UNIVERSITY OF GHANA

COLLEGE OF BASIC AND APPLIED SCIENCES

GLUTATHIONE S-TRANSFERASE GENE POLYMORPHISM AND ANTIOXIDANT ENZYMES ACTIVITY IN HIV/AIDS PROGRESSION IN GHANAIAN PATIENTS

BY

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A THESIS SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES IN PARTIAL FULFULMENT OF THE AWARD OF MASTER OF PHILOSOPHY DEGREE IN MOLECULAR CELL BIOLOGY OF INFECTIOUS DISEASES

DEPARTMENT OF BIOCHEMISTRY, CELL AND MOLECULAR BIOLOGY

JULY 2016

DECLARATION

I, Joshua Agbemefa Kuleape hereby declare that this thesis is the outcome of my own research project under the supervision of Dr. Osbourne Quaye of the Department of Biochemistry, Cell and Molecular Biology, University of Ghana and Dr. Evelyn Yayra Bonney, Noguchi Memorial Institute for Medical Research. To the best of my knowledge, this thesis has not been presented for the award of any degree or published elsewhere. Any mention of other authors' works have been duly acknowledged and properly referenced.

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ABSTRACT

Glutathione and enzymes such as glutathione S-transferase (GST), glutathione reductase (GR) and superoxide dismutase (SOD) play crucial roles in defending the cell against reactive oxygen species (ROS) which have been implicated in HIV replication. It has been shown that cells infected with HIV generate large amounts of inflammatory products such as superoxide anions, peroxynitrites and other ROS, and is accompanied by the deregulation of antioxidant enzymes. The pro-oxidative stress environment in the cell results in reduction of the protective potential of the antioxidants. GST family of enzymes is involved in a two-stage detoxification process of a wide range of environmental toxins, carcinogens and antiretroviral (ARV) drugs. The GST enzymes play important roles in oxidative stress pathways, and polymorphisms in different GST genes mediate susceptibility and outcome in different diseases. Little information is however known about the polymorphisms in Ghanaian patients. Hence the aim of this study was to investigate GST gene polymorphisms and antioxidant enzymes activities in Ghanaian HIV/AIDS patients. A total of 242 individuals comprising 105 HIV-infected patients on ART, 77 HIV patients who are ART naïve and 60 HIV seronegative people were recruited for the study. The impact of the virus on HIV/AIDS disease progression was assessed by measuring CD4+ cell count and viral load. It was observed that the $CD4^+$ count in the ART naïve patients (298 ± 243 cells/mm^3) was significantly less when compared to the HIV patients on ART (604 ± 294 cells/mm^3) and control subjects (946 ± 125 cells/mm^3). The lower CD4+ count was due to progression of the disease in these individuals. Viral load was significantly lower in the ART patient group (30379 ± 15073 copies/mm^3) than the ART naïve group (209882 ± 75045 copies/mm^3). The study further investigated the impact

of the virus and/or antiretroviral treatment on liver function and hematological profile in the study population. Liver function tests results showed elevated levels of ALP, AST and GGT in both HIV patient groups which suggest liver toxicity. Liver toxicity in these patients could be due to the virus in ART naïve patients whereas in those on treatment, it could be due to the antiretroviral drugs. Haematological profile results showed that the ART naïve individuals had normal RBC count of [4.0 ± 0.8 (10^{12} /L)], but low hemoglobin (10.8 ± 2.2 g/dL) as well as low hematocrit (32.7 ± 6.1 %) and mean corpuscular volume levels (81.2 ± 8.5 fL) compared to ART treatment patients and the seronegative control group. Hence, the ART naïve patients could be suffering from iron-deficiency anemia. The results from the liver function and hematological profile suggest a possible ineffective clearance of ROS. In confirmation of the proposed ineffective clearance of ROS, activities of superoxide dismutase (SOD) and glutathione reductase (GR), and the levels of reduced glutathione (GSH) were investigated. Significantly low SOD and GR activities, as well as GSH levels were observed in the ART naive patients and those with CD4 count below 200 cells/mm^3. More importantly, this study is the first report of the frequencies of GSTM1 and GSTT1 deletions in Ghana which were shown to be 21.9% and 19.8%, respectively in HIV patients and those who had a homozygous deletion of both GSTM1 and GSTT1 were at risk of their CD4 count falling below 350 cells/mm^3.

DEDICATION

I dedicate this work to my father and late mother for all their sacrifices in getting me this far in life.

ACKNOWLEDGEMENT

I wish to express my profound gratitude to God Almighty as well as my supervisors, Dr. Osbourne Quaye, Department of Biochemistry, Cell and Molecular Biology and Dr. Evelyn Y. Bonney, Noguchi Memorial Institute for Medical Research for their patience, supervision and mentorship. I would also like to thank WACCBIP and Noguchi Postdoctoral Masters Fellowship for funding this work. WACCBIP paid my fees and provided a monthly stipend for which I am very grateful. A special appreciation goes to Mr. Emmanuel Tagoe for the idea behind the study, his selfless support, motivation and encouragement. Thank you very much Sir.

My sincere appreciation goes to Dr Jonathan Adjimani and my sister, Yvonne Kuleape for the time and resources they made available to me to get me throughout the course. To Mr. Seth Agyemang of Immunology Department, central Laboratory of Korle-Bu Teaching Hospital, I say thank you, for your time, patience and support during the laboratory analysis of samples. I also thank the patients and staff of the Fevers unit of Korle-Bu Teaching Hospital for their help during my sample collection at the unit.

To my brother, Ishmael Tagoe, I say thank you for all your selfless support. I am indeed grateful. A heartfelt appreciation goes to Samuel Mawuli Adade, Maame Ekua Acquah and Precious Cramer and the entire Virology Laboratory, Department of Biochemistry, Cell and Molecular Biology, UG, for their help throughout this study. A special thanks to the leadership and members of Molecular Biology and Infectious Diseases Laboratories for their support and advice during the project. I extend my gratitude to the lecturers,

laboratory technicians and supporting staff of Department of Biochemistry, Cell and Molecular Biology.

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LIST OF ABBREVIATIONS AND ACRONYMS

3TC – Lamivudine

ABC – Abacavir

AIDS – Acquired Immune Deficiency Syndrome

ALP – Alkaline Phosphatase

ALT – Alanine Transaminase

ARV – Antiretroviral

ART – Antiretroviral treatment

AST – Aspartate Transaminase

AZT – Azidothymidine (Zidovudine)

CAT - Catalase

CD4 – Cluster of differentiation 4

CDC – Center for Disease Control

CRF – Circulating Recombinant Form

d4T – Stavudine

ddI – Didanosine

DNA – Deoxyribonucleic Acid

dsDNA – double stranded DNA

EFV – Efavirenz

EIA – Enzyme Immunoassay

FTC – Emtricitabine

GGT – Gamma Glutamyl Transferase

GPx - Glutathione peroxidase

GR – Glutathione reductase

GSH – Reduced glutathione

GSSG – Disulfide glutathione

GST – Glutathione S-transferase

GSTA - Glutathione S-transferase alpha

GSTK - Glutathione S-transferase

GSTM - Glutathione S-transferase Mu

GSTP - Glutathione S-transferase Pi

GSTS - Glutathione S-transferase sigma

GSTT - Glutathione S-transferase theta

GSTZ - Glutathione S-transferase zeta

HAART – Highly Active Antiretroviral Therapy

HCT – Haematocrit

HIV – Human Immunodeficiency Virus

HTLV – Human T-lymphotrophic Virus

IκB – Inhibitory kappa B

IDV – Indinavir

LAV – Lymphadenopathy Associated Virus

LTR - Long terminal repeats

MCH – Mean Cell Hemoglobin

MCHC – Mean Cell Hemoglobin Concentration

MCV – Mean Cell Volume

NF-κB – Nuclear factor kappa B

NFV – Nelfinavir

NNRTI – Non-Nucleoside Reverse Transcriptase Inhibitors

NRTI – Nucleoside Reverse Transcriptase Inhibitors

NVP – Nevirapine

PBMC - Peripheral blood mononuclear cells

PBS - Primer binding site

PI – Protease Inhibitors

PPT - Poly-purine tract

PUFA – Polyunsaturated Fatty acids

RBC – Red Blood Cell

RDT – Rapid diagnostic tests

RNA – Ribonucleic Acid

mRNA - messenger RNA

ssRNA – single stranded Ribonucleic Acid

ROS – Reactive Oxygen Species

RTI - Reverse transcriptase inhibitors

SIV – Simian Immunodeficiency Virus

SNP - Single nucleotide polymorphisms

SOD – Superoxide Dismutase

SQV – Saquinavir

TDF – Tenofovir

TNF-α – Tumour Necrosis Factor-alpha

UNAIDS – United Nations Programme on HIV/AIDS

WBC – White Blood Cell

WHO - World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND

Since its discovery more than three decades ago, HIV/AIDS has become one of the leading causes of death worldwide, and an estimated 35 million people were reportedly living with HIV as at 2013 (UNAIDS, 2014). Sub-Saharan Africa has borne the brunt of the infection since the highest number of individuals living with the virus resides there. The incidence of HIV and AIDS related deaths is due to a continuous immunodeficiency associated with the depletion of $CD4^+$ T lymphocytes, which leads to a compromised immune system. HIV infection is for life since the body cannot get rid of the virus.

Deficiencies in the ability of antioxidants to defend the host and amplified levels of products from oxidation have been detected in HIV patients who are asymptomatic and in patients with AIDS (Pace & Leaf, 1995). This confirmation suggests oxidative stress is accelerated in the patients (Pace & Leaf, 1995). Oxidative stress arises from the inability of antioxidant defenses to effectively clear the reactive oxygen species (ROS) produced from oxidative metabolism (Allard *et al.*, 1998). The condition is frequently described in HIV-1-infected patients, as studies have reported raised up levels of extracellular and intracellular ROS (Allard *et al.*, 1998). Studies have revealed that oxidative stress favors viral replication, and thus an increase in viral load (Droge *et al.*, 1994). Additionally, viral proteins such as gp120, Vpr and Tat are known to induce cellular oxidative stress (Alcamo, 1993; Westendorp *et al.*, 1995). Moreover, antiretroviral treatment (ART) was introduced in the absence of an HIV vaccine to delay viral replication and immune system deterioration in order to improve the quality and span of life. However, antiretroviral drugs

have many side effects. Protease inhibitors (PIs), for example, have been shown to boost production of ROS in cells such as adipocytes, macrophages, endothelial cells in umbilical vein, β-cells of the pancreas, and vascular smooth muscle cells (Lei *et al.*, 2010; Wu *et al.*, 2010; Salmen & Berrueta, 2012). The ROS produced enable the disruption of the integrity of the intestinal barrier, signifying that ROS promote side effects caused by HIV PIs (Wu *et al.*, 2010; Salmen & Berrueta, 2012).

Oxidative stress in infected cells enables viral replication which is regulated by nuclear factor-kappa B (NF-κB) transcriptionally and enables inflammatory cytokine release; thus, maintaining immune activation and the advancement of the disease (Westendorp *et al*., 1995). NF-κB also activates the inflammatory cytokines of the immune system (Reshi *et al.*, 2014). Besides membrane damage, serum from HIV-infected and AIDS patients have high hydroperoxide and malondialdehyde levels, which are the consequence of lipid peroxidation (Reshi *et al.*, 2014). They also display an increase in resting oxygen consumption, as formation of free-radicals is linked to the metabolism of oxygen (Reshi *et al.*, 2014). This is buttressed by the fact that ROS is produced in the neutrophils of HIV-infected patients (Jarstrand & Akerlund, 1994), whose ROS detoxification systems undergo intense modifications. In clinical studies, markers for increased oxidative stress in plasma such as superoxide dismutase and circulating CD4+ T-lymphocytes correlate with progression of disease in HIV infected individuals (Ames *et al.*, 1993).

Superoxide dismutase (SOD) levels and activity in children suffering from HIV infection have been shown to be diminished (Reshi *et al*., 2014). Conversely, the antioxidant enzyme catalase, has increased activity as AIDS progresses in HIV patients

(Pasupathi *et al.*, 2009). Glutathione peroxidase levels in RBCs and plasma also decreases (Reshi *et al*., 2014). This indicates that the antioxidant defense system in the body becomes feebler as the disease advances (Reshi *et al*., 2014). Internal and external imbalances of the cell have an effect on it undergoing programmed cell death. The reduced levels of catalase and glutathione result in more hydrogen peroxide (H_2O_2) storage and further elevates hydroxyl radicals and lipid peroxide levels which signal the cell to undergo apoptosis (Reshi *et al*., 2014). Under *in-vitro* conditions, the decrease in antioxidants and subsequent increase in H_2O_2 result in apoptosis in the cell culture system (Reshi *et al*., 2014). This disparity in ROS promotes CD4 cell death and impairs the functions of other constituents of the immune system.

The unrelenting oxidative load results in an enhanced rate of consumption of glutathione (GSH). GSH is a potent endogenous antioxidant that aids the clearing of ROS. Over 90% of the total glutathione in healthy cells and tissue occurs in the reduced form (GSH) whilst less than 10% is in the disulfide form (GSSG). A rise in GSSG-to-GSH ratio is suggestive of oxidative stress. Moreover, studies have demonstrated that the amount of glutathione in HIV seropositive people is reduced in their plasma, peripheral blood mononuclear cells (PBMCs), fluid in lung epithelial lining, lymphocytes and monocytes (Westendorp *et al*., 1995). The diminished levels of GSH negatively affects T cell function (Roederer *et al.*, 1993) and enhances expression of HIV proteins (Kalebic *et al.*, 1991); hence proposing an association between deficiency in GSH and progression of the disease. Herzenberg *et al.* (1997) confirmed this by reporting increased death rates among HIV-infected persons with lower GSH levels and better survival when GSH was replenished (Wanchu *et al*., 2009).

Glutathione S-transferase (GST) is a drug metabolizing enzyme that has a high level of conjugation specificity for glutathione (GSH). They are a family of enzymes that are essential to the biosynthesis and metabolism processes of many substances. They consist of four groups of enzymes namely; Alpha (GSTA), Mu (GSTM), Pi (GSTP) and Theta (GSTT), which control the detoxification of compounds in drugs and carcinogens, produced from oxidative stress, and inhibit oxidative damage to tissues. The most studied GST genes are GSTM1 and GSTT1, and they have been described as polymorphic in humans. Polymorphisms in GST are connected to higher risk of oxidative stress (Watson *et al.*, 1997; Zhao *et al.*, 2000). These polymorphisms can be classified in two groups: the homologous deletion genotype (also called the null genotype) and one or two undeleted alleles (called the non-null or present genotype).

Disparities in the sequences of the GSTM1 and GSTT1 genes have been linked with different expression of GST gene, and it affects the enzyme's activity and increases susceptibility to certain cancers (Moulik *et al.*, 2.014). Therefore, investigating polymorphisms in GSTM1 and GSTT1 genes is imperative for the assessment of disease progression. Many studies have revealed polymorphic GST alleles to be connected to altered risk or outcome of several diseases. The GST variants alter the catalytic activity of the enzymes. Consequently, persons producing less definite detoxification enzymes could be at a higher risk of adverse outcome of disease (Sun *et al.*, 2014). Clearly, there is a substantial need to clarify the role of polymorphic genes in HIV disease progression. Hence the study was aimed at elucidating the frequency of the null GST isoforms as well as the effect of antioxidant enzymes activities in Ghanaian HIV/AIDS patients.

1.1.1 Justification/Hypothesis

There is little or no information on the frequency of the null GST isoforms among Ghanaians. It is increasingly recognized that GST deficiencies worsen oxidative stress condition in HIV/AIDS patients. Therefore, we hypothesized that those HIV patients with the null GSTM1 and GSTT1 isoforms will have deficiencies in GST activity as well as increased oxidative stress. Hence, knowledge of GST and oxidative stress status will help provide a comprehensive approach to improve health care delivery to HIV/AIDS patients. The current study was designed to provide adequate baseline data on effects of GST deficiencies on liver function and oxidative stress in Ghanaian HIV/AIDS patients. This information will help with the design of a more extensive study, the findings of which will aid the management of the HIV disease in patients.

1.1.2 Aim

To investigate Glutathione S-Transferase gene polymorphism and antioxidant enzymes activity in HIV/AIDS progression in Ghanaian patients

1.1.3 Specific objectives

1. Effect of HIV and/or ART on hematological profile, oxidative stress markers and liver function of study population.
2. Investigate glutathione S-transferase gene polymorphism in patient and control groups.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 HISTORY OF HIV/AIDS

Acquired immunodeficiency syndrome (AIDS) is caused by human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2). Human immunodeficiency virus (HIV) and its subtypes are etiologic agents of AIDS, and they are retroviruses. Human retroviruses were first discovered in the 1980's, by which time animal retroviruses like feline leukaemia virus had already been detected (Avert, 2006). HIV belongs to genus lentiviruses which are regarded as being associated with diseases of immunosuppression, the central nervous system and have long incubation periods after infection till signs of illness become obvious (Avert, 2006). Both HIVs resulted from several cross-species spread of simian immunodeficiency viruses (SIVs) which naturally infects African primates. A lot of these transfers led to the spread of viruses in humans to only a limited extent. However, the main cause of the AIDS pandemic, HIV-1 group M was caused by a single transmission event, which involved SIVcpz from chimpanzees found in southeastern Cameroon. HIV/AIDS was officially accepted as a new disease in 1981 when growing numbers of young homosexual men succumbed to unfamiliar opportunistic infections and rare malignancies (CDC 1981; Greene 2007). HIV spreads through sexual intercourse, needle sharing, and during birth as well as blood transfusions (Hladik and McElrath 2008; Cohen *et al*., 2011); nonetheless, about 80% of adult HIV-1 infections occur after exposure at mucosal surfaces, and hence, HIV/AIDS is predominantly a sexually transmitted disease (Hladik and McElrath 2008; Cohen *et al*., 2011). The first known case of HIV-1 infection in a human was identified in 1959 in a blood sample collected from a man in Kinshasa,

Democratic Republic of the Congo. Information from analysis of the blood sample genetically suggested that the infection may have been acquired in the late 1940s or early 1950s.

In 1982 public health officials started using the term "acquired immunodeficiency syndrome," or AIDS, to refer to the incidences of opportunistic infections, Kaposi's sarcoma (a kind of cancer), and *Pneumocystis jirovecii* pneumonia in people who used to be healthy. The surveillance of HIV/AIDS cases began that year in the United States. In 1983, scientists discovered the virus that causes AIDS and an international scientific committee initially called it human T-cell lymphotropic virus-type III/lymphadenopathy-associated virus (HTLV-III/LAV), however, it was later renamed human immunodeficiency virus (HIV). Millions of dollars have been spent on research in order to understand the reasons for the sudden emergence of HIV, the spread of the epidemic, and its unique pathogenicity. Researchers in 1986 discovered a structurally similar but antigenically different virus which also caused HIV/AIDS in people in West Africa (Clavel *et al*., 1986). Interestingly, the novel virus, called human immunodeficiency virus type 2 (HIV-2), was related to HIV-1, but remained closely related to a simian virus responsible for immunodeficiency in captive macaques (Chakrabarti *et al*., 1987; Guyader *et al*., 1987). After this, more viruses, jointly labelled simian immunodeficiency viruses (SIVs) with a suffix to designate their species of origin, were found in diverse primates from sub-Saharan Africa. These included African green monkeys, sooty mangabeys, mandrills and chimpanzees.

Amazingly, these viruses were mostly non-pathogenic in their natural hosts, even though they clustered together with the human and simian AIDS viruses in a distinct

phylogenetic lineage of lentiviruses. Close simian relatives of HIV-1 and HIV-2 were found in chimpanzees (Huet *et al*., 1990) and sooty mangabeys, respectively (Hirsch *et al*., 1989). These connections provided the initial indication that AIDS had emerged in both humans and macaques as a result of cross-species infections with lentiviruses from different species of primates (Sharp *et al*., 1994). Later studies established that SIVmac was not a normal pathogen of macaques (which are Asian primates), but had been produced unintentionally in US primate centers by injecting several species of macaques with blood from sooty mangabeys that were naturally infected (Apetrei *et al*., 2005; Apetrei *et al*., 2006). In the same way, it became clear that HIV-1 and HIV-2 were the consequence of zoonotic transferences of viruses that infected primates in Africa (Hahn *et al*., 2000).

From the time when it was first identified more than 30 years ago, HIV-1 group has infected more than 60 million people and accounted for over 35 million deaths worldwide (Merson *et al*., 2008; UNAIDS, 2014). Developing countries have borne the greatest brunt of HIV/AIDS morbidity and mortality, with sub-Saharan African countries recording the highest prevalence rates (Sharp & Hahn, 2011). Antiretroviral therapy has improved access to treatment and decreased the toll of AIDS-related deaths. However, the likelihood of developing curative treatments and an effective vaccine are unclear (Barouch 2008; Richman *et al*., 2009). Thus, HIV/AIDS will linger as a significant threat to public health for years to come.

HIV infection is often diagnosed through rapid diagnostic tests (RDTs), which detect the presence or absence of HIV antibodies. Most often these tests provide same day

test results; essential for same day diagnosis and early treatment and care. There is no cure for HIV infection. However, effective antiretroviral (ARV) drugs can control the virus and help prevent transmission so that people with HIV, and those at substantial risk, can enjoy healthy and productive lives.

2.2 HIV/AIDS STATISTICS WORLDWIDE

HIV continues to be a major global public health issue, having claimed more than 35 million lives so far. According to the WHO progress report on HIV in 2014, 1.2 [0.98–1.6] million people died from HIV-related causes globally (WHO, 2014). There were approximately 36.9 [34.3–41.4] million people (including 2.6 million children) living with HIV at the end of 2014 with 2.0 [1.9–2.2] million people becoming newly infected with HIV globally (WHO, 2014). Sub-Saharan Africa was the most affected region, with 25.8 [24.0–28.7] million people living with HIV in 2014. Moreover, sub-Saharan Africa accounted for almost 70% of the global total of new HIV infections (WHO, 2014). It is estimated that only 54% of people with HIV know their status (UNAIDS, 2014; WHO, 2014). In 2014, approximately 150 million children and adults in 129 low and middle-income countries received HIV testing services (UNAIDS, 2014; WHO, 2014). The WHO report projected that by mid-2015, 15.8 million people living with HIV would be receiving antiretroviral therapy (ART) globally. Between 2000 and 2015, new HIV infections have fallen by 35%, AIDS-related deaths fell by 24% with some 7.8 million lives saved as a result of international efforts that led the global achievement of the HIV targets of the Millennium Development Goals (UNAIDS, 2016). Expanding ART to all people living with HIV and expanding prevention choices can help avert 21 million AIDS-related deaths and 28 million new infections by 2030 (UNAIDS, 2016).

2.3 HIV/AIDS IN GHANA

Like other countries worldwide, HIV/AIDS is present in Ghana. In response to the epidemic, the government established the Ghana AIDS Commission (GAC) and the National AIDS/STI Control Programme (NACP). GAC coordinates efforts amongst NGO's, international organizations and other parties to support the education about and treatment of AIDS throughout Ghana and alleviating HIV/AIDS issues in Ghana. NACP undertakes technical aspects of curbing the HIV/AIDS menace in the country by procuring and equipping health facilities and workers with equipment, reagents, test kits, consumables and requisite training to diagnose, manage and monitor the cases.

Ghana's system of HIV surveillance for women attending antenatal clinics has functioned well since its establishment in 1994. Yearly Sentinel Surveys of 69 antenatal clinics located in 40 sentinel sites across the country was initiated on the premise that prevalence of HIV among women with their first pregnancies is a good proxy indicator of the spread of the infection among the populace. According to the UNAIDS HIV/AIDS estimates for 2014, Ghana's HIV prevalence was 1.6%. A total of 250,000 [190,000 - 330,000] people were living with HIV; of which 230,000 [180,000 - 300,000] were adults. Children aged 0 to 14 living with HIV were 21,000 [16,000 - 28,000]. The prevalence rate among adults aged 15 to 49 years was 1.5% [1.1% - 2.0%]. Women aged 15 years and above living with HIV were 140,000 [110,000 - 180,000]. Deaths due to AIDS totalled 9,200 [7,000 - 13,000] and 120,000 [85,000 - 250,000] young people aged 0 to 17 years were orphaned due to AIDS.

HIV prevalence at the regional level ranged from 0.6% in the Northern region to 3.7% in the Eastern region. The Greater Accra region recorded a prevalence of 3.1%

followed by Ashanti region (2.8%), Brong Ahafo (2.6%), Western (2.4%), Volta (2.2%), Upper East (1.4%), Central (1.4%) and Upper West (1.3%) in that order (Ghana Health Service, 2014).

2.4 CLASSIFICATION OF HIV INFECTION

The Centres for Disease Control and Prevention (CDC) classification system for HIV Infection is the health classification structure used to categorize HIV disease and infection. The government uses the system to handle epidemic statistics and outline who receives support. The CDC included pulmonary tuberculosis, persistent pneumonia, and invasive cervical cancer to the list of clinical conditions in the AIDS surveillance case definitions put out in 1987.

The AIDS surveillance case definition was also expanded to include all HIV seropositive individuals with CD4+ T-lymphocyte counts below 200 cells/µL or a CD4+ percentage of less than 14 or has one of the following illnesses; Candidiasis of bronchi, trachea, or lungs; esophageal Candidiasis; invasive cervical cancer; disseminated or extrapulmonary Coccidioidomycosis; extrapulmonary Cryptococcosis; chronic intestinal Cryptosporidiosis which has persisted more than 1 month; Cytomegalovirus disease (other than liver, spleen or lymph nodes); HIV-related Encephalopathy; Herpes simplex: chronic ulcer(s) (for more than 1 month); or bronchitis, pneumonitis, or esophagitis; disseminated or extrapulmonary Histoplasmosis; chronic intestinal Isosporiasis for more than 1 month; Kaposi's sarcoma; immunoblastic or primary brain Lymphoma Burkitt's; Mycobacterium avium complex; other species of Mycobacterium, disseminated or extrapulmonary; Pneumocystis carinii pneumonia; recurrent Pneumonia; Progressive multifocal

leukoencephalopathy; recurrent Salmonella septicemia; Toxoplasmosis of the brain; Tuberculosis; Wasting syndrome due to HIV. Significant variation exists in the relative risk of death after different AIDS defining clinical conditions. It must be noted that HIV seronegative individuals may develop these conditions but they are required to present laboratory evidence against HIV infection (CDC, 1993; WHO, 2007).

2.5 TYPES OF HIV

HIV has two main forms called HIV-1 and HIV-2. HIV-1 was discovered in 1983 by Luc Montagnier and his team at the Institute Pasteur, Paris. HIV-2 was first identified among patients in Cameroon in 1985. It is more related to SIV (Simian Immunodeficiency Virus) than HIV-1 and less virulent (UNAIDS, 2014).

2.5.1 HIV-1

Data from molecular epidemiology have shown that HIV-1 is the most common and pathogenic strain of HIV. It is related to viruses found in primates living in West Africa. The virus has been divided into a major group (Group M) and three minor groups, namely, Group N, O and P. They are quite rare and only found in Cameroon, Gabon and Equatorial Guinea. In group M, nine genetically distinct subtypes of HIV-1 have been identified. These are subtypes A, B, C, D, F, G, H, J and K. Furthermore, dissimilar subtypes can combine genetic material to form a hybrid virus, referred to as a 'circulating recombinant form' (CRFs), of which quite a few have been identified (UNAIDS, 2014).

The main subtype of HIV found in the Americas, Western Europe and Australasia is subtype B. Consequently, mainstream HIV clinical research has been focused on populations dominated by subtype B. The problem is that this subtype accounts for just

12% of HIV infections globally. Conversely, less research is available for subtype C, even though just under half of all HIV infected people have subtype C. It is quite common in the countries with high prevalence such as those in Southern Africa, in addition to the horn of Africa and India. The greatest assortment of subtypes is found in Cameroon and the Democratic Republic of Congo - the countries where the HIV-1 epidemic originated. However, these geographical patterns in the distribution of subtypes are changing over time, due to migration and the mixing of populations (UNAIDS, 2014).

2.5.2 HIV-2

This virus is relatively unknown and it is found mostly in West Africa. It is less infectious and its progression is slower than HIV-1. HIV-2 has greater homology to simian immunodeficiency virus (SIV) than to HIV-1. The widely used antiretroviral drugs are effective against HIV-2; however, optimum treatment is poorly understood. The HIV-2 subtypes have been grouped from A through F. The difference in genetic homology is up to 25% among these subtypes. Enzyme immunoassay (EIA) and Western blot assays can be used in the detection of all six; similar to those for HIV-1. Infection with HIV-2 in the long run leads to AIDS. Individuals can be co-infected with HIV-1 and HIV-2 (UNAIDS, 2014).

2.6 STRUCTURE OF HIV

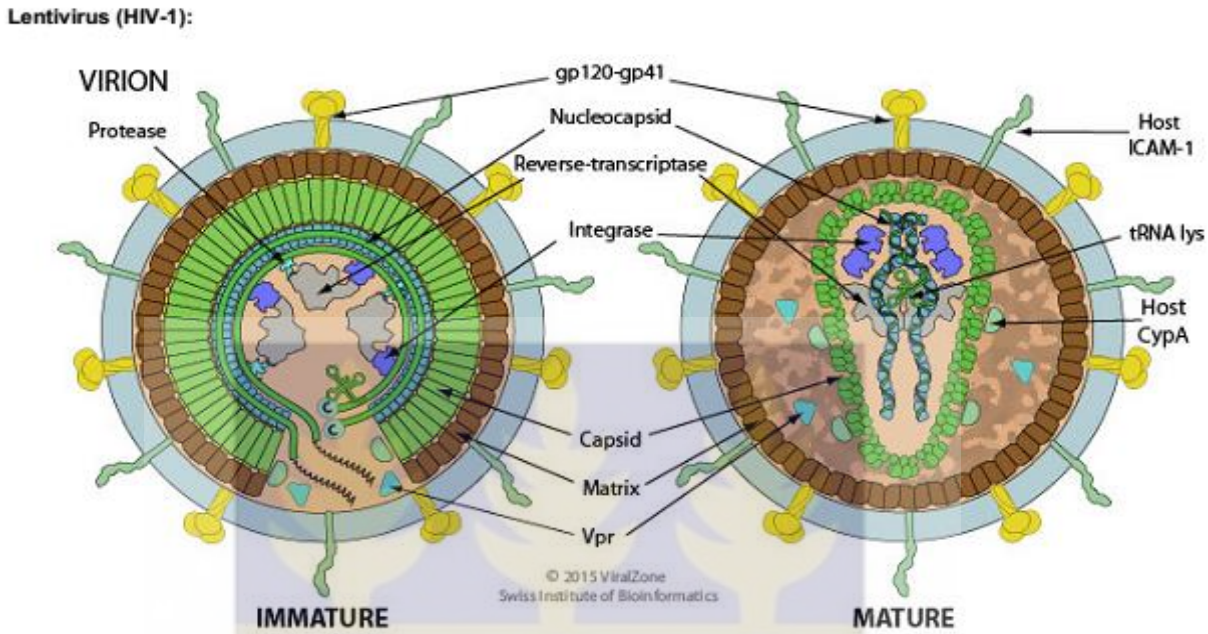

Figure 2. 1. General structures of an immature and mature HIV (ViralZone, 2015)

2.7 HIV GENOME

The single stranded RNA (ssRNA) (+) genome of HIV is about 9.75 kb in size with a 5' cap and a 3' poly-A tail. It has two long terminal repeats (LTRs) which are 600 nucleotides in length at the 5' and 3' ends. The U3, R, and U5 regions are contained within the LTRs. The genome also has a primer binding site (PBS) at the 5' end and a poly-purine tract (PPT) at the 3'end. The integrated provirus makes use of the promoter elements in the 5' LTR aid transcription. The resultant messenger RNA (mRNA) is not spliced and serves as genetic material that is either packed into virions or translated to make gag and gag-(pro) pol proteins. The partly spliced mRNAs code for env, which is cut into SU and TM envelope proteins, and other proteins like vif, vpu, and vpr. Conversely, the fully spliced mRNAs code for Rev, Tat and Nef proteins. Rev guides partially spliced and unspliced RNAs to exit the nucleus of diseased cells (Lu & Summers, 2011).

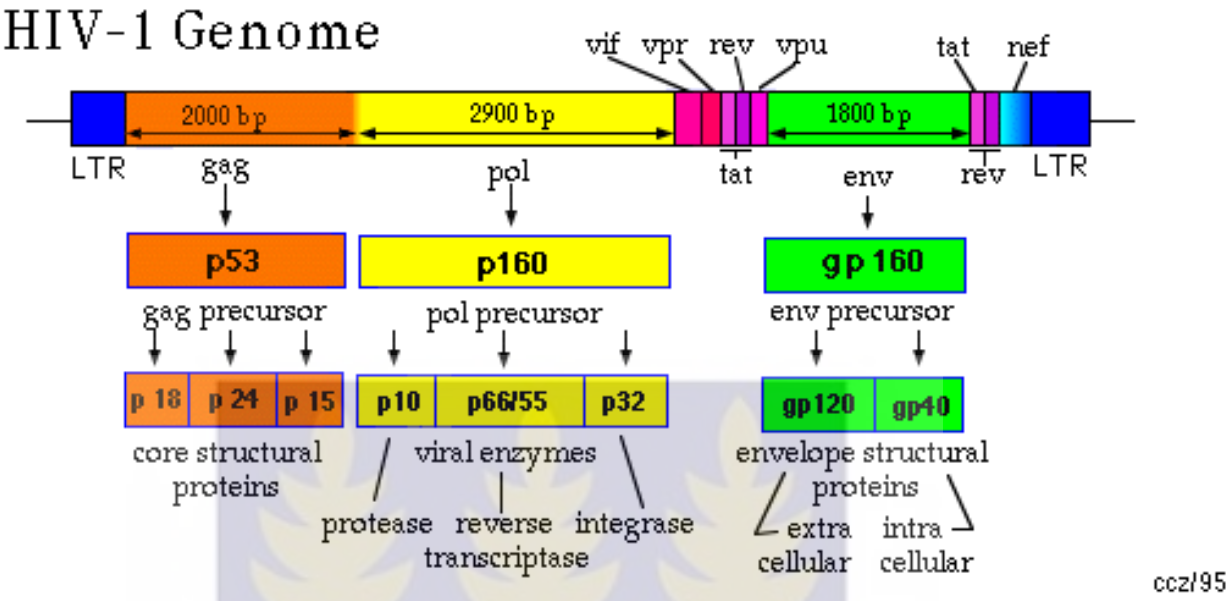

Figure 2. 2. Genome of HIV (Retroviridae, 2016)

2.8 LIFECYCLE OF HIV

HIV has a unique mechanism of replicating that is prone to mutations and it has accounted for the survival and success of the viruses. When the virus enters a cell, there is a release of the ssRNA and reverse transcriptase from the viral envelope. The ssRNA is changed into double-stranded DNA (dsDNA) by the action of reverse transcriptase and mediated by the host-cell RNA polymerase II. This dsDNA is then fused into the host's genome as a provirus. The virus now depends almost entirely on the host-cell replication machinery for the making of the viral DNA. This is followed by transcription of the viral DNA to form mRNA. After this, mRNA is translated into a protein, which is then cut by proteases to produce the distinct proteins that make up the virus. The mRNA also encodes the gag and pol gene products which are wrapped up into the virion particle as progeny genomic RNA (Figure 2.3).

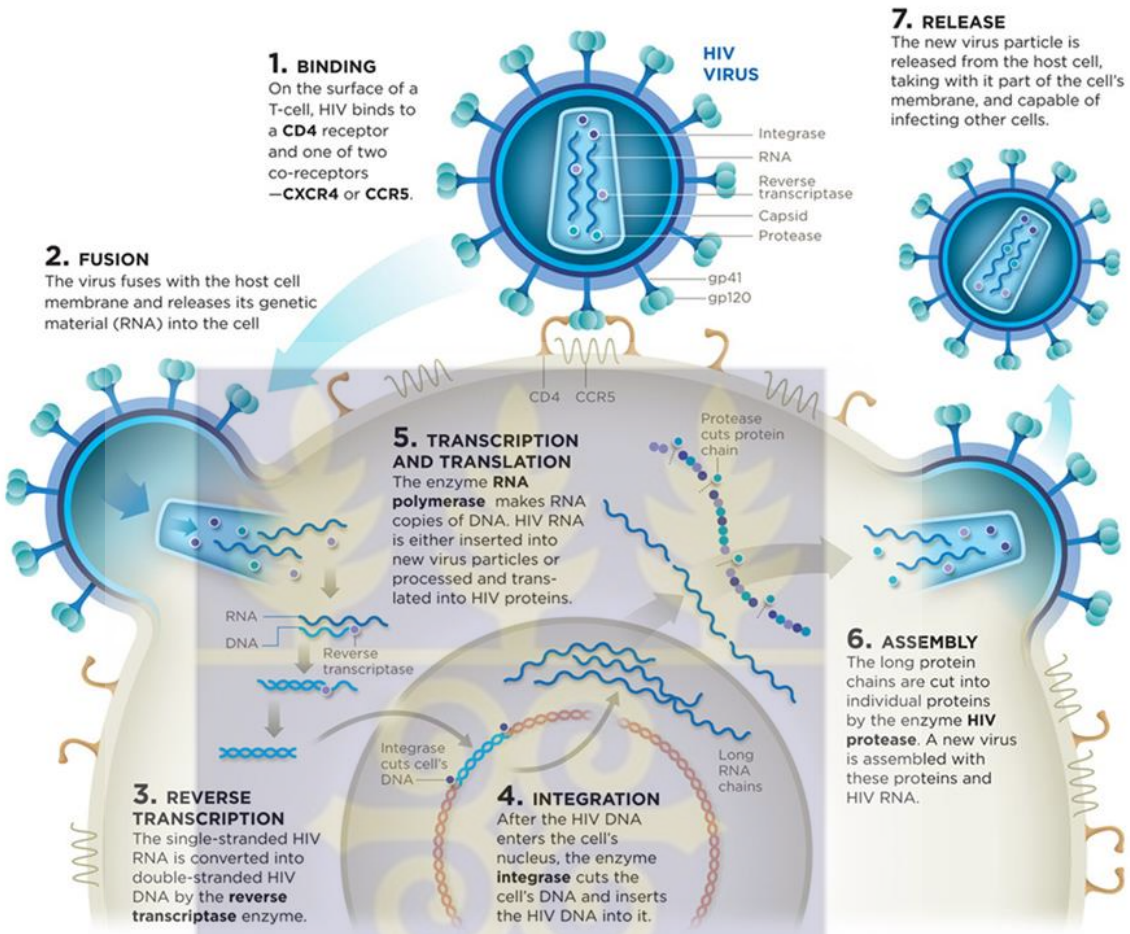

Figure 2.3. The HIV lifecycle (Pinterest, 2015)

2.9 ANTIRETROVIRAL DRUGS

Antiretroviral drugs (ARVs) are medicines used for the treatment of HIV infection and were first introduced in 1986. The combination of several of such drugs, typically three or four is termed highly active antiretroviral therapy (HAART) (British HIV Association Writing Committee, 2001; USDHHS, 2004). In the year 2001, a special session on HIV/AIDS organized by United Nations general assembly backed the complementarity of HIV care and avoidance, and advised governments to make available the highest level of care, including ART to people living with HIV/AIDS (WHO, 2002). HAART is, therefore,

recommended for the treatment of all patients with HIV/AIDS. However, due to the complexity of selecting and following a regimen as well as severity of the side-effects and the importance of compliance to prevent viral resistance; emphasis of involving patients in therapy choices, analyzing risks and potential benefit is important (Dybul *et al*., 2002).

2.9.1 Classes of Antiretroviral Drugs

There are different groups of antiretroviral drugs responsible for targeting different points of the HIV replication cycle.

2.9.1.1 Nucleotide and nucleoside reverse transcriptase inhibitors (NRTIs)

These drugs target reverse transcription by being inserted into the newly produced viral DNA and thwarting its further elongation. Drugs in this category include zidovudine (AZT), didanosine (ddl), lamivudine (3TC), tenofovir (TDF), stavudine (d4T), abacavir (ABC) and emtricitabine (FTC) (Weller and Williams, 2001).

2.9.1.2 Non-nucleoside reverse transcriptase inhibitors (NNRTIs)

These drugs stop reverse transcriptase openly by binding to the enzyme and tempering with its function. Some include nevirapine (NVP) and efavirenz (EFV) (Weller and Williams, 2001).

2.9.1.3 Protease inhibitors (PIs)

Protease inhibitors (PIs) prevent viral assembly by targeting protease activity. This enzyme is used by HIV to cut newly formed proteins for final assembly of fresh viral particles. Saquinavir (SQV), indinavir (IDV) and nelfinavir (NFV) belong in this class of drugs (de Soultrait *et al*., 2002).

2.9.1.4 Integrase inhibitors

Drugs such as raltegravir belong in this category and impede the activity integrase, which is in charge of integrating viral DNA into the host cell DNA (de Soultrait *et al*., 2002).

2.9.1.5 Entry inhibitors

Maraviroc and enfuvirtide are responsible for obstructing the attachment, merging and entry of HIV-1 into the host cell by blocking one of several targets (Kilby *et al*., 1998).

2.9.1.6 Maturation inhibitors

These drugs prevent the last step in the processing of gag wherein the capsid polyprotein of the virus is cut, thereby stopping its conversion into a mature capsid protein, p24. Since the viral particles have a faulty core, the virions that are released comprise mostly of non-infectious particles. There are no drugs in this class currently available, although BMS-955176 has been declared a promising drug (Kilby *et al*., 1998).

2.9.2 Combination Therapy

The replication cycle of HIV from entry of the virus into a cell, through release of new viral particles, to the infection of other cells can be as short as a day and half (Perelson *et al*., 1996). The virus lacks enzymes to proofread in order to correct inaccuracies in converting viral RNA into DNA via reverse transcription. This error prone replication enables the virus to mutate quickly, bringing about high genetic variability of HIV. Combining several antiretroviral drugs creates manifold obstacles to viral replication. It reduces the viral load and decreases the likelihood of more mutations. When a mutation makes the virus resistant to one of the drugs being administered, the others continue to

subdue duplication of that mutation. All antiretroviral drugs are unable to singly suppress the infection for long and hence these medicines have to be taken in combination so as to have a long-lasting effect. Consequently, the administered combinations comprise of two nucleoside analogue reverse transcriptase inhibitors (RTIs) and a single non-nucleoside-analogue RTI or protease inhibitor (USDHHS, 2004). This is referred to as highly active antiretroviral treatment (HAART).

Combination therapy is subject to negative as well as positive synergy, and this limits the number of effective combinations. For instance, Didanosine and AZT block each other, so taking them separately is better than having them in the combination. Factors such as dosing schedules and severe side-effects of the drugs often limit treatment options for some patients. As a result of this, pharmaceutical companies have combined these complex course of therapy into simpler prescriptions known as fixed-dose combinations. For instance, two pills can be made to contain two or three medicines each, and administered with ease twice daily. As a result, there is increased effectiveness in the treatment in the long-term. Lack of adherence on the part of patients is main reason for the development of drug resistance. Patients that comply with the regimen use the drugs for decades without developing any resistance. This significantly increases likelihood of survival in the long-term.

2.9.2.1 Initiation of HAART

Guidelines for antiretroviral treatment have changed several times. A conventional approach initiated the regimen when $CD4^+$ T cell count was below 200 cells/mm^3 initially then reviewed to below 350, then below 500; however, the current gold standard is to treat all. The current guiding principles for ART from the World Health Organization (WHO)

mirror the 2003 modifications to the guidelines and recommend that in resource-inadequate settings such as unindustrialized countries, HIV seropositive adults and young people should begin ART when the infection has been established and one of these conditions is present (WHO, 2003):

Clinically progressing disease;

WHO Stage I or II HIV disease with $CD4^+$ cell counts less than 200 cells/mm^3

WHO Stage III disease with $CD4^+$ cell counts below 350 cells/ mm^3;

WHO Stage IV HIV disease, regardless of the $CD4^+$ cell count.

2.9.2.2 Adverse effects of antiretroviral drugs

Provision of antiretroviral treatment is resource-intensive and expensive, and most of the seropositive individuals cannot access treatment services without government subventions. The side effects of the treatments vary by ethnicity, drug, patient and drug interactions with substances like alcohol and other drugs. Some individuals may be hypersensitive to some of the drugs. Examples of a number of common contrary indications experienced by patients on some antiretroviral drugs include: liver failure, abdominal pain, anemia, diarrhea, asthenia, hepatitis, flatulence, headache, dizziness, hyperpigmentation of nails, palms or soles, hypercholesterolemia, hyperbilirubinemia, ingrown nails, insomnia, lipodystrophy, mental confusion, alopecia, jaundice, malaise, migraines, mitochondrial toxicity, mood swings, myalgia, chronic fatigue syndrome, nausea, myopathy, low number of white blood cells, nightmares, oral ulcers, pancreatitis, numbness, peripheral neuropathy, rash, xerostomia (dry mouth), somnolence (drowsiness), change in taste perception, vomiting, xeroderma and renal failure or insufficiency (McNicholl, 2004).

2.10 OXIDATIVE STRESS

Reactive oxygen species are a normal consequence of a variety of essential biochemical reactions such as mitochondrial oxidative phosphorylation. Consequently, there is the need to a balance between free radical formation and antioxidant defence. Oxidative stress occurs when this balance between pro-oxidants and antioxidants is disrupted. Thus, oxidative stress is fundamentally an imbalance between the production of reactive oxygen species and the capacity of the organism's natural protective mechanisms to handle the reactive compounds and prevent their harmful effects. ROS are very reactive hence their accumulation beyond the needs of the cell may disturb cellular structure and functions by reacting with molecules like DNA, proteins, and lipids (Ames *et al.*, 1993; Mitra *et al.*, 2002; Cui *et al.*, 2004; Schaller, 2005).

2.10.1 Types of ROS

2.10.1.1 Singlet oxygen

Singlet oxygen ($^1O_2$) is mainly involved in photochemical reactions. Even though it does not have any unpaired electrons and is not a free radical, it is very reactive. It is formed *in vivo* through the activation of oxygen enzymatically; for instance, through lipo-oxygenase activity during the biosynthesis of prostaglandins (Cadenas and Sies, 1984). The highly reactive nature of this ROS brings about several carcinogenic, genotoxic and mutagenic effects when it interacts with polyunsaturated fatty acids (PUFAs) and DNA (Di Mascio *et al*., 1994).

2.10.1.2 Superoxide anion

Superoxide is a radical formed as a result of molecular oxygen being reduced when it accepts a single electron. It can also be formed from the dissociation of the hydroperoxyl radical which is unstable at physiological pH. *In vivo*, it is mainly formed through electron leakage in the mitochondria and microsomes along the electron transport chain. This phenomenon of electron leakage increases with increasing utilization of oxygen (McCord and Omar, 1993). Superoxide radicals can also be formed by the oxidation of epinephrine and norepinephrine using a metal ion catalyst, and by means of the action of enzymes like xanthine oxygenase, indoleamine dioxygenase and tryptophan hydroxylase. Metabolic pathways in activated phagocytes produce superoxide radicals to fight bacterial infections (Curnutte and Babior, 1987).

2.10.1.3 Hydrogen peroxide

Hydrogen peroxide and superoxide are transformed in the presence of transition metals; chiefly iron and copper (Halliwell and Gutteridge, 1990) in the Fenton or Haber-Weiss reactions to form highly reactive hydroxyl radicals. This ability coupled with hydrogen peroxide's membrane permeability gives them the ability to disrupt the integrity of molecules within the cell (Cochrane, 1991).

2.10.1.4 Hydroxyl radical

The most aggressive member of ROS is the hydroxyl radical and can cause widespread harm to several types of molecules like nucleic acids, lipids, and proteins. In DNA, the HO• can induce several effects including base and sugar modifications, cross-linking between bases, cross-linking between DNA and protein, strand breaks, and adducts

formation (Cochrane, 1991). This action on proteins results in extensive protein–protein cross-linking. The oxidation of PUFAs by hydroxyl radicals, also known as lipid peroxidation, is one of the most austere attacks on the integrity of the cell (Gutteridge, 1995).

2.10.1.5 Peroxyl radical

Peroxyl radicals are formed mainly from lipid peroxidation, which is initiated by removal of a single hydrogen atom from polyunsaturated lipids. Even though the process has been established to be beneficial in certain biological processes, peroxidation of PUFAs may unfavorably affect many functionally significant parameters like membrane permeability, fluidity, controlled conveyance of metabolites across the membrane and electrical potential (Halliwell & Gutteridge, 1989).

2.10.2 Antioxidants

According to Halliwell & Gutteridge (1989), antioxidants are substances which at fairly low concentrations compete with oxidant substrates in order to considerably delay or prevent their oxidation. They include enzymes like catalase, glutathione peroxidase (GPx), and superoxide dismutase (SOD); in addition to non-enzymatic compounds such as vitamins C & E, beta-carotene, and glutathione. They exert their effect by taking out or reducing the levels of either oxygen, ROS, or metal ions catalyzing the oxidation (Fe^{3+}, Cu^{2+}, etc.). They also interfere with the chain reaction that extends oxidation to neighboring molecules and enhance the endogenous antioxidant defenses of the cell. Therefore, antioxidants interfere with any of the three major steps of the oxidative process: initiation, propagation, or termination (Cui *et al*., 2004).

2.10.2.1 Classification of antioxidants

Antioxidants are categorized into two groups, depending on whether they are hydrophilic or hydrophobic. Generally, water soluble antioxidants react with oxidizing agents in the cytosol and the blood plasma, whereas those that are lipid-soluble safeguard cell membranes from lipid peroxidation (Sies, 1997). These compounds may either be obtained from diet or produced in the body. Antioxidants present in body fluids and tissues have different concentrations for instance, high amounts of glutathione can be found within cells, even though others such as uric acid are more evenly distributed. The extent to which an antioxidant protects a cell is dependent on its concentration, reactivity with the reactive oxygen species under consideration and the state of the antioxidants it interacts with (Vertuani *et al*., 2004). Below are some examples of antioxidants.

2.10.2.2 Ascorbic acid

Ascorbic acid is found in both animals and plants. Since humans lack one of the enzymes needed to make ascorbic acid, it must be gotten from diet and is thus considered a vitamin (Smirnoff, 2001). It is kept in its reduced form in cells by reacting with reduced glutathione, catalyzed by protein and glutaredoxins (Meister, 1994). Ascorbic acid is a reductant and can neutralize ROS such as hydrogen peroxide, peroxyl radical, singlet oxygen, hypochlorite and hydroxyl radical by reduction (Padayatty *et al*., 2003). Studies on human plasma lipids have confirmed that ascorbic acid is far more effective in preventing lipid peroxidation started by a peroxyl radical initiator than other plasma antioxidants such as protein thiols, urate, bilirubin and α-tocopherol. It traps peroxyl radicals efficiently in

the aqueous phase before they are able to initiate lipid peroxidation thus shielding bio-membranes from peroxidative damage (Smirnoff, 2001).

2.10.2.3 Glutathione

Glutathione is a cysteine-containing peptide synthesized in cells from its constituent amino acids and found in most aerobic organisms (Meister, 1994). It has antioxidant properties as the thiol group in the cysteine moiety is a reductant and can be oxidized and reduced reversibly. In cells, glutathione is kept in the reduced form by glutathione reductase and subsequently reduces products of metabolism and enzyme systems, such as glutathione peroxidases, ascorbate in the glutathione-ascorbate cycle and glutaredoxins in addition to direct reactions with oxidizing agents (Meister, 1994). Glutathione is one of the most important cellular antioxidants because of its central role in maintaining the cell's redox state and high concentration.

2.10.2.4 Tocopherol

Vitamin E is the collective name for a group of eight related tocopherols which are fat soluble vitamins and have antioxidant properties (Herrera and Barbas, 2001). The most studied is α-tocopherol and it has the highest bioavailability; with the body favorably absorbing and metabolizing this form (Brigelius-Flohe and Traber, 1999). Several studies have claimed α-tocopherol to be the most important lipid-soluble antioxidant, and that it prevents oxidation of membranes by reacting with lipid radicals formed from the lipid peroxidation chain reaction (Traber and Atkinson, 2007). It takes out the free radical intermediates and inhibits the propagation reaction. The reaction yields oxidized α-tocopheroxyl radicals which are recycled into the active reduced form by other

antioxidants like retinol, ascorbate or ubiquinol through reduction (Seiler *et al*., 2008). Other studies showed that α-tocopherol efficiently protected glutathione peroxidase 4 - deficient cells from apoptosis (Seiler *et al*., 2008). Glutathione peroxidase 4 is the only enzyme known to efficiently reduce lipid-hydroperoxides within biological membranes.

2.10.2.5 Enzyme systems

The superoxide free radical produced by processes like oxidative phosphorylation is first changed to H_2O_2 and subsequently reduced to water. This detoxification process is the result of the action multiple enzymes (Figure 2.4); with superoxide dismutase (SOD) catalyzing the opening reaction followed by catalases (CAT) and several peroxidases removing hydrogen peroxide. The SOD, catalase and glutathione peroxidase are the main intracellular enzymatic antioxidants, whereas the extracellular antioxidants are mainly scavengers (Cui *et al*., 2004).

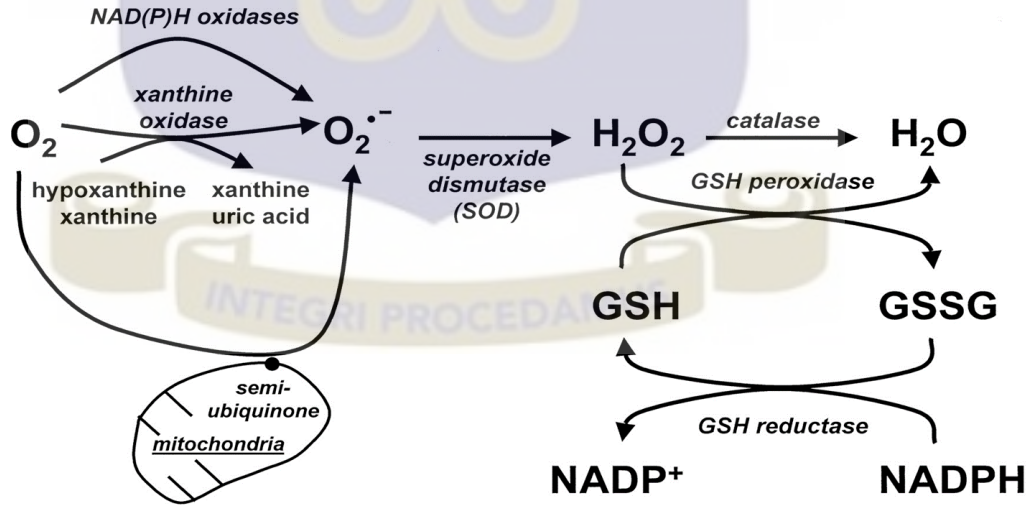

Figure 2.4: Enzyme systems working to clear ROS (Droge, 2002)

2.11 HIV AND OXIDATIVE STRESS

HIV infection is marked by a depletion of cellular $CD4^+$ T cells and seropositive individuals have been shown to experience prolonged oxidative stress. Disturbances to the antioxidant defense system in patients include depletions in ascorbic acid, selenium, carotenoids, superoxide dismutase, tocopherol and glutathione (Tersmette and Schuitemaker, 1993). It has also been confirmed that the reduction of lymphocytes, predominantly of the $CD4^+$ T cell subset during the infection might be as a result of programmed cell death (Gougeon and Montagnier, 1993). Since most of the inducers of apoptosis are either oxidizing agents or cellular oxidative metabolism activators suggests that the production of ROS may possibly induce apoptosis. Pace and Leaf (1995) suggested that oxidative stress mediated by the generation of ROS induces apoptosis. Hence oxidative stress promotes the replication of HIV.

In HIV infection, monocytes are constantly stimulated bringing about the formation of monokines such as tumor necrosis factor alpha (TNF-α); which boosts ROS production by neutrophils and monocytes, and has been confirmed to play a part in the start of apoptosis. TNF-α and ROS enhance the discharge of nuclear factor κB (NF-κB) from factor IκB in the cytoplasm; this results in the translocation of NF-κB to the nucleus and its subsequent binding to DNA, a process which has been implicated in increased HIV transcription (Greenspan *et al.*, 1994).

2.11.1 Evidence for *in vivo* Oxidative Stress in HIV Infection

It is difficult to directly measure oxidative stress under clinical conditions. Nevertheless, indirect scientific evidence shows that HIV infection is linked with increased production of ROS as well as higher antioxidants consumption. The levels of intracellular

glutathione (GSH) in lymphocytes and PBMCs of asymptomatic HIV patients were found to be slightly less than those of healthy controls (Tersmette and Schuitemaker, 1993). On the other hand, GSH levels in individuals with AIDS and AIDS-related complications were severely reduced but zidovudine treatment restored the GSH to measurable levels (Buhl *et al*., 1989). GSH is able to remove intracellular H_2O_2 by supplying a substrate for GSH peroxidase, the enzyme responsible for clearing H_2O_2. It has been shown that this enzyme is dependent on selenium whose levels are diminished in HIV seropositive individuals (Dworkin *et al*., 1986). Furthermore, HIV patients usually have reduced levels of acid soluble thiol, a vital indicator of the activity of antioxidants in the blood (Eck *et al*., 1989). Patients have been shown to excrete higher amounts of malondialdehyde in their urine than seronegative individuals, and this reflects amplified levels of lipid peroxidation.

2.12 GST GENE POLYMORPHISMS

The GSTs are enzymes that catalyze the phase II xenobiotic metabolism of environmental carcinogens, reactive oxygen species and chemotherapeutic agents (Hayes & Pulford, 1995). The mechanism is characterized by the coupling of these hydrophobic and electrophilic compounds with glutathione, in order to form more soluble products that can be excreted (Lavender *et al*., 2009). Most of the reactions between glutathione and electrophilic compounds that are catalyzed by GSTs form detoxification products, even though in some cases the metabolic products formed are more reactive than the original one. Due to their detoxifying action, it has been suggested that these enzymes play an important role in preventing various diseases (Wunsch & Gattas, 2001). At least eight distinct classes of soluble GST that are mostly produced in the liver of mammals have been

identified and designated alpha, mu, pi, sigma, theta, kappa, and zeta. They are encoded by GSTA, GSTM, GSTK, GSTO, GSTP, GSTS, GSTT and GSTZ genes (Gattás *et al*., 2004). Moreover, genetic variations in these enzymes may impact individual predisposition to some diseases linked with the injurious effects of oxidative metabolism.

The GSTA1 gene, found on chromosome 6 (6p12.1), has two alleles, GSTA1 * A and GSTA1 * B. Each allele has three single nucleotide polymorphisms (SNPs) that are connected (-567T, -69C, -52G and -567G, -69T, -52A, respectively). Even though the polymorphism has been widely studied, the exact mechanism of its effect on gene expression has not been fully elucidated. It is, however, known that the change of base G to A at position -52 prevents the transcription factor (Sp1) from binding to the promoter site and results in reduced expression of the gene GSTA1 (Morel *et al*., 2002). Polymorphisms connected to the decreased expression of the GSTA1 enzyme have been shown to result in the buildup of carcinogens in the body and increasing the risk of cancers (Så *et al.*, 2014). The GSTM1 located on chromosome 1 (1p13.3) gene is highly polymorphic and its three allelic variants GSTM1 *0, GSTM1 *A, and GSTM1 *B have been extensively studied in different cancers (Zupa *et al*., 2009). The alleles GSTM1 *A and GSTM1 *B encode enzymes that are metabolically active. The alleles are differentiated by the change of a base G to C in the coding region at position 534; so there is a switch of lysine for asparagine (N534K). Enzymatic function is not affected by this change (Så *et al.*, 2014). The proteins from these two genes merge to form homo- and hetero-dimerically active enzymes. The polymorphic nature of this locus makes it interesting and the high incidence of the null phenotype (40-60%) in most of the populations studied has been shown to vary among ethnic groups (Ebeshi *et al.*, 2011). Some studies reported lower frequencies of the null

allele in Nigerians and Indians, which contrasts with a higher incidence among Chinese and Micronesians (Ebeshi *et al.*, 2011; Parsons *et al.*, 2013).

The other alleles also show differences among ethnic groups: the rate of GSTM1*A is higher while that of GSTM1*B is lesser in Indians, Nigerians, English and French than in Chinese and Japanese populations. The null allele, GSTM1 *0, is the end result of a deletion of the GSTM1 gene caused by fusion of two homologous regions that flank the gene. Several studies have suggested that homozygosity for GSTM1 *0 is connected with an increased risk of several pathologies, including some malignancies, by reason of no protein expression (Hatagima *et al.*, 2000; Hatagima, 2002). Thus, GSTM1 polymorphism together with other detoxifying enzyme polymorphisms could be possibly used to detect high-risk individuals in clinical surveillance programs (Hatagima, 2002). However, this is complicated by the considerable differences in the GSTM1 allele frequencies detected among different ethnic groups. These frequencies are known in many populations but there are limited data from African populations (Hatagima *et al.*, 2000). The human GSTT1 gene is 8.1 kb in length and it has been mapped on chromosome 22 (22q11.2). Analogous to GSTM1 *0, the GSTT1 gene has a null allele (GSTT1 *0). Regardless of the deficiency in the detoxification of carcinogenic compounds due to gene deletion, arguments still persist about which allele (GSTT1 *0 or GSTT1 positive) would be associated with the risk for cancer. This is because unlike GSTM1, GSTT1 has a role in both detoxification and activation processes (Rossini *et al.*, 2002). Two different alleles in GSTT1 have been recognized that make the gene functional. Individuals with no less than one functional allele for GSTM1 and GSTT1 are grouped into the positive conjugator types, and called GSTM1-positive and GSTT1-positive, respectively (Gattás *et al.*, 2004). Studies have

demonstrated the associations of GSTM1 and/or GSTT1 null genotypes with bladder, lung, and colorectal cancer, in addition to head and neck squamous cell carcinoma and represent an area of intensive research. The significance of the polymorphisms of these enzymes regarding DNA adducts levels or urinary metabolites of xenobiotics have also been extensively discussed. The differences in the distribution of the GSTM1 and GSTT1 null phenotypes as a result of total or partial gene deletion causing the lack of an active enzyme, has been described in different populations, especially in ethnically well-defined groups (Gattás *et al*., 2004).

The GSTP1 gene on chromosome 11 (11q13.2) has a G → A base substitution at nucleotide position 313, producing a switch of isoleucine for valine at codon 105 (Moyer *et al*., 2008). The substitution at this position modifies the kinetic properties of the enzyme with possible changes in its function in the detoxification process. Hence, in case there is prolonged exposure to carcinogens, it can cause irreversible damage to DNA, favoring carcinogenesis (Så *et al.,* 2014). This polymorphism has been widely explored, and research studies have shown that the genotype GSTP1 105 Val / Val may be linked with increased risk for pancreatic cancer (Så *et al.,* 2014).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 CHEMICALS AND REAGENTS

The reagents for the quantification of CD4 cells which included BD FACSCount CD4/CD3 Reagent Kit, BD FACSCount Control Kit, BD FACSClean, BD FACSRinse and paper roll-thermal were obtained from BD Biosciences, Brussels, Belgium. The QIAamp DNA Blood Mini Kit and QIAGEN Multiplex PCR Kit were bought from Qiagen, Hilden, Germany. Total glutathione assay, superoxide dismutase kits and nuclease free water were purchased from Sigma-Aldrich, Missouri, USA. The glutathione reductase kit was procured from Abcam, Cambridge, UK. Standard chemicals and consumables were obtained from various commercial sources.

3.2 STUDY SITE AND POPULATION

Study participants were recruited from the Fevers Unit of the Korle Bu Teaching Hospital, Accra, Ghana. Questionnaires were used to collect data on socio-economic status and medical history of the patients. A total of 242 adults (18 years and above) were recruited for the study and they included 60 HIV negative individuals as controls, 105 HIV infected people on ART and 77 HIV infected but ART naïve individuals. The study was approved by the Institutional Review Board (IRB) of the Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Ghana and all participants signed a consent form after the objectives of the study were explained to them.

3.2.1 Inclusion and Exclusion Criteria

The study required patients to be HIV positive with or without ART but not taking multivitamins or mineral supplements for at least 3 months prior to the collection of the blood samples. Patients had to be clinically stable with no active opportunistic infections. People were excluded from the study if they consumed alcohol or smoked or had initiated antioxidant vitamin therapy. Individuals with diabetes or kidney problems or liver dysfunction were also excluded from the study.

3.3 SAMPLE AND PREPARATION

Overnight fasting blood samples were collected from each recruited individual under aseptic conditions and a portion was placed into a vacutainer plain gel tube and allowed to clot. The sample was then centrifuged at 3500 rpm for 5 minutes, serum separated and stored at -20^{o} C. The serum was used for biochemical analysis. The other portion was placed into EDTA tubes and used for hematological, trace elements, some oxidative stress markers and gene polymorphism analysis. In addition to this, the individuals were made to stand on an Omron full body sensor to determine their body mass index, body fat and visceral fat.

3.4 METHODS

3.4.1 Markers of Disease Progression

3.4.1.1 CD4 count

The automated Becton Dickson (BD) FACSCount machine (Becton, Dickson and Company, Brussels, Belgium) was used to determine the absolute count of CD4 in whole blood. After blood was aseptically collected by venipuncture, the EDTA tube was inverted

5 to 10 times to ensure adequate mixing. This was followed by pipetting 50 µL of the blood into a reagent tube labelled with the corresponding patient number, capped and vortexed for 6 seconds. This step allowed the fluorochrome-labelled antibodies and fluorescent nuclear dye in the reagents to bind specifically to white blood cell surface antigens and nucleated blood cells respectively. The procedure was repeated for all patient samples and the tubes incubated for 30 minutes at room temperature in the work station. Each sample tube was uncapped and 50 µL of fixative solution added. Tubes were recapped and vortexed for another 6 seconds. The samples were then run on the BD FACSCount machine to quantify the CD4 cells present in the blood. The laser light in the machine caused the labeled cells to fluoresce. This fluorescent light provided the information required for the instrument to identify and count the CD4 T lymphocytes. In addition, the reagent tubes contained a known number of fluorescent reference beads to which a precise volume of whole blood was added. The software automatically identified the lymphocyte populations of interest and calculated the CD4 counts (cells/µL) by comparing cellular events to bead events.

3.4.1.2 Viral load

The COBAS AmpliPrep/COBAS TaqMan machine (ROCHE, USA) was used to quantify HIV RNA in the plasma samples as per the producer's protocol. The entire procedure was automated with the COBAS AmpliPrep Instrument preparing the sample whilst amplification and detection was done by the COBAS TaqMan Analyzer.

3.4.2 Haematological Profile

Full blood cell counts were determined using the ABX Pentra 120 DX automated hematology analyzer (Horiba ABX, Montpellier, France) as per the manufacturer's protocol. The analyses recorded were red blood cell (RBC) count, hemoglobin concentration, hematocrit, mean corpuscular volume (MCV), white blood cell (WBC), lymphocyte, neutrophil, and platelet counts.

3.4.3 Liver Function Test

The automated Biosystems A25 Chemistry Analyzer was used to measure the levels of serum albumin, total protein, alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate transaminase (AST) and gamma glutamyl transferase (GGT). The manufacturer's protocol was used.

3.4.4 Oxidative Stress Markers

3.4.4.1 Superoxide dismutase

The SOD activity was measured by the inhibition reaction (Randox, 1996). A volume of 20 µL of sample solution was added to each sample and blank 2 well, whilst 20 µL of double distilled water was added to each blank 1 and blank 3 well. This was followed by the addition of 200 µL of WST Working Solution to each well, and mixed. Then, 20 µL of Dilution Buffer were added to each blank 2 and blank 3 well. About 20 µL of Enzyme Working Solution was added to each sample and blank 1 well, and then mixed thoroughly. The plate was incubated at 37 °C for 20 minutes; after which the absorbance was read at 450 nm using a microplate reader. The SOD activity (inhibition rate %) was

calculated using the following equation: SOD activity (inhibition rate %) = {[(Ablank 1 - Ablank 3) – (Asample - Ablank 2)]/ (Ablank 1 - Ablank 3)} x 100. All samples were in triplicates.

3.4.4.2 Glutathione reductase

The assay was performed with the method described by Osuji *et al*. (2012). Serum samples were pre-treated to destroy GSH before the assay. To a 100 µL sample, 5 µL of 3% H_2O_2 were added, mixed and incubated at 25 °C for 5 minutes. Then 5 µL of catalase were added, mixed and incubated at 25 °C for another 5 minutes. A 20 µL volume of the pre-treated samples was loaded into a 96 well-plate and the volume brought to 50 µL with assay buffer. A TNB standard curve was prepared by adding 0, 2, 4, 6, 8, 10 µL of the TNB Standard into 96 well-plate in duplicate to generate 0, 10, 20, 30, 40, 50 nmol/well standard. The final volume was brought to 100 µL with assay buffer. The reaction mix was prepared by mixing enough reagents for the number of assays to be performed. Each well contained a total volume of 50 µL reaction mix; made up of 40 µL GR assay buffer, 2 µL DTNB solution, 2 µL NADPH-GNERAT™ solution and 6 µL GSSG solution. A volume of 50 µL of the reaction mix was added to each test sample and mixed well. Immediately OD_{405nm} was measured at time, T_1 and it was named reading A_1. The reaction was incubated at 25 °C for 10 minutes or longer if the GR activity was low. The reaction was protected from light and OD_{405nm} was measured again at T_2 and it was named reading A_2. The change in absorbance (ΔA_{405nm}) was calculated as: $\Delta A_{405nm} = A_2 - A_1$

The TNB standard curve was plotted and the ΔA_{405nm} value for each sample extrapolated to the standard curve to get ΔB nmol of TNB. The GR activity was calculated by the formula:

$$\text{GR Activity} = \frac{\Delta B}{(T_2 - T_1) \times 0.9 \times V} \times \text{Sample Dilution Factor} = \text{nmol/min/ml} = \text{mU/ml}$$

Where: ΔB is the TNB amount from TNB standard Curve (in nmol).

T_1 is the time of the first reading (A1) (in min).

T_2 is the time of the second reading (A2) (in min).

V is the pre-treated sample volume added into the reaction well (in mL).

0.9 is the sample volume change factor during sample pre-treatment procedure.

3.4.4.3 Glutathione assay

Serum reduced GSH was analysed with the method described by Sedlak and Lindsay. The serum sample was deproteinized with the 5% 5-sulfosalicylic acid solution, centrifuged to remove the precipitated protein, and then assayed for glutathione. The first 2 wells of the 96 well-plate contained only 10 µl of the 5% 5-sulfosalicylic acid solution as a reagent blank. A volume of 10 µl of the prepared glutathione standard solutions was added into separate wells of the plate. About 10 µl serum sample were added in duplicate to separate wells. After this, 150 µl of the working mixture were added to each well with a multichannel pipette and mixed by pipetting up and down. The 96 well-plate was incubated at room temperature for 5 minutes and 50 µl of the diluted NADPH solution added and mixed. The plate reader was set to 405 nm with kinetic read at 1 minute intervals for 5 minutes. The absorbance from the glutathione standard solution was used to determine the standard curve from which the ΔA_{405}/min equivalent to 1 nmole of reduced glutathione per well was calculated. The amount of GSH in the plasma samples was calculated as follows:

$$\text{nmoles GSH per ml of sample} = \frac{\Delta A_{405}/\text{min(sample)} \times \text{dil}}{\Delta A_{405}/\text{min(1 nmole)} \times \text{vol}}$$

Where; ΔA_{405}/min(sample)= slope generated by sample (after subtracting the values generated by the blank reaction).

ΔA_{405}/min(1 nmole)= slope calculated from standard curve for 1 nmole of GSH

dil= dilution factor of original sample

vol= volume of sample in the reaction in ml

3.4.5 Glutathione S Transferase Gene Polymorphism

Genomic DNA for genotyping was extracted from 200 µL of whole blood using the QIAamp DNA Mini kit as per the manufacturer's protocol. A Thermo Fischer Nanodrop Lite was used to check to purity and yield of the DNA obtained. After this, a multiplex PCR assay was performed using a kit bought from Qiagen. Briefly, 2.5 µL of DNA were amplified in a 50 µL multiplex reaction which contained a mixture of the PCR master mix and 2.5 µL of each of the following GSTM1 primers (forward - 5' GAA CTC CCT GAA AAG CTA AAG C 3' and reverse - 5' GTT GGG CTC AAA TAT ACG GTG G 3'), and of the following GSTT1 primers (forward - 5' TTC CTT ACT GGT CCT CAC ATC TC 3' and reverse - 5' TCA CCG GAT CAT GGC CAG CA 3'). The exon 7 of the CYP1A1 gene was also amplified (5' GAA CTG CCA CTT CAG CTG TCT 3' and 5' CAG CTG CAT TTG GAA GTG CTC 3') as an internal control (Figure 3.1). The cycling conditions included an initial melting temperature of 94ºC (5 min) followed by 35 cycles of amplification (2 min at 94ºC, 1 min at 59ºC, and extension for 1 min at 72ºC). A final 10-min extension step at 72ºC terminated the process. The final PCR product from co-

amplification of GSTM1 (215 bp) and GSTT1 (480 bp) was visualized on an ethidium bromide-stained 2.0% agarose gel. The participants were grouped as either positive or null genotypes.

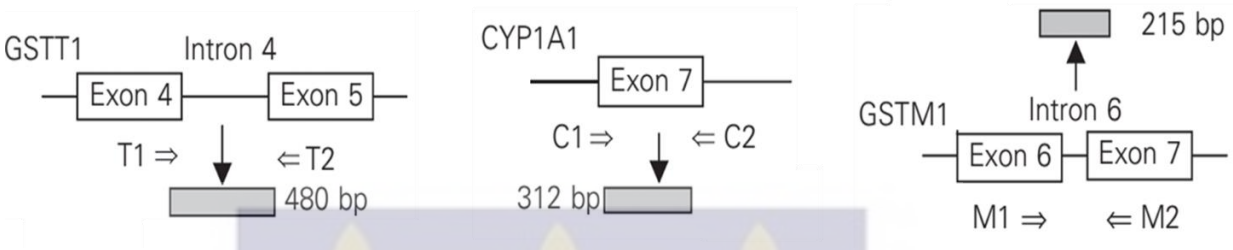

Figure 3.1: Positions on the genes that were amplified in the PCR (Gattás *et al*., 2004).

3.5 STATISTICAL ANALYSIS

The Shapiro-Wilk normality test was used to distinguish parametric and non-parametric data. Parametric data were represented as mean ± standard deviation. One-way ANOVA was used to analyze differences among the data; after which a post-hoc analysis was performed. All non-parametric data were presented as median (lower quartile-upper quartile). Kruskal-Wallis test was used to assess differences in median and then Dunn's multiple comparison test was used for post-hoc analysis. Chi-square test and Fischer's exact test were applied to assess the association between the categorical variables according to pre-established cut-off points. Odds ratios (ORs) and 95% confidence interval (CI) were calculated. Pearson's and Spearman's tests were used to study the correlations between oxidative stress and markers of disease progression. The statistical significance level was set at p-value <0.05. IBM SPSS 22 and GraphPad Prism v6 were used for the analysis.

CHAPTER FOUR

4.0 RESULTS

4.1 DEMOGRAPHIC AND BASIC CLINICAL CHARACTERISTICS OF STUDY POPULATION

A total of 242 individuals were recruited for the study and this included 182 HIV seropositive individuals, 105 of which were on ART and 77 ART naïve. Females were 157 and males totaled 85. ART patients were older (45.6 ± 0.9 years $P \leq 0.01$) than the ART naive (41.1 ± 1.4 years) and control groups (38.7 ± 1.9 years) respectively (Table 4.1). There were no significant differences in the BMI, body fat and visceral fat among the three groups.

Table 4.1: Basic clinical data of study population stratified by ART use

	Patient groups			
	ART ± SE (N=105)	ART naïve ± SE (N=77)	Control ± SE (N=60)	P value
Age (years)	45.6 ± 0.9	41.1 ± 1.4	38.7 ± 1.9	0.01
Male n (%)	45(42.9%)	21(27.3%)	19(31.7%)	
Female n (%)	60(57.1%)	56(72.7%)	41(68.3%)	
BMI (kg/m^2)	25.0 ± 0.4	23.6 ± 0.7	24.7 ± 0.6	0.136
Body fat (%)	26.7 ± 1.2	27.9 ± 1.4	28.4 ± 1.5	0.622
Visceral fat	6.7 ± 0.3	5.7 ± 0.4	6.0 ± 0.4	0.108
Systolic BP (mmHg)	120.4 ± 2.3	119.9 ± 2.1	119.0 ± 3.2	0.062
Diastolic BP (mmHg)	74.4 ± 1.3	71.6 ± 1.2	72.6 ± 1.8	0.058

Study participants were categorized into three groups: ART, ART naïve and controls. Data are presented as mean ± standard error of mean (SEM). P-values were obtained from One-way ANOVA by comparing the results for the two HIV patient groups with the seronegative controls. BMI = body mass index. ART= antiretroviral treatment. BP = blood pressure.

4.2 MARKERS OF HIV PROGRESSION

The CD4 count among the individuals on ART (603.8 ± 28.7, P ≤ 0.005) was significantly higher than those who are ART naïve (296.4 ± 27.0) (Table 4.2). Conversely, viral load among the ART patients (30716 ± 12461, P ≤ 0.001) was significantly less than those who were not on ART (193641 ± 65464). About 79% of patients on ART had CD4 counts more than 350 cells/mm^3, whilst 41.5% of the patients who were not on treatment had CD4 counts below 200 cells/mm^3 (Table 4.2).

Table 4. 2: CD4 count and viral load of the HIV seropositive group

	Patient groups		
	ART ± SE (N=105)	ART naïve ± SE (N=77)	P value
CD4 count (cells/mm^3)	603.8 ± 28.7	296.4 ± 27.0	0.005*
Viral load (copies/mm^3)	30716 ± 12461	193641 ± 65464	< 0.001*
	CD4 groups		
< 200 (cells/mm^3)	5(4.8%)	32(41.5%)	
200-349 (cells/mm^3)	17(16.2%)	16(20.8%)	<0.001**
> 350 (cells/mm^3)	83(79%)	29(37.7%)	

*P value was determined from t-test whereas the **P value was determined from the Pearson Chi-square test

4.3 HEMATOLOGICAL PROFILE

Hemoglobin levels were decreased among the ART naïve patients (10.79 ± 0.25, $p < 0.001$) when compared with those on ART and the control group (Table 4.3). Bonferroni's multiple comparison test showed that the difference was significant, but there was no significant difference between the patients on ART and the control group. The mean hematocrit, MCV and MCH levels showed a similar trend as the hemoglobin levels but the multiple comparison test showed significant differences among the three groups. Although the number of RBCs of those on ART (3.84 ± 0.05, $p < 0.001$) was significantly lower than the ART naïve patients and control group, the values were within the reference range of 3.5 - 5.5 ($10^{\wedge 12}$/L). The ART naïve patients had significantly higher neutrophil (1.37 ± 0.14, $p < 0.02$), monocytes (0.51 ± 0.08, $p < 0.001$), platelets (261.5 ± 12.2, $p < 0.001$) and lymphocyte (3.44 ± 0.19, $p < 0.001$) levels than those on ART and the controls. Furthermore, HIV seropositive individuals with CD4 count below 200 had significantly lower HB (10.36 ± 0.46, $p < 0.001$), hematocrit (31.51±1.30, $p < 0.001$), MCV (82.24±1.37, $p < 0.001$), MCH (26.64±0.50, $p < 0.001$) and lymphocyte (2.71±0.22, $p < 0.001$) levels than those with CD4 count more than 200. Bonferroni multiple comparison test showed the difference between the groups. Conversely, the levels of monocytes and platelets in the group were significantly higher than those in the other two categories.

Table 4.3: Hematological parameters of the study population and CD4 groups

Parameter	Patient groups		Control ± SE (N=60)	CD4 count (cells/mm^3)		
	ART ±SE (N=105)	ART naïve ± SE(N=77)		<200 (N=37)	200-349 (N=31)	≥350 (N= 174)
HB (g/dL)	12.70 ± 0.15	10.79 ± 0.25*	12.80 ± 0.23	10.36 ± 0.46*	11.96±0.36	12.38±1.54
RBC (10^12/L)	3.84 ± 0.05*	4.05 ± 0.09	4.51 ± 0.08	3.86±0.14	3.98±0.13	3.94±0.06
WBC (10^9/L)	4.82 ± 0.13	5.59 ± 0.40*	3.05 ± 0.49	4.60±0.27	5.28±0.90	5.28±0.14
Hematocrit (%)	37.98 ± 0.48	32.70 ± 0.70*	42.22 ± 0.66	31.51±1.30*	35.96±0.99	37.10±0.45
MCV (fL)	100.00 ± 1.21	81.15 ± 0.96*	94.00 ± 1.13	82.24±1.37*	90.50±2.20	95.62±1.39
MCH (pg)	33.36 ± 0.39	26.17 ± 0.35*	28.46 ± 0.38	26.64±0.50*	29.07±0.93	31.86±0.46
MCHC (g/dL)	33.44 ± 0.22	32.32 ± 0.21	30.27 ± 0.15	32.21±0.31***	32.34±0.40	33.39±0.20
Platelets (10^9/L)	252.5 ± 3.3	261.5 ± 12.2*	164.3 ± 9.4	270.8±17.5	228.6±17.9	259.1±4.5
Neutrophils (10^9/L)	1.25 ± 0.08	1.37 ± 0.14**	0.83 ± 0.06	1.22±0.15	1.36±0.26	1.31±0.09
Lymphocytes (10^9/L)	3.31 ± 0.11	3.44 ± 0.19*	1.28 ± 0.08	2.71±0.22*	3.00±0.36	3.65±0.11
Monocytes (10^9/L)	0.19 ± 0.02*	0.51 ± 0.08	0.22 ± 0.02	0.66±0.13*	0.31±0.09	0.22±0.02
Eosinophils (10^9/L)	0.057 ± 0.008	0.048 ± 0.01	0.077 ± 0.02	0.04±0.01	0.03±0.01	0.06±0.01

P-values were obtained from One-way ANOVA by comparing the HIV patient groups with the seronegative controls, and the two groups with CD4 counts below 350 to those with CD4 counts above 350. Significant p-values obtained from the One-way ANOVA were *P<0.001, **P<0.02, ***P<0.002. Hb: hemoglobin, RBC: red blood cells, WBC: white blood cells, MCV: Mean Cell Volume, MCHC: Mean Cell Hemoglobin concentration, MCH: Mean Cell Hemoglobin.

4.4 LIVER FUNCTION TESTS

The levels of ALP, AST and GGT were significantly increased in the HIV positive individuals but more pronounced in those who were not on ART (Table 4.4). Bonferroni multiple comparison test showed that the differences in these enzymes levels were between all three groups. The levels of albumin, ALP and total protein, although statistically different from the control group, were within the reference range. The seronegative individuals had the enzyme levels within the reference ranges. However, when stratified by CD4 count group, individuals with CD4 count below 200 cells/mm^3 had significantly elevated ALP (333.2±157.5 U/L) and AST (53.5±36.8 U/L) levels when compared with those in the CD4 count between 200-349 and CD4 >350 (Table 4.5). HIV patients with CD4 <200 also had elevated GGT levels but this was not statistically different from the other two groups.

Table 4.4: Liver function test results of the study population

	Patient groups			
Parameter	ART ± SD (N=105)	ART naïve ± SD (N=77)	Control ±SD (N=60)	P value
Albumin (g/L)	45.54±8.96	38.89±6.88	37.91±5.22	<0.001
ALP (U/L)	216.3±127.9	271.1±155.5	149.0±67.30	<0.0001
ALT (U/L)	19.93±10.0	21.93±11.8	30.82±11.50	<0.001
AST (U/L)	39.51±16.9	42.87±23.15	27.00±11.20	<0.0001
GGT (U/L)	68.58±33.12	60.68±31.83	38.30±14.17	<0.037
Total protein(g/L)	97.46±14.57	104.58±13.55	72.93±16.31	<0.0001

P-values were obtained from One-way ANOVA by comparing the HIV patient groups with the seronegative controls. ALP=Alkaline phosphatase; ALT=Alanine transaminase; AST=Aspartate transaminase; GGT= Gamma glutamyl transferase.

Table 4. 5: Liver function tests categorized by CD4 count

Parameter	CD4 count group			P value
	CD4 <200 N=37	200-349 N=33	CD4 >350 N=112	
Albumin(g/L)	36.7 ± 8.5	40.9 ± 7.4	45.2 ± 8.2	<0.0001
ALP (U/L)	333.2 ± 157.5	257.6 ± 143.9	204.1 ±116.9	<0.001
ALT (U/L)	29.3 ± 13.2	17.9 ± 9.9	18.8 ± 8.1	<0.013
AST (U/L)	53.5 ± 36.8	40.3 ± 27.2	37.0 ± 17.1	<0.002
GGT (U/L)	84.4 ± 14.1	72.5 ± 24.1	57.1 ± 5.5	<0.001
Total protein(g/L)	101.6 ± 19.8	101.5 ± 11.5	99.8 ± 14.6	0.753

P-values were obtained from One-way ANOVA by comparing the two patient groups with CD4 counts below 350 to those with CD4 counts above 350. ALP=Alkaline phosphatase; ALT=Alanine transaminase; AST=Aspartate transaminase; GGT= Gamma glutamyl transferase.

4.5 OXIDATIVE STRESS MARKERS

4.5.1 Glutathione Reductase

ART naïve patients had significantly lower GR activity [0.031 (0.016-0.055), H=76.27, p<0.0001] than those on ART [0.071 (0.038-0.118)] and seronegative controls [0.133 (0.108-0.172)] (Figure 4.1A). Post hoc analysis showed that the differences were between all the groups. When the GR activity was classified by CD4 count, patients with CD4 count <200 [0.037, H=22.89, p<0.0001)] recorded low enzyme activities when compared to those with CD4 count of 200-349 [0.045 (0.018-0.091)] and CD4 count >350 [0.096 (0.040-0.140)] (Figure 4.1B). The Dunn's test showed that the difference was between individuals with CD4 count below 200 and those with CD4 counts above 350. The Pearson's correlation showed significant association between GR activity and CD4 count at the level of 0.01 (Table 4.6).

4.5.2 Reduced Glutathione

The Kruskal-Wallis test showed that reduced glutathione levels in the sera of HIV patients who were not on ART [2.18 (1.31-3.05), H=73.54, p<0.0001] was significantly less than those on ART [2.45 (1.73-3.87)] and the seronegative controls [4.23 (3.33-5.48)] (Figure 4.1C). Dunn's multiple comparisons test confirmed that the significant difference was between all the groups. When stratified by CD4 count, patients with CD4 count below 200 [2.04 (1.28-2.98), H=19.92, p<0.0001] had lower GSH levels than individuals with CD4 of 200-349 [2.37 (1.58-3.25)], and CD4 more than 350 [3.12 (2.08-4.26)] (Figure 4.1D). The multiple comparison test showed that the significant difference was only between CD4 <200 and CD4 >350. GSH levels positively correlated with CD4 count and this association was significant (p<0.001) (Table 4.6).

4.5.3 Superoxide Dismutase

Superoxide dismutase activity was significantly reduced in ART naïve HIV patients [59.3 (51.8-68.4), H=26.54, p<0.0001] than in those on ART [67.7 (52.4-81.5)] and the control group [75.0 (63.0-83.6)] (Figure 4.1E). Using Dunn's multiple comparison test, the difference was between ART and ART naïve, as well as ART naïve and control. SOD activity among the CD4 groups was significantly less in the CD4 <200 group [59.9 (53.4-68.8), H=7.41. p < 0.02] than the CD4 >350 group (Figure 4.1F). SOD activity positively correlated with GSH levels (p < 0.0001) and GR activity (p < 0.003) (Table 4.6).

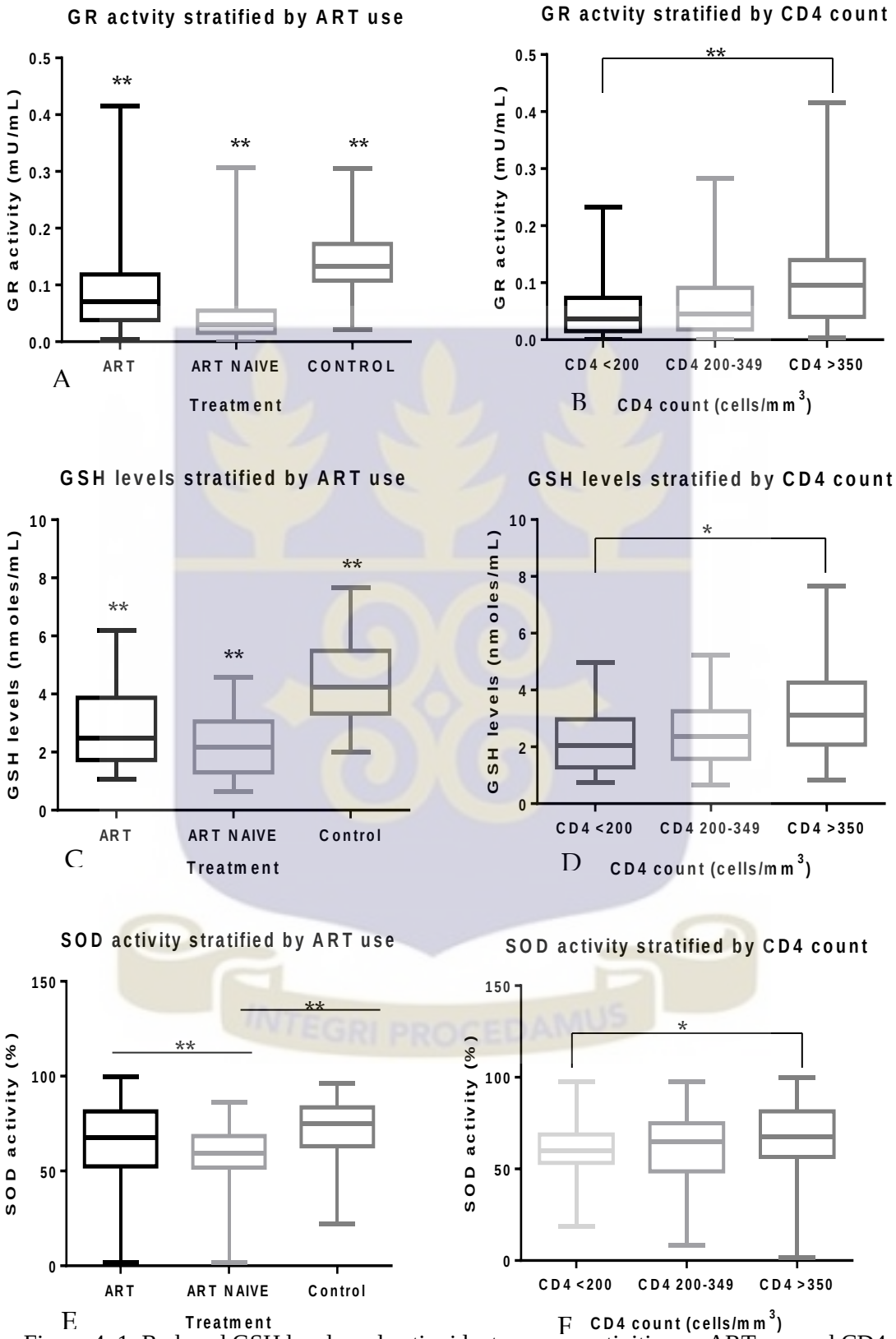

Figure 4. 1: Reduced GSH levels and antioxidant enzyme activities on ART use and CD4 count. Decreased GSH levels and antioxidant enzyme activities observed in ART naïve and CD4 <200 groups. *p<0.0001 and **p<0.0001

4.5.4 Correlation between Antioxidants and CD4 Count

Significant positive correlations were observed between CD4 count and GR as well as GSH. This means that an increase in CD4 count also increases the activity and levels of GR and GSH respectively. Amazingly, no significant association was observed between SOD activity and CD4 count. The antioxidants also had positive correlations with each other.

Table 4.6: Pearson's correlation coefficients of antioxidant and enzymes activities

Parameters	CD4 count	SOD	GR	GSH
CD4 count	1.00	0.09	0.235**	0.176*
SOD	0.09	1.00	0.188***	0.297****
GR	0.235**	0.188***	1.00	0.252****
GSH	0.176*	0.297****	0.252****	1.00

P values: **p<0.001; *p<0.02; ***p<0.003; ****p<0.0001. CD4 = cluster of differentiation, SOD = superoxide dismutase, GR = glutathione reductase, GSH = reduced glutathione.

4.6 GLUTATHIONE S-TRANSFERASE GENE POLYMORPHISM

4.6.1 Identification of GST Polymorphism Genotypes

Amplicons from samples positive for GSTM1 and GSTT1 genotypes yielded bands of 215 bp and 480 bp respectively, while the internal positive control (CYP1A1) PCR product corresponded to 312 bp. Absence of 480 and 215 bp bands indicated homozygous null genotypes of GSTM1 and GSTT1, respectively. Lanes 2, 14, 24, 28, 29, 40 and 43 were positive for GSTT1 deletion genotype. Lanes 6, 18, 20, 21, 23, 33, 37, 39, 41, 42 and 44 had GSTM1 deletion genotype (Figure 4.2). Lanes 31 and 32 represented individuals with a deletion of both genotypes. The remainder had both genes present.

Figure 4. 2: PCR products from the co-amplification of GSTM1 (215 bp), GSTT1 (480 bp) and the internal control, CYP1A1 gene (312 bp). The gel was ethidium bromide stained 2% agarose gel. N = negative control, M = 100bp molecular weight marker.

4.6.2 GST Gene Polymorphisms Stratified by ART Use

A total of 53 (21.9%) individuals had the GSTM1 deletion and among them, 25 were on ART, 17 were ART naïve and 11 were healthy controls. Among those with the GSTT1 deletion, 20 were on ART, 16 ART naïve and 12 HIV seronegative controls. The majority, 120 (49.6%) of the study population had both GSTM1 and GSTT1 present, and this included 51 on ART, 42 ART-naïve and 27 seronegative individuals. A total of 21 (8.7%) had both genes deleted, which included 9 HIV patients on ART, 2 ART-naïve and 10 seronegative persons (Figure 4.3).

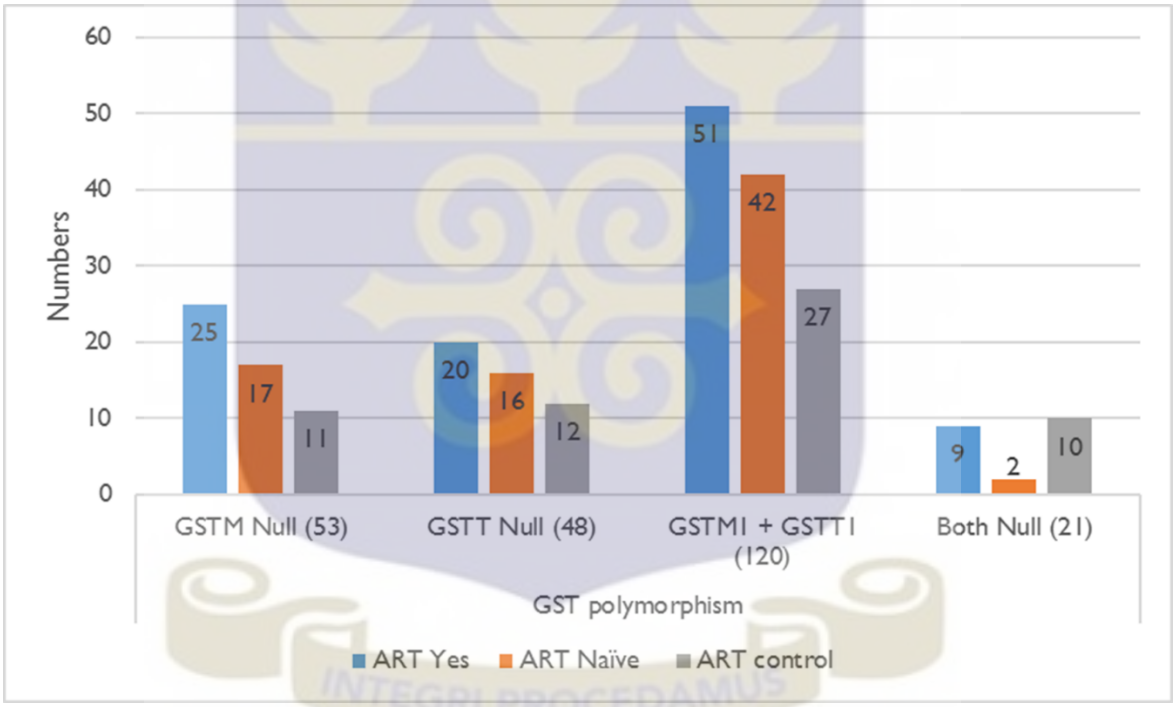

Figure 4. 3: Distribution of GST polymorphisms among patient and control groups

4.6.3 GST Gene Polymorphisms among CD4 Groups

HIV patients with the GSTM1 deletion were 42 and this included 9 with CD4 <200, 6 with CD4 count >200 but <350, and 27 with CD4 count >350. Those with the

GSTT1 deletion totaled 36 (Figure 4.4) and they included 5 with CD4 <200, 9 with CD4 >200 but <350 and 22 with CD4 count >350. Among the HIV patients with both genes present, 23 had CD4 count <200, 15 had CD4 count of 200-350 and 55 had CD4 count >350. Among those with both genes deleted, none had CD4 <200, 1 person had CD4 count of 200-350 and those with CD4 count >350 totaled 10.

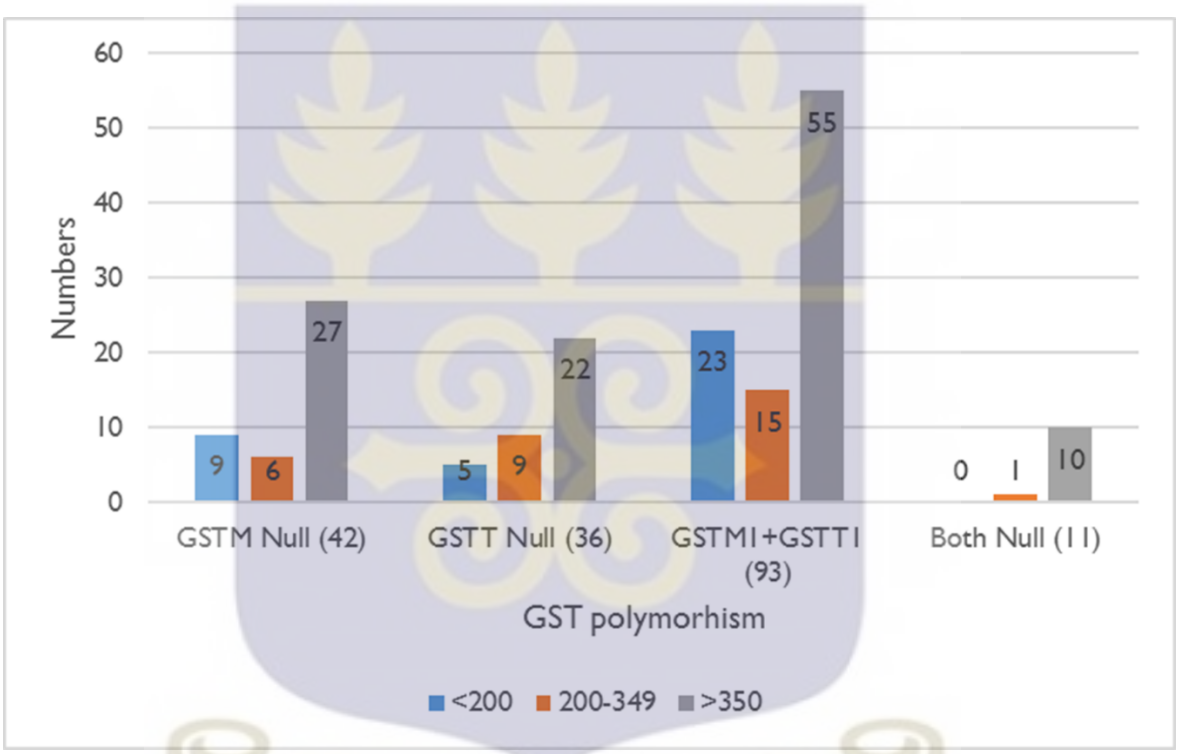

Figure 4. 4: GST polymorphisms among the patient CD4 count categories

4.6.4 Distribution of GST Gene Polymorphisms by Gender

More females had the GSTM1 deletion (N=40) than males (N=13) and the same trend was observed for the other genotypes (Figure 4.5). The majority of males and females had both GSTT1 and GSTM1 genes present.

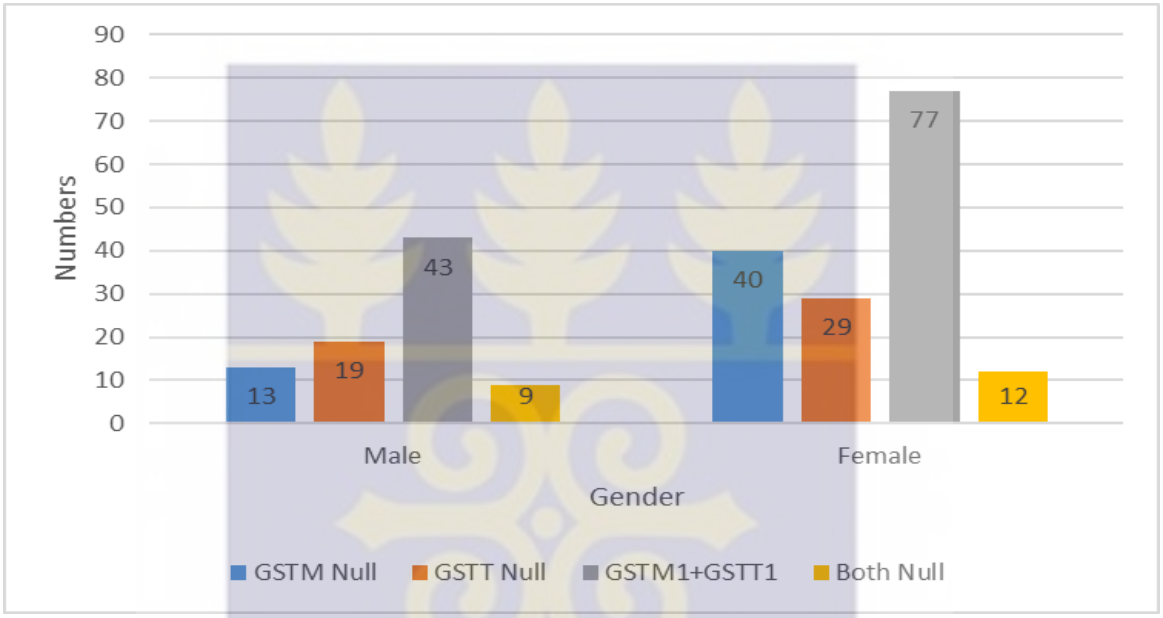

Figure 4. 5: GST genotypes distribution by gender

4.6.5 Effect of GST Polymorphism on HIV and CD4 Count

When the single genotype deletions were assessed with CD4 count, persons with the GSTM1 deletions were not associated with increased risk of having CD4 count less than 350 (Table 4.7). Individuals with the GSTT1 deletion also were not at any risk of having CD4 count below 350. However, combined genotypic analysis showed that those who had both genes deleted were at an increased risk of having CD4 count below 350 (Table 4.7). But individuals with both genes present were not at risk of having their CD4 count falling below 350.

Table 4.7: Interaction of GSTM1 and GSTT1 genotypes and association with CD4 count

Single Genotype	Odds Ratio	95% CI	P-value
GSTM1 null	1.01	0.51-2.00	1.00
GSTT1 null	1.07	0.53-2.14	0.86
Combined Genotype			
GSTM1+GSTT1	1.42	0.81-2.50	0.25
Both null	0.11	0.02-0.87	0.0103*

P values were from the Fischer's exact test.

4.6.6 GST Genotypes and Liver Function Tests

AST levels were significantly elevated in persons having the GSTM1 deletion (43.31 ±15.40) when compared with those of the other genotypes (Table 4.8). No significant differences were observed for ALP, ALT, GGT, total protein and albumin levels, which were within their respective reference ranges.

Table 4. 8: Liver function test results among the GST genotypes

Parameter	GST genotype ± SD			
	GSTM1 null	GSTT1 null	GSTM1+GSTT1	Both null
Albumin (g/L)	41.12±8.43	43.43±6.56	40.79±8.83	42.64±8.19
ALP (U/L)	221.8±147.6	188.8±107.7	231.4±132.6	191.4±79.8
ALT (U/L)	23.48±13.07	20.32±9.84	24.08±14.71	24.46±13.14
AST (U/L)	43.31±15.40**	35.21±18.11	37.65±21.50	27.43±10.14
GGT (U/L)	65.61±10.87	47.49±17.23	62.10±7.84	46.97±12.33
Total protein(g/L)	91.54±19.41	96.11±17.30	94.42±18.47	89.90±25.17

P values obtained from One-way ANOVA by comparing the results with those who had both genes present. **p<0.048. ALP=Alkaline phosphatase; ALT=Alanine transaminase; AST=Aspartate transaminase; GGT= Gamma glutamyl transferase.

4.6.7 Antioxidant Enzymes Activities and GSH Levels among GST Genotypes

The GST genotypes did not have any significant differences in the activities of SOD and GR as well as GSH levels (Table 4.9). However, persons with a deletion of both GSTM1 and GSTT1 generally had higher SOD and GR activities. They also had increased GSH levels. Data were non-parametric hence they were represented as median (lower quartile-upper quartile).

Table 4. 9: Antioxidants classified according to GST genotypes

	GST genotypes			
Parameters	GSTM1 null	GSTT1 null	GSTM1+GSTT1	Both null
SOD (%)	65.1(56.7-77.5)	64.6(45.7-79.0)	66.0(56.5-77.3)	73.6(57-87.2)
GR (mU/mL)	0.09(0.05-0.15)	0.05(0.03-0.12)	0.05(0.02-0.13)	0.1(0.04-0.11)
GSH(nmol/mL)	2.96(1.83-3.98)	3.02(2.14-4.20)	2.88(1.55-3.94)	3.28(2.06-4.9)

Data are presented as median (lower quartile-upper quartile). P -values were obtained from Kruskal-Wallis.

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 DISCUSSION

HIV infections are the basis of chronic inflammation in seropositive individuals which result from high levels of plasma inflammatory cytokines and ROS production. Hepatic and hematological anomalies are among some of the common complications of HIV infection. The increase in oxidative stress observed in the HIV/AIDS patients may be clinically significant for the reason that there is experimental evidence linking oxidative stress with stimulating viral replication. *In vitro* experiments have shown that ROS trigger nuclear factor kB to induce the expression and replication of HIV-1 in human T cells. The deficit of total antioxidant status as well as polymorphisms in glutathione S-transferase markedly increases oxidative stress, possibly adversely affecting the immune system. The study therefore set out to investigate GST gene polymorphisms and antioxidant enzymes activities in Ghanaian HIV/AIDS patients.

In this study females were 64.9% of the study population; accounting for 57% of those on ART, 72.7% ART naïve and 68.3% of the seronegative controls. Due to the high proportion of female to male HIV patients, some studies have suggested an increased risk in females getting infected. According to the CDC HIV Surveillance Report (CDC, 2014), the risk of a woman contracting HIV during intercourse is higher than it is for the man. Besides, some of the women may not be aware of their male sexual partner's risk factors for HIV; which may include multiple sexual partners, and drug abuse. Moreover, HIV policies in many countries have mandated pregnant women to undergo an HIV test and this account for the reason why more women have tested positive than males. The World

Health Organization (WHO) AIDS Epidemic Report of 2004 stated that sub-Saharan African women were more severely affected by HIV/AIDS and those of reproductive age constituted about 57% of adults living with HIV, and this accounted for up to 80% of HIV infected women in the world (Dabis and Ekpini, 2002; WHO, 2004).

The significant difference in age between patients on ART and those who are ART naïve also backs the notion that HIV/AIDS infection may be acquired at an early age; possibly during the reproductive years of life. The strict eligibility criteria that qualifies an HIV patient to initiate ART (WHO recommends CD4 <350 cells/mm^3) could also be a contributing factor to the significant differences in age between those on ART and the ART naïve group. This means that there could be a time lag between the time an individual tests positive for HIV and the time ART is initiated.

Furthermore, the study population had normal BMI and body fat; suggesting that they were not overweight or obese (Table 4.1). Several studies in children and adults have shown that obese persons had increased oxidative stress levels compared to individuals whose weight was normal and this association was further supported by the occurrence of other risk factors linked with metabolic syndrome (Van Guilder *et al*., 2006; Araki *et al*., 2010). Thus, the effect of weight on the findings which would have been a potential confounder was eliminated.

Significantly higher average CD4 counts were observed in patients on ART as compared to their naive counterparts. It was also noted that ART naïve patients were at increased risk of having CD4 <200 cells mm^{-3} (Table 4.2) in comparison to those on ART who generally had improvements in their CD4 counts to levels above 350 cells mm^{-3}. The low CD4 count in the individuals not on ART suggests a weaker immune system than the

ART group and hence, they were at significant risk of acquiring opportunistic illnesses (Quaye *et al.*, 2000; Merson *et al.*, 2008). ART is administered in order to raise CD4 count levels while providing protection against opportunistic infections (Corbeau & Reynes, 2011; Costagliola *et al*., 2014). This explains the generally high CD4 count in those taking ARTs. Some individuals on ART also had CD4 count below 200 cells/mm^3 (Table 4.2). Studies have shown that the counts vary a lot between people with regards to diverse factors such as recent vaccinations, exercise, lack of sleep, and time of day or smoking (Bøyum *et al*., 1996; Sullivan *et al*., 2000; Nielsen *et al*., 2016). It is therefore possible that these factors could have contributed to the low CD4 counts observed in some of the ART patients. Hence it has been suggested that a one-time CD4 count may not give reliable results, so repeated measures of the counts, once every three to six months is more reliable (Gale *et al*., 2013).

Conversely, viral load of those on ART was significantly lower than in the ART naïve group. While CD4 count was used to assess the reaction of the body to the virus, the viral load test evaluated the number of viral particles present in the blood. The low viral load in the ART group suggests that the virus was not actively reproducing and that the immediate risk of disease progression was low (Costagliola *et al*., 2014). However, the high viral load in those who were ART naïve meant that the virus was active and the infection could progress (Costagliola *et al*., 2014).

Anemia may occur at any stage of the infection and its prevalence in addition to severity are correlated with disease progression (Evans & Scadden, 2000). ART naïve and patients whose CD4 counts were below 200 cells/mm^3 had iron deficiency anemia (Table 4.3). These findings agree with those of other authors who also found decreases in blood

hemoglobin levels as the infection progressed (Kreuzer & Rockstroh, 1997; Obirikorang & Yeboah, 2009; Johannsenn *et al*., 2011). The normal hematological profile of those on ART suggests that the treatment appears to correct anemia associated with HIV infection (Moore & Forney, 2002; Shet *et al*., 2009). A study conducted in rural Uganda found that the average hemoglobin improved from 11.3 g/dL at baseline to 12.8 g/dL after 12 months on ART (Forna *et al*., 2009). The decrease in opportunistic infections such as tuberculosis and candidiasis in addition to the reduction of inflammatory cytokines such as tumor necrosis factor (TNF) which are normally responsible for the suppression of red blood cell production could be mechanisms that may account for the improvement of anemia after initiation of ART (Evans & Scadden, 2000; Idigbe *et al*., 2005; Redig & Berliner, 2013). The study also had more women than men and so, it is possible that the iron deficiency anemia observed could also be due to folate deficiency as well as multiple deliveries.

Furthermore, Nagababu *et al.* (2008) reported that increased oxidative stress occurs in severe anemic RBCs than in normal cells. So, this means that the ART naïve individuals could be having problems in effectively clearing ROS produced from oxidative metabolism. The damage to the RBCs explains the shorter RBC life-span associated with anemia.

The liver is the main organ for detoxification and maintaining metabolic homeostasis. It also metabolizes compounds that generate ROS; hence a defective liver function could suggest the antioxidant defense system may not be working effectively (Droge, 2002; Muriel & Gordillo, 2016). In order to ensure that any toxicity observed was due to the ART drugs and/or HIV, all subjects were tested for hepatitis B virus which is a risk factor. Alcohol intake was included in the exclusion criteria. The other hepatitis

viruses, and other conditions which affect the liver such as liver flukes were not tested; hence these could be potential confounders to the findings. It was observed that the levels of ALP, AST and GGT were significantly elevated in both HIV patient groups but was more pronounced in the ART naïve group (Table 4.4). This suggests that for those on ART, the toxicity could be due to the drugs whilst for the ART naïve patients, the HIV could be responsible. Some studies and case reports have linked elevations of liver enzymes with specific HIV drugs as liver toxicity is one of the common serious side effects of ART. This means that some HIV medicines have greater potential than others to raise liver enzyme levels. Sulkowski *et al*. (2000) reported that most patients placed on full dose ritonavir were at increased risk of having grade 3 hepatic enzyme elevations; however, the risk reduced when lower doses were used. Wit *et al*. (2002) showed that HIV drugs predisposes patients to hepatotoxicity, possibly because of impaired hepatocyte defense mechanism as hepatic glutathione (GSH) levels were reduced. Since GSH is the main antioxidant defense, it also protects against liver toxicity. This is because N-acetylcysteine, a cysteine donor, replenishes GSH stores and is the accepted therapy for acetaminophen-induced liver injury. Moreover, work done by De Rosa *et al.* (2000) associated HIV infection with depleted GSH stores but levels were restored upon treatment with N-acetylcysteine.

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. The incidence of iron deficiency anemia and liver injury observed in patients who were not on treatment and those whose CD4 counts were below 200 cells/mm^3 suggested impaired clearance of ROS (De Rosa *et al*., 2000; Sulkowski *et al*., 2000). Hence the activities of superoxide

dismutase (SOD) and glutathione reductase (GR) as well as reduced glutathione (GSH) levels were used to assess oxidative stress in the study population. The study showed decreased SOD and GR activities as well as low GSH levels among the ART naïve patients and those with CD4 count <200 cells/mm^3 (Figure 4.1) but the values increased as CD4 count improved. SOD is the main defense against oxidative damage caused by superoxide anion radical and three isoenzymes 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 established that HIV Tat protein down regulates the synthesis and induction of MnSOD (Flores *et al*., 1993). Total SOD activity was measured in this study and included activities of CuZnSOD and MnSOD; therefore, it is possible that the reduction in total SOD activity was due to reduction in the synthesis of MnSOD by HIV Tat protein. The effect of Tat protein on EC-SOD is not clearly understood but EC-SOD activity is strongly influenced by inflammatory cytokines such as TNF-alpha (Marklund, 1992; Flores *et al.*, 1993; Brady *et al.*, 1997). Furthermore, GR is responsible for converting oxidized glutathione, GSSG, to the reduced form. Hence the low GR activity suggests elevated GSSG levels and therefore increased oxidative stress, which in general upregulates inflammatory cytokine activities (Brady *et al.*, 1997).

The increase in selected antioxidant markers among the ART group indicates the progressive improvement in antioxidant status on treatment with ART. On the other hand, the observed decrease of these markers in the ART naïve subjects could be attributed to rapid depletion of the endogenous enzymes by high levels of circulating chemically reactive species and ROS associated with the ART naïve patients and low CD4 counts (Osuji *et al.*, 2012). The lowered antioxidant status may affect the ability of HIV infected

individuals to scavenge free radicals and ROS, which might enhance disease progression. Our results clearly show that antioxidant depletion occurs in HIV seropositive individuals in comparison with controls and with the progression of disease. This is confirmed by the correlation between CD4+ T cell counts and the antioxidants, GR and GSH (Table 4.6). The lower the CD4+ T cells count the lower the levels of these antioxidant markers. Antioxidant enzymes are sensitive to oxidative stress so the variations observed in these enzyme levels suggest possible cell damage and weakened antioxidant defense in HIV-infected individuals.

In this present study, it was observed for the first time in Ghana that the frequency of deletion of GSTM1 was 21.9% whilst the GSTT1 was 19.8% (Figure 4.3). Parsons *et al*. (2013) observed that the GSTM1 genotype that codes for a functional enzyme was not equally distributed by race. Another study in Nigeria also found different frequencies among three ethnic groups (Ebeshi *et al*., 2011).

Some of the study participants were found to have a homozygous deletion of both GSTM1 and GSTT1 and they accounted for only 8.7% (Figure 4.3). The functional GSTM1 enzyme has been shown to play a role in cancer prevention (Ntais *et al*., 2005). Therefore, the low frequency of GSTM1 gene deletion implies that Ghanaians in general will be better protected than Asians and Whites from the effects of reactive metabolites such as, benzo(a)pyrene, known to cause lung cancer (Ebeshi *et al*., 2011). On the other hand, some of the pathways involving GST enzymes have been suggested to produce toxic intermediates; hence, GSTM1 and GSTT1 expression as well as exposure to certain compounds might pose risks rather than confer protection (Barahmani *et al*., 2009; Ebeshi *et al*., 2011). For instance, when GST is exposed to methylchloride, the latter is converted

to formaldehyde which is more toxic (Josephy, 2010). The deleterious effects as well as the advantages of GST polymorphisms could be the reason why nature preserves the null phenotypes in populations.

Furthermore, when the GST polymorphisms were stratified by CD4 counts (Figure 4.4), it was observed that HIV patients who had both GSTM1 and GSTT1 deleted were at increased risk of having their CD4 counts fall below 350 cells/mm^3. This shows a weak but significant association between carriers of the GSTM1 and GSTT1 homozygous deletion and an unfavorable immune function. Moreover, this indicates that full loss of GSTT1 and GSTM1 activities confers a risk of CD4 count dropping whereas the presence of at least one functional allele is enough to confer protection. They also had slightly high antioxidant enzyme activities but it was due to chance since it was not significant. Several studies that have linked the homozygous deletion of both GSTM1 and GSTT1 to cancer susceptibility found the risk was weak since the odds ratio was less than 2 (Rebbeck, 1997; Masood & Kayani, 2013).

It was also noted that the people who lacked only the GSTM1 functional enzyme had elevated levels of GGT and AST; which could be attributed to the HIV drugs since most of them were on treatment. The relatively high antioxidant enzymes activities and GSH level in these individuals could be due to increased levels of compensatory antioxidant enzymes when exposed to oxidative stress conditions.

5.2 CONCLUSIONS

The findings of this study show that HIV/AIDS patients who are not on treatment have iron deficiency anemia. Furthermore, combined HIV associated and drug induced liver toxicity were observed in ART naïve and ART-treated HIV/AIDS patients. Additionally, increased

oxidative stress was observed in the HIV patients as compared to the seronegative controls but it was more severe in patients who were ART naïve and had CD4 counts below 200 cells/mm^3. Moreover, this study, the first to investigate GST gene polymorphisms in Ghana, observed that the frequencies of deletion of GSTM1 and GSTT1 were 21.9% and 19.8% respectively and that HIV patients who had a homozygous deletion of both GSTM1 and GSTT1 were at risk of CD4 count below 350 cells/mm^3.

5.3 RECOMMENDATIONS

This study needs to be repeated with a larger sample size to verify the effect of the homozygous deletion of both GSTM1 and GSTT1 on disease progression. This is because, individuals who failed the CD4 count in this study were dropped and this accounted for the large disparity in the numbers for each group due to limited funds.

Moreover, study population was not tested for Hepatitis C and other hepatic viruses, and therefore testing for the presence of these hepatic viruses is imperative.

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APPENDICES

APPENDIX I

NMIMR-IRB CONSENT FORM TEMPLATE
Title: Glutathione S-Transferase gene polymorphism and antioxidant enzymes activity in HIV/AIDS progression in Ghanaian patients

Principal Investigator: Joshua Kuleape

Address: Department of Biochemistry, Cell and Molecular Biology, University of Ghana. P. O. Box LG 54 Volta Road Legon

General Information about Research
Our body makes chemicals to destroy dead cells and germs. When these chemicals are produced in high amounts, they have serious effects on the functions of many organs and life in general. The body again produces other substances to remove these chemicals in order to prevent their harmful effects. In situations where the chemicals are higher than the body's defense system, one is said to be diseased. It has been shown that HIV/AIDS patients lack the ability to remove harmful substances from their body and this worsens the disease condition. In this study, we want to investigate the strength of the body's ability to remove these harmful substances and the impact on HIV/AIDS patients.

In view of this, I would like to seek permission to include you in my study. If you agree to take part in this research, you will spend few minutes answering some questions, after which 5 ml of blood will be taken from you. No risk is expected but you may feel a bit uncomfortable when the needle is used to prick your skin to collect blood. All confidential information obtained shall be respected and used for the prime purpose of the study. At the end of the study, a copy of the results and any useful information obtained will be made available to the hospital without any delay. I would indeed be grateful if you agreed to be part of this important study which would be of much benefit to health care of HIV/AIDS patients.

Possible Risks and Discomforts
There will be some discomfort when the needle pricks you for blood collection.

Possible Benefits
The project will not benefit you individually but successful completion of the study will provide vital information to promote effective management of HIV/AIDS in the country.

Confidentiality
Any information that you provide will be protected and kept confidential to the best of my ability. You will not be named in any report.

Compensation
You will not be compensated for participating in the study.

Voluntary Participation and Right to Leave the Research
Please understand that taking part in this research is entirely voluntary and that you may refuse to take part or withdraw at any point.

Contacts for Additional Information
If you have any questions or concerns about this study, please contact Mr Joshua Kuleape on 0540820905, Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Legon.

Your rights as a Participant
This research has been reviewed and approved by the Institutional Review Board of Noguchi Memorial Institute for Medical Research (NMIMR-IRB). If you have any questions about your rights as a research participant you can contact the IRB Office between the hours of 8am-5pm through the landline 0302916438 or email addresses: nirb@noguchi.mimcom.org

VOLUNTEER AGREEMENT

The above document describing the benefits, risks and procedures for the research title "Investigating the effect of Glutathione S-Transferase gene polymorphism and antioxidant enzymes activity in HIV/AIDS progression in Ghanaian patients" has been read and explained to me. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree to participate as a volunteer.

_____ _____

Date Name and signature or mark of volunteer

If volunteers cannot read the form themselves, a witness must sign here:

I was present while the benefits, risks and procedures were read to the volunteer. All questions were answered and the volunteer has agreed to take part in the research.

_____ _____

Date Name and signature of witness

I certify that the nature and purpose, the potential benefits, and possible risks associated with participating in this research have been explained to the above individual.

_____ _____

Date Name Signature of Person Who Obtained Consent

QUESTIONNAIRE

TOPIC: **Glutathione S-Transferase Gene Polymorphism and Antioxidant Enzymes Activity in HIV/AIDS Progression in Ghanaian Patients**

DERMOGRAPHIC DATA

1. ID……. 2. Age……..….. 3. Sex ……....

4. Visceral fat……5. Body fat……..…… .6. BMI……..

7. Systolic Blood Pressure……...8. Diastolic Blood Pressure……

9. Waist circumference……..... 10. Hip circumference ……....

11. What is your highest level of education?
a. Elementary (Primary)

b. Middle school & J.H.S.

c. Secondary (SHS & Vocational)

d. Tertiary (Polytechnic, University, Professional studies)

e. none

12. What is your occupation?
    a. Professional ___ Trader___ Farmer___
    b. Retired ___
    c. Stays at home___
13. Have you been diagnosed of any cardiovascular disease?
    a. Yes
    b. No
    c. Don't know
14. If yes when?
    a. Before antiretroviral therapy
    b. After antiretroviral therapy
    c. Don't know

15. Have you been diagnosed of any liver disease?
    a. Yes
    b. No
    c. Don't know

16. If yes when?
    a. Before antiretroviral therapy
    b. After antiretroviral therapy
    c. Don't know
17. Are you on any other medication aside the antiretroviral therapy
    a. Yes
    b. No
18. If yes specify_____
19. Do you smoke?
    a. Yes
    b. No
20. Do you experience any of the following?
    a. Swellings yes ___ no___ don't know___
    b. Body pain yes___ no___ don't know___
    c. Fever yes___ no___ don't know___
    d. Skin redness yes___ no___ don't know___
21. If yes, when did you notice?
    a. Before antiretroviral treatment
    b. After antiretroviral treatment
    c. Don't know

22. Are you on antiretroviral drug?
    a. Yes
    b. No
23. What type of antiretroviral therapy are you receiving?

Please select, by ticking, from the list of drugs, the drugs you are taking currently. Tick the appropriate therapy:

Class	**Generic name**
Nucleoside Analogue Reverse Transcriptase Inhibitors (NRTIs)	Abacavir (ABC), Didanosine (ddI), Stavudine (d4T), Lamivudine (3TC), Zidovudine (AZT), Tenofovir (TDF)*
Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)	Efavirenz (EFZ), Nevirapine (NVP), Delavirdine
Protease Inhibitors (PI)	Fosamprenavir (FAPV), Atazanavir (ATV), Darunavir (DRV), Indinavir (IDV), Lopinavir (LPV), Nelfinavir (NFV), Ritonavir (RTV), Saquinavir (SQV)

24. If yes, for how long have you been on antiretroviral therapy?
    a. Less than 3 months
    b. between 3 months -1 year
    c. between 1-2 years
    d. More than 2 year

25. Have you noticed any change in your body fat distribution since you started the regime?
    a. Yes
    b. No
    c. Don't know

Participant's ID……

26. If yes, answer the following by choosing the most appropriate; under CHANGE indicate 'd' for decrease and 'i' for increase under SCORE indicate mild = 1, moderate = 2 and severe = 3

Body part	Change	Score
Face		
Arms		
Abdomen		
Legs		
Buttocks		
Neck		

LIPODYSTROPHY ASSESSMENT

Indicate with 1= Mild, 2= Moderate and 3= Severe

Type /site	**Lipoatrophy**	**Hypertrophy**
Facial		
Arms		
Abdomen		
Legs		
Buttocks		
Neck		

APPENDIX II

Sample size calculation

$$n = \frac{z^2 \times p(1-p)}{\varepsilon^2}$$

Where n is sample size

P is the proportion (frequency of GSTM1 deletion in Nigerians)

α = 0.05

ε = margin of error (0.06)