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DEPARTMENT OF MOLECULAR MEDICINE

CORRELATION OF SERUM LEVEL OF INFLAMMATORY MARKERS WITH CD4 COUNT IN HAART NAÏVE HIV PATIENT AND PATIENT ON HAART

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MASTER OF SCIENCE IN IMMUNOLOGY

BY

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DECLARATION

The research work described in this thesis was carried out by me at the Department of Molecular Medicine, School of Medical Sciences, KNUST. The work has not been submitted for any other degree.

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ABBREVIATIONS AND DEFINITIONS

AIDS	Acquired Immune Deficiency Syndrome
AZT	Zidovudine
CD4	Cluster of Differentiation
CHRPE	Committee on Human Research, Publication and Ethics
CMI	Cell-Mediated Immune response
CMV	Cytomegalovirus
CRP	C-Reactive protein
COX-2	Cycloxygenase 2
EBV	Epstien-Bar virus
ELISA	Enzyme-Linked-Immuno-sorbent Assay
HAART	Highly Active Antiretroviral Therapy
Hb	Hemoglobin
HCT	Hematocrit
HI	Humoral immune response
HIV	Human Immuno-deficiency Virus
HRP	Horseradish Peroxidase

IFN-α	Interferon-alpha
IL	Interleukin
IL-2	Interleukin-2
IL-6	Interluekin-6
LPS	Lipopolysaccharide
LTR	Long Terminal Repeat
MBP	Mannose-binding Protein
MCH	Mean Cell Haemoglobin
MCHC	Mean Cell Haemoglobin Concentration
MCV	Mean Cell Volume
MHC	Major Histocompatibility Complex
MT	Microbial Translocation
MTCT	Mother to Child Transmission
NACP	National AIDS Control Programme
Nef	Negative factor
NFkB	Nuclear Factor kappa B
NK	Natural Killer

PAMPs	Pathogen-association molecular pattern
PBMC	Peripheral Blood Monocular Cell
PLWHAs	People Living with HIV/AIDS
PRR	Pattern Recognition Receptors
RBC	Red Blood cell
ROS	Reactive Oxygen Species
SIV	Simian Immuno-deficiency Virus
TH1	T helper 1
TH2	T helper 2
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNF-α	Tumor Necrosis Factor alpha
WBC	White Blood Cell
WHO	World Health Organization

ABSTRACT

BACKGROUND/INTRODUCTION

Objective: The objective of the study was to evaluate the correlation of the inflammatory markers with absolute CD4 count in on HAART and HAART naïve HIV patients.

Methods: one hundred and fifty participants, comprising of 50 on-treatment and 50 treatment naïve HIV patients, and 50 sero-negative subjects were recruited for the study. Serum CRP, IL-6 and TNF- α were measured using ELISA, CD4 count analysis was done using the BD FACS count and hematology parameters were measured using an XS500i automatic blood cell analyzer (Sysmex Corporation Co. Ltd, Japan).

Results: IL-6 and TNF- α concentrations in HIV-seropositive group were significantly higher than those in seronegative group, and mutually correlated. No significant differences in serum IL-6, CRP and TNF-α level between on HAART, and HAART naïve, groups were observed. Median levels of proinflammatory cytokines (IL-6, CRP and TNF-Alpha) significantly increased with decreasing CD4 count. Subjects with CD4 count less than 200 cells/mm3 were associated with significantly higher median levels of IL-6, CRP and TNF-Alpha compared to those with CD4 count of 500 cells/mm3 and more. The data observed indicated an association between CD8 and CD3 and between the ratio of CD4/CD8 and the ratio of CD4/CD3. A significant and negative correlation of CD8 was observed with the ratio of CD4/CD8 and CD4/CD3 and TNF-alpha with Hb; HCT and RBC but not WBC in all the studied participants. A negative correlation between TNF-alpha and Hb, HCT, RBC and WBC count and also between TNF-alpha and the ratio of CD4/CD8 among HAART naïve HIV participants were observed.

Meanwhile Hb, HCT, RBC and WBC were not significantly associated with both IL-6 and CRP.

Conclusion: Regardless of the source and initial stimulus, continued immune activation and inflammation results in the over-production of IL-6, CRP and TNF-α and thus create

an oxidised environment replication of new virion of HIV. The attack of these viruses will decrease CD4 T cell which will speed up the progression of HIV pathogenesis.

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CHAPTER ONE INTRODUCTION

1.1 STUDY BACKGROUND

Immune activation and inflammation has been identified as the leading cause of pathogenesis of heart disease (Hansson 2005), stroke (Grau 1997, Vaughan 2003), and Alzheimer's disease (Aisen and Davis 1994, Wyss-Coray 2006). When the immune system senses the presence of antigens or foreign particles, the immune cells increase in number and produce molecules that will destroy the pathogen as well as the cells that accommodate these pathogens. Although essential for host defense mechanism, prolonging for a long time becomes deleterious to the host cell, as in chronic infection like Human immuno-deficiency virus (HIV) and Simian Immunodeficiency Virus (SIV). Several conditions may trigger immune activation and the destruction caused to the immune system may not be the same in all diseases and may differ in different patients. Lymphomas, a tumor, are observed in HIV induced AIDS patients when their immune system is subjected to Chronic activation (Dalgleish 1992, Habeshaw, Hounsell and Dalgleish 1992). Chronic immune activation has also been noted to speed up immunological ageing and cardiovascular, renal, and liver diseases and malignancies which are age related ailment seen in HIV infection (French et al. 2009, Brenchley, Price and Douek 2006a, Ciccone et al. 2010).

The body defense mechanism lessens when HIV directly attacks CCR5 CD4 activated T cells. This triggers the adaptive immune system to produce HIV-specific $CD4^+$cells and $CD8^+$ cells. Whiles $CD4^+$ T cells helps in the defense mechanism, $CD8^+$ T cells eliminates the virus infected cells together with the virus. The virus intends infect new $CD4^+$ T cells to ensure survival. The continuous attack and destruction of $CD4^+$ T cells initiate a destructive cycle.

The heightened systemic immune activation leads to the numerical and functional loss of CD4+ T lymphocytes (Aukrust et al. 1995a, Bass et al. 1992, Mahalingam et al. 1993).This causes gradual breakdown of the immune system with sustained immune activation and inflammation. At the mucosal lymphoid site especially the gastrointestinal tract were majority of the CCR5 CD4 activated T cells are located, drastic depletion of the activated CD4 T cells will lead to the erosion of the epithelial lining. This bleach will cause the translocation of the pathogens (bacteria, peptidoglycan, lipopolysaccharides, and flagellin) that are harbored in the gastro-intestinal tract in to the intracellular tissue fluid a process called microbial translocation. The intracellular mast cell degranulates to release inflammatory mediator, histamine, which activates resident macrophages through Pattern recognition receptors (PRRs) and intracellular sensors to release proinflammatory cytokines (Brenchley et al. 2006b). The immune cells, that are attracted to site of infection by the TNF-α released from resident macrophages, are incited when exposed to these bacteria and their products to secrete a lot more of TNF-α and IL-6 (Bergamini et al. 1999). The expansion of activated T effector cell may be accompanied by the production of pro-inflammatory cytokines like TNF-α and IL-6. Increased level of TNF-α decreases trans epithelial resistance in mucosal tissue (Stockmann et al. 2000), thereby promoting microbial translocation and further activation. The chronic pro-inflammatory environment especially the TNF-α and IL-6 exerts a suppressive effect on the architecture thymus. The thymus is unable to produce enough functional Th1 and Th2 T cells. Th1 T cell secrete IL-2 which aids in the proliferation of Th1T cell and CD8 T cells and Th2 T cell secrete IL-6 which helps in the proliferation of B cells into plasma cell to produce antibodies. Therefore increased levels of the inflammatory cytokines interfers with the function of B cells, T cells, dentritic cells and monocytes (Braun et al. 1988). This affects the ability of the

immune system to produce new immune cells at the level of the bone marrow, thymus and the lymph node (Dion et al. 2004, Zhou et al. 2003). Therefore the increase of inflammatory cytokines (TNF-α, CRP and IL-6) may results in decreased levels of $CD4^+$T cells and $CD8^+$ T cells. Increased secretion of Il-6 from macrophage and adipocytes (Lau et al. 2005) triggers the liver (Pepys and Hirschfield 2003) to secret C-reactive protein. HIV disease at the acute phase is therefore characterized by massive increment of cytokine production (Stacey et al. 2009).

The inability of the immune system to make new cells at the levels of bone marrow, thymus, and lymph nodes (Haynes et al. 1999, Schacker et al. 2002, Dion et al. 2004, Estes et al. 2007) compromise its ability to constitute effective defense mechanism to adequately respond to opportunistic infection.

Interestingly, HIV patients on High Active Anti-retroviral Therapy (HAART) have been reported to have low plasma viral load and a stepwise buildup of their immune system (Hammer et al. 1997, Lederman et al. 1998). This enormous shift in viral and cellular dynamics in HIV patients placed on HAART inures to an overall reduced rate of immune activation. The viral receptors on the cell surface of $CD8^+$ (Bisset et al. 1998, Evans et al. 1998, Giorgi et al. 1998) and $CD4^+$ (Bisset et al. 1998) T cells and monocytes (Amirayan-Chevillard et al. 2000) in peripheral blood becomes inactive , this reduces the cellular expression of CCR5 and CXCR4 viral coreceptors (Andersson et al. 1998) and soluble adhesion molecules (Mastroianni et al. 2000) resulting in a low secretion of proinflammatory cytokines(Amirayan-Chevillard et al.
2000). This will reduce suppressive effect of inflammatory cytokines (IL-6 and TNF-α) on thymus. This enables the thymus to produce enough Th1 which restores IL-2 secretion to rejuvenate the non-functional CD4 T cells. This in effect ensures the

enormous restoration of peripheral $CD4^+$ T-lymphocyte population (Weiss et al. 1999). With this, HAART to a greater extent of inhibiting viral multiplication revamps the immune system of HIV patients.

Reduction in HIV viral load commensurate with lower numbers activated T cells and low levels of many inflammation indicators including CRP, IL-6 and TNF-α; conversely, stopping treatment can worsen inflammation. Moreover, CD4 T-cell counts less than 200 cells/µl does little to make better the health condition of HIV patient when placed on ART (Lewden et al. 2008). Increased immune activation in HIV patient place on long-term suppressive ART (Chege et al. 2011, French et al. 2009, Piconi et al. 2010) has been associated with increased mortality (Sandler et al. 2011, Hunt et al. 2011) and both AIDS and non-AIDS-defining illnesses (Tincati et al. 2009, Vassallo et al. 2013). This suggests that increased morbidity and mortality in HIV patients may have strong tides with chronic immune activation.

1.2 PROBLEM STATEMENT

The survival rates of HIV-infected patients are threatened since they are exposed to a lot of opportunistic infection the longer they live even when their CD4 count improves. HIV being a chronic infection is associated with continuous immune activation and inflammation. T cells and other immune cells supplant most of the activities of neutrophils as they become dormant during chronic inflammation. The continuous immune activation experience in chronic infection, as in HIV, speeds up T cells maturation which shortens the time required for their growth and division through the cell life cycle. As a result the T cells may not be well developed and may undergo apoptosis or lose their ability to multiply. The aberrant immune activation coupled with the increase levels of pro-inflammatory cytokines, weakens the immune system and

cause damage to the whole body. Such patients may experience cardiovascular, liver, kidney, bone and neurologic diseases. The high level of inflammatory makers creates an oxidized for the replication of new virion. Increase in viral load will result in low level of CD4 T cells which is associated with the survival of HIV patient.

The Combined effect of two or more antiretroviral therapy boost the immune function and reduce complications associated with AIDS but does not guarantee full recovery in HIV patients.

The present study is therefore designed to evaluate the correlation of the inflammatory markers with absolute CD4 count in on HAART and HAART naïve HIV patients. It is likely that knowledge gained on how inflammation affects HIV disease would help us understand other chronic inflammatory diseases.

1.3 JUSTIFICATION

HIV has become a public health concern since its discovery in 1983(Barre-Sinoussi et al. 1983) and a global health issue with a sum of 33.3 million HIV-infected people as at 2009.

The stigmatization associated with HIV infection coupled with lack of health care facilities, economic difficulties and scarcity of equipment to manage HIV infection to name a few makes people living with HIV/AIDS (PLWHAs) report late for treatment. Although absolute CD4 count of such patients may improve when placed on highly active antiretroviral therapy (HAART) which may translate in dramatic change in disease course, long term therapy leads to development of resistance and toxicity. This may account for decrease $CD4^+$ T cell and increase inflammatory markers in

HIV patients. Chronic inflammation has been noted as one of the leading cause of mortality and morbidity in HIV patients. High levels TNF-α is understood to speed up pathogenesis in HIV patient (Matsuyama, Kobayashi and Yamamoto 1991) by supporting the NF-κB pathway transcription of new virion which are able to escape macrophages and T lymphocyte(Matsuyama et al. 1991, Clouse et al. 1989, Folks et al. 1989, Okamoto et al. 1989). In monocytic cells, IL-6 co-operates with TNF-α to augment HIV-1 replication at transcriptional and posttranscriptional levels (Poli et al. 1990). CRP have been related to cardiovascular disease (Ridker et al. 2002) and atherosclerotic disease as well as some of the common diseases in PLWHAs. Thus the complications associated with the replication and pathogenesis with regards to increase levels of inflammatory cytokines in HIV patient cannot be underestimated.

In Ghana, strategies targeted at improving the health of PLWHAs place more emphasis on initiating them on antiretroviral drugs but research has proven that proinflammatory markers (TNF-α, CRP and IL-6) play a pivotal role in the pathogenesis of HIV. But there are no readily available data on the correlation of inflammatory markers and absolute CD4 count in HIV patients in Ghana. It is therefore imperative to evaluate TNF-α, CRP and IL-6 as inflammatory markers in HIV patients in Ghana.

We envisage that such findings will help improve the care and management of PLWHAs.

1.4 STUDY HYPOTHESIS

Proinflammatory cytokines have correlation with absolute CD4 count of On HAART and HAART naïve HIV patients.

1.5 STUDY AIMS AND OBJECTIVES

1.5.1 Main Aim of the Study

The study aims to determine the correlation between the levels of inflammatory cytokines and the CD4 count of HIV patients before and during treatment.

1.5.2 Specific Objectives

1. To determine proinflammatory markers among HIV patients on HAART and HAART naïve patients
2. To determine lymphocyte subset among HIV patients on HAART and HAART naïve patients
3. To determine correlation between proinflammatory markers in relation to the lymphocytes subset.
4. To determine the effect of proinflammatory cytokines on the lymphocyte subset and hematological parameters.

1.6 SCOPE OF THE STUDY

The details of the study included taken blood sample of on HAART and HAART naïve HIV patients together with seronegative HIV group. CD4 count and Full Blood Count (FBC) alongside with Tumor Necrosis Factor Alpha (TNF-α), Interluekin-6 (IL-6) and C-reactive protein (CRP) were determined for the three groups, using ELISA.

CHAPTER TWO LITERATURE REVIEW

2.1 THE STRUCTURE OF HIV

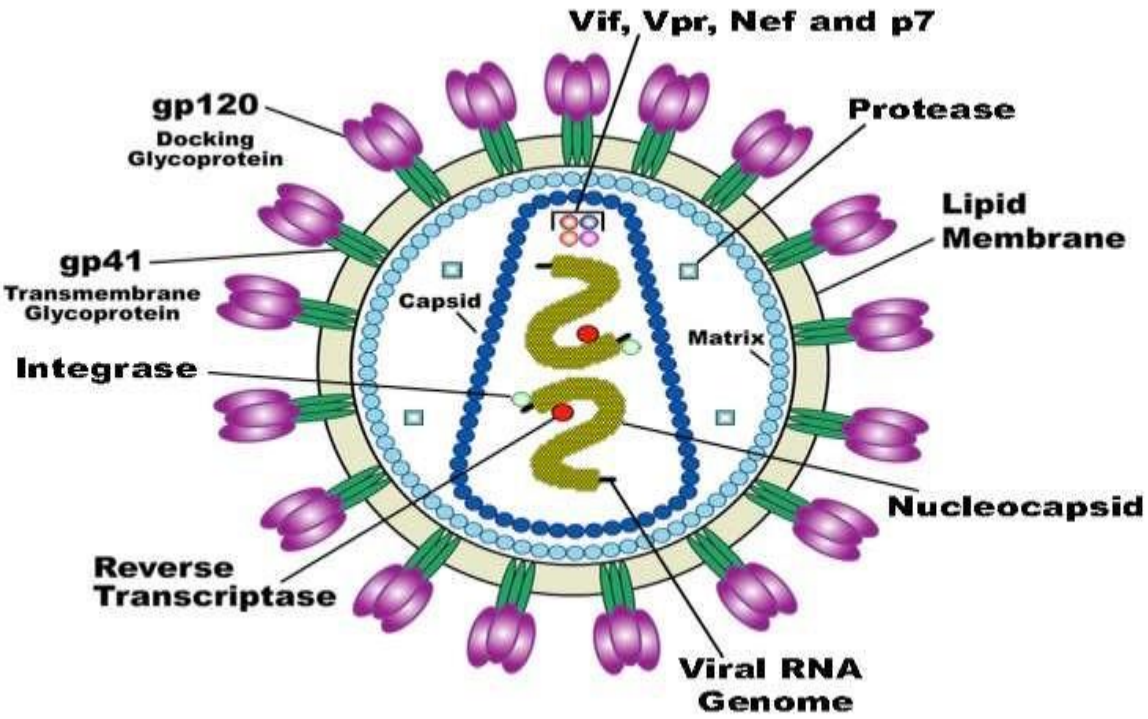

Figure 1.1: A diagrammatic representation of HIV

Source: US National Institute of Health (2005)

The viral envelop of HIV may alternate from spherical shape to oval or sometimes irregular outlines but it is known to be roughly circular in shape. At maturity the virus comprises of a bar-shaped electron dense core which enclose the viral genome. The viral genome consist of 9200 nucleotides base pair of short strands of ribonucleic acid (RNA) surrounded by enzyme reverse transcriptase, protease, ribonuclease, and integrase, all encased in an outer lipid envelop. Projections on the surface of the outer lipid envelop contain gp120, and help the virus to binds to the target cell. When observed under an electronic microscope a budding virus particle of 90-100 nanometers in diameter is seen in the plasma membrane of an infected $CD4^+$ lymphocyte (Greene 1993, Ferguson et al. 2002).

2.2 TYPES OF HIV

HIV-1 and HIV-2 are the only types discovered with the same mode of transmission which leads to AIDS pathogenesis. But transmission of HIV-2 seems a little slower than HIV-1, which accounts for an extended period between infection and the later stage of AIDS in HIV-2 patients. HIV-1 strain is more common worldwide than HIV2 strains, with West Africa recording the highest number of HIV-2 strains. HIV-1 strains can be classified into Group M which is the major group and Group O, the catch all category. Group M are dotted throughout the world and have 10 subtypes (A-J) of viruses. The subtypes are distributed as follows subtypes A and D in SubSahara Africa region; subtype C in South Africa; subtype E in Central Africa

Republic with subtype B commonly found in industrialized world but less common in Africa.

2.3 TRANSMISSION MECHANISMS

HIV infection is mostly transmitted through sexual intercourse whether homosexual or heterosexual engagement from the body fluid of the infected individual to his/her partner. Mothers having the virus can also transmit it to their babies during pregnancy, at the point of delivery or through breast feeding that is Mother-To-ChildTransmission (MTCT). Drug users can also be infected with the virus when they administer injections using a single infected syringe.

2.4 REPLICATION OF HIV

The absence of deoxyribonucleic acid (DNA) in Retroviruses makes it impossible for it to make a copy of itself outside the infected host cells. Pathogenesis in HIV infected

person may include a lot of factors but not limited to virus life cycle, cellular environment of the host, and the viral load of the infected individual. The virus upon gaining access into the body attaches itself to the host cells through surface CD4 receptor. After which it empties it viral genome by fusion or endocytosis into the host cells. It then integrates its viral genome into the DNA of the host and then makes similar copies thereof. The rate of infection may depend on the number of HIV virion in the infected individual and the number of cells having the appropriate CD4 receptors when they make contact (Ferguson *et al.*, 2002).

2.5 PATHOGENESIS OF HIV INFECTION.

HIV destroys the immune system by invading the CD4 cells in the blood. The more CD4 cells that are destroyed by the virus, the weaker the immune system becomes. Reduction in CD 4 T cell level are as results of cytotoxic effect of CD8 lymphocyte, programmed cell death of infected cells and the viral attack. The virus may target immune cells like macrophages, and dendritic cells (Cunningham et al. 2010)

Pathogenesis in HIV infection primarily begins with the attack of the virus on activated $CCR5^{+}CD4^{+}$ T cells (Siliciano and Siliciano 2000). The lymph node harbors milliard of these types of lymphocytes, it becomes inflamed with increase adhesive molecule upon incessant attacks from the virus. This may result in lymphadenopathy syndrome at the early stage of HIV infection. Similar attack happens at mucosal membrane of the gastrointestinal tract with also harbors a lot of activated $CD4^{+}$ memory T cells with HIV co-receptor CCR5 (Veazey, Marx and Lackner 2003,

Brenchley et al. 2004). As the infection progress with time a lot of memory $CD4^{+}$ T cells in the lymphoid and circulation site are discriminately destroyed which completely eliminates the presence of naive and memory phenotype $CD4^{+}$ T cells

(Roederer et al. 1995). The rate of depletion of $CD4^+$ T cells in the peripheral blood is much slower as compare to the excessive deterioration at the mucosal sites. Nevertheless, the depletion observed in the peripheral blood gives us a clue on the progress of HIV pathogenesis. The high extent of depletion of CD4+ T cells both in the mucosal sites and the peripheral blood circulation are accompanied with a rise of systemic immune activation (Aukrust et al. 1995a, Bass et al. 1992, Mahalingam et al. 1993) which results in high levels of serum inflammatory cytokines. When $CD4^+$ T cells number fall far below normal, the immune system ability to fight the HIV virus becomes lessens and that exposes the body to other infections. Certain stage of infection records complete absence $CD4^+$ T cells both in the lymphoid and circulation sites. At this stage the infected HIV individual starts experiencing AIDS condition (Douek et al. 2002). AIDS ushers the gradual breakdown of the immune status as a result of the reduced level of CD4 T cell and high levels of inflammatory cytokines which suppresses other immune cells from replenishing lost ones.

Contrarily to $CD4^+$ T cell, $CD8^+$ T-cell rather increase in their numbers due to the expansion of memory $CD8^+$ T cells usually HIV-reactive cells during the early stage of HIV infection. Expansion of $CD8^+$ T cell may reduce at the later stage of HIV infection (Margolick et al. 1995). Although naïve $CD8^+$ T cell may decrease during the beginning of HIV infection, absolute count of CD8 T cells only declines when HIV disease progresses (Roederer et al. 1995).

This therefore makes it expedient to get credible CD4 count results in order to estimate immunity buildup and properly manage the health of persons infected with HIV (Turner, Hecht and Ismail 1994, Fei, Paxton and Chen 1993, Hoover et al. 1992, Mandy et al. 2002). It is important that clinicians request for CD4 count to be done for

HIV infected patients every 3-6 months, so that those with low levels of CD4 count are placed on antiretroviral therapy (De Gruttola, Gelman and Lagakos 1993). Presentation of low absolute CD4 counts and the perturbed high level of cytokines indicates break down of the immune system of HIV patient.

2.6 EPIDEMIOLOGY OF HIV INFECTION

Since its discovery in 1983 (Barre-Sinoussi et al. 1983),HIV/AIDS has become a major public health concern and a global health issue with about 33.3 million people infected with the virus worldwide as at 2009. About 30 million people had died out of HIV pandemic for the past 30 years. In sub-Saharan Africa about 22.9 million people were living with HIV as at 2012 (2013).

Although the least affected with HIV/AIDS in Sub-Sahara Africa, some countries of West Africa had seen some significant appreciation in HIV prevalence rate. Nigeria as at 2012 had HIV prevalence rate of 3.6% which translated to about 3.3 million of its total population living with HIV. Cameroon and Gabon had HIV prevalence rate of 5.3 and 5.2 respectively.

Ghana first reported Human Immune-Deficiency Virus / Acquired Immune Deficiency Syndrome (HIV/AIDS) cases in 1986, mainly among women who had travelled outside the country. Total cumulative cases of 37,298 was recorded at the end 1999. Close to 90% of the 37,298 were between the age of 15-49 and 63% were female. HIV prevalence increased from 1.7 % to 1.9% from 2008 to 2009 but decreased to 1.5 for both 2010 and 2011 fiscal year. In 2011 the national HIV prevalence of 1.5% translated to about 225,478 people living with HIV/AIDS in

Ghana, out of which 100,334 were men and 125,144 women. The country's HIV prevalence rate of as at 2015 stands at 1.6%.

2.7 HIV AND IMMUNE RESPONSE

2.7.1 Immune activation in HIV infected individuals

The presence of HIV in the host cell induces response which culminates into immune activation. Immune activation though important for host in wading effective immune response against HIV, its repeated occurrence creates an oxides environment for the virus to replicate. Active molecular and cellular processes are among the several events that characterize immune activation in HIV patient.

During the early stage of HIV infection a lot of people initiate an effective immune response but it becomes ineffective over time. CD4 and CD8 T cells form the pillar of cellular response whiles humoral response involves the antibody production and its related activities. CD8 T cells exercise its cytotoxic effect on the virus as well as secrete some chemokines during the primary stage of HIV infection. Some HIV patient are able to maintain high level of $CD8^+$ specific T cell which control viral replication but it falls after infection in most patients. CD8 T cells can therefore be used to ascertain pathogenesis in HIV infected persons. Clinical studies conducted by Golgi and colleagues concluded that excessive immune activation leads to immune dysfunction in chronic HIV patients. The findings showed that CD38 a maker of CD8 T cell and HLA-DR expression negatively correlate better with the worse condition of HIV infected persons than viral load (Liu et al. 2011, Giorgi et al. 1999, Deeks et al. 2004, Giorgi et al. 2002). So as $CD4^+$ T-cell numbers decrease in HIV condition, HLA-DR+ and CD38+ CD8+ T lymphocytes rather appreciates in numbers (Giorgi et al. 1999). Further studies has also established that viral load cannot separately determine the rate of progression to AIDS, and that immune activation better reflects variations in $CD4^+$ T-cell stronger and independent of viral load(Hunt et al. 2008, Rodriguez et al. 2006, Hazenberg et al. 2003, Jiang et al. 2009).

Several researches conducted in the past two decades such as those stated below confirms that immune activation is associated with pathogenesis in HIV patients;

Firstly the survival rate of HIV patient is closely related to the levels of activated $CD8^+$ T cells than CD4+ T cells or viral load (Giorgi et al. 1999); secondly high viremia in some HIV patient is not an indication of HIV pathogenesis (Choudhary et al. 2007, Rotger et al. 2011) ; thirdly in 2002 Sousa et al findings indicated a direct negative correlation between immune activation and CD4+ T-cell depletion in HIV-2 patient but not virus replication rate(Sousa et al. 2002); fourthly immune activation is highly related to high incidence of diseases and death in successfully HAARTtreated patients with undetectable viremia (Sauce et al. 2011); and finally T – cell activation is higher in elite controllers of HIV (HIC) and even higher in HAARTsuppressed individuals than healthy donors (Hunt et al. 2008). In conclusion immunological studies conducted in mice by Tesselaar et al indicated that the mice immune system breaks down when it experienced a lot of chronic immune activation even in absence of viral infection (Tesselaar et al. 2003).

2.7.2 Cells associated with chronic immune activation.

A lot of cells play different roles during chronic immune activation and inflammation in HIV infection. Notable among them are T-regulatory (Treg) cells and T cells that produce TGF-β and IP-10 respectively (Estes et al. 2006, Durudas et al. 2011). IFN-1 are mostly secreted by plasmacytoid dendritic cells (pDCs) but at the later part of infection some other cells may produce this cytokine (Nascimbeni et al. 2009, Harris et al. 2010)**.** TNF-α is normally produced by macrophages, monocytes and lymphocytes in response to lipopolysaccharide but can be overproduced by M-DC8+ monocytes (Dutertre et al. 2012).

Cells like B cells, neutrophils and natural killer T (NKT) cells can augment inflammatory and immunosuppressive conditions by secreting chemokines which alter cellular distributions. Cervical epithelial cells can also secrete chemokines when exposed to HIV-1. (Berlier et al. 2006, Li et al. 2009)

2.7.3 Causes of immune activation

Immune activation and inflammation comes to play base on several processes that emanate from viral replication directly or indirectly.

The entry of the HIV virus into the host elicits antiviral immune response from the host cell ranging from innate to adaptive or vice versa which induces systemic immune activation. Immune response at the innate level begins with the stimulation of Toll-like receptor (TLR) of the plasmacytoid dendritic cells (Fonteneau et al. 2004, Beignon et al. 2005) which triggers the activation of adaptive HIV-specific immune responses. $CD8^+$ T cells initiate an unyielding immune response at the early stage of HIV infection which is maintained for a longer period due to the replicative effect of the virus. $CD8^+$ HIV-specific T cells accounts for about 20% of the total $CD8^+$ T cells population in HAART naïve HIV patient at chronic stage of infection (Betts et al. 2001, Papagno et al. 2002). Contrarily to the HIV specific $CD4^+$ T cell accounting for only 3% of the circulating $CD4^+$ T cell (Betts et al. 2001), which reflects the deterioration effect of the virus attack on them (Douek et al. 2002). Meanwhile important as virus-specific adaptive immune response may serve, it can equally catalyze chronic T-cell activation once the virus escape immune defense which can be inimical to the host immune system.

Secondary intracellular signaling induces by the binding of envelope protein gp120/160 to CD4 and/or CCR5 can trigger immune activation (Ascher and Sheppard 1991,

Herbeuval et al. 2005b, Sailaja et al. 2007). Immune activation can be augmented through the activation of lymphocyte by accessory protein Nef (Wang et al. 2000, Simmons, Aluvihare and McMichael 2001), or through the attachment accessory protein Nef to residence macrophages (Swingler et al. 1999).

Thirdly, microbial translocation a condition in HIV infection also causes immune activation (Brenchley et al. 2006a, Brenchley et al. 2006b). Microbial translocation is characterized by the depletion of majority of CD4 T cells and monocyte/macrophage located in the intestinal lamina propria by the HIV virus. As a results of this, microbial organism (bacteria) and/its products in the intestinal lumen makes its way into the systemic circulation. When LPS or other microbial components enter the bloodstream as a result of this breach, they bind to TLR4, activating T cell. This may culminate into systemic activation and differentiation of lymphocytes and monocytes and the establishment of a proinflammatory milieu. The high levels of plasma LPS levels recorded during microbial translocation can be directly associated with high level of immune activation (Brenchley et al. 2006b). Other pathogens, including but not limited to those causing Opportunistic Infection (OI) such helminth infection may speed up the rate of HIV pathogenesis to AIDS at the tail end of HIV infection (Bafica et al. 2004, Borkow and Bentwich 2006).

Fourthly, CD4 regulatory T cells (T_{reg}) which suppress immune activation by either direct cell-to-cell contact, secretion of cytokines, or restraining the activities of dendritic cell are expended and/ or dysfunction by the HIV virus (Aandahl et al. 2004, Andersson et al. 2005). The depletion or dysfunction of these cells further increases immune activation.

Lastly the persistent virus like cytomegalovirus (CMV) that appears recurrently in health donors (Papagno et al. 2002) reactivates and replicates due to the repeated antigenic stimulation during HIV infection. The various immune activation causes by the virus and other pathogens during HIV infection put too much pressure on the immune system thereby weakening it.

2.7.4 Consequences of immune activation and inflammation in HIV infection When conditions like immune activation and inflammation last longer due to the presence of an antigen in this case HIV, it poses a threat to the immune system and human health. The presence of HIV in the host cell causes persistent T cell activation and which provide the right environment for the virus to replicate effectively and destroy the activated $CD4^+$ T lymphocytes together with the host cells that harbors it (Cullen and Greene 1989). The rise in the level of intracellular nuclear factor kappa B due to persistent immune activation enhances the production of new virion that target naïve T cell. This establishes a vicious cycle where immune activation promotes the virus replication and the virus replication causes immune activation. The outcome of which is infection, activation and killing of HIV specific CD4 T cells (Douek et al. 2002). During activation of the T cell both CD4 and CD8 lineages differentiate from naïve T cells to antigen experience T cells (Hazenberg et al. 2003, Silvestri and Feinberg 2003, Grossman et al. 2002). Activation, expansion and apoptosis varied between $CD4^+$ and $CD8^+$ (Ferreira et al. 2000, Homann, Teyton and Oldstone 2001, Foulds et al. 2002).Contrary to the maintance of a stable pool of memory cells by the $CD8^+$ cell, majority of the memory $CD4^+$cell dies off due to the extensive expansion during activation. This creates a gap in the $CD4^+$ T cell pool. HIV-specific CD4 T cells may decrease as low as 0.01-1% compare to the total CD4 T cells in the peripheral blood (Haase et al. 1996, Lassen et al. 2004, Douek et al. 2002).

In effect chronic immune activation speeds up maturation of the T-cell pool through it life cycle which greatly affect the number of memory $CD4^+$ T Cell available to recognized the virus(Okoye et al. 2007). As a results of this the immune system of HIV-infected patient normally develops prematurely (Appay and Rowland-Jones 2002). Pro-inflammatory and pro-apoptotic cytokines like IL-1β, TNFα and IL-6 are release from activated T effector cells anytime it expands during the vicious cycle of chronic immune activation further incites T cell activation(Decrion et al. 2005).IL-1β and TNFα are noted to erode trans epithelial resistance in mucosal tissues (Stockmann et al. 2000), hence facilitating microbial translation and further activation. Immune cells such as B cells, NK, T-cells, dendritic cells, and monocytes are affect by chronic pro-inflammatory environment (Lane et al. 1983, Braun et al.

1988, Crowe and Kornbluth 1994, Martinez-Maza et al. 1987, Poccia et al. 1996, Zhou et al. 2003) , and may hamper the ability of the immune system to replace damage immune cells at the levels of bone marrow, thymus, and lymph nodes (Haynes et al. 1999, Schacker et al. 2002, Dion et al. 2004).

2.8 INFLAMMATION

Inflammation is the immune response of the body to infection and injury.

Inflammation aims at eliminating the pathogen or causative agent, removing necrotic cells and tissues and initiating repair process. Whenever the body sustains injury, mast cell at the site of injury degranulates to release histamines, bradykines, leukotrienes which cause the endothelial cell to contract. This widens the venule and allows a lot of blood to flow to the site of infection. The dilation of the capillary increase it permeability for fluids and protein in the blood to cross to the interstitial space. Neutrophils and macrophages (monocytes in blood) also migrate across the capillary into the interstitial space. Histamine then incites the resident macrophages through

pattern recognition receptors and intracellular sensors to release TNF-α and other cytokines. The TNF-α exerts a ‘‘domino effect’ ’this aids in the release of lipid mediators, cytokines and chemokines. This attracts more inflame cells to the site of infection. The acute inflammatory response results in swelling, heat, redness and pain at the site of infection. Acute inflammation resolves in hours or few days to restore equilibrium. But sometimes acute inflammation can generates into chronic inflammation if the pathogen persist for a longer period. High levels of TNF-α secreted during chronic inflammation results in excessive production of Reactive Oxygen Species (ROS). Oxidative stress in HIV patient create oxidized environment for the virus to replicate (Das et al. 1990). Plasmacoid, lymphocytes, fibroblast and macrophages are the major immune cells involved in chronic inflammation with primary mediators like reactive oxygen species (ROS), hydrolytic enzymes, INFcytokines and growth factors. Chronic inflammation can last for several days or even years and may differ for different patient. The outcome of chronic inflammation is tissue destruction, thickening and scaring of connective tissue (fibrosis) and death of cells or tissues (necrosis).

For the purpose of this research we will be looking at these inflammatory markers TNF-α, IL-6 and CRP.

2.8.1 Role of Tumor Necrosis Factor alpha (TNF-α) in HIV pathogenesis

Tumor necrosis factor a glycoprotein of 185-amino acid was isolated in 1975. It is connected to the cell surface of mononuclear phagocytes by 212 amino-acid peptide. TNF is located on chromosome 7p21 in human and functions in inciting growth of some tumors. Macrophages are the main source of TNF although T cells, NK cells and mast cells when activated may secrete TNF. Interferon also supplements TNF synthesis. TNF attracts more inflammatory cells to the site of infection and elevated levels causes’ heat,

swelling, redness and pain at the site of infection. It also exerts systemic protective effect on the hypothalamus region of brain which causes fever. TNF have a suppressive effect on appetite and regenerative ability of the bone marrow stem cell division and a depressive effect on myocardial contractility through the perfusion of its tissues. . High levels TNF-α is understood to speed up pathogenesis in HIV patient (Matsuyama et al. 1991) by supporting the NF-κB pathway transcription of new virion (Matsuyama et al. 1991, Clouse et al. 1989, Folks et al. 1989, Okamoto et al. 1989).

2.8.2 Role of Interluekin-6 (Il-6) in HIV pathogenesis

In human IL-6 is encoded as IL-6 gene and acts as pro-inflammatory and antiinflammatory myokine. Macrophage, T cell, fibroblast and vascular endothelial cells release IL-6 when the body experience inflammation, burns and tissue damage. It induces osteoclast formation when released from osteoblast. Just as TNF and IL-1, IL6 also causes fever. IL-6 aids in the attraction of neutrophils to the site of infection and also initiate differentiation of B cells. Mobilization of energy in the fatty and muscle tissue instigated by IL-6 raise the body temperature. IL-6 also stimulates the hypothalamus to synthesize Prostaglandin E_2 which ensures changes in the set point of the body temperature. As a myokine, IL-6 elevation triggers muscle contraction.

High levels of IL-6 in children can results in the development of encephalitis. IL-6 is noted to be high in retroviral infection, autoimmune diseases and benign or malignant tumor patients and promotes HIV (Poli et al. 1990, Poli and Fauci 1992). It mediates T-cell activation, stimulates proliferation of keratinocytes (Sehgal 1990), and, at the beginning of acute inflammation, mediates the acute phase responses (Ishihara and Hirano 2002).There is increasing evidence that Il-6 is an important factor in pathogenesis of atherosclerotic disease and associated abnormalities of the serum lipid

(Rifai et al. 1999, Gierens et al. 2000). In monocytic cells, IL-6 co-operates with TNF-α to augment HIV-1 replication at transcriptional and posttranscriptional levels (Poli et al. 1990).

2.8.3 Role of C-reactive protein in HIV pathogenesis

During Acute phase response of chronic inflammation caused by bacteria, virus and other pathogens, there is an increase secretion of Il-6 from macrophage and adipocytes(Lau et al. 2005). The rise in Il-6 level triggers the liver (Pepys and Hirschfield 2003) to secret C-reactive protein (CRP). CRP is a ring-shaped, pentameric protein synthesize by activated hepatocytes in response to acute inflammation. It is located at chromosome, 1q21-q23 and have a molecular mass of 25106 Da and 224 amino acids. CRP ensures phagocytosis by macrophage through it attachment to the phosphocholine on the surface of the antigen and then links it to the macrophage CRP receptor to complete opsonin-mediated phagocytosis. It may increase 50,000 times its initial level within two hour during inflammation and sustains for 48 hours. At a serum concentration of 10mg/L in healthy human, it can increase as high as 40mg/L during viral infection; 40-200mg/L in bacterial infection and >200mg/L during severe bacterial infection and burns(Clyne and Olshaker 1999). CRP can be a good indicator of immune activation in response to inflammatory damage or infection and has been shown to increase in HIV-1 infected individuals (Lau et al. 2006, Pepys and Hirschfield 2003). CRP have been related to

cardiovascular disease (Ridker et al. 2002) and atherosclerotic disease as well as some of the common diseases in PLWHAs.

2.9 ANTIRETROVIRAL DRUG AND HIV

Before its advent in 1997 several immunomodulation therapeutic strategies had been used to suppress immune activation in HIV replication. The use of antiretroviral drugs is noted to drastically reduce viral load in the plasma and help the immune system to progressively improve on it defense mechanism (Hammer et al. 1997, Lederman et al. 1998). Patients with low basal viral load (Renaud et al. 1999, Connick et al. 2000) ,genetic factors (Rodriguez et al. 2006), younger age (Lederman et al. 2000, Kalayjian et al. 2003), and the small percentage of naive cells (Gandhi et al. 2006) have a greater chance of redeeming or appreciating their CD4 T-cell levels when initiated on ART. Conversely complications like residual viral replication (Wood et al. 2000), altered thymic function (Teixeira et al. 2001), older age (Viard et al. 2001), immune activation (Hunt et al. 2003, Giorgi et al. 1999), apoptosis, and viral coinfections (Greub et al. 2000, Al-Harthi et al. 2006) may hinder CD4 T cell restoration even when placed on ART. Although ART acts in reducing T cell activation in HIV patient, it has been noted to increase in many HIV patient who had many years been on ART with minimal sign of CD4 recovery (Hunt et al. 2003, Gandhi et al. 2006, Valdez et al. 2002, Kaufmann et al. 1999, Goicoechea et al. 2006). Such patient with suppress viremia but low level of $CD4^+$ T-cells have high level of pro-inflammatory cytokine (French et al. 2009)and independently predicts cardiovascular disease and mortality (Kuller et al. 2008).

For close to 30 years (1997) since the manufacturing of Zidovudine (AZT) drug for HIV treatment about 30 new drugs have been introduced. These antiretroviral drugs can be categorized into five classes. Each implores different procedure in suppressing viral replication. Combining more than two different classes for one medication effectively lowers viremia and boosts the immune system. The classes include:

Nucleoside/Nucleotide Reverse Transcriptase Inhibitors (NRTIs): Sometimes called "nukes." These classes of drugs interfere with the reproductive process of the HIV virus. The NRTI's dissembles itself as one of the building block of the viral DNA. As a result of this the viral reverse transcriptase enzyme fails to make new copies of itself.

Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs): These are also called "non-nukes." NNRTIs prevent the virus from replicating its own DNA by directly interfering with the enzyme reverse transcriptase. Therefore the enzyme reverse transcriptase is not able to effectively carry out its duty in ensuring that new virions are replicated.

Protease Inhibitors (PIs): As the name implies it inhibits/blocks protease from effectively incising the long strand of viral genetic material into short functional units.

Entry/Fusion Inhibitors: These classes of drugs align itself to the receptor sites on either the CD4 T cell or HIV. In so doing prevents the virus from infecting other cells.

Integrase Inhibitors: integrase as the name implies is the viral enzyme that aids the virus to effectively combine it genetic material with the host DNA. The drug therefore blocks the integrase and prevent the virus from incorporating it genome into the host DNA. This terminates the replicative ability of the virus.

When more than two classes of the above drug are combined together as one drug it is referred as Fixed-dose combinations. These drugs (ART) are very effective for HIV treatment. This helps reduce the chance of the virus developing resistance for a particular class of drug.

CHAPTER THREE MATERIALS AND METHODS

3.1 STUDY DESIGN

The study is hospital-based, case control study, where we sought to evaluate inflammatory markers and the CD4 count of HIV patients and normal healthy participants asked to undergo blood screening for donation as a control. The test group was classified into two subgroups; HIV patients' on HAART and HAART naïve HIV patients. After informed consent has been sought from the participants, their personal information and clinical histories were recorded before their blood samples were taken for laboratory investigations.

3.2 STUDY SITE

The Komfo Anokye Teaching Hospital (KATH), situated in the Ashanti Region, Kumasi, is one of the leading tertiary hospitals and a major referral Centre in the country. KATH serves the Ashanti, Brong Ahafo, Northern, Upper East and Upper West regions of Ghana and also serves as the teaching Hospital for both medical and paramedic students of Kwame Nkrumah University of Science and Technology. It has an HIV clinic with over 10,000 clients at various levels and stages of treatment. The Clinical Diagnostic Department of the Hospital has various units equipped with the requisite equipment/machines to check the various parameters needed in the treatment and management of HIV client including, Biochemistry, Haematology, Parasitology and serology units.

3.3 STUDY POPULATION

The study was conducted from June to November 2014. 150 participants in all were recruited for this study, hundred participants were recruited from the HIV clinic at

KATH out of which 50 were on ART and the other 50 participants were treatment naïve. The 50 control group was selected from client visiting the blood bank unit of KATH to make voluntary donation or replacement of blood given to their relations. Blood samples taken were analyses at the serology and hematology unit at KATH. The patients were informed that the study was confidential, and that the information provided by them will not affect their laboratory results or the management of their disease status.

3.3.1 Inclusion criteria:

Participants with the under listed criteria were selected for the study

1. HIV patients between 18-45years.
2. Newly diagnosis patient for at most six months for HAART naive.
3. HIV patient on HAART for at most one year.
4. HIV patients without Immuno-compromised infection (example Tuberculosis, HCV, HBV).

3.3.2 Exclusion criteria:

Participants with the under listed criteria were not selected for the studies

1. Patients with Hemoglobin levels less than 8g/dl.
2. Those having any Immuno-compromised infection (example Tuberculosis, HCV, HBV)

3.3.3 Control Selection

Fifty (50) blood donors were recruited as the control group from the Regional Blood Transfusion Centre KATH, Kumasi. The exclusion criteria for the control group were the same as those of the patients aside being negative for HIV.

ETHICAL ISSUES

The study protocol was sent for review and approval from the Committee on Human Research, Publications and Ethics, School of Medical Sciences, Kwame Nkrumah University of Science and Technology and Komfo Anokye Teaching Hospital, in Kumasi. Permission was sought from the Head of Diagnostic Directorate to undertake the study at the serology unit of the Komfo Anokye Teaching Hospital. Subjects who accepted to be part of the study were made to sign an informed consent form.

3.4 SAMPLE COLLECTION

Seven milliliters (7mls) of venous blood was drawn from each of the participant by venipuncture; three milliliter (3mls) into K_3 EDTA vacutainer tube and four milliliters (4mls) in coagulated tube. A complete blood count and CD4 counts were done on each sample in the anti-coagulated tube. However, the blood samples in the BD Vacutainer® SST™ Gel Separator Tubes were allowed to clot and serum obtained by centrifuging it at 3000 revolutions per minute (rpm). The serum samples were pipetted into cryogenic storage tubes and accumulated for a month at -20˚C prior to immunological analyses.

3.5 HAEMATOLOGICAL ANALYSES

The hematological parameters that were analyzed were: hemoglobin concentration, total white blood cell count, lymphocyte count, neutrophil count and platelet count.

XS-500i automatic blood cell analyzer (Sysmex Corporation Co., Ltd., Japan) was used in the determination of these parameters.

The XS-500i automatic blood cell analyzer determines the hemoglobin concentration just as the colorimetric method whiles the platelets and the WBCs are counted similarly as the impedance method. A control specimen was run before the samples of the participants. The EDTA tube containing the blood specimen was inverted about 5 – 10 times to ensure that the content of the tube was well mixed. The tube was uncapped and then placed under the sample probe of the analyzer. The aspirate button was pressed for the analyzer to aspirate the required volume of blood for the analyses. After about 20sec, the result was ready and printed from the analyzer. This procedure was followed for all the samples of the participants.

3.6 IMMUNOLOGICAL ASSAY

The immunological markers that were determined were absolute cell counts of CD4,CD8 and CD3 T lymphocytes in unlysed whole blood, serum CRP,TNF-α and IL-6. The CD4, CD8 and CD3 T lymphocytes counts were determined using the Becton Dickinson (BD) FACSCount system (Becton, Dickinson and Company, Califonia, USA). Serum CRP, TNF and IL-6 were measured using ELISA.

3.6.1 BD FACS Count System By Flow Cytometry For The Quantification Of CD4 And CD3 Lymphocyte.

The BD FACSCount machine is a complete system that comes with its own reagent, control and inbuilt software for the analysis of the sample ran. Only whole blood sample are ran on the machine with no lyse or wash step required. The machine determines $CD4^+$, $CD8^+$ and CD3 T lymphocytes and counts them by using flow cytometry for quantification. The lymphocyte of interest is then calculated by the inbuilt software. The precise and accurate figure given for the $CD4^+$ T lymphocyte will help determine the progress of HIV pathogenesis. Other results like the absolute CD3 count, $CD8^+$ T lymphocyte and the CD4+/ $CD8^+$ helps determines the immune

status of the patient.

Principle of the Test

The BD FACSCount reagent contains antibodies that are flourochrome labelled which binds with the antigen on the surface of the lymphocyte in the whole blood that is added to it. The fluorescent nuclear dye binds to the nucleated blood cells. The sample is then run on the machine after a fixative in added to it and incubated for some time. The BD FACSCount machine have an in-built laser light which cause the fluorochrome-labelled cells to fluoresce as they pass by the laser light. The CD4 lymphocytes and other lymphocytes are counted depending on the fluoresce light that are emitted and quantified by the machine. The in-built software of the machine then calculate and generate CD4 count per sample ran.

Methodology

The CD4 count reagent was inverted up and down for 10 sec in order to suspend the bead and then uncorked with the coring station. After which 50µl of the sample (whole blood) was added to the corresponding tube. The mixture was vortexed and incubated at room temperature in the dark for 60-120 minutes. 50µl of the fixative solution was added, caped and incubated for 15 mins. It was then immediately analyzed on the FACScount instrument after vortexing upside down for 5 sec.

The in-built software then analyze and quantifies the $CD4^+$ lymphocyte, $CD8^+$ lymphocytes, $CD4^+$/ CD8+ ratio and the absolute CD3 count of each sample.

3.7 Determination of Tumor Necrosis Factor- Alpha, C - reactive protein and Interleukin- 6 Concentrations

Principle for the Test

ELISA Assay Kit (Greenstone Swiss Company limited, China) was used for the analysis. Sandwich ELISA principle was employed in measuring the serum levels of TNF-α, IL-6 and CRP. Monoclonal antibody to human antibody immobilized on the micro titer plate bind to the human antigen in the sample or standard. After incubation with a specimen, the excess specimen is washed out and a horseradish peroxidase (HRP) conjugated anti-human antibody is added. This antibody binds to the human TNF-α captured on the plate. After another incubation, the excess antibody is washed out and a tetramethylbenzidine (TMB)-peroxide substrate colour developer is added. The peroxidase present will react with the peroxide substrate and TMB chromogen, resulting in blue colour development. The enzymatic reaction is terminated by the addition of an acidic stop solution (sulphuric acid) which turns the blue colour to yellow. The absorbance of the solution, measured at 450nm, correlates to the concentration of the human antigen bound to the well. The same principle was used in the determination of TNF-α, C-reactive protein and IL-6.

Methodology

All reagents were brought to stand on the bench for 30 minutes before it was used for the analysis. The samples were prediluted in a 1:150 ratio with a zero standard.

After the dilution, 40µl of the standards (TNF-α or IL-6 or CRP) and 10µl patient sample were pipetted into the respective micro titer wells. The plate was tapped gently to mix the content; the plate was sealed and incubated at 37 °C for 30 mins. The wells were washed five (5) times with diluted wash buffer (diluted 30 folds with distilled water). After drying, 50µl horseradish peroxidase (HRP)-Conjugate reagent was added to each well, except blank well. The plate was then covered and incubated at 37ºC for

30 minutes. The liquid was discarded after incubation and washed with a buffer solution repeatedly for five times and dried by patting. After that 50 µl of tetramethylbenzidine (TMB)-peroxide substrate colour developer was added to each well and incubated for 10 min at 37 ºC. Finally 50 µl of stop solution is added to each well to stop the reaction. Absorbance was read at 450nm within 15min with Mindray MR-96A reader.

Serial dilutions of standard of TNF-α, IL-6 and CRP were done as listed in the table below.

Table 3.1: A table representing serial dilutions of TNF-α standard

400 µg/L	5 standard	150 µl original density standard + 150 µl Standard diluent
200 µg/L	4 standard	150 µl 5 standard + 150 µl Standard diluent
100 µg/L	3 standard	150 µl 4 standard + 150 µl Standard diluent
50 µg/L	2 standard	150 µl 3 standard + 150 µl Standard diluent
25 µg /L	1standard	150 µl 2 standard + 150 µl Standard diluent

Table 3.2: A table representing serial dilutions of CRP standard

2400 µg/L	5 standard	150 µl original density standard + 150 µl Standard diluent
1200 µg/L	4 standard	150 µl 5 standard + 150 µl Standard diluent
600 µg /L	3 standard	150 µl 4 standard + 150 µl Standard diluent
300 µg /L	2 standard	150 µl 3 standard + 150 µl Standard diluent
150 µg /L	1 standard	150 µl 2 standard + 150 µl Standard diluent

Table 3.3: A table representing serial dilutions of IL-6 standard

20 ng/L	5 standard	150 µl original density standard + 150 µl Standard diluent
10 ng/L	4 standard	150 µl 5 standard + 150 µl Standard diluent

5 ng/L	3 standard	150 µl 4 standard + 150 µl Standard diluent
2.5 ng/L	2 standard	150 µl 3 standard + 150 µl Standard diluent
1.25 ng/L	1standard	150 µl 2 standard + 150 µl Standard diluent

3.8 DATA ANALYSIS

The data obtained was analyzed using the Statistical Package for Social Scientist (SPSS) Statistical Software (version 17.0, SPSS Inc., Chicago, IL, USA). Continuous variables which were expressed as mean ± standard deviation (SD) and nonparametric were express as median with interquartile range, whiles categorical variables were expressed in proportions. The confidence intervals of the proportions were calculated using the binomial test. Correlations were evaluated using the

Pearson's correlation analysis. For all statistical comparisons, the level of significance was set at $p<0.05$.

CHAPTER FOUR RESULTS

Hundred and fifty participants, comprising of one hundred HIV patients (fifty on antiretroviral therapy (on-treatment) and 50 who had not been given any antiretroviral therapy (treatment naïve) patients) and 50 HIV sero-negative patients were recruited for this project. The biochemical/immunological parameters analyzed were TNF-α, CRP and Il-6 in all the three groups and the results are shown in Tables 4.4 and 4.5. Tables4.6, 4.7, 4.8 relate the three cytokines with CD4 count including figures 4.1, 4.2, and 4.3. Table 4.3 shows CD4 count analyzed in the various groups. Table 4.2 shows analyses of full blood count in the participants and Table 4.9 relates some of the hematological parameters to the proinflammatory cytokines. Table 4.1 shows the demographic characteristics of studied participants.

4.1 DEMOGRAPHIC CHARACTERISTICS

The mean age of the HIV patients (38.57 ± 1.13 for those on treatment and 37.25 ± 1.19 for treatment naive) were significantly higher (P<0.0001) respectively than that of the controls (22.35 ± 1.03). The female participants (42 for those on treatment and 36 for the treatment naïve) far outnumber the male participants (8 for on-treatment and 14 for treatment naïve). However for the controls the number of males (37) was almost three times the number of females (13) (Table 4.1).

Table 4.1: Sociodemographic Characteristics of study participants

		HIV Patients		
	Control (n=50)	On HAART (n=50)	HAART Naïve(n=50)	P-value
Age (years)	22.35 ± 1.03	38.57 ± 1.13†††	37.25 ± 1.19†††	< 0.0001
Gender n (%)				
Male	37(74.0%)	8(16.0%)	14 (28.0%)	
Female	13(26.0%)	42(84.0%)	36(72.0%)	

Values are presented as mean ± standard deviation (SD) and frequency (percentage)

4.2 HAEMATOLOGICAL PROFILE OF THE STUDY POPULATION

Full blood count analysis indicated that White Blood Cells (WBC), Red Blood Cells(RBC), Hematocrit (HCT), and Hemoglobin (Hb) were significantly lower among treatment and treatment naïve HIV infected participants compared to control (p<0.05). Meanwhile Mean Platelet Volume (MPV) were significantly higher among treatment and treatment naïve HIV infected participants compared to control (p<0.05). Absolute

and differential eosinophil count was significantly lower whiles absolute and differential monocyte count were significantly higher in treatment naïve HIV infected participants compared to control ($p<0.05$) (Table 4.2).

Table 4.2: Hematological Profile of studied participants

FBC	Control (n=50)	HIV		P-value
		On HAART (n=50)	HAART Naïve(n=50)	
WBC (10^3/µL)	6.12 ± 0.24	5.19 ± 0.24††	5.35 ± 0.23†	**0.0118**
RBC (10^6/µL)	5.07 ± 0.08	4.20 ± 0.09†††	4.226 ± 0.09†††	**< 0.0001**
Hb (g/dl)	14.36 ± 0.20	12.26 ± 0.28†††	12.13 ± 0.27†††	**< 0.0001**
HCT (%)	44.85 ± 0.73	37.41 ± 0.73†††	36.74 ± 0.96†††	**< 0.0001**
MCV (fL)	88.97 ± 1.22	90.27 ± 1.62	87.31 ± 1.65	0.3842
MCH (pg)	28.50 ± 0.35	29.55 ± 0.65	28.80 ± 0.44	0.2991
MCHC(g/dl)	32.11 ± 0.28	32.69 ± 0.28	33.18 ± 0.33†	**0.0438**
PLT (10^3/µL)	241.0 ± 7.80	244.2 ± 12.05	261.2 ± 18.21	0.5194

RDW-SD (fL)	47.68 ± 0.82	49.66 ± 1.02	47.11 ± 1.24	0.1977
RDW-CV (%)	15.17 ± 0.24	14.82 ± 0.32	14.78 ± 0.32	0.5882
PDW (fL)	13.86 ± 0.34	14.47 ± 0.29	13.77 ± 0.36	0.2824
MPV (fL)	10.99 ± 0.14	10.18 ± 0.18††	13.77 ± 0.36†††	**< 0.0001**
P-LCR (%)	32.32 ± 0.97	32.08 ± 1.83	28.73 ± 2.06	0.1900
PCT (%)	0.26 ± 0.01	0.2513 ± 0.01	0.2592 ± 0.02	0.9039
WBC differential count				
NEUT (10^3/µL)	2.24 ± 0.13	2.083 ± 0.19	2.202 ± 0.16	0.7906
LYM (10^3/µL)	3.020 ± 0.13	2.37 ± 0.10	4.274 ± 1.73	0.4126
MONO (10^3/µL)	0.5784 ± 0.03	0.57 ± 0.04	0.65 ± 0.05††	0.3540
EOSIN (10^3/µL)	0.26 ± 0.05	0.36 ± 0.10	0.12 ± 0.03†	0.0546
BASO (10^3/µL)	0.04 ± 0.003	0.05 ± 0.009	0.03 ± 0.008	0.0738
Absolute WBC count				
NEUT (%)	36.10 ± 1.52	37.30 ± 2.03	39.17 ± 2.21	0.5195
LYM (%)	49.96 ± 1.57	48.53 ± 2.19	46.86 ± 2.30	0.5534
MONO (%)	9.282 ± 0.31	11.56 ± 0.54†††	10.70 ± 0.58††	**0.0010**
EOSIN (%)	3.70 ± 0.53	5.88 ± 1.43	2.06 ± 0.39†	**0.0139**

BASO (%)	0.65 ± 0.05	0.98 ± 0.19	0.51 ± 0.11	**0.0189**

Values are presented as mean ± SD. † p<0.05; †† p<0.001; †††p<0.0001 HIV patients (on-treatment and treatment naïve groups) compared to control

4.3 CD4 COUNT ANALYSIS FOR THE STUDY POPULATION

Mean levels of CD8, CD4, and the ratio of CD4/CD8 were significantly lower among treatment and treatment naïve HIV infected participants compared to control (p<0.0001). Moreover, the ratio of CD4/CD3 was significantly lower among treatment and treatment naïve HIV infected participants compared to control (p<0.0001) **(Table 4.3)** but difference between treatment and treatment naïve was not significant**.**

Table 4.3: Levels of Lymphocyte Subset in studied group

		HIV Patient		
	Control (n=50)	On HAART (n=50)	HAART Naïve (n=50)	P-value
CD3 (cells/mm^3)	1867 ± 99.72	1639 ± 96.56	1755 ± 139.3	0.3674
CD8 (cells/mm^3)	662.0 ± 45.84	1091 ± 74.81†††	1210 ± 125.3†††	**< 0.0001**
CD4 (cells/mm^3)	1084 ± 55.40	498.5 ± 43.85†††	506.7 ± 60.58†††	**< 0.0001**
CD4/CD3	0.59 ± 0.01	0.30 ± 0.02†††	0.28 ± 0.03†††	**< 0.0001**

CD4/CD8	1.73 ± 0.06	0.53 ± 0.06†††	0.55 ± 0.08†††	**< 0.0001**

Values are presented as mean ± SD. † p<0.05; †† p<0.001; †††p<0.0001 HIV patients (On-treatment and treatment naïve groups) compared to control

4.4 PROINFLAMMATORY CYTOKINES

Table 4 shows median levels of proinflammatory cytokines in the study population. There was a significantly higher median levels of IL-6, CRP and TNF-α in the order of Control<on-treatment group< treatment naïve group (p<0.0001) **(Table 4.4).**

Table 4.4: Level of Proinflammatory markers in general studied population

		HIV Patient		
Biomarkers	Control (n=50)	On HAART (n=50)	HAART Naïve (n=50)	P-value
IL-6 (ng/L)	4.454(1.274 - 8.725)	14.98(5.875 - 24.93)†††	17.66(13.00 - 24.10)†††	< 0.0001
CRP (µg/L)	156 (117.1 - 582.2)	1450(251.3 - 2688)†††	1453(328.8 - 2510)†††	< 0.0001
TNF-α (ng/L)	40.28 (24.73 - 81.00)	297.4 (150.2 - 419.7)†††	318.3 (123.0 - 497.9)†††	< 0.0001

Values are presented as Median (25th to 75th quartile range). † p<0.05; †† p<0.001; †††p<0.0001 HIV patients (On-treatment and treatment naïve groups) compared to control

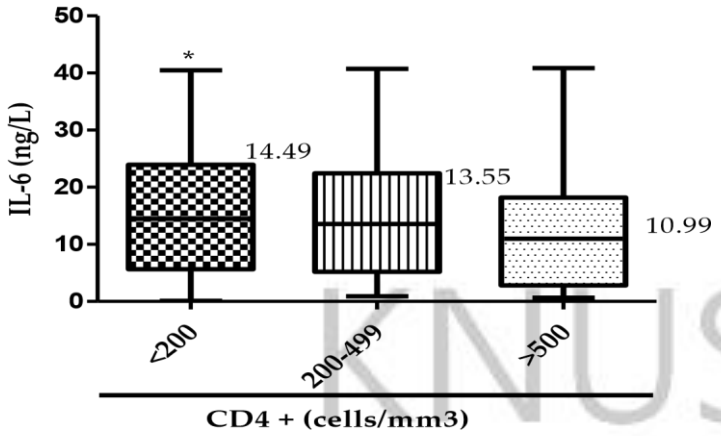

Figure 4.1: IL- 6 relation to the severity of CD4 count among the studied population.

***P<0.05; **p<0.001 ***p<0.0001. Each CD4 count <200 and 200-499 compared >500cells/mm3.**

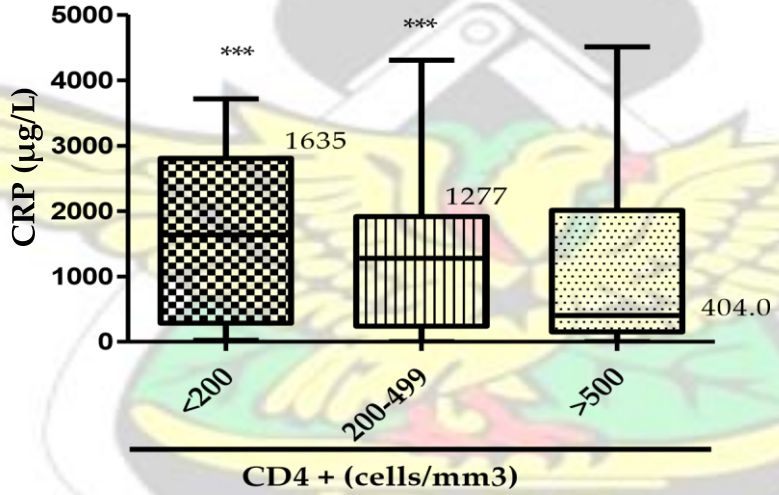

Figure 4.2: CRP relation to the severity of CD4 count among the studied population.

***P<0.05; **p<0.001 ***p<0.0001. Each CD4 count <200 and 200-499 compared >500cells/mm3.**

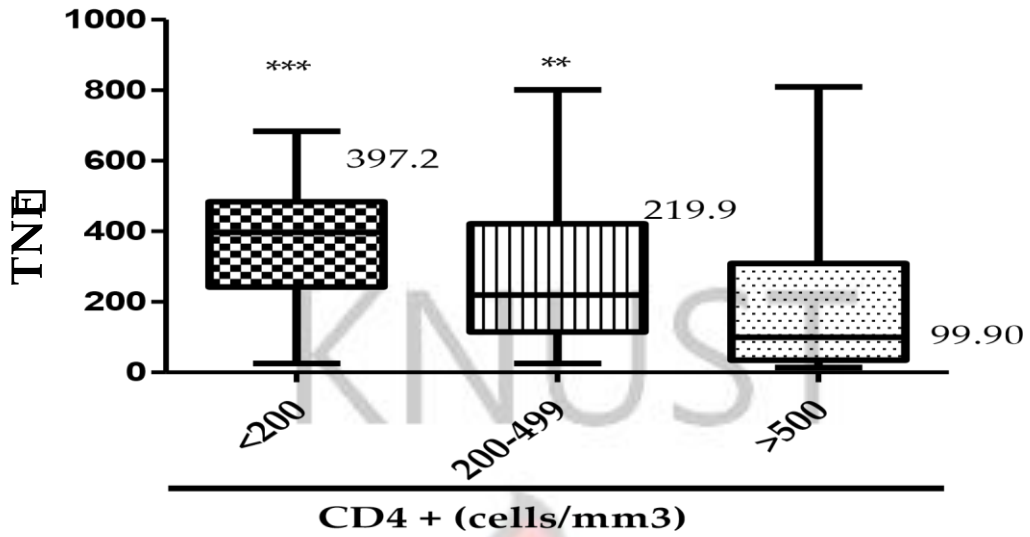

Figure 2.3: TNF- α relation to the severity of CD4 count among the studied population. ***P<0.05; **p<0.001 ***p<0.0001. Each CD4 count <200 and 200-499 compared >500cells/mm3**.

4.4.1 Proinflammatory cytokines in relation to the severity of CD4 count

Median levels of proinflammatory cytokines (IL-6, CRP and TNF-Alpha) significantly increased with decreasing CD4 count. Subjects with CD4 counts less than 200 cells/mm3 were associated with a significantly higher median levels of IL-6, CRP and TNF-Alpha compared to those with CD4 count of 500 cells/mm3 and more ($p<0.05$) in all the three groups.

Table 4.5: Proinflammatory cytokines stratified by gender

Study groups	Male (n=59)	Female (n=91)	p-value
Control			
IL-6	2.90 (0.97 - 6.78)	8.33 (3.98 -14.41)	0.0091
CRP	156 (123.8 -542.5)	260.8 (96.88 - 1442)	0.4518
TNF-Alpha	40.86(24.45 - 77.32)	39.7(20.33 - 112.0)	0.8944

On HAART			
IL-6	10.44(3.57 - 25.00) †††	15.23 (7.48 - 24.93) †††	0.4196
CRP	1653 (282.8 - 2513)†††	1398 (328.8 - 2510) †††	0.8634
TNF-Alpha	180.3 (150.1 - 345.0)†††	302.3 (151.4 - 428.6)†††	0.435
HAART Naïve			
IL-6	17.54(12.51 - 23.28)†††	17.66(12.99 - 24.68)†††	0.9569
CRP	1894 (426.0 - 3264)†††	1450 (187.9 -2648)†††	0.3473
TNF-Alpha	321(270.2 - 427.4)†††	318.3(108.0 - 500.9)†††	0.9054

Values are presented as Median (25th to 75th quartile range). † p<0.05; †† p<0.001; †††p<0.0001 (On- treatment and treatment naïve groups compared to control)

4.5 CORRELATION OF PROINFLAMMATORY CYTOKINES WITH IMMUNE LYMPHOCYTE CELLS

There was a significant positive correlation between TNF-alpha and the ratio of CD4/CD8 (r=0.315; p=0.026) among HAART naïve HIV patients **(Table 4.8)**. CD4 count levels of HAART naïve subjects negatively correlated with CD8 (r=-0.334; p=0.018) but positively correlated with CD4/CD3 ratio (r=0.395; p=0.005) and CD4/CD8 ratio (r=0.476; p<0.0001) **(Table 4.8)**. Meanwhile there was a significant positive correlations between CD8 and CD3 and between the ratio of CD4/CD8 and the ratio of CD4/CD3 (p<0.0001). A significant and negative correlation of CD8 was observed with the ratio of CD4/CD8 and CD4/CD3 (p<0.05) in all the studied participants **(Table 4.6, 4.7 and 4.8).**

Table 4.6: Pearson moment correlation of proinflammatory cytokine with immune lymphocyte cells in Control

		CD4	IL-6	CRP	TNF-α	CD3	CD8	CD4/CD3	CD4/CD8
CD4	r	1	-0.125	-0.126	-0.041	-0.038	-0.081	0.204	0.160
	p-value		0.385	0.382	0.778	0.792	0.576	0.156	0.266
IL-6	r		1	0.027	0.018	-0.006	-0.025	-0.023	-0.020
	p-value			0.850	0.904	0.969	0.863	0.876	0.890
CRP	r			1	-0.010	0.133	0.070	0.073	0.049
	p-value				0.947	0.356	0.627	0.614	0.735
TNF-α	r				1	0.163	0.158	0.142	0.026
	p-value					0.257	0.272	0.325	0.860
CD3	r	1	0.936**	-0.143	-0.133				
	p-value		**0.000**	0.323	0.357				
CD8	r					1		-0.389**	-0.426**
	p-value							**0.005**	**0.002**
CD4/CD3	r							1	0.911**
	p-value								**0.000**

CD4/ CD8	r							1	
	p-value								

r=correlation coefficient. **. Correlation is significant at the 0.01 level (2-tailed).

Table 4.7: Pearson moment correlation of proinflammatory cytokine with immune lymphocyte cells in On HAART group

		CD4	IL-6	CRP	TNF-α	CD3	CD8	CD4/ CD3	CD4/ CD8
CD4	r	1	0.090	0.111	-0.142	0.122	0.019	0.082	0.045
	p-value		0.535	0.444	0.324	0.397	0.896	0.570	0.756
IL6	r		1	0.043	0.069	0.114	-0.100	0.201	0.266
	p-value			0.767	0.634	0.432	0.488	0.161	0.062
CRP	r			1	0.126	0.126	0.181	-0.187	-0.219
	p-value				0.384	0.381	0.209	0.193	0.126
TNF-α	r				1	-0.169	-0.104	-0.031	0.032
	p-value					0.241	0.471	0.828	0.823
CD3	r					1	0.846**	0.121	0.078
	p-value						**0.000**	0.403	0.592

CD8	r	1	-0.281*	-0.352*
	p-value		**0.048**	**0.012**
CD4/ CD3	r		1	0.908**
	p-value			**0.000**
CD4/ CD8	r			1
	p-value			

r=correlation coefficient. **. Correlation is significant at the 0.01 level (2-tailed).

***. Correlation is significant at the 0.05 level (2-tailed).**

Table 4.8: Pearson moment correlation of proinflammatory cytokine with immune lymphocyte cells in HAART naïve

		CD4	IL-6	CRP	TNF-α	CD3	CD8	CD4/ CD3	CD4/ CD8
CD4	r	1	0.263	-0.060	0.226	-0.115	-0.334*	0.395**	0.476**
	p-value		0.065	0.678	0.115	0.428	**0.018**	**0.005**	**0.000**
IL6	r		1	0.155	0.233	-0.009	-0.142	0.177	0.231
	p-value			0.281	0.103	0.952	0.325	0.219	0.107
CRP	r			1	0.070	0.137	0.063	0.025	0.026
	p-value				0.629	0.344	0.665	0.864	0.858
TNF- α	r				1	-0.114	-0.227	0.271	-0.315*
	p-value					0.432	0.112	0.057	**0.026**

CD3	r	1	0.863**	0.075	0.137
	p-value		**0.000**	0.605	0.343
CD8	r		1	-0.337*	-0.326*
	p-value			**0.017**	**0.021**
CD4/	r			1	0.939**
CD3	p-value				**0.000**
CD4/	r				1
CD8	p-value				

r=correlation coefficient. *Correlation is significant at the 0.05 level (2-tailed). **Correlation is significant at the 0.01 level (2-tailed).

4.6 CORRELATION OF PROINFLAMMATORY CYTOKINES WITH HB, HCT, RBC AND WBC

Table 9 shows correlation of proinflammatory markers with Hb, HCT, RBC and WBC count among HAART naïve HIV participants. TNF-alpha was significantly higher and positively correlated with Hb (r=0.310; p=0.028); HCT (r=0.307; p=0.030) and RBC (r=0.323; p=0.022) but not WBC (r=0.117; p=0.420). Meanwhile Hb, HCT, RBC and WBC were not significantly associated with both IL-6 and CRP (p<0.05) **(Table 4.9).**

Table 4.9: Correlation of proinflammatory cytokine with Hb, HCT, RBC and WBC

	IL-6	CRP	TNF-alpha
HB	r=--0.020; p=0.891	r=-0.193; p=0.179	**r=-0.310; p=0.028**

HCT	r=0.025; p=0.865	r=0.130; p=0.368	**r=-0.307; p=0.030**
RBC	r=-0.043; p=0.768	r=-0.048; p=0.743	**r=-0.323; p=0.022**
WBC	r=0.012; p=0.932	r=0.026; p=0.855	r=0.117; p=0.420

r=correlation coefficient

CHAPTER FIVE DISCUSSION

This study sought firstly: to assess proinflammatory markers among HIV seropositive on HAART and HAART naïve patients at the HIV clinic in KATH, and secondly, to assess the correlation between proinflammatory markers, lymphocyte subsets and their hematological parameters. Proinflammatory markers were analyzed using ELISA. Our results indicated that the proinflammatory markers correlated with some of the lymphocyte subset and hematological parameters.

5.1 LYMPHOCYTE SUBSETS

The study observed a significant decrease of CD4 T cell in seropositive HIV studied group compared to seronegative group (Table 4.3).Generally CD4 count in HIV patient have been observed to decline as the disease progresses (Fahey et al. 1990, Burcham et al. 1991) and is a vascular risk factor among HIV-infected individuals (Hsue et al. 2004). The increased rates of apoptosis within blood lymphocyte subsets in HIV positive individuals (Gougeon et al. 1996) could contribute to lower lymphocyte levels. Secondly, the interactions between the virus g120 and the CD4 T cell of the host cell induce by IFN can result apoptosis of the host cell due to the deleterious effect of the virus activity on the $CD4^+$ T cell (Herbeuval et al. 2005a). Thirdly the thymosupressive

effect of pro-inflammatory cytokines (IL-6) (Sempowski et al. 2000) can affect the thymus output. This leads to fibrosis of the lymphatic tissue damaging the architecture and preventing normal T cell homeostasis (Schacker et al. 2002, Schacker et al. 2005). Due to this there is a reduction in the number of new cell that is churns out by the thymus in seropositive HIV patient (Douek et al. 1998). The ability of HIV infected patient in replacing lost T cells becomes compromised. As the naïve T cell pool are not been replenished, with time there will not be any T cell to supplant the old CD8 and CD4 T cell when they die out. At such situation attack by new strain of the virus receive less resistance from the CD8 T cells resulting in the reduction in the numbers of the CD4 T cells. There was no significant difference between the $CD4^{+}$T cell of the On-treatment and treatment naïve group probably because, effective HAART regimes results in similar decay of plasma HIV-RNA (Huang et al. 2001).

There was a significant high level of $CD8^{+}$ T cell in seropositive HIV compared to control group (Table 4.3). In HIV infected individuals $CD8^{+}$subset in the blood from site such as the lung are unable to migrate into high endothelia venule-containing lymphoid tissue due to insufficient expression L-selectin homing receptor(s) or an inability to transduce G-protein-associated signals leading to its accumulation in the peripheral blood. $CD8^{+}$ T cell are also known to expand more extensively than $CD4^{+}$ T cells during immune response. This might have accounted for the overall increase in $CD8^{+}$ T cell in HIV infected individual. As the blood $CD4^{+}$ T cells declines, CD8+ T cell levels are either maintained or increased, this will therefore result in CD4/CD8 ratio decreasing in order to maintain homeostatic mechanisms. This trend was consistent with this study results (table 4.3) which indicates a significant decrease of

CD4/CD8 ratios of the seropositive HIV group compared to the control group. However, the ratio of CD4/CD3 was significantly lower among treatment and treatment

naïve HIV infected participants compared to control. The low level of CD4/CD3 exhibit a steady progression of the HIV infection (Landay, OhlssonWilhelm and Giorgi 1990) and is used to monitor some forms of immunodeficiency

(Nicholson 1989, Nikulin 1994, Schmidt 1989). The association between low CD4+ T-cell count may possibly be explained by chronic inflammation (Lau et al. 2006) in immunosuppressed HIV-infected individuals. Meanwhile there was a significant positive correlation between CD8 T cell and CD3 T cells and between the ratio of

CD4/CD8 and the ratio of CD4/CD3. A significant and negative correlation of CD8 T cell was observed with the ratio of CD4/CD8 and CD4/CD3 in all the studied participants (Tables 4.6, 4.7 and 4.8).

5.2 PRO-INFLAMMATORY CYTOKINES

This study observed a significant increase TNF-α, CRP and IL-6 in HIV patients (Table 4.4). In effect, the cycle of HIV infection, immunosuppression, opportunistic disease, high TNF levels, and further induction of HIV expression could explain at least in part the progression of disease in HIV infected individuals. This study findings show six fold increase of TNF-α in HIV seropositive patient compare to the control group. Generally HIV infected persons are exposed to microbial translocation due to the preferential attack of the CD4 T cell at the mucosal tissue site like the gastrointestinal tract. The presence of the bacteria in the interstitial space triggers immune response leading to the release of TNF-α by macrophage during the inflammation process. As this condition persists in HIV infection due the replicative effect of the virus there is a continuous rise in the level of TNF-α in the serum. High level of TNF-α have been noted to aid in the expression of HIV in infected cells (Lahdevirta et al. 1988, Michie et al. 1988) which confirms that pathogenesis of HIV is associated with high level of

TNF-α. This increased level of TNF-α induces the production of IL-6 by monocytic cells (Davis et al. 1987).

This could have accounted for the significant increase in IL-6 level recorded in seropositive HIV groups than that of the control group, (Table 4.4). Other cells may also secrete IL-6 during inflammation and tissue damage together with vascular endothelial cells and fibroblasts in HIV infected patient. Persistent IL-6 production could therefore be an additional risk factor for the development of hyperlipidemia in patient receiving HAART. Co-operation between IL-6 and TNF-α results in the upregulation of HIV production and potentiates the TNF-α- induced transcription of NFκB (Poli et al. 1990, Poli, Kinter and Fauci 1994). The high level of IL-6 induces the hepatocytes of the liver to secrete of C- reactive protein (Gauldie et al. 1989).

Serum C-reactive protein concentration raise as the arterial walls inflames during atherosclerosis which is common in HIV infected patients. The C-reactive protein concentration increased by more than eight folds in the seropositive HIV groups compared to the control group in our study. CRP is capable of acting as an opsonin in the host response to infection and at the same time, limiting excessive damaging inflammatory response to the host (Black, Kushner and Samols 2004). Individual with High level of CRP have an increased risk for cardiovascular disease relative to those with lower CRP concentrations (Ridker et al. 2002, Ridker 2003).

5.3 HEMATOLOGICAL PARAMETERS

The study observed that lower levels of some hematological parameters were associated with seropositive HIV group than the control group (Table 4.2). Generally

HIV ailments have been associated with hematological abnormalities (Harbol et al. 1994, Ballem et al. 1992) with prevalence and severity of the anemia correlating with

the progression of HIV infection (Sullivan et al. 1998). An HIV patient with CD4 count of 200 cells/μL or less and anemic has a low survival rate. Increase in viral load results in low CD4 count and high levels of pro-inflammatory cytokine. The suppressive effect of some of the cytokines (TNF-α and IL-6) on the bone marrow can lead to anemia. The results of this study analyses on anemic condition of seropositive HIV patient were consistent with the study of anemia as a prognostic factor in HIV infection (Morfeldt-Manson et al. 1991).

Hemoglobin (HB) level was lower in seropositive HIV patient compared to control group. There was not much difference between on-treatment and treatment naïve group. Ledru et al in his study in West Africa identify hemoglobin as a diagnostic indicator in the management of HIV patient (Ledru et al. 1998). Clinicians managing HIV patient normally lookout for the Hb level of their client to decide the next action to take. Regular checking of Hb level therefore gives a clue of HIV progression and also helps the Clinician to determine which patient needs or may requires further treatment. The suppressive effect of the proinflammatory cytokine on the bone marrow, internal bleeding, lack of proper diet intake are some but few causes of low hemoglobin recorded in HIV patients.

Red Blood Cell (RBC) decreased in HIV infected patient as compare to the control group. But there was no significant difference between on-treatment and treatment naïve patients. This may be due to the decrease in RBC production as result of opportunistic infection, direct effect of the HIV infection, mylosuppressive medication and decrease in production of erythropoietin. It can also be inefficient production of RBC as a result of folic acid and vitamin B12 deficiencies. And finally increased RBC destruction resulting from autoimmune hemolytic anemia, thrombotic

microangiopathy, disseminated intravascular coagulation may have accounted for the low RBC observed. The study result was consistent with the findings of Antelman et al (Antelman et al. 2000).

There were lower white blood cells (WBC), recorded in HIV positive individuals as compared to the seronegative group. The immune system of a host in a bid to defend itself against infection and intrusion of foreign particle employs white blood cells in it defense mechanism. Therefore during inflammation, bacterial or viral infection one would expect some changes in the WBC level. The decrease in white blood cells observed in HIV patients may be due to the deleterious effect of the HIV virus on the immune system.

Meanwhile MPVs were significantly higher among treatment and treatment naïve HIV infected participants compared to control ($p<0.05$), which is an indication of a certain inherited disorder.

5.4 EFFECT OF INFLAMMATORY MARKERS ON THE LYMPHOCYTE SUBSET AND THE HEMATOLOGICAL PARAMETERS

Median levels of proinflammatory cytokines (IL-6, CRP and TNF-Alpha) significantly increased with decreasing CD4 count as shown in Fig 4.1,4.2 and 4.3, which clearly indicates that the concentration of these proinflammatory cytokines have an effect on the disease progression of HIV (Aukrust et al. 1995b, Lau et al. 2006).

HIV been a chronic infection is characterize by chronic immune activation which result in proliferative senescence of the T-cell pool particularly at the level of $CD4^{+}$T memory cell (Okoye et al. 2007). The expansion of activated T effector cell may be accompanied by the production of pro-inflammatory cytokines like TNF-α and IL-6.

Increased level of TNF-α decreases transepithelial resistance in mucosal tissue (Stockmann et al. 2000), thereby promoting microbial translocation and further activation. The chronic pro-inflammatory environment especially the TNF-α and IL-6 exerts a suppressive effect on the architecture thymus. The thymus is unable to produce enough functional Th1 and Th2 T cells. Th1 cell secrete IL-2 which aids in the proliferation of Th1 cell and CD8 T cells and Th2 T cell secrete IL-6 which helps in the proliferation of B cells into plasma cell to produce antibodies. Therefore increased levels of the inflammatory cytokines interfers with the function of B cells, T cells, dentritic cells and monocytes (Braun et al. 1988). This affects the ability of the immune system to produce new immune cells at the level of the bone marrow, thymus and the lymph node (Dion et al. 2004). This to a larger extent may have resulted in the increase of inflammatory cytokines (TNF-α, CRP and IL-6) as CD4+T cells depreciate. This might also have accounted for the significant negative correlation observed between TNF-alpha and the ratio of CD4/CD8 among treatment naïve HIV patients (Table 4.8).

Moreover, in response to inflammatory cytokines, increasing IL-6 causes the liver to produce high amount of Hepcidin which stops ferroportin from been released from iron stores. Iron utilization is impaired, with decreased serum iron and transferrin concentrations and an increased synthesis of ferritin (Goodnough and Marcus 1997) decreasing the ability of the bone marrow to respond to erythropoietin. The subsequent effect is increased lactoferrin resulting in anemia (Tanaka et al. 1999). TNF-α suppresses bone marrow stem cell division and decreasing the ability of the bone marrow to respond to erythropoietin. This will adversely aggravate the anemic condition of HIV patients. This is in support of the study findings which indicate a negative significant correlation between TNF-α and Hb, HCT and RBC in HAART naive patients (table 4.9).

This study indicated slight difference between pro-inflammatory cytokine; hematological parameters and CD4 count of on HAART and HAART naïve participants. This may be as a result of the baseline level of the CD4 count of the on HAART participants when they were placed on the antiretroviral therapy.

In Ghana, at the period the study HIV patients were placed on HAART when their CD4 counts were below 350 cells. But most of the patient that begins taking ART medication have their CD4 count below 200 cells/µl. Lewden et al in a cross-sectional found reduced life expectancy in HIV infected patients that are placed on ART with CD4 T-cell below 200 cells/µl (Lewden et al. 2008). At such stage most of the T-cells burnt out prematurely and may undergo apoptosis or lost their ability to divide. Therefore capacity of the immune system to restore peripheral CD4 + T cell lymphocyte population remains compromised. So the reversal of the functional impaired CD4 T cells which will aid the restitution of IL-2 secretion becomes apparently difficult. This therefore extends the time that immune system could reverse the immune-pathological process of the HIV infection. Secondly our study considered HAART naïve participants that were newly diagnosed in 2014 and HAART patient most of whom had been on ART for not more than a year. Therefore not much of an improvement required of the immune response of HIV patient on ART was seen in the on HAART patients.

Thus immune activation in such patient on ART may show no significant difference from HAART naïve patients (newly diagnose). This might have accounted for the insignificant difference of the pro-inflammatory cytokines (TNF-α, CRP and IL-6), lymphocytes subset and hematological parameters observed between the on-treatment and treatment naïve participant of our study. That notwithstanding we observed a significant positive correlation between TNF-alpha and the ratio of CD4/CD8 ($r=0.315$; $p=0.026$) among treatment naïve HIV patients (Table 4.8). CD4 count levels of HAART

naïve subjects negatively correlated with CD8 (r=-0.334; p=0.018) but positively correlated with CD4/CD3 ratio (r=0.395; p=0.005) and CD4/CD8 ratio (r=0.476; p<0.0001) (Table 4.8). This may indicate a need for antiretroviral therapy at an early stage of HIV infection in order to manage the effect of inflammatory cytokines (TNF-alpha) on the lymphocyte subset of HAART naïve participants.

CHAPTER SIX CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSION

Many human immunodeficiency viruses (HIV)-infected patients suffer from chronic immune activation resulting from an increase in proinflammatory cytokines, which may lead to a systematic reduction in $CD4^+$ T cell and anemia.

From the study results it was evident that HIV infection is more prevalent among females than males. It was clear in our study that the pathogenic effect of HIV virus resulted in significantly low mean levels of CD4, CD8, CD4/CD3 and CD4/CD8 ratio among on HAART and HAART naïve patients compared to control group ($p< 0.0001$).

Hematological abnormalities were also recorded in HIV infected participant compared to control group. This gives a clear indication of the deleterious effect of the HIV virus which results in decreased in red blood cell (RBC) production and increased in RBC destruction which will lead to anemia.

The present study confirmed that there are elevated levels of proinflammatory cytokines (IL-6, CRP and TNF-Alpha) in seropositive HIV groups compared to seronegative group.

The data supports a modest correlation between the level of inflammatory markers and the degree of immunosuppression in HIV patients. Increasing levels of inflammation for individuals with progression to AIDS may have a potential link with cardiovascular disease, especially as seropositive patient continues to live on HAART.

We thus conclude that HIV progression to AIDS is not a single cellular event but a combination of other previously less observed factors like chronic inflammation which this study has confirmed. The overall effect is the selective and progressive destruction of cells through apoptosis and finally cell death. Early initiation on HAART may also help improve CD4 count which may lead to the reduction of proinflammatory cytokine secretion.

6.2 RECOMMENDATIONS AND SUGGESTIONS

Based on the results of this study the following ideas are highly recommended:

We recommend that care managers of PLWHAs should include inflammatory cytokines in the laboratory investigations of HIV patients.

A future study should evaluate total antioxidant capacity, free radicals in addition to other inflammatory markers and also establish the reasons for these elevations in inflammatory markers and the interventions required to lower them. And for such research we would advise that participants on-treatment recruited should have been on ART for more than two years. This will help ascertain the effect of oxidative stress and inflammatory markers in the pathogenesis of HIV patients and further enhance effective management of People living with HIV/AIDS (PLWHAs) in Ghana.

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APPENDIX I

KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY

SCHOOL OF MEDICAL SCIENCES

DEPARTMENT OF MOLECULAR MEDICINE PARTICIPANT

INFORMATION LEAFLET

Title of the Research:

CORRELATION OF SERUM LEVEL OF INFLAMMATORY MARKERS WITH CD4 COUNT IN HAART NAÏVE HIV PATIENT AND PATIENT ON HAART

Names and Affiliations of Researchers:

This research is being conducted by Mr. Kwame Osei Wiredu and Prof. (Mrs.) Margret T. Frempong, Department of Molecular Medicine, School of Medical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi.

Background:

This research is about the effect of inflammatory cytokines in HIV patients. More than 34 million people now live with HIV/AIDS out of which 3.3 million of them are under the age of 15 **(UNAIDS World AIDS Day Report 2012).**

HIV infects helper T cells ($CD4^+$ T cells), macrophages, and dendritic cells (Cunningham et al., 2010). These cells activate both B-cell and cytotoxic T-cell immune responses. Without helper T-cells, the body cannot make antibodies properly, nor can infected cells containing HIV be properly eliminated. When $CD4^+$ T cell numbers decline below a critical level, cell-mediated immunity is lost, and the body becomes progressively more susceptible to opportunistic infections. HIV been a chronic infection is associated with inflammation. This results in persistent activation of the T-cells weakening the immune system. Long-term immune activation and sustained high levels of pro-inflammatory cytokines can cause damage throughout the body.

Combination ART is the most effective strategy for reducing excessive immune activation and inflammation. Numerous studies have shown that suppressing HIV viral load decreases the number of activated T-cells and reduces blood levels of several inflammation biomarkers; conversely, stopping treatment can worsen inflammation.

In Ghana, there is no information on the correlation of inflammatory makers and lymphocytes subsets in on-treatment and treatment naïve HIV patients. This will help ascertain the effect of inflammatory makers on lymphocyte subsets in the pathogenesis of HIV patients and further enhance effective management of People living with HIV/AIDS (PLWHAs) in Ghana.

Purpose of the Research:

The purpose of this research is to find out whether inflammatory markers have any correlation with the lymphocyte subset of HIV patients before and during treatment.

Procedure of the Research:

To accomplish the purpose of this research about Seven millilitres (7mls) of venous blood will be drawn each participant into a vacutainer; three millilitre (3mls) into anticoagulated tube for full blood count and CD4 count analysis and four millilitres (4mls) in coagulated tube for analysis of inflammatory markers. Hundred (100) HIV patients were randomly selected from the study population with fifty each for newly diagnosed and those on ART for 6 months or more together with fifty (50) normal control individuals from the blood donors at the Blood Bank.

Risk:

The blood sample taking can be inconvenient to you. You may develop burning sensation or swelling at the site where the blood will be drawn from since a syringe and needle would be used.

Benefit:

You would get to know effect of inflammatory cytokines on your CD4 count. The outcome of this research in Ghana would also help in the management of People living with HIV/AIDS (PLWHAs) in the future.

Confidentiality:

All information which will be collected from you in this research will be given code numbers. Data collected will not be linked to you in anyway. No name or identifier will be used in any publication or reports from this research. However, as part of our responsibility to conduct this research properly, we may allow officials from the ethics committee to have access to your records.

Voluntariness:

Taking part in this research should be out of your own free will. You are not under any obligation to take part in this research. This research is entirely voluntary.

Alternatives to Participation:

If you choose not to participate in this research, services provided to you in this company will not be affected in any way.

Withdrawal from the Research:

You may choose to withdraw from the research at any time without having to explain yourself to the researchers. You may also choose not to answer any question you find uncomfortable or private.

Consequence of Withdrawal:

There will be no consequence, loss of benefit or care to you if you choose to withdraw from this research. Please note however that, some of the information that may have been obtained from you, before you chose to withdraw, may have been modified or used in analysis of reports and publications. These cannot be removed anymore. We do promise to comply with your wishes as much as practicable.

Contacts:

If you have any question concerning this research, please do not hesitate to contact Mr. Kwame Osei Wiredu (054777 2777/ 0501384488) and Prof. (Mrs.) Margret T. Frempong (0208 186 136).

Further, if you have any concern about the conduct of this research, your welfare or your rights as a research participant, you may contact:

The Office of the Chairman

Committee on Human Research and Publication Ethics

Kumasi

Tel: 0322 063 248 or 0205 453 785

APPENDIX II

KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY

SCHOOL OF MEDICAL SCIENCES

DEPARTMENT OF MOLECULAR MEDICINE

CONSENT FORM

Statement of person obtaining informed consent:

I have fully explained this research to _____ and have given sufficient information about the research, including that on procedures, risks and benefits, to enable the prospective participant to make an informed decision to or not to participate.

DATE: _____ NAME: _____

Statement of person giving consent:

I have read the information on this research or have had it translated into a language I understand. I have also talked it over with the interviewer to my satisfaction.

I understand that my participation is voluntary (not compulsory).

I know enough about the purpose, methods, risks and benefits of the research to decide that I want to take part in it.

I understand that I may freely stop being part of this research at any time without having to explain myself.

I have received a copy of this information leaflet and consent form to keep for myself.

NAME: _____

DATE:_____SIGNATURE/THUMBPRINT: _____

Statement of person witnessing consent (Process for Non-Literate Participants):

I _____ (Name of Witness) certify that information

given to _____ (Name of Participant), in the local language, is a true reflection of what I have read from the research Participant Information Leaflet, attached.

WITNESS' SIGNATURE: _____

KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY

FACULTY OF HEALTH SCIENCES SCHOOL OF MEDICAL SCIENCES

DEPARTMENT OF MOLECULAR MEDICINE

RESEARCH QUESTIONNAIRE

GENERAL INFORMATION

1. Sample ID:……
2. Age: □18-24 □25-30 □31-35 □36-40 □ 41-45
3. Place of residence:……..
4. Marital status: □Single □Marriage □Divorced
5. Educational level: □Primary □Secondary □Tertiary □None
6. Occupation: □Self-employed □Employee
7. If employed, are you working with: □Private □Public
8. Which drugs are you on: **NNRTI** □Nevirapine □Efavirenz □Etravirine

   NRTI □Lamivudine □Zidovudine □Abacavir □Didanosine

   PLS □Indinavir □Atazanavir □Ritonavir
9. Duration of therapy:…….
10. (a) Switch from first to second line? □ Yes □No

10. (b) Reasons for the switch.

……

……

……

……

……

11. (a) Have you ever defaulted: □No □Yes

11.(b)If yes why

……

……

……

……

11. (c) Time interval. □ Week …… □ month…….

12. The last CD4 count done if available. …….