample when read at 500 nm.

2.6.6 LDL-Cholesterol

The LDL-Cholesterol concentration (LDL-C) is calculated from the total cholesterol concentration (TC), HDL-Cholesterol concentration (HDL-C) and the triglycerides concentration (TG) according to Friedewald found below (Friedewald *et al.*, 1972).

$$\boldsymbol{LDL = TC - HDL - \frac{TG}{2.2}} \quad \boldsymbol{mmol/l}$$

2.6.7 Statistical Analysis

The OUTLIERS preliminary test for detection of error values was initially applied for statistical analysis. The results were given as mean ± Standard error of mean (SEM). Statistical comparisons were analysed using the one way ANOVA followed by Bonferroni's Multiple Comparison test. Correlations were evaluated using the Pearson's correlation test. The Receiver Operator Characteristic (ROC) was used to analyse the sensitivity and specificity of the assays used in the analysis of the samples. For all statistical comparisons, the level of significance was set at $p<0.05$. All data analysis in this research was done using GraphPad Prism for Windows version 4.02 (GraphPad Software, San Diego, CA, USA) and MedCalc ® for Windows version 9.4.2.0.

Chapter 3

RESULTS

3.1 MARKERS OF OXIDATIVE STRESS

3.1.1 Ferric Reducing Ability of Plasma (FRAP)

In the FRAP test, there was a significant difference ($F_{3, 228}$=119.9; p<0.0001) between the test subjects and the control group. The mean serum FRAP levels of the test subjects were 563.4±17.70 µmol/l, 463.3±13.28 µmol/l and 342.0±9.93 µmol/l for CD4 >500 mm^{-3}, 200-499 mm^{-3} and <200 mm^{-3} respectively and that of the control subjects was 923.3±36.91 µmol/L as shown in Fig 3.1A. Using the Bonferroni's Multiple Comparison Test, there was significant difference between the control group and various groups of the subjects (p<0.0001). There was also significant difference amongst the various groups of the subjects when they were compared against each other (p< 0.0001). From the Pearson's correlation, it was observed there was a strong positive correlation (R=0.618; p<0.001) between the serum FRAP levels of the test subjects and their respective CD4 count as shown in Fig. 3.1B.

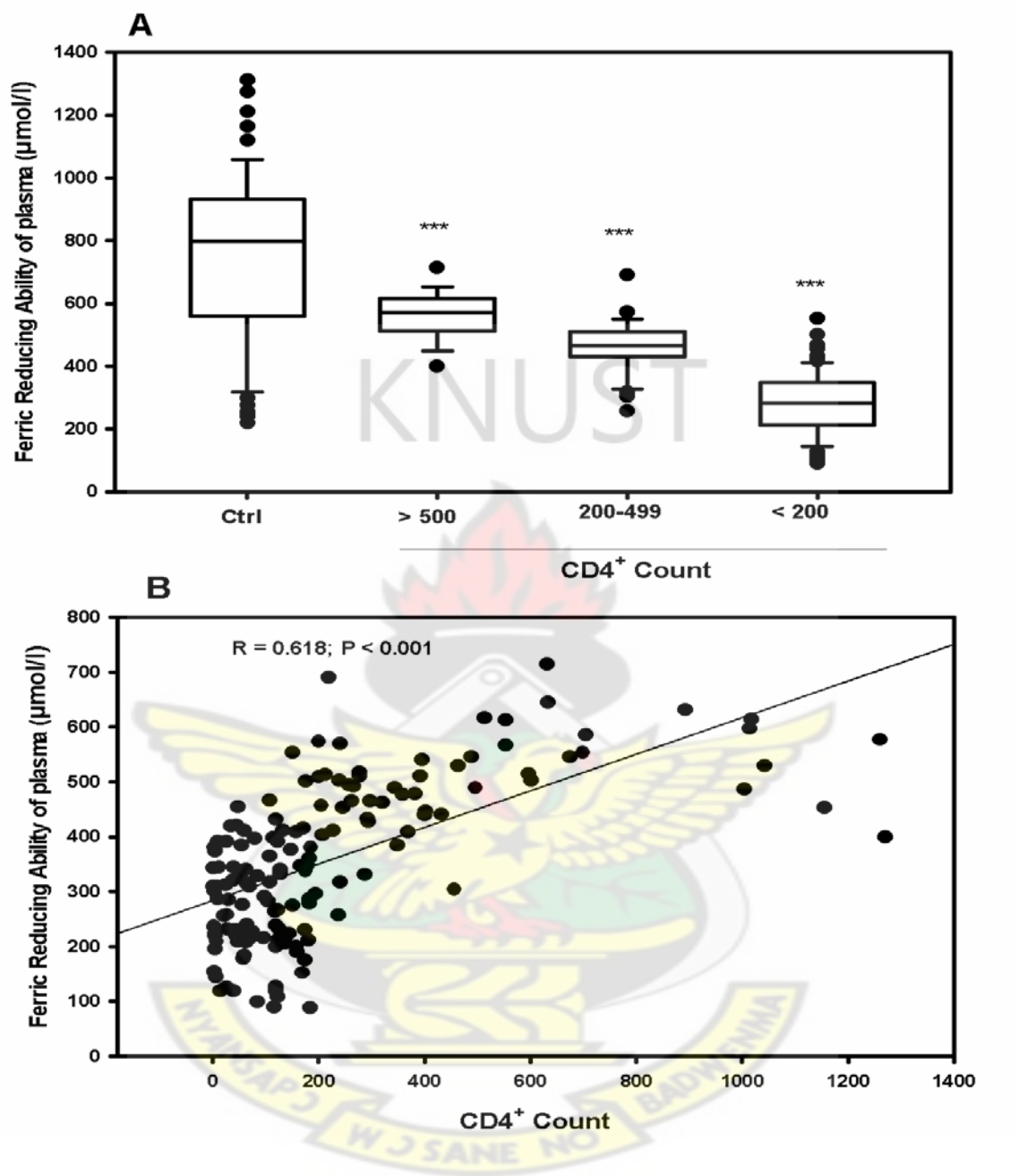


Figure 3.1 (A) Comparison of FRAP concentration in controls and CD4 stages. (B) Linear regression between CD4 count and serum FRAP of the Test subjects. The results are presented as means ± SEM. ***$P \leq 0.0001$ determines the level of significance when the respective subjects were compared to control.

3.1.2 Malondialdehyde (MDA)

Analysis of serum MDA gave a significant difference between the Test subjects ($F_{3, 228}$=109.8; p<0.0001) and the control. The mean serum MDA levels of the Test subjects were 2.68±0.09 mmol/l, 2.77±0.10 mmol/l and 3.17±0.13 mmol/l for CD4 >500 mm-3, 200-499 mm^{-3} and <200 mm^{-3} respectively and that of the control subjects was 1.54±0.04 mmol/l as shown in Figure 3.2A. Comparison of the control group and the various test groups gave a significant difference (p<0.0001). From the Pearson's correlation, it was deduced that there was a significant negative correlation (r= 0.063; p< 0.001) between the serum MDA levels of the test subjects and their respective CD4 counts as shown in Figure 3.2B.

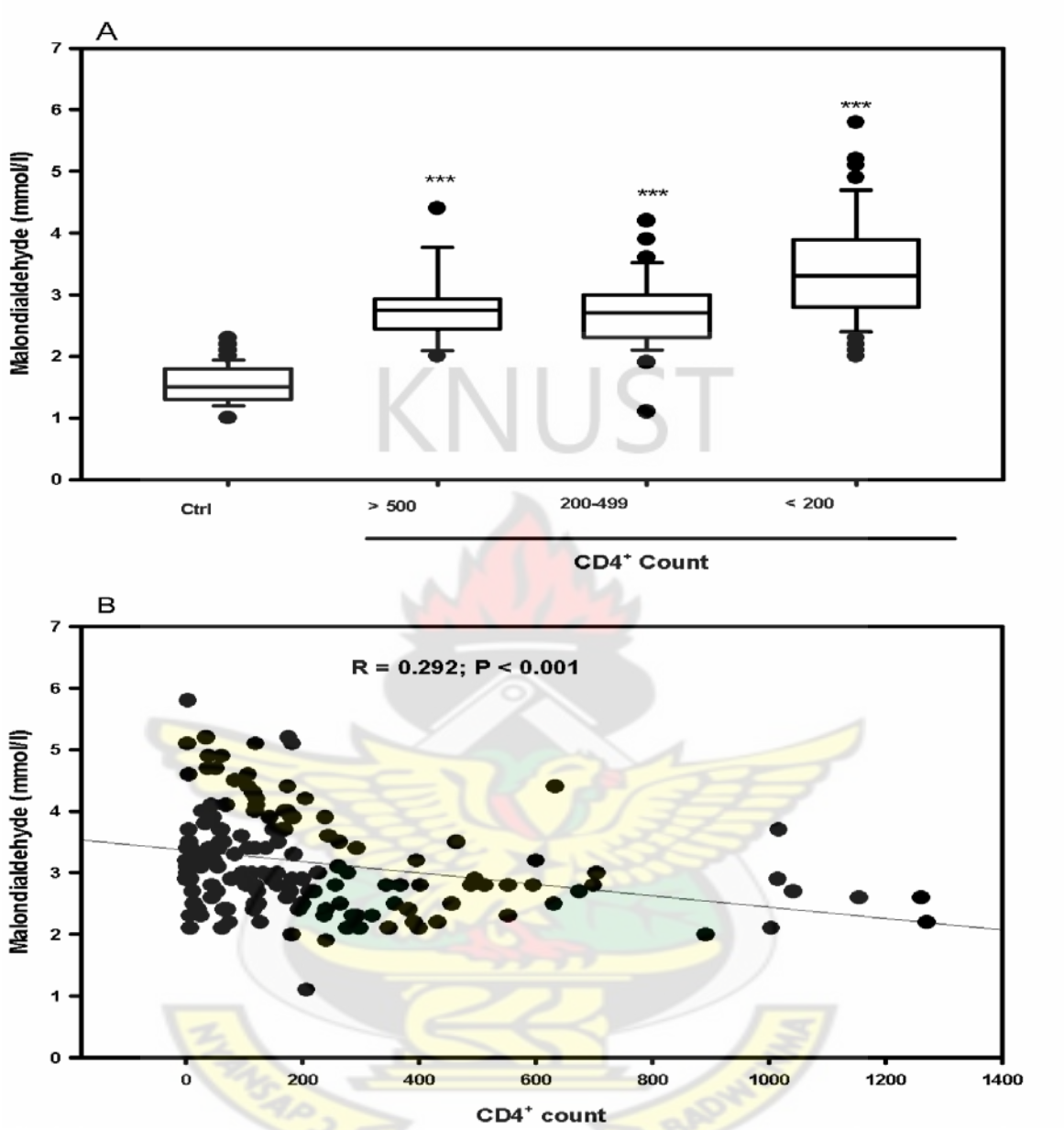


Figure 3.2 Comparison of MDA concentration in controls and CD4 stages. (B) Linear regression between CD4 count and serum MDA of the Test subjects. The results are presented as means ± SEM. ***$P \leq 0.0001$ determines the level of significance when the respective subjects were compared to control.

3.1.3 Superoxide Dismutase (SOD)

Blood SOD revealed a significant difference between the Test subjects ($F_{3,\ 228}$= 677.2; $p<0.0001$) and the control group. The mean blood SOD of the test subjects were 155.72±2.787 U/ml, 116.19±2.412 U/ml and 85.98±0.8924 U/ml for CD4 >500 $mm^{-3,}$ 200-499 mm^{-3} and <200 mm^{-3} respectively and that of the control subjects was 217.16±3.914 U/ml (Figure 3.3A). Correlation analysis revealed a significant difference (R= 0.812; $p<0.001$) between the blood SOD levels of the subjects and their respective CD4 count as shown in Figure 3.3B.

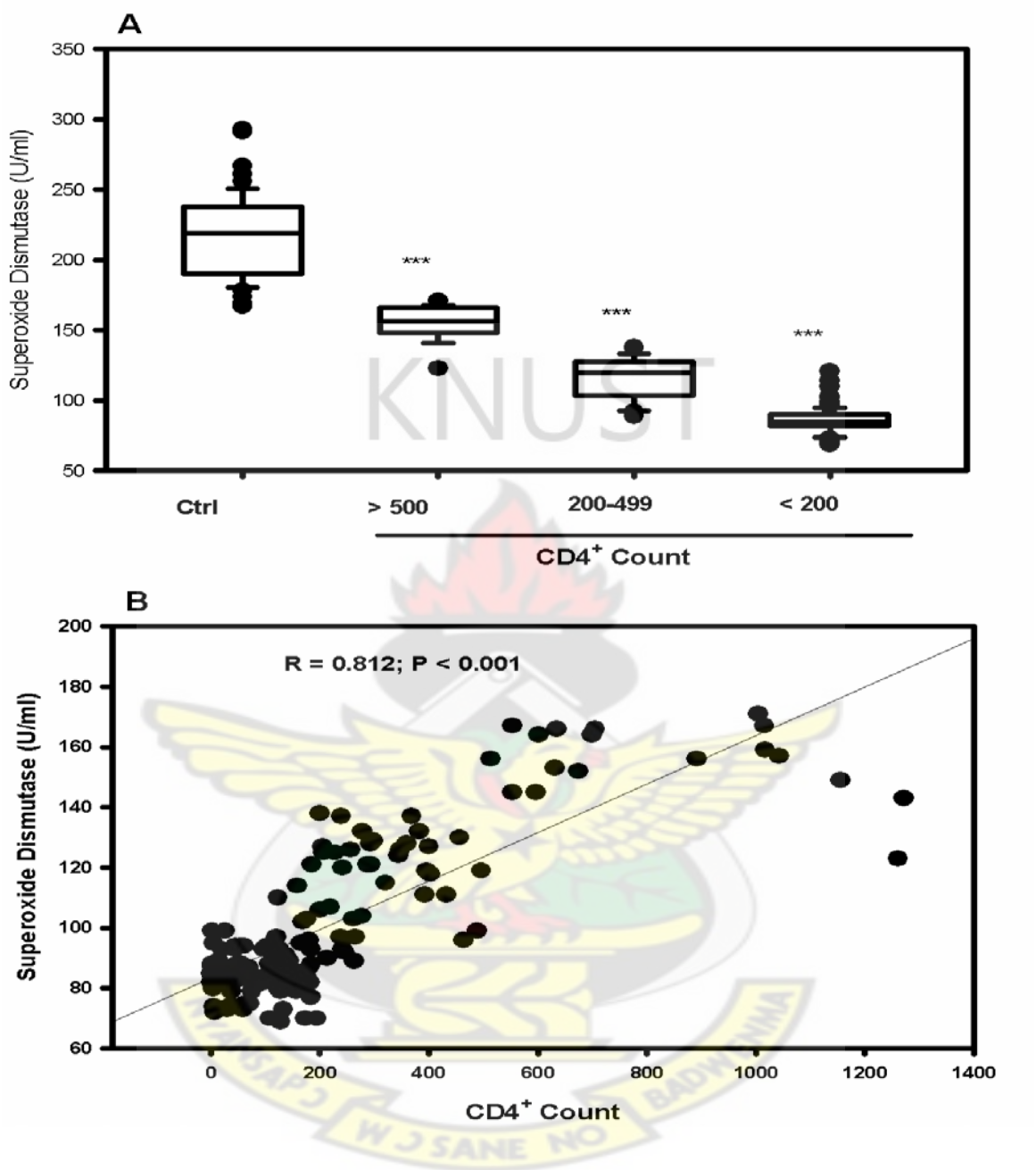


Figure 3.3 Comparison of SOD concentration in controls and CD4 stages. (B) Linear regression between CD4 count and blood SOD of the Test subjects. The results are presented as means ± SEM. ***$P \leq 0.0001$ determines the level of significance when the respective subjects were compared to control.

3.1.4 Glutathione Peroxidase

Using the one-way ANOVA analysis, it revealed a significant difference between the Test subjects ($F_{3, 228}$= 20.3, p<0.0001) and the control subjects. The mean blood Glutathione Peroxidase were 5599±179.4 U/l, 4234±124.0 U/l and 3547±52.16 U/l for CD4 >500 mm^{-3}, 200-499 mm^{-3} and <200 mm^{-3} respectively and that of the control subjects was 5126±283.54 U/l (Figure 3.4A). Correlation of the blood glutathione peroxidase levels of the Test subjects with their respective CD4 counts revealed a significant correlation (R=0.652; p< 0.001) which is shown in Figure 3.4B.

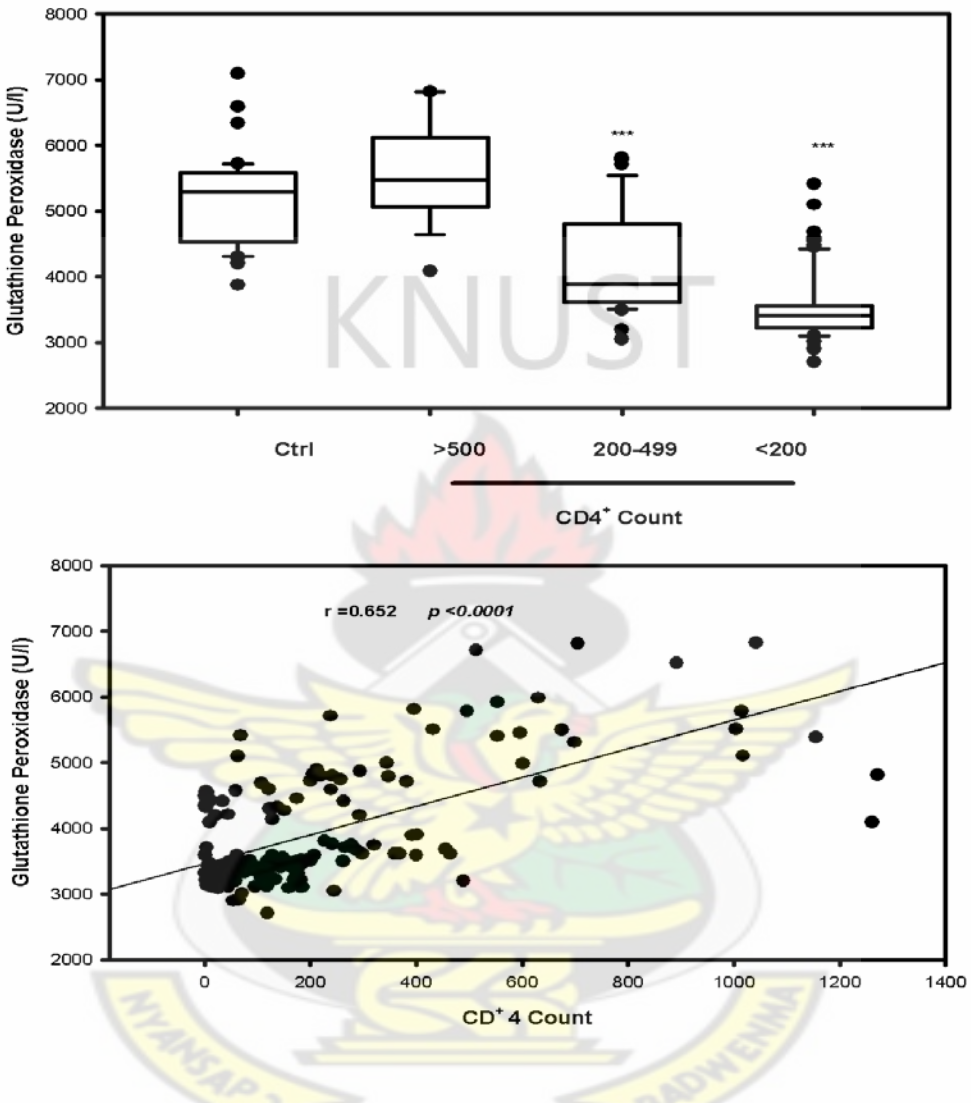


Figure 3.4 Comparison of GPx concentration in controls and CD4 stages. (B) Linear regression between CD4 count and blood GPx of the Test subjects. The results are presented as means ± SEM. ***P ≤ 0.0001 determines the level of significance when the respective subjects were compared to control.

3.1.5 Vitamin C

The data obtained from the study showed a significant difference ($F_{3,\ 228}$= 35.2, p<0.0001) between the serum vitamin C levels of the test subjects and that of the control subjects using the one-way ANOVA analysis. The mean serum Vitamin C levels of the test subjects were 52.72±4.496 µmol/l, 40.85±2.509 µmol/l, 27.66±1.201 µmol/l for CD4 >500 mm^{-3}, 200-499 mm^{-3} and <200 mm^{-3} respectively and that of the control subjects was 58.29±2.352 µmol/l as shown in Figure. 3.5A. Using the Bonferroni's Multiple Comparison Test, there was significant difference between the control group and various groups of the subjects (p<0.0001). There was also significant difference amongst the various groups of the subjects when they were compared against each other (p< 0.0001). From the Pearson's correlation, it was observed that there was a strong positive correlation (r= 0.472; p< 0.001) between the serum Vitamin C levels of the test subjects and the CD4 count (Fig. 3.5B).

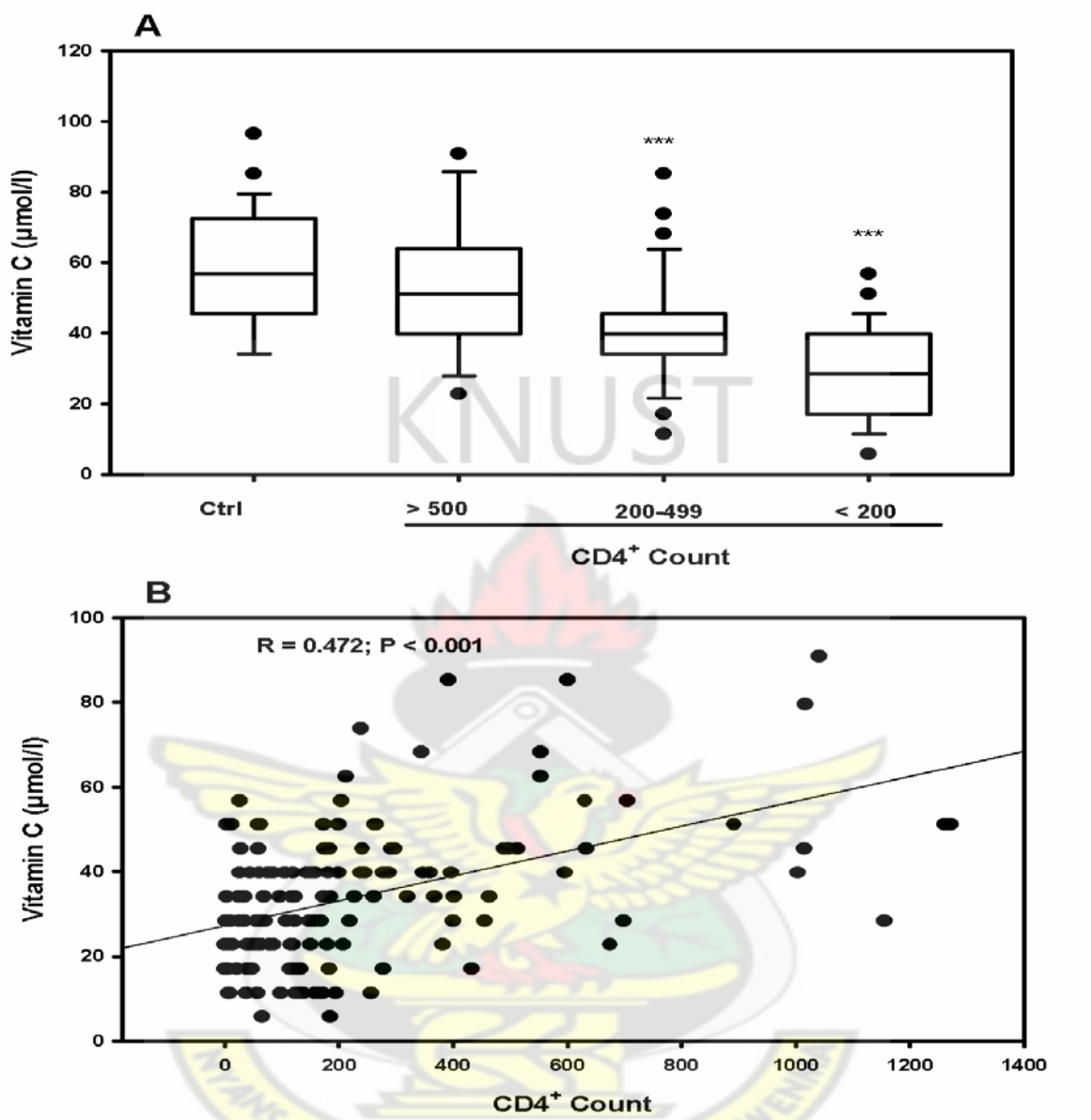


Figure 3.5 Comparison of Vitamin C concentration in controls and CD4 stages. (B) Linear regression between CD4 count and serum Vitamin C of the Test subjects. The results are presented as means ± SEM. ***$P \leq 0.0001$ determines the level of significance when the respective subjects were compared to control.

3.1.6 Vitamin E

Results obtained from the study showed significant difference ($F_{3, 228}$= 35.2, p<0.0001) between the serum vitamin E levels of the test subjects and that of the control subjects using the one-way ANOVA analysis. The mean serum Vitamin E levels of the test subjects were 19.71±1.25 µmol/l, 13.96±0.45 µmol/l, 7.32±0.29 µmol/l for CD4 ≥500 mm^{-3}, 200-499 mm^{-3} and ≤200 mm^{-3} respectively and that of the control subjects was 21.14±0.56 µmol/l as shown in Fig. 3.6A. Using the Bonferroni's Multiple Comparison Test, there was significant difference between the control group and those with CD4 count ≤200 mm^{-3} and those with CD4 count between 200-499 mm^{-3} (p<0.0001). There was however no significant difference between the control group and those with CD4 count ≥500 mm^{-3}. There was also significant difference amongst the various groups of the subjects when they were compared against each other (p< 0.0001). From the Pearson's correlation, it was observed that there was a strong positive correlation (r= 0.766; p<0.0001) between the serum Vitamin E levels of the test subjects and the CD4 count (Fig. 3.6B).

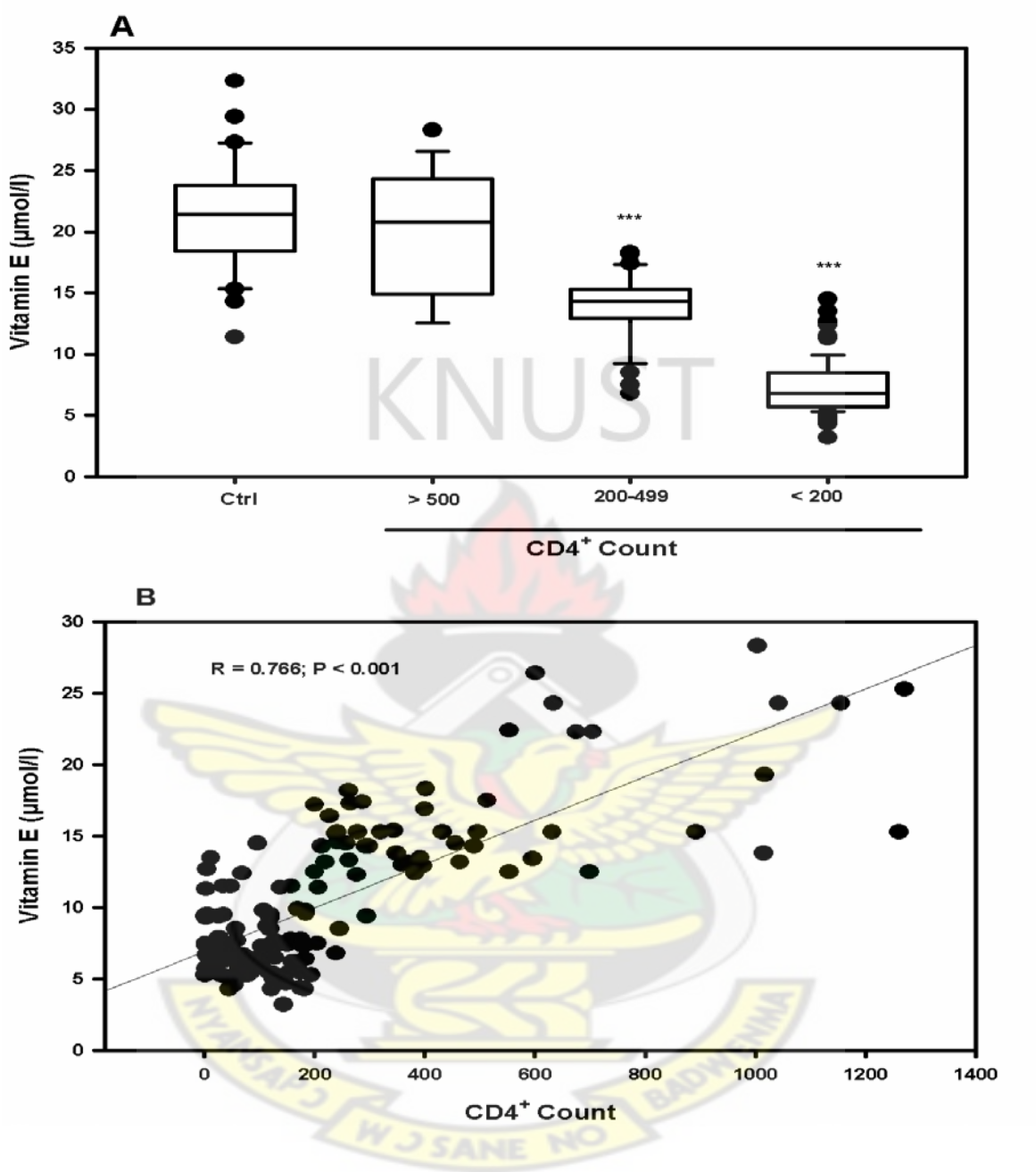


Figure 3.6 Comparison of Vitamin E concentration in controls and CD4 stages. (B) Linear regression between CD4 count and serum Vitamin E of the Test subjects. The results are presented as means ± SEM. ***$P \leq 0.0001$ determines the level of significance when the respective subjects were compared to control.

3.2 HAEMALOGICAL INVESTIGATION

3.2.1 Haemoglobin and Haematocrit

The haematological parameters of the study population and the control are given in Table 3.1. Blood haemoglobin levels decreased as the HIV infection progressed as shown in Table 3.1. At a cut-off of 11.0g/dl, 82.40% of the test subjects were found out to be anaemic. One-way ANOVA revealed a significant difference between the test subjects and the control group ($F_{3, 228}$=13.24; p<0.0001). A further post-test analysis using the Bonferroni's Multiple Comparison Test revealed that there was a significant difference between the control group and the subjects with CD4 count <200 and CD4 count between 200 and 499 (p<0.0001). There was no significant difference between the control group and those with CD4 count greater than 500. There was also a significant difference between the subjects with CD4 count greater than 500 and those with CD4 count less than 200 (p<0.001). Using the Pearson's correlation, there was a significant and positive correlation (r^2=0.1755; p<0.0001) between the blood haemoglobin level and their respective CD4 counts as shown in Figure 3.7A. Thus as the blood CD4 count of the subjects decreased there was a decrease in their respective blood haemoglobin levels. Blood haematocrit has similar trend as the blood haemoglobin. The mean haematocrit values are shown in table 3.1. A correlation analysis between the CD4 count of the test subjects and their respective haematocrit levels revealed a significant and positive correlation (r^2=0.1869; p<0.0001) as shown in Figure 3.7B.

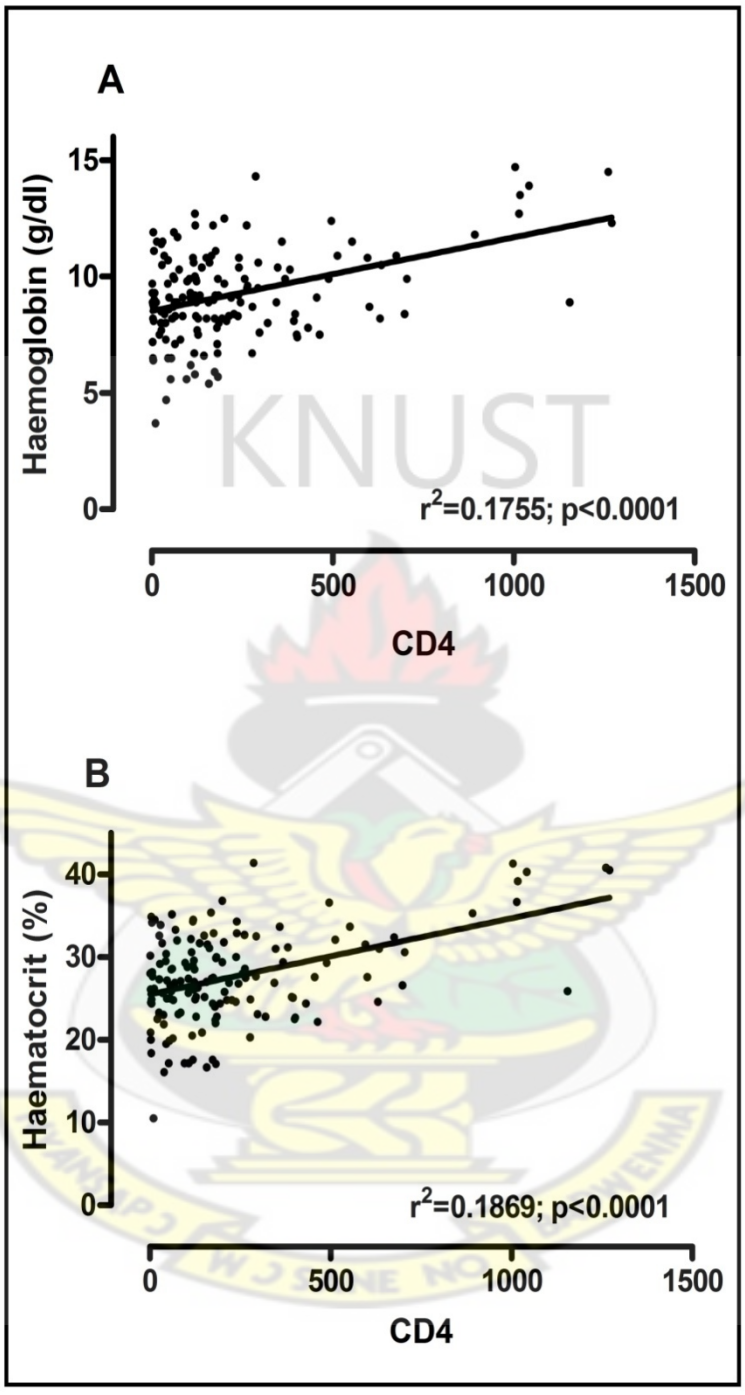


Figure 3.7 Linear correlation between CD4 count and blood (A) Haemoglobin and (B) Haematocrit, in patients with HIV infection. (r= correlation coefficient).

Table 3.1 Haematological Characteristics of the Test and Control subjects

Parameters	Control	Test subjects	CD4 Count (mm^3)		
			<200	200-499	>500
Hb (g/dl)	14.29±0.77	9.25±0.17***	8.83±0.22***	10.03±0.31***	11.3±0.44
HCT (%)	41.15±2.15	27.65±0.45***	23.53±0.85***	28.28±0.77***	33.54±1.35
WBC (x 10^6/L)	6.28±0.69	5.20±0.23	4.90±0.22	5.14±0.31	5.71±0.44
MCV (fl)	86.53±2.58	81.41±0.76	80.49±9.61**	82.38±9.71*	84.40±6.96
MCHC (g/dl)	30.34±1.09	27.25±0.27**	26.92±3.46	27.57±3.66	28.41±2.87
MCH (pg)	35.03±0.51	33.84±0.41	34.03±1.58	33.45±1.23	33.59±1.32

Data are presented as group means (±SEM). Significantly different from control: *P<0.05, **P<0.01, ***P<0.001 by Bonferroni's Multiple Comparison test. Hb: haemoglobin, HCT: haematocrit, WBC: white blood cells, MCV: Mean Cell Volume, MCHC: Mean Cell Haemoglobin concentration, MCH: Mean Cell Haemoglobin

3.2.2 White blood cell (WBC) count

Analysis of the WBC count of the data obtained for the test subjects and the control group revealed no significant difference ($F_{3,\ 228}$=1.637; p=0.1830) (Table 3.1) between the test subjects and the control group. Comparing the various groups of the test subjects to the control group also revealed no significant difference. Using the Pearson's correlation, there was no significant correlation (R=0.1678; p=0.0401) between the WBC counts of the test subjects and their CD4. Thus as the CD4 count of the test subjects decreased there was no significant difference in their WBC count as compared to the control group.

3.3 BIOCHEMICAL ASSAYS

3.3.1 Total Protein and Albumin

Serum total protein in the test subjects were significantly different ($F_{3,\ 228}$= 28.27; P<0.0001) from the control group. There was a significant increase in the serum protein among the test subjects as the CD4 count decreased (Table 3.2). There was a significant difference when the three test groups were compared amongst themselves. Correlation studies of serum protein (Table 3.3) revealed a significant and negative correlation between the test group and the control group (R= -0.36; p<0.0001). Thus as the HIV infection progressed, there was a significant increase in the serum protein levels. Serum albumin levels of the test subjects decreased as the HIV infection progressed (Table 3.2) comparing the test subjects to the control group, there was a significant difference ($F_{3,\ 228}$=3.867; p=0.0106) between them. There was however no significant difference when the various test groups were compared to each other. There was a positive correlation (R= 0.41; p<0.0001) when the serum albumin levels of the test subjects were compared with their respective CD4 count (Table 3.3).

Table 3.2 Effect of HIV progression on Biochemical Parameters

Parameters	Control	Test subjects	CD4 Count		
			<200	200-499	>500
Total Protein (g/l)	73.49±0.95	86.69±1.07***	86.56±1.60**	93.12±2.59**	89.73±3.80**
Albumin (g/l)	37.45±1.401	34.62±1.87*	32.61±0.86**	34.65±1.13*	36.6±1.90
Total Cholesterol (mmol/l)	5.21±0.10	2.43±0.05***	2.14±0.46***	2.64±0.46***	3.61±0.47***
Triglycerides (mmol/l)	0.98±0.05	1.83± 0.03***	1.90±0.41***	1.81±0.35***	1.47±0.25***
HDL-Cholesterol (mmol/l)	1.38±0.05	1.05±0.04***	0.90±0.41***	0.97±0.42**	1.36±0.21
LDL-Cholesterol (mmol/l)	1.65±0.05	1.02±0.03***	0.96±0.38***	0.91±0.40***	1.27±0.30**

Data are presented as group means (±SEM). Significantly different from control: *P<0.05, **P<0.01, ***P<0.001 by Bonferroni's *post hoc* test

Table 3.3 Pearson's correlation coefficients of Biochemical Parameters for Subject group (upper right-hand side) and the control group (lower left-hand side)

Parameters	CD4	TP	Alb	TC	TG	HDL-C	LDL-C
CD4 (mm^3)		-0.36***	0.41***	0.67***	-0.27**	0.27**	0.21**
TP (g/l)	0.04		0.16*	0.28**	-0.02	0.15	-0.02
Alb (g/l)	-0.03	0.28*		0.32***	0.02	-0.11	0.14
TC (mmol/l)	0.07	0.33*	0.09		-0.18	0.16*	0.2
TG (mmol/l)	-0.18	0.04	-0.05	0.10		-0.31	-0.14
HDL-C (mmol/l)	-0.20	-0.10	-0.15	0.02	-0.20		0.13
LDL-C (mmol/l)	0.01	0.19	0.14	0.11	0.08	0.07	

* Correlation is significant at the level 0.05, ** Correlation is significant at the level 0.001, *** Correlation is significant at the level 0.0001. HDL: High Density Lipoprotein, LDL: Low Density Lipoprotein, CD: Cluster of differentiation. CD4: Cluster of Differentiation, TP: Total Protein, ALB: Albumin, TC: Total Cholesterol, TG: Triglycerides, HDL-C: High Density Lipoprotein Cholesterol, LDL-C: Low Density Lipoprotein Cholesterol.

3.3.2 Lipid Profile

When the serum Lipid profile parameters were analysed using the one way ANOVA analysis followed by the Bonferroni's Multiple Comparison Test, serum Total Cholesterol ($F_{3,\ 228}$=384.8; p <0.0001), HDL-Cholesterol ($F_{3,\ 228}$= 18.05; p <0.0001) and LDL-Cholesterol ($F_{3,\ 228}$= 48.10; p <0.0001) all decreased as HIV infection increased. Only serum Triglycerides ($F_{3,\ 228}$= 74.22; p <0.0001) increased as HIV infection progressed. Serum Lipid profile levels of the three test groups were all significant when compared with the control group with the exception of HDL-Cholesterol which was not significant for the $\geq$ 500mm^{-3}(Table 3.2). Correlation analyses revealed a significant and positive correlation for all the serum Lipid profile parameters with the exception of serum Triglycerides which gave a negative correlation (Table 3.3).

3.4 RECEIVER OPERATOR CHARACTERISTICS (ROC) ANALYSIS OF MARKERS OF OXIDATIVE STRESS

Receiver Operator Characteristics analysis was done on all the markers of Oxidative stress analysed to ascertain which of the parameter(s) would be the best predictor(s) of markers of oxidative stress in HIV/AIDS progression. Receiver Operator Characteristics (ROC) of the markers of Oxidative Stress is shown in Table 3.4. SOD and GPx were the best predictive markers of Oxidative Stress as seen in the area under the curve with Vitamin C and FRAP being the least predictive markers (Table 3.4). Sensitivity, Specificity and other parameters of the various markers are shown in Table 3.4. All oxidative stress markers were able to distinguish between the test subjects and the control group (p<0.0001).

Pair wise comparisons of the markers of Oxidative Stress were done to find out how the markers fared against each other (Table 3.5). Differences between the Areas under the Curve (AUC), standard error and other parameters were

analysed. Least difference in the AUC was found between GPx and SOD (p=0.378) with GPx-Vitamin C pair giving the biggest difference (p=0.001). The sensitivity and specificity of all the oxidative stress markers were plotted. SOD had the almost perfect curve followed by GPx with FRAP giving the least fit curve (Figure 3.8).

Table 3.4 Receiver Operator Characteristics (ROC) of Markers of Oxidative Stress

Parameters	Vitamin C	FRAP	MDA	GPx	SOD	Vitamin E
AUC	0.86	0.87	0.98	0.99	0.99	0.93
Standard error	0.035	0.035	0.007	0.010	0.002	0.026
95% CI	0.813 to 0.912	0.815 to 0.913	0.960 to 0.998	0.964 to 0.999	0.981 to 1.000	0.889 to 0.964
P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Cut-off	≤45.46	≤554.00	>2.00	≤4103.00	≤166.00	≤15.30
Sensitivity	84.42	91.56	97.4	96.73	98.05	87.66
95% CI	77.7 - 89.8	86.0 - 95.4	93.5 - 99.3	92.5 - 98.9	94.4 - 99.6	81.4 - 92.4
Specificity	73.91	76.09	93.48	95.65	100	91.3
95% CI	58.9 - 85.7	61.2 - 87.4	82.1 - 98.6	85.1 - 99.3	92.2 - 100.0	79.2 - 97.5
+LR	3.24	3.83	14.94	22.25		10.08
-LR	0.21	0.11	0.028	0.034	0.019	0.14
+PV	91.5	92.8	98	98.7	100	97.1
-PV	58.0	72.9	91.5	89.8	93.9	68.9

AUC: Area under the curve, CI: Confidence Interval, P: Level of Significance, +LR: Positive likelihood ratio, -LR: Negative likelihood ratio, +PV: Positive predictive value, -PV: Negative predictive value, FRAP: Ferric Reducing ability of Plasma, MDA: Malondialdehyde, GPx: Glutathione Peroxidase, SOD: Superoxide Dismutase

Table 3.5 Pair wise Comparison of Markers of Oxidative Stress using the Receiver Operator Characteristics Analysis

Parameters	Difference between areas	Standard error	95% CI	z statistic	*p* value
FRAP-MDA	0.120	0.0354	0.051 to 0.189	3.388	0.001
FRAP-GPx	0.124	0.0341	0.057 to 0.191	3.622	0.001
FRAP-SOD	0.133	0.0354	0.063 to 0.202	3.748	0.001
FRAP-Vitamin C	0.003	0.0438	-0.083 to 0.088	0.061	0.951
FRAP-Vitamin E	0.066	0.0373	-0.007 to 0.139	1.770	0.077
MDA-GPx	0.004	0.0119	-0.019 to 0.027	0.310	0.756
MDA-Vitamin C	0.117	0.0359	0.047 to 0.188	3.266	0.001
MDA-Vitamin E	0.054	0.0265	0.002 to 0.106	2.032	0.042
GPx-SOD	0.009	0.0101	-0.011 to 0.029	0.882	0.378
GPx-Vitamin C	0.121	0.0355	0.051 to 0.191	3.406	0.001
GPx-Vitamin E	0.058	0.0264	0.006 to 0.109	2.185	0.029
SOD-Vitamin C	0.130	0.0353	0.061 to 0.199	3.681	0.001
SOD-Vitamin E	0.067	0.0260	0.015 to 0.118	2.551	0.011
Vitamin C- Vitamin E	0.063	0.0404	-0.016 to 0.143	1.569	0.117

MDA: Malondialdehyde, SOD: Superoxide Dismutase, GPx: Glutathione Peroxidase, FRAP: Ferric Reducing ability of Plasma CI: Confidence Interval, P: Level of Significance

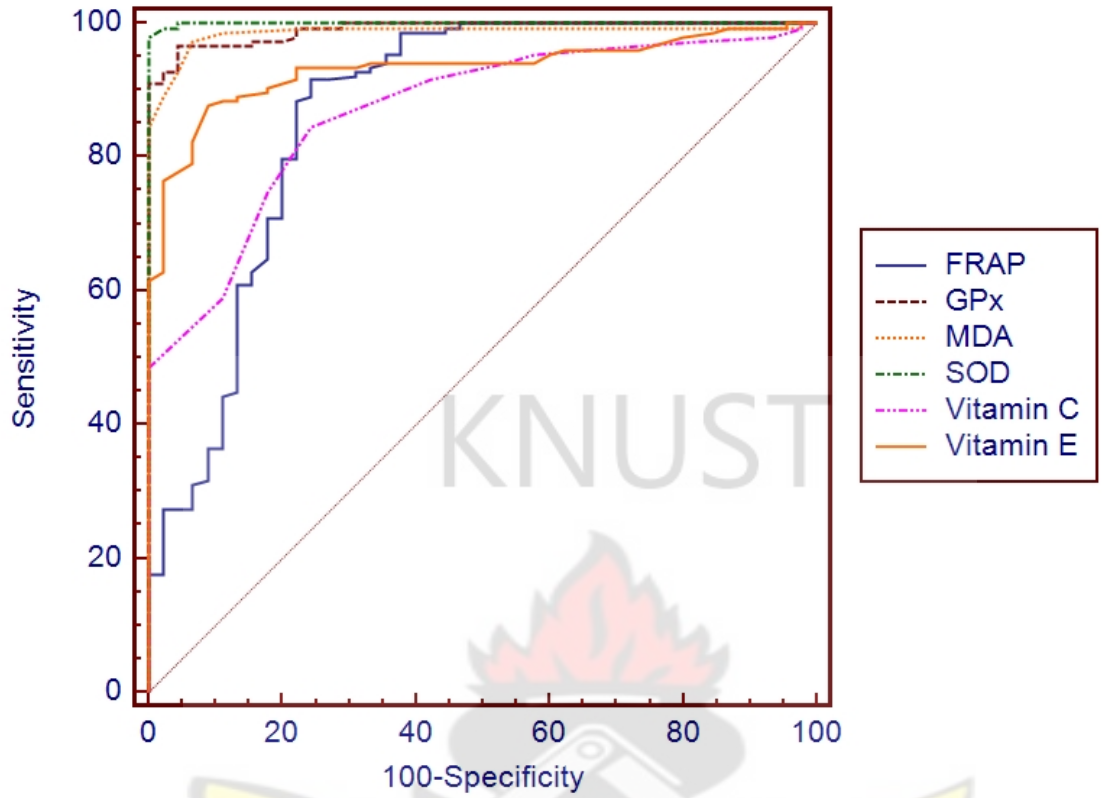


Figure 3.8 ROC curves of the various markers of Oxidative stress

Chapter 4

DISCUSSION

The various phases of HIV infection, including the early asymptomatic phase, are closely associated with quantifiable laboratory findings (el-Sadr *et al.*, 1987; Melmed *et al.*, 1989; Fahey *et al.*, 1990). Surrogate markers of HIV infection are, by definition, measurable traits that correlate with development of clinical AIDS. Although several candidate markers have been described, only a few have shown promise. Ideally, assessment of these markers should fulfill majority of the following criteria:

(i) permit identification of patients at highest risk of disease progression,

(ii) aid in estimating the duration of infection,

(iii) assist in disease staging,

(iv) predict development of indicator diseases (opportunistic infections of AIDS), and

(v) Follow, in vitro, the therapeutic efficacy of immunomodulating or antiviral treatments. In addition, these markers must be easily quantifiable, reliable, clinically available, and affordable.

The most characteristic feature of AIDS is a selective depletion of the CD4+ T-helper-inducer subset of T cells. The degree of CD4+ T-cell depletion is currently the single most important laboratory finding taken into consideration when recommendations are made regarding therapy with antiretroviral drugs. Most experimental therapeutic protocols enroll patients on the basis of CD4+ T-cell counts and/or the presence or absence of viral antigenemia. These surrogate markers of clinical AIDS development are used to formulate decisions on the

timing of medical intervention, to predict actuarial outcomes, and to plan future health care expenditures.

4.1 OXIDATIVE STRESS AND HIV PROGRESSION

Human immunodeficiency virus (HIV) infection is usually characterized by a variable, although long, period of latency prior to the development of AIDS (Garcia-Blanco and Cullen, 1991; Rosenberg and Fauci, 1991). Recent infection is associated with a low frequency of infected cells and low levels of viral gene expression and production of viral progeny (Ho *et al.*, 1989). Progression to AIDS is associated with an increased level of viremia and p24 antigenemia (Schnittman *et al.*, 1991). One of the factors that limit progression to full-blown AIDS is possibly time and extent of activation of the latent provirus (Greene, 1991). The identification of the mechanisms that lead to viral activation is a major focus of research activity, probably due to its potential to point to new therapeutic options. Oxidative stress is a potent inducer of viral activation and DNA damage in infected cells, as well as one of the long-term consequences of HIV infection, i.e., immunosuppression (Lloyd and Schneider, 1990.). Formation of highly reactive oxygen-containing molecules is normal consequence of a variety of essential biochemical reactions. Oxidants such as superoxide radicals (O_2), hydrogen peroxides (H_20_2), hydroxyl radicals (HO), and lipid peroxides (LOOH) have been shown to play an ever more important role in human disease (Saltman, 1989). Oxidants that are produced by stimulated neutrophils and/or macrophages can induce cellular injury and lysis of nearby bystander cells both in vivo and in vitro (Johnson *et al.*, 1981). As a means of dealing with this, most cells have enzymes and other molecules with antioxidant capabilities that can protect against the adverse effects of free radical reaction. Oxidative stress occurs when the balance between pro-oxidants and anti-oxidants is distorted. In such cases, active oxygen species and free radicals are so reactive and short-lived that their levels are difficult to measure directly. For these reasons, most methods measure the products of

oxidative stress. According to a model of disease pathogenesis proposed by Fuchs (1991), HIV and opportunistic infections directly or indirectly lead to an oxidative stress. The pro-oxidative conditions cause activation of free radical-producing immune cells, enhancement of viral replication and weakening of the antioxidant defence system. This cycle becomes autocatalytic and facilitates disease progression. During infection, abnormal oxidative stress may result from either or both of (1) Increased replication and/or pathogenesis of an infecting agent causing over-production of reactive oxygen species and (2) Failure of normal defence mechanisms, leading to decreased elimination of reactive substances.

4.1.1 FRAP

The results of this study demonstrated that the antioxidant capacity of human serum as measured by FRAP decreases in HIV positive patients as compared to the HIV negative control (Figure 3.1A). The FRAP also correlated positively with CD4 of the HIV positive subjects (Figure 3.1B). The mechanism behind the significant reduction in total FRAP is likely to be due to increased oxidative stress induced by an increase in free radicals production (i.e. imbalance between antioxidant capacity and pro-oxidants). Therefore antioxidants can be used in HIV infection to effectively inhibit the severity of infection and to improve their survival. Antioxidant therapy in these patients before they progressed to AIDS may ameliorate the effect of the disease.

4.1.2 Malondialdehyde

This study has shown that in HIV-infection there is evidence of significantly greater release of MDA in severe cases, compared with mild disease as shown in Figure 3.2A. This study suggests that the oxidative stress which leads to MDA formation and release is a reflection of both severities of HIV-infection and of anti-oxidant deprivation, because the mild and severe groups based on CD4 counts had

progressive diminishing levels of anti-oxidant (i.e. SOD, GPx, FRAP, ascorbic acid and alpha-tocopherol). Levels of MDA were significantly increased in HIV seropositives as confirmed by previous study (Muller et al., 1996). The observed increase of oxidative stress processes in these patients resulting from cytotoxic products may modify proteins and DNA by addition reactions (Siems et al., 1998). An explanation for this may relate to the depletion of neutrophils which occurs in the late disease stages and which may be influenced by the treatments used by patients (Favier *et al.*, 1994; Allard *et al.*, 1998a). The results of this study strongly suggest that one of the main reasons for high MDA levels in HIV seropositive patients could be decreased activity of the defense system protecting tissues from free radical damage as seen in the trend of MDA levels as HIV infection progressed. Lipid peroxidation is a free radical-related process that may occur under enzymatic control (e.g., for the generation of lipid-derived inflammatory mediators) or non-enzymatically in biologic systems. This latter form is associated mostly with cellular damage as a result of oxidative stress, which also involves cellular antioxidants (Romero *et al.*, 1998). The high HIV-positive/HIV-negative ratio of MDA concentration and the significant negative correlation between MDA and CD4 count (Figure 3.2B) strongly indicate the occurrence of oxidative stress and lipid peroxidation as a mechanism of tissue damage in cases of chronic HIV-infection.

4.1.3 Superoxide Dismutase

Superoxide dismutase is the main defence against superoxide radical-mediated oxidative damage and three isoenzymes of SOD have been identified: a copper/zinc form (CuZnSOD), a manganese form (MnSOD), and an extracellular isoenzyme (EC-SOD), which is found in plasma (Hassan, 1988). It has been showed from this study that even at the early stages of HIV infection when the CD4 count is greater than 500 mm^{-3}, the SOD activity is decreased (Figure 3.3A). Moreover, the decrease of SOD was parallel to that of CD4 lymphocytes as seen by the significant positive correlation with CD4 count as shown in figure 3.3B. (Flores *et*

al., 1993) found that the HIV Tat protein down-regulates the synthesis and overrides the induction of the MnSOD, which is induced by oxidative stress. Total SOD activity was measured in this study, which includes activities of CuZnSOD and MnSOD, it may be presumed that the decrease of total SOD activity was dependent on inhibition of MnSOD by HIV Tat protein. Though the effect of Tat protein on EC-SOD has not been properly described, EC-SOD activity is strongly influenced by inflammatory cytokines such as TNF-alpha (Marklund, 1992; Flores *et al.*, 1993; Brady *et al.*, 1997). TNF-alpha was not measured in this study, however, the increase in inflammatory process in this study may suppose that TNF-α produced by activated immune cells of HIV-infected patients, may depress EC-SOD expression.

4.1.4 Glutathione Peroxidase

Plasma glutathione peroxidase-levels were initially higher in HIV-positive individuals as compared to the HIV-negative control group and then later fell significantly below that of the control group as the CD4 count declined in this study as shown in figure 3.4A. The initial increase in GPx observed in this study is in agreement with previous work (Delmas-Beauvieux *et al.*, 1996; Batterham *et al.*, 2001; Trotti *et al.*, 2002). It is likely that GPX activity first increases under lipid peroxidation via an adaptative response, and then that GPX activity decreases as a result of its consumption, as was observed as the CD4 count decreased. This corresponds to an aggravation of the disease with development of opportunistic infections and thus an increased production of ROS. HIV infection also caused an increase in oxidative stress during erythropoiesis, which, in turn, increases the level of GPX-1 expression, the predominant form of the enzyme found in erythrocytes (Delmas-Beauvieux *et al.*, 1996). Another possibility is that increased GPX activity in whole blood could be due to a putative, HIV encoded GPX expressed in HIV-infected cells (Zhao *et al.*, 2000). The positive correlation between

the CD4 counts and the glutathione peroxidise level as shown in figure 3.4B in this study is likely due to the fact that, an increased consumption of GSH by the GSH-Px reaction and/or decreased release into the circulation from the liver, the site of GSH production. Considering the role of GSH in immune function the loss of thiol compounds, especially of GSH, represents a critical feature of HIV-disease. In fact, low concentrations of GSH in CD4+ and CD8+ lymphocyte subsets (Roederer *et al.*, 1992; Droge, 1993), in peripheral blood mononuclear cells (PBMC) (de Quay *et al.*, 1992), or in plasma and bronchoalveolar lavage (Buhl *et al.*, 1989) of individuals infected with HIV have been reported.

4.1.5 Vitamin C (Ascorbic acid)

The results of this study indicated that serum ascorbate was lower in subjects with HIV infection (Figure 3.5A). This means that such subjects have higher vitamin C requirements than do persons without HIV infection. Although oxidized ascorbate can be recycled, increased oxidative stress is assumed to increase ascorbate depletion (Halliwell and Gutteridge, 1990). Thus, plasma ascorbate may be decreased by the chronic immune activation of HIV infection even if dietary intake is at a level judged to be adequate for healthy persons (Kruzich *et al.*, 2004). The lower plasma ascorbate in HIV-positive than in HIV-negative is in agreement with previous observation (Lacey *et al.*, 1996; Allard *et al.*, 1998a; Treitinger *et al.*, 2000). The results in the current study suggest that vitamin C utilization is increased by HIV infection. Higher intakes during HIV infection should help prevent oxidative damage and maintain normal immune function. Vitamin C is a water soluble vitamin and can function as both an intracellular and extracellular antioxidant (Bendich, 1990). Vitamin C deficiency results in depressed cell-mediated immune response; in some human studies, T and B lymphocyte proliferative responses were increased upon supplementation with vitamin C (Bendich, 1990). Vitamin C has been shown to inhibit HIV replication in acutely and chronically infected T cells and to inhibit HIV reactivation in T cells

stimulated by TNF-alpha (Harakeh *et al.*, 1990; Harakeh and Jariwalla, 1995). In this study, the depressed plasma ascorbate levels found in the HIV-infected patients in addition to the positive correlation with the CD4 counts suggest that they are less protected against viral replication. Oxidative stress may enhance viral replication via activation of nuclear transcription factor NF-kB by reactive oxygen species (Baruchel and Wainberg, 1992). NF-kB also acts as a transcription factor for many inflammatory cytokines (Brach *et al.*, 1992; Meyer *et al.*, 1994). Diverse antioxidants inhibit HIV-LTR transactivation by blocking NF-kB activation in lymphoblastoid T and monocytic cell lines (Israel *et al.*, 1992). Ascorbic acid reduced the levels of extracellular reverse transcriptase activity and the expression of p24 antigen in an HIV-infected T-lymphocyte cell line (Harakeh *et al.*, 1990). The observed decreased in ascorbic acid observed in the study could lead to an increase in the nuclear transcription factor NF-kB which would result in increased viral replication. Vitamin C and E supplements have been shown to decrease oxidative damage and tended to decrease disease severity in HIV-positive Canadian adults (Allard *et al.*, 1998a) and high intakes of vitamin C has also been associated with a lower risk of progression to AIDS in US (Tang *et al.*, 1993). Studies in Tanzanian (Fawzi *et al.*, 2004) and Kenyan (McClelland *et al.*, 2004) also indicate lower risk of death and increase in CD4 T lymphocyte respectively due to HIV-infection following multi-nutrient supplements containing vitamins C and E.

4.1.6 Vitamin E (Alpha-tocopherol)

As confirmed by this study, previous works had indicated lower plasma alpha-tocopherol concentrations in subjects (Figure 3.6A) with HIV infection or AIDS than in healthy controls (Beach *et al.*, 1992; Baum *et al.*, 1995; Jordao Junior *et al.*, 1998). The significant positive correlation between CD4 count and alpha-tocopherol (Figure 3.6B) is also consistent with the report that plasma alpha-tocopherol concentrations decrease with time during HIV infection as CD4 count declined (Pacht *et al.*, 1997). Earlier works suggest that vitamin E may increase immune response to antigens, improve host resistance against challenge with

microorganisms, and enhance B and T cell lymphocyte functions, as well as phagocytic function (Odeleye and Watson, 1991; Tang *et al.*, 1997). The effects of vitamin E on immune function and the potential benefits of vitamin E therapy in HIV-infected patients have been documented (Tengerdy, 1990; Odeleye and Watson, 1991; Meydani, 1992). The majority of evidence put forth by these studies suggests that vitamin E is not only necessary for proper functioning of the immune system, but also has important immunostimulatory properties. Studies in both humans and animal models have shown that vitamin E supplementation, in several fold excess of the recommendations, significantly increases humoral and cell-mediated immune responses to antigens and enhance phagocytic functions (Tengerdy, 1990). More specifically, high doses of vitamin E along with a nutritionally adequate diet have been repeatedly shown to enhance in vitro and in vivo antibody production, phagocytosis, lymphoproliferative responses, and resistance to viral and infectious diseases (Odeleye and Watson, 1991). The progressive diminishing in the vitamin E level in this study could lead to viral replication and faster progression from HIV to AIDS.

The prevalence of overtly or marginally low serum vitamin E levels has been documented to range between 8% and 20% in studies of HIV positive individuals (Baum *et al.*, 1992; Beach *et al.*, 1992; Coodley *et al.*, 1993; Favier *et al.*, 1994). None of these studies have examined the consequences of low serum vitamin E levels on HIV-1 disease progression nor have the mechanisms leading to vitamin E depletion during HIV-1 infection been clearly elucidated. In this study 74.6% of the studied population was found to have low concentrations of serum vitamin E (< 15.30 µmol/l). The disparity could be due to (1) the differences in the stage of the HIV-infection in the study population as most of the subjects in this study had their CD4 count lower than 200 mm^{-3}, (2) the difference in the study population as age, sex, diet, genetic composition are some of the factors that can modulate the immune system (i.e. CD4 count).

4.2 HAEMATOLOGICAL PARAMETERS AND HIV PROGRESSION

The incidence of anemia was strongly and consistently associated with the progression of HIV disease as measured by CD4 count (Table 3.1). This association is most likely explained by the increasing viral burden as HIV disease progresses, which could cause anemia by increased cytokine mediated myelosuppression. Alternatively, anemia may be a surrogate marker for some aspect of disease progression not captured by controlling for CD4 count and clinical AIDS diagnosis. Our study results are consistent with previous studies which showed that low haemoglobin levels increase the risk of AIDS (Hoover *et al.*, 1995; Rabeneck *et al.*, 1997), increase the risk of death in patients with AIDS (Swanson and Cooper, 1990; Saah *et al.*, 1994; Turner *et al.*, 1996b) and increase the risk of death among patients with advanced immunodeficiency (Chene *et al.*, 1997; Spino *et al.*, 1997), both in cross-sectional (Hoover *et al.*, 1992) and longitudinal studies (Hoover *et al.*, 1995). Several staging systems have suggested that important information can be provided by haemoglobin levels (Turner *et al.*, 1996a; Chene *et al.*, 1997; Spino *et al.*, 1997). Significant and positive correlation was found when blood haemoglobin level was compared with their respective CD4 count (Figure 3.7) of the subject group. Our results also show that haemoglobin levels provide prognostic information independent of that provided by the CD4 lymphocyte count.

According to the study, blood haemoglobin less than 11g/dl and blood haematocrit less than 33% was classified as anaemia. This cutoff was chosen for defining anemia to exclude hereditary causes for mild anemia, such as thalassemia trait, and to allow for use of a single cutoff that would clearly exclude normal hemoglobin concentrations for both men and women. Most of the subjects group (82%) had anaemia according to this cutoff. The subject group with CD4<200 all had blood haemoglobin less than the cutoff. Although the cut-offs we used to define anaemia differ from those in a study by Moore et al (1998), these results agree well with this recent study, which used the latest measure of haemoglobin

and CD4 lymphocyte count in a similar way to our analysis. From the Red cell indices analysis, most of the test subjects had low MCV and MCHC as compared to the control group. Our findings are consistent with those of (Massawe *et al.*, 1999) who found that the majority of test subjects presented with microcytic hypochromic anaemia which is common in most chronic diseases (Table 3.1).

All of our data was collected before common use of the new highly active antiretroviral therapies. Before the introduction of HAART, investigators reported that the annual loss of CD4 lymphocyte counts was 40–80 x 10^6 cells/l (Margolick *et al.*, 1992; Montella *et al.*, 1992; Easterbrook *et al.*, 1993; Sheppard *et al.*, 1993). It was interesting to note that the change in haemoglobin in our study was gradual and a continual process. There have been considerable changes in the availability of antiretroviral regimens and in the way they are combined in the EuroSIDA study (Kirk *et al.*, 1998). There was preliminary evidence from this analysis that patients starting HAART were less likely to have anaemia following treatment, although the duration of follow-up was quite limited (Kirk *et al.*, 1998).

The pathogenesis of HIV-associated anaemia is unclear and is likely to be multifactorial in nature (Hambleton, 1996; Coyle, 1997). Possible causes are bleeding (gastrointestinal malignancy/severe infection), insufficient dietary intake (vitamins such as cobalamin and folate, iron, and general malnutrition), haemolytic anaemia (i.e. malignancies, infections, splenomegaly, and immune dysfunction), and changes in erythropoietin synthesis and/or bone marrow suppression. Suppression of the bone marrow in HIV-infected patients may be initiated in several ways. These include an action of HIV itself (infection of the progenitor of the red blood cell synthesis in late-stage disease), direct pathogenic involvement of the bone marrow (malignant lymphoma, atypical mycobacteriosis), prophylactic or therapeutic treatment against opportunistic diseases or malignancies (i.e. sulphamethoxazole, ganciclovir, methotrexate, adriamycin) or simply non-specific effects relative to chronic or acute infections.

Haemoglobin is both easy and inexpensive to measure and it was measured before the initiation of ART. Clearly patients who developed severe anaemia before the initiation of ART were at an increased risk of death. Monitoring haemoglobin levels could be used to alert clinicians to those patients who require more regular clinical follow-up or who may need treatment for their anaemia. Moore *et al* (1998) found that treatment of anaemia with erythropoietin was associated with an improved prognosis possibly by allowing higher doses or prolonged use of drugs such as zidovudine and ganciclovir. It is important to note that data on use of erythropoietin were not available for this analysis, nor were data on the frequency of transfusion for anaemia and its relationship with prognosis. In a recent cross-sectional study, haemoglobin measurement was shown to play an important role in the basic management of HIV disease in West Africa (Ledru *et al.*, 1998). Given its strong relationship with AIDS-defining illness and death, haemoglobin levels could be measured easily where resources for more sophisticated laboratory markers such as viral load or even CD4 lymphocyte count were not available (given that measurement of the CD4 lymphocyte count requires flow cytometry, an expensive technique unavailable in many developing countries) (Stein *et al.*, 1992). Regular measurements could help to determine which patients are at greatest risk of disease progression, allowing these patients to be identified for closer monitoring or therapeutic intervention.

4.3 BIOCHEMICAL PARAMETERS AND HIV PROGRESSION

The findings from that study was consistent with the finding of our study where albumin in the test subjects decreased as the HIV infection progressed as shown in Table 3.2. Comparing the CD4 count of the test subjects with their respective albumin revealed a significant and positive correlation (Table 3.3) which goes to buttress the point that there was a significant decrease in the serum albumin level as the HIV infection progressed.

It has been reported that the baseline serum albumin level can be considered an independent predictor of mortality in HIV-1-infected women and can be used as an additional marker of HIV-1 disease progression (Feldman *et al.*, 2003). Mehta et al (2006) examined the albumin levels among HIV-infected individuals at entry into a community-based cohort, as well as the albumin concentrations measured before and after HIV seroconversion. They found that an albumin level of <35 g/l was associated with faster progression to AIDS. This is a very common occurrence, and has been well described since the beginning of the epidemic. A low albumin typically occurs at advanced stages of HIV disease, and reflects poor nutritional status. Several hypothetical mechanisms may explain decreases in albumin levels among HIV-1-infected individuals. Other potential mechanisms may include anorexia or acute renal dysfunction. Alternatively, massive cellular apoptosis and inflammation in gut-associated lymphoid tissue (GALT) in early HIV-1 infection (Centlivre *et al.*, 2007) may lead to decreased protein absorption, enteropathy with associated protein loss, or decreased hepatic protein synthesis. Increasing evidence points to microbial translocation as a cause of systemic immune activation in chronic HIV infection, and early mucosal damage may also be important (Brenchley *et al.*, 2006). It should be emphasized here that a better understanding of the cause of decreases in serum albumin in early HIV-1 infection may suggest interventions to slow disease progression in HIV-1-infected individuals.

There was a significantly higher serum total protein in the test subjects than in the control group (Table 3.2) in our study. This corroborates earlier finding by (Feldman *et al.*, 2003). Although serum total protein estimation has limited diagnostic importance when compared to albumin because of the compensatory increases in other serum proteins (the globulins) during infections, its relevance in the evaluation of patients with some clinical conditions such as malnutrition,

malignancy, renal and liver diseases and immune disorders cannot be ignored (Gray *et al.*, 1985).

The elevated total protein and globulin fraction can occur in otherwise healthy HIV-positive individuals. High serum total protein probably reflects a generalized, polyclonal gammopathy, with increased antibody production, which is an attempt on the part of the immune system to compensate for cellular immunodeficiency. The high total serum protein concentration (Table 3.2) in the test group could be due to many factors. Generally the protein level of serum is increased when there is an infection in the absence of malnutrition. This is because there is antibody production to contain and prevent the spread of the infection. These antibodies, which are mainly γ-globulins, will increase the serum total protein concentration. Chronic infections are known to lead to a high level of total protein frequently as a result of high level of immunoglobulins. In the case of HIV/AIDS the high serum protein levels is normally attributed to tissue degeneration and breakdown (Stahl *et al.*, 1982).

In wasting processes, such as HIV/AIDS there is increased tissue protein breakdown. Negative nitrogen balance develops, which may be partly due to excessive secretion of glucocorticoids and a proteolysis–inducing factor, Interleukin-1, which causes skeletal muscle catabolism. The catabolism of muscle and general body tissue to provide proteins for glucose production through gluconeogenesis is suspected to add to the generally high level of serum total protein in the face of reduced albumin concentrations in HIV/AIDS patients. The dehydration that accompanies diarrhoea and the frequency of diarrhoea in HIV/AIDS especially in the tropics is also suspected to lead to "normal" and "high" serum total proteins (Bullock and Rosendal, 1988).

Hypertriglyceridaemia was the first dyslipidaemia to be reported in HIV infected patients, but other lipid abnormalities such as hypocholesterolaemia or hypo HDL-cholesterolaemia have also been reported. Previous studies have demonstrated that patients with AIDS exhibit highly abnormal total lipid concentrations in plasma (Mullamitha and Pazare, 1999). Few authors (Rogowska-Szadkowska and Borzuchowska, 1999; Ducobu and Payen, 2000) who determined the levels of plasma triglycerides, total cholesterol and HDL cholesterol in HIV infected individuals by the level of immunological deficiency according to the CD4 lymphocyte count, also came to the same conclusion that, with an increase of immunological deficiency and clinical development of HIV infection, lipid profile disorders, indicated by an increase in triglyceride level and decreased concentrations of HDL cholesterol intensified as well. Consistent with earlier reports, our study also showed similar findings in which the decrease of CD4 count due to disease progression was accompanied by a decrease in total cholesterol, HDL cholesterol and LDL cholesterol, and an increase in triglyceride levels as shown in Table 3.2. Our findings are also consistent with reports from (Ducobu and Payen, 2000) who stated that HIV infection induced an early decrease of cholesterol and a late increase of triglyceride with a reduction of HDL. These changes were proportional to the lowering of CD4 count, which reflected the severity of infections, as was the case in our study. (Shor-Posner et al., 1993) reported similar findings in which they showed significant low levels of total cholesterol, HDL, LDL cholesterol in HIV infected patients when compared to seronegative controls ($P < 0.05$). This low level of total, HDL, LDL cholesterol was reported to be associated with elevated levels of beta-2 microglobulin. The low levels of cholesterol are prevalent even during early stages of HIV and were associated with a specific alteration in immune function (Shor-Posner et al., 1993). Our results (Table 3.2) show that triglycerides increase in HIV positive patients at a late stage of disease. This hypertriglyceridaemia, found by other authors (Grunfeld et al., 1989; Mildvan et al., 1992) is correlated to opportunistic infections and to

interferon-α. The relationship between triglycerides (TG) and interferon-alpha (IFN- α) in HIV-positive patients has been previously found by (Grunfeld et al., 1991). IFN- α may increase TG by two main mechanisms: a decrease in TG clearance; and an increase of de novo hepatic lipogenesis and VLDL production. This hepatic lipogenesis may be stimulated by two types of cytokines: tumour necrosis factor- alpha (TNF- α), Interleukin 1 and 6 (IL- 1 and IL-6) that increase hepatic levels of citrate; and IFN- α that does not increase hepatic citrate. Both decreased TG clearance and increased hepatic VLDL overproduction have been found in HIV-positive patients, and the hepatic increased lipogenesis correlates to IFN- α (Grunfeld et al., 1992). Acute infections might increase TG by the way of hormones (steroids) or cytokines other than TNF- α or IFN- α. A decrease in cholesterol, especially in HDL-C occurs in HIV-positive patients long before hypertriglyceridaemia. These disturbances of cholesterol metabolism have been found by others (Falkenbach et al., 1990; Shor-Posner et al., 1993). TNF-α has been found to play a role in the peroxidation of plasma lipoproteins and lipids in experimental animals and in patients by stimulating the production of oxygen reacting species (McDonagh et al., 1992). Lipid peroxidation may, in part, explain the alterations of cholesterol metabolism in HIV-positive patients. These modifications might have important implications in immune dysfunction.

4.4 RECEIVER OPERATOR CHARACTERISTICS

From the Receiver Operator Characteristics (ROC) analysis, the Area Under the Curve (AUC) indicates that all the markers of Oxidative Stress could be used as predictive markers in HIV infection with Malondialdehyde (MDA), Glutathione Peroxidase (GPx), Superoxide Dismutase (SOD) and Vitamin E having the best AUC of 0.98, 0.99, 0.99 and 0.93 (Table 3.4). From this study, SOD was adjudged to be the best oxidative stress predictor in HIV infection. This conclusion stem from the fact that it gave the best sensitivity, specificity, positive predictive value and

negative predictive value of 98.05, 100.00, 100.00 and 93.9 percent respectively. This was followed by MDA, GPx, Vitamin E, FRAP and the least being Vitamin C.

From the pair-wise comparison of the predictive powers of Oxidative Stress markers FRAP, Vitamin E and Vitamin C could be used interchangeably as their predictive strength do not differ significantly as reflected by their p value and difference between AUC as shown in Table 3.5. Judging by the same parameters MDA, GPx and SOD could be used interchangeably as their predictive strength does not differ significantly. Since no literature has documented the predictive value of these oxidative stress markers as such there is no data to compare this finding to, a larger study prospectively designed for this purpose would be required.

The clear difference in the oxidative stress markers measured in this study in severe (CD4 < 200 mm^{-3}) compared to moderate (CD4 200-499 mm^{-3}) and to mild HIV (CD4 > 500 mm^{-3}) is in agreement with either a process in the severe cases resulting in greater burst of reactive oxygen species release and hence a greater rise in lipid peroxidation products or, a similar level of reactive oxygen species but with greater tissue damage in the presence of other unknown factors. The presence of significant difference between mild to moderate and severe disease of both enzymatic and vitamin antioxidant activity in this study supports the hypothesis that the release of ROS participate in the production of MDA and oxidative stress in severe HIV infection. The factor affecting severity appears to be due in part to the dietary deficiency of antioxidants, which was different in the CD4 classification of the severity.

Chapter 5

CONCLUSIONS AND RECOMMENDATION

It is clear from this study that during the progression of HIV infection, there is a concomitant induction of cellular oxidative stress characterized by; An increase in Malodialdehyde (MDA), a product from lipid peroxidation as HIV infection progresses indicating an increase in lipid peroxidation in HIV infection. There is also a general decline in Ferric Reducing Ability of Plasma (FRAP) reflecting a general decrease in antioxidant capacity of the HIV infected subjects. An overall decline in the level of antioxidant enzymes viz superoxide dismutase (SOD) and glutathione peroxidase (GPx). This demonstrates a derangement in the antioxidant system of People Living with HIV/AIDS (PLWHAs). There was also a general decrease in the scavenging antioxidants measured i.e. Vitamin C and E. this signified a derangement in the antioxidant system of HIV infected individuals. These Reactive Oxidative Species (ROS) induce cellular imbalance which has been found to present in various stages of HIV infection. We propose among other things that;

Many human immunodeficiency viruses (HIV)-infected patients suffer from chronic oxidative stress resulting from an increase in plasma ROS and a systematic reduction in the concentrations of antioxidants. During HIV infection, ROS can induce pro-inflammatory cytokines, including tumour necrosis factor (TNF) and interferons which subsequently promote further ROS generation.

Intracellular ROS is involved in the activation NF-kB, a known transactivator of HIV-LTR. An increase in NF-kB activation would result in an increase in HIV replication via the activation at the LTR of the viral genome. Diverse antioxidants inhibit HIV-LTR transactivation by blocking NF-kB in most cell lines. Besides, these ROS are likely to work in synergy with other haematological and biochemical indices like the lipid profile and blood haemoglobin to reflect a broad spectrum

effect at cellular level which perhaps leads to programmed cell death and progression of HIV infection. We thus conclude that HIV progression to AIDS is not a single cellular event but a combination of other previously less observed factors like ROS which this study has confirmed. The overall effect is the selective and progressive destruction of cells through apoptosis and finally cell death.

It is likely from this study that adverse effect of ROS can be alleviated by administration of Antioxidants as a combination therapy with Antiretroviral drugs which at cellular level will alleviate the effect of ROS and lead to the proper management of People Living with HIV/AIDS. Again, the data obtained from the haematological and biochemical assays could be used as pre-intervention data for the management of HIV/AIDS patients in Ghana.

It is thus recommended from this study that studies on the benefits of antioxidants as adjuncts of antiretroviral therapy should be carried out. This would enhancement of the management of People living with HIV/AIDS (PLWHAs) in Ghana.

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APPENDIX

Reagents used in the assay of Glutathione Peroxidase

Content	Concentration in the test
Reagent	
Glutathione	4.0mmol/l
Glutathione Reductase	≥ 0.5U/l
NADPH	0.34mmol/l
Buffer	
Phosphate buffer	0.05mol/l; pH 7.2
EDTA	4.3mmol/l
Cumene hydroperoxidase	0.18mmol/l
Diluting agent	
Haemoglobin Reagent	
Potassium Phosphate	10.3mmol/l
Potassium Ferricyanide	6.08mmol/l
Potassium Cyanide	7.68mmol/l
Surfactant	0.1% v/v

Reagents used in the assay of Superoxide dismutase

Content	Concentrations of solutions
Mixed substrate	
Xanthine	0.05 mmol/l
INT	0.025 mmol/l
Buffer	
CAPS	40 mmol/l; pH 10.2
EDTA	0.94 mmol/l
Xanthine oxidase	80 U/l
Standard	
NaCl solution	0.9 %
Phosphate buffer	0.01 mmol/l