Influence of inflammatory markers on HIV pathogenesis

By

Jani Bernadette Rutendo

A project submitted in partial fulfillment of the requirements for

the MSc Biotechnology degree

Department of Biochemistry
Faculty of Science

University of Zimbabwe
Box MP 167
Mt Pleasant
Harare
Zimbabwe

November 2015
ABSTRACT

HIV infection can now be treated effectively with combination antiretroviral medications, and mortalities due to AIDS have been greatly reduced. The increase in the number of deaths and incidences of heart and liver diseases in people whose CD4 counts are above 200 is evidence that there is more happening inside the body than just the cell counts and viremia. HIV infection induces chronic inflammation and immune activation which predisposes patients to cardiovascular diseases (CVD). The objective of this study was to determine the prevalence of risk factors for CVD among HIV-infected people, and to investigate any association between such risk factors, stage of HIV disease, and use of antiretroviral therapies among HIV-positive outpatients at an HIV treatment clinic in Harare, Zimbabwe. To achieve this there was need to look at the lipid profiles, markers of inflammation and markers of endothelial dysfunction, to get a picture of what will be taking place with or without optimal virologic control by HAART. We compared three groups namely HIV unexposed and uninfected, HIV patients on highly active antiretroviral therapy (HAART) and HIV infected but HAART naïve. The population was controlled for traditional risk factors that are confounding factors of CVD. All participants had average lipid and glucose values within normal ranges, but there was a small difference between the ART and ART naïve- for total cholesterol (TC) and high-density lipoprotein (HDL). IL-6 levels were elevated in HIV infected patients compared to healthy controls, and higher in HAART naïve patients than those on HAART. We did not find significant differences in TNF-α levels by HIV or ART status. There were high levels of plasmatic VCAM and ICAM in HAART naïve patients compared to those on HAART. Results showed that HIV infection leads to an inflamed environment and endothelial damage, which is optimally controlled by HAART but still does not normalise. These markers of immune activation together with information on lipid profiles can be used to predict the risk for CVD in HIV patients. Framingham risk showed 1.4% prevalence of high CHD risk within the next ten years.
ACKNOWLEDGEMENTS

I would like to thank God for his grace that has taken me this far. My sincere gratitude goes to my supervisor Professor T. Mduluza whose expertise and enthusiasm in the field of immunology has made this work a rewarding exercise. I am grateful to the Letten foundation, with special mention to Proffessor Babill Stray Pederssen for sponsoring this work. I would like to thank my colleague Elizabeth Gori. Many thanks go to the University of Namibia School of Medicine and to Prof Quaye for the assistance and supervision. I would like to acknowledge the Biochemistry staff for the opportunity and to my classmates-thank you all.
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<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular Diseases</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular Cellular Adhesion Molecule</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular Adhesion Molecule</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin six</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour Necrosis Factor Alpha</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary Heart Diseases</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>CD4</td>
<td>Cluster of Differentiation 4</td>
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<tr>
<td>HAART</td>
<td>Highly Active Antiretroviral Therapy</td>
</tr>
<tr>
<td>Cart</td>
<td>Combinational Antiretroviral Therapy</td>
</tr>
<tr>
<td>PIs</td>
<td>Protease Inhibitors</td>
</tr>
<tr>
<td>CAMs</td>
<td>Cellular Adhesion Molecules</td>
</tr>
<tr>
<td>FMD</td>
<td>Flow-mediated dilatation</td>
</tr>
<tr>
<td>UEU</td>
<td>Unexposed and uninfected</td>
</tr>
<tr>
<td>MRCZ</td>
<td>Medical Research Council of Zimbabwe</td>
</tr>
<tr>
<td>TC</td>
<td>Total Cholesterol</td>
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<tr>
<td>HDL</td>
<td>High Density Lipoprotein</td>
</tr>
<tr>
<td>LDL</td>
<td>Low Density Lipoprotein</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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1. INTRODUCTION

The natural course of Human Immunodeficiency Virus (HIV) was historically thought to include a period of latency. This period when the virus was said to be inactive, often for years seemed to be a respite from the harsh effects that HIV can have on the body. However recent studies have shown in fact, HIV is not silent during this latency period (Kaminski et al., 2010).

Previously, it was assumed that the level of protection was directly proportional to the CD4 count. This would mean the higher the CD4 counts, the lower the risk for AIDS-defining opportunistic infections and other diseases. Lately, people with higher CD4 counts are suffering from serious conditions like heart, liver, and kidney diseases (Serrano-Villar et al., 2014). The increase in the number of deaths in people whose CD4 counts are above 200 is a clear sign that CD4 levels alone may not indicate what is happening inside the body.

The increased use of antiretroviral therapy (ART)-mediated viral suppression remains troubled by abnormally elevated markers of immune activation, inflammation, and coagulation in many HIV-infected individuals. Sustained combination antiretroviral therapy (ART) worldwide has led to a decrease in AIDS-related illnesses in people infected with HIV (Smith et al., 2010). The most common cause of death in the past ten years have become non-AIDS illnesses, including cardiovascular disease (CVD), liver failure and cancer (Palella et al., 2006). Pathogenesis of these conditions is due to chronic immune activation and inflammation along with traditional risk factors such as smoking, obesity, diabetes, and dyslipidemia. ART has brought about many gains yet still, life expectancy, remains shorter for HIV+ individuals than in the general population. Untreated HIV infection causes
inflammation and, despite ART, it does not normalize. The causes of persistent immune activation are not fully understood.

Despite the decline in morbidity and mortality due to highly active antiretroviral therapy (HAART), cases of metabolic derangements, including dyslipidaemia, insulin resistance, abnormalities of glucose metabolism and changes in fat distribution have been on the rise (Smith et al., 2010; Palella et al., 2006). All these changes occur simultaneously with HIV infection, increasing the risk of cardiovascular (CV) disease. Concern has also been raised on the long term safety of antiretroviral (ARV) therapy administration (such as abacavir or protease inhibitors (PIs)) especially in an era where treatment of non-HIV-related serious events is on the rise (Islam et al., 2012).

There is general consensus that antiretroviral drugs induce a long-term risk of CHD, although the levels of that risk are somewhat controversial (Freiberg et al., 2013; Islam FM et al., 2012). With the evidence of non-traditional CVD risk factors increasing, it is important to improve measures to identify HIV infected people who are at risk of CVD. Biomarkers have proved to be useful in predicting clinical events and are increasingly employed in monitoring health, identifying individuals at risk (Ingelsson et al., 2008). So far, research in CVD risk factors has identified a number of predictive biomarkers, such as sP-selectin, IL-6, intercellular adhesion molecule 1 (ICAM-1), vascular adhesion molecule 1 (VCAM-1), total cholesterol, and highly active protein (CRP) among others (De La et al., 2013). If successfully determined as independent risk factors, the biomarkers could also be employed in the HIV infected population to identify people at risk. As of now, no consensus has been reached.

The intention of this study was to describe profiles of surrogate markers for the long-term risk of CHD among HIV-positive outpatients at an HIV treatment clinic in Harare, Zimbabwe.
2. LITERATURE REVIEW

2.1 HIV AND INFLAMMATION

Inflammation is a broad term for what happens in the body when the immune system is activated to counter a threat. A healthy immune response is key to good health, but on-going immune activation and inflammation due to a persistent threat such as chronic HIV infection can lead to many different problems throughout the body (Neuhaus et al., 2010). One theory is that as HIV chronically infects the body, cells and tissues are destroyed and then heal, activating the immune system. That leads to an overstimulated immune system that can become burned out or weakened. So, even though a lab result may show a high CD4 count,
the amount of inflammation in the body may be causing damage on a cellular level. And that can lead to heart, liver, kidney disease, and greater levels of bone loss.

At the moment one of the crucial issues to the HIV community is the wringer of whether or not the untimely development of coronary artery disease is due to HAART-associated lipid disorders (Annillo et al., 2014). There exists an association between endothelial injury and disease-related biochemical abnormalities that are implicated in HIV pathogenesis. For that reason the study of endothelial function in HIV infection and its modifications by HAART is an exciting new field in clinical research.

Figure 2. The relationship between inflammation and virologic control.
2.2 HIV AND THE CARDIOVASCULAR SYSTEM

Infection with HIV has been shown to increase the risk of coronary heart diseases (CHD), although it is difficult to separately assess the part played by the virus and that of the therapy.

Figure 3. Pathogenesis of atherothrombosis in HIV infection.
HIV infection and its complex interaction with the immune system plays a crucial role leading to increased cytokine expression and vascular damage (Verma and Anderson, 2002).

"Endothelial function" refers to a number of physiological functions of the vascular endothelium that are achieved via secretion of diverse bioactive substances. This makes the endothelium an active participant in healthy homeostasis of the vascular wall. Endothelium is involved in important homeostatic mechanisms of non-thrombotic vascular surfaces, vascular tone regulation and immunomodulation (Torriani et al., 2014). Several different clinical conditions, such as, hypertension, dyslipidaemia diabetes mellitus, contribute to endothelial dysfunction thought to be a major link between infection, inflammation and atherosclerosis (Vallance et al., 1997; Verma and Anderson, 2002).

The HIV epidemic introduces a new agent that has been associated with endothelial dysfunction. Several observations in pathophysiologic studies in humans and animals led to the formulation of the response-to-injury hypothesis of atherosclerosis, which initially proposed that endothelial denudation was the first step in atherosclerosis (Blann and Taberner, 1995; Nolan et al., 2003). Endothelial injury is associated with disease-related biochemical abnormalities that are implicated in HIV pathogenesis. For example, entry of virus into endothelial cells could possibly occur through CD4 antigen, galactosyl-ceramide receptors or chemokine receptors (Cohen et al., 1998; Ostrowski et al., 1998). Endothelial activation may also occur either by cytokines secreted in response to mononuclear or adventitial cell activation by HIV virus or by the effects of gp120 and Tat, both secreted HIV-associated proteins, on endothelium (Moir et al., 1999; Verma and Anderson, 2002).
Intercellular adhesion molecule (sICAM-1) and P-selectin are documented factors implicated in myocardial infarction and atherosclerosis. High levels of these adhesion molecules represent early markers of the development of atherosclerosis (Blankenberg et al., 2003). Of interest, beside soluble adhesion molecules, other factors such as fibrinolytic factors, tissue plasminogen activator (t-PA) and plasminogen activator inhibitor (PAI-1) have also been considered to be markers of endothelial dysfunction (Ridker et al., 1998)

Figure 4: Cardiovascular implications from untreated human immunodeficiency virus infection.
2.3.1 ENDOThelial Dysfunction AS A PREDICTOR OF CARDIOVASCULAR EVENTS.

The extent of endothelial dysfunction appears to correlate with the traditional risk factor “burden”. This means that combined or repeated injury to the vascular endothelium results in greater dysfunction (Vallance et al., 1997; Blann and Taberner, 1995). Factors causing vascular injury as well as repair mechanisms modulate endothelial function and are potentially mediated via circulating endothelial progenitor cells (Verma and Anderson, 2002). Thus, the concept has been put forth that endothelial vasodilator function is a reflection of overall vascular health, or a barometer of the injury/repair inflicted by multiple environmental and genetic factors, and, therefore, could potentially serve as a useful diagnostic and prognostic tool in individual patients (Ridker et al., 1998).

It is important to realize that the presence of a similar risk factor profile does not necessarily imply the presence of an equivalent degree of endothelial dysfunction. Thus, the observation that assessment of endothelial function can provide an independent prediction of future cardiovascular risk demonstrates not only the crucial pathophysiologic role of this entity to existing vascular disease but also how the disease is likely to progress with time (Blankenberg et al., 2003).

Endothelial dysfunction is associated with diabetes mellitus, hypertension, dyslipidemia, tobacco use and other metabolic disorders and is a predictor of future cardiovascular events. Endothelial dysfunction has also been associated with HIV infection and HIV therapy (Blann and Taberner, 1995).
### 2.3.2 AVAILABLE METHODS FOR ASSESSMENT OF ENDOTHELIAL FUNCTION

Endothelial function assessment could be performed either invasively or non-invasively. Non-invasive models include study of biomarkers that are present on the surface of endothelial cells or are expressed in response to several stimuli and have an important role in the process of leukocyte rolling, firm adhesion and trans endothelial migration, biomarkers are either present on the surface of endothelial cells or are expressed in response to several stimuli and have an important role in the process of leukocyte rolling, firm adhesion and trans endothelial migration (Gokce et al., 2003). Soluble CAMs are considered reliable biomarkers of atherosclerosis development and severity and to add to the predictive value of classic risk factors for coronary artery disease in healthy individuals and in patients (Blankenberg and Barbaux, 2003).

Another non-invasive technique is the use of ultrasonography to assess the degree of flow-mediated dilatation (FMD) of the brachial artery following an ischaemic stimulus. Endothelial function at the brachial artery provides a surrogate measure of the coronary circulation and a correlate of the severity of coronary artery disease (Corretti et al., 1998). Accordingly, abnormal brachial artery endothelial function has been associated with a wide spectrum of cardiovascular risk factors, including dyslipidaemia, smoking, diabetes and hypertension. Endothelial dysfunction is considered the key step in the development of atherosclerosis and it is known to be an early predictor of future cardiovascular events in patients without and with known cardiovascular disease (Blankenberg and Barbaux, 2003). Endothelial function can also be studied invasively, by studying blood flow responses (Corretti et al., 2002).
2.3.3 ICAM and VCAM

*Intercellular adhesion molecules (ICAMs)* and *Vascular cell adhesion molecule-1 (VCAM-1)* are part of the immunoglobulin superfamily. They are important in inflammation, immune responses and in intracellular signalling events. These ICAMs may exist in soluble forms in human plasma, due to activation and proteolysis mechanisms at cell surfaces (Gahmberg *et al.*, 1997).

Recruitment of circulating leukocytes at sites of atherosclerosis is mediated through a family of adhesion molecules. The function of circulating forms of these adhesion molecules remains unknown, but their levels may serve as molecular markers of subclinical coronary heart disease (CHD). Among the identified adhesion molecules, the expression and biological properties of VCAM-1, endothelial-leukocyte adhesion molecule-1 (E-selectin), and ICAM-1 are well characterized. Circulating forms of VCAM-1, E-selectin, and ICAM-1 have been detected in plasma and are elevated during inflammatory conditions in which detailed pathology studies have documented increased expression of cellular adhesion molecules on endothelial cells and other tissue types (Gearing and Newman, 1993). The origins of circulating VCAM-1, E-selectin, and ICAM-1 are unclear, but they may arise from shedding or proteolytic cleavage from endothelial cells (Pigott *et al.*, 1992). This our study was based on the hypothesis that circulating levels of VCAM-1, E-selectin, and ICAM-1 may be useful markers for increased expression of cellular adhesion molecules in atherosclerosis.
2.4 ARV THERAPY: SOLUTION OR ADDITIVE RISK FACTOR FOR CV DISEASE?

The introduction of highly active antiretroviral therapy has significantly improved the quality of life of HIV infected patients. However, in recent years, several clinical studies have hinted at an increased risk for cardiovascular disease (CAD), particularly among patients receiving protease inhibitors (Rhew et al., 2003). Although the DAD Study Group found that the relative risk of cardiovascular disease increases with the duration of antiretroviral therapy, the absolute risk for cardiovascular disease remain low for most patients, except those with multiple traditional risk factors for coronary artery disease, and is far outweighed by the benefits of antiretroviral therapy in terms of reduced risks of AIDS and death in most HIV infected patients (Neumann et al., 2005).

Protease inhibitors, a main component of antiretroviral therapy, induce many metabolic derangements, such as dyslipidemia, insulin resistance and other metabolic disorders and may expose HIV infected patients to an increase risk for coronary artery disease (Fantry, 2003). Antiretroviral therapy may cause endothelial dysfunction by a direct effect on the endothelial cells or by indirect means such as working with the HIV virus on endothelial cells, or through its effects upon the lipid and glucose metabolism (AIDS Treatment News (277)1997). However, there are still conflicting results regarding the effects of HIV infection and its therapy on endothelial dysfunction, as assessed by brachial artery flow mediated vasodilatation, some showing a worsening and others showing improvement of endothelial function (Cotter B. Endothelial dysfunction in HIV infection, In press).

It is not proper to conclusively attribute certain effects to HIV therapy before assessing traditional cardiovascular risk factors for CAD in these individuals., Factors such as diabetes
mellitus, hypertension, dyslipidemia, tobacco use, sedentary life style, obesity and family
history need to be assessed first in HIV infected patients before because they are also
confounding factors for CVD.

The recommended treatment regimen for HIV today is the combination of three or more
antiretroviral drugs to effectively reduce the viral load. In Zimbabwe, the ART regimens
mostly used are nucleotide reverse transcriptase inhibitors (NRTIs) such as tenofovir (Gilead
Sciences, Foster City, CA, USA) in combination with non-nucleoside reverse transcriptase
inhibitors (NNRTIs) such as nevirapine (Boehringer Ingelheim, Ridgefield, CT, USA) or in
combination with protease inhibitors such as lopinavir and ritonavir, together known as
Kaletra/Alluvia (Abbott Laboratories, North Chicago, IL, USA). There is conflicting
evidence from studies performed in both African and Western countries as to whether ART
has effects that increase the risk of CHD.

The HIV epidemic has hysterically changed the pattern and model for the development of
drug therapy in the last 15 years. For now the objective is not sorely the reduction of plasma
viral load, but also to restore the immune weakness due to disease progression (Fauci, 2003;
Young et al., 2006).
2.5 INFLAMMATORY MARKERS AND MORTALITY

The big question is whether increased inflammation affects the lifespan of people with HIV. Early studies suggest it could be linked to all causes of death among people with HIV. So it would appear that inflammation may be causing damage early in the course of HIV disease, despite lower viral loads and higher CD4 counts, and that it may play a role in both HIV-related cancers and death (Neuhaus et al., 2010)

Table 1. Inflammatory markers and clinical associations

<table>
<thead>
<tr>
<th>Marker</th>
<th>Function</th>
<th>Clinical association</th>
</tr>
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<tr>
<td>Soluble CD14 (sCD14)</td>
<td>Co-receptor for the detection of bacterial LPS; marker of monocyte response to LPS.</td>
<td>Shown to independently predict mortality and progression of vascular disease in HIV.</td>
</tr>
</tbody>
</table>
| Soluble CD163 (sCD163)                      | Monooyte and macrophage-specific scavenger receptor. Extracellular TLR activation leads to shedding of sCD163. Surface sCD163 acts as an innate immune receptor for bacteria. Shedding may be a mechanism to ↓ acute & severe monocyte activation/inflammation. | Shown to decline with effective cART and spikes with interruption.  
Associated with neurocognitive function, arterial inflammation, non-calcified coronary plaques. |
| Soluble tumor necrosis factor receptor (sTNF-RI & RII) | Proinflammatory cytokines                                                | Associated with non-AIDS events and diabetes                                          |
| Interleukin 6 (IL-6)                        | Secreted by T cells and macrophages Pro-inflammatory cytokine & anti-inflammatory myokine. Stimulates immune response to tissue damage → inflammation. | Predictive of all-cause mortality in HIV; correlates with residual viremia and immune dysfunction in cART controlled individuals. Associated with CV events |
| Soluble vascular cell adhesion molecule (sVCAM-1) | Signal activation/damage to endothelium predisposing to atherosclerosis. Levels ↑ decades before development of atherosclerosis | Associated with vascular disease in HIV ↑ in adolescents infected through risk behaviors vs. controls |
| High-sensitivity C-reactive protein (hsCRP)  | Binds phosphocholine on surface on damaged cells and microbes, activating the complement system, and enhancing phagocytosis. Acute phase reactant. Rises quickly in response to injury and declines rapidly with a short half-life | Predictor of MI in HIV, and associated with vascular disease. |
It will take more studies before we know how to prevent heart, liver, and kidney disease in people with HIV. But one thing seems clear: HIV isn't sitting silently during its "latency period." Indeed, it is quite active, leaving a significant imprint on the body's immune and inflammatory systems (Kaminski et al., 2010).

Virologists and immunologists have recognised that both the virus (HIV) and the immune system (the human host) are inseparable players in the process leading to AIDS. There are differences of opinion as to whether the better treatments will be those directed against the virus or those to help the immune system; in practice, a combination of the two is likely to be needed for people whose immunity has already been substantially impaired by HIV (Henrard, 1995).
Figure 5: Summary of immunological events between HIV infection and progression into AIDS
2.5.1 INTERLEUKIN 6

In the general population, raised levels of inflammatory markers are stronger predictors of fatal than nonfatal cardiovascular disease (CVD) events. People with HIV have elevated levels of interleukin-6 (IL-6), high-sensitivity C-reactive protein (hsCRP), and D-dimer; HIV-induced activation of inflammatory and coagulation pathways may be responsible for their greater risk of CVD. Elevated IL-6 levels have been linked to increased risk of cardiovascular disease (CVD), cancer and death. Interleukin 6 (IL-6) is a proinflammatory cytokine that regulates various physiological processes (Heinrich and Andus, 1990). It plays a key role in the acute phase response and in the transition from acute to chronic inflammation. Evidence has accrued to suggest that dysregulation of IL-6 production is a major contributor to the pathogenesis of chronic inflammatory and autoimmune diseases (Tanaka and Kishimoto, 2014). Human immunodeficiency virus (HIV) infection has long been shown to induce expression and secretion of IL-6 by monocytes and macrophages (Breen et al., 1990). Even when virologically suppressed, treated HIV-infected persons have significantly higher plasma levels of IL-6 than well-matched uninfected controls. Activated inflammation, as demonstrated by persistently higher IL-6 levels, may have profound and far-reaching clinical implications. Recent reports involving both HIV-infected and HIV-uninfected persons have linked increased plasma IL-6 levels to a variety of adverse clinical outcomes, including anaemia, cancer, cardiovascular disease, and death (Nordell et al., 2014). Interleukin 6 (IL-6) is a multifunctional cytokine that has a major role in initiation and promulgation of the inflammatory and immune responses. In this study level of IL 6 were measured as a marker of inflammation as previously proved by other authors that IL 6 is a strong marker (Knudsen et al., 2004; Prati and Goyal, 2013).
2.5.2 TUMOUR NECROSIS FACTOR ALPHA

HIV-1 infection results in the depletion of CD4+/CD8+T cells and alters the cytokine network in the infected individuals. Tumour necrosis factor alpha (TNF-alpha), a proinflammatory cytokine, plays a critical role in HIV-1 pathogenesis. HIV-1 utilizes the TNF-alpha signaling pathway for expanding its reservoir. To evaluate the effects of HIV on immune responses the levels of cytokines can be quantified from plasma and stimulated peripheral blood mononuclear cells (PBMCs) from individuals infected with HIV. Several lines of evidence suggest that increased TNF activity is not only a marker of disease activity but may be directly involved in the immunopathogenesis of HIV infection. Thus, TNF-activation may enhance HIV replication by mechanisms involving the activation of nuclear factor-κB and may contribute to development of immunodeficiency and some clinical manifestations in HIV-infected persons (Godfried et al., 1994). Tumour necrosis factor-α (TNF-α) is a multifunctional circulating cytokine derived from endothelial and smooth muscle cells as well as macrophages associated with coronary atheroma(Warner and Libby,1989).Initially identified as a factor that promoted haemorrhagic necrosis in transplanted tumors, TNF-α is involved in several cardiovascular processes. For example, TNF-α levels are markedly elevated in advanced heart failure (Tipping and Hancock, 1994;McMurray et al.,1991. Persistent TNF-α activation may have an important pathogenic role in HIV infection hence it would be useful to know if HAART can down-regulate or even normalize the sustained activity of the TNF system. We measured levels of TNF alpha in plasma of HIV positive patients who are on HAART and the HAART naïve, compared these to the HIV negative controls and also examined a possible correlation of TNF activation with virologic and immunologic treatment failure using CD4 counts and viral load measurements.
2.6 CONTROVERSIAL ISSUES AND GAPS IN KNOWLEDGE

Epidemiologic evidence suggests that HIV-infected people remain at higher than usual risk for “ordinary” illnesses even if their HIV infection is optimally controlled (Neuhaus et al., 2010). Therefore the aim is to be able to investigate the patterns of biochemical markers that may support such evidence.

Human immunodeficiency virus (HIV)–infected individuals are living longer in the era of antiretroviral therapy. As a result, they are increasingly prone to the development of concomitant chronic disease (Friis-Møller et al., 2004). Coronary heart disease (CHD) is the leading cause of death in the United States and Europe. Recent studies suggest that CHD rates may be increasing among HIV-infected patients and thus appropriate screening strategies for CHD in this population are needed (Lohse et al., 2005). Recently, approaches to screening and assessment of cardiovascular disease (CVD) in HIV-infected individuals were discussed at a State of the Science Conference. Although insufficient evidence now exists to recommend a screening strategy for CHD in HIV that differs from that recommended in the non-HIV population, emerging risk factors and surrogate markers for atherosclerosis unique to the HIV population suggest specific strategies that may be useful in this population (Morise and Jalisi, 2003). Two broad screening categories are most commonly used. The first screening strategy seeks to define the pre-test likelihood of disease by identifying the presence of predisposing risk factors such as hypertension, elevated serum cholesterol, cigarette smoking, and physical inactivity. The second screening strategy aims at the detection of established CHD, even in its earliest stages (Gokce et al., 2003).
However, several non-invasive surrogate biological markers (biomarkers) have been demonstrated to monitor the inflammatory process and lipid metabolism (Ferri, 2006). Inflammatory biomarkers include pro-inflammatory cytokines, chemokines, products of hepatic circulation, and immunoglobulin molecules. Lipid biomarkers include the traditional lipid profile, other lipoproteins, LDL fractions, and HDL subfractions. (Wu JT and Wu LL., 2006).

There is little information on differences among c ART regimens in their ability to reduce and potentially normalize the chronic inflammation (CDC, 2010). Several abstracts began to shed some light on this very important and inadequately studied area of antiretroviral research. Although many studies have suggested that some ART regimens may improve inflammation better than others, ART alone may not fully normalize inflammation in all patients. There is need to search for therapies to use in conjunction with ART starting with agents that have demonstrated both anti-inflammatory properties and safety in other disease states (Chan, Sviridov and Dart, 2009).

The large amount of data on inflammation in HIV may lead to a few definitive conclusions that can influence clinical management.
2.7 JUSTIFICATION OF THE STUDY

There is a paucity of data in Africa regarding the measure of inflammation and platelet activation profile in healthy and in HIV infected people whether on treatment or not. This is against a background where HIV infected individuals have been demonstrated to have sustained low grade inflammation and hypercoagulable state even in successful ART. Profiles of inflammation modulating cytokines are not well known in Zimbabwean populations on c ART. When these markers are measured for every individual coming for an HIV test or individuals already on ART say every six months, the individual’s risk for disease progression and related complications independent of clinical or social factors will be known. This allows for new patient management strategies to be adopted. Such early diagnosis of the relative risk of developing cardiovascular disease or other non AIDS illnesses allow for therapeutic approaches guided NOT only by viral load and CD4 count but by the inflammatory state of an individual as well. Immunotherapy such as use of recombinant cytokines to correct Th1/Th2 balance and or powerful anti-inflammatory agents could represent attractive treatments. These therapies co administered with cART, can result in reduced viral load, increased CD4 count, correct Th1/Th2 balance, repair of the injured endothelium and mitigation of all other factors due to response to injury. The life span of the individuals on HAART can be as long as that of their age and gender matched healthy individuals.
2.8 OBJECTIVES

2.8.1 Main Objective

To determine the prevalence of risk factors for cardiovascular disease (CVD) among HIV-infected people, and to investigate any association between such risk factors, stage of HIV disease, and use of antiretroviral therapies.

2.8.2 SPECIFIC OBJECTIVES

- Measure and compare selective markers of:
  - immune activation and inflammation (IL 6, TNFα),
  - endothelial damage (vCAM and iCAM),

between groups on HAART and HAART naïve age and gender-matched patients.

- Correlate inflammatory states with:
  - antibody profiles of HIV infected individuals.
  - viral load and CD4 count.
3. MATERIALS AND METHODS

3.1 Recruitment of participants and data collection

Recruitment was carried out at an HIV treatment clinic in Newlands Harare, Zimbabwe. HIV-infected patients attending the clinic for scheduled visits on selected days between March 2013 and August 2014 were sequentially approached. A Standard non Communicable Disease questionnaire was used to collect demographic data which include those factors that may affect the levels of inflammatory markers in people such as age and body mass index, smoking or history of strokes and heart diseases. Only compliant participants were recruited. Ethical approval was received from Joint Parirenyatwa Research Ethics Committee &MRCZ.

3.2 INCLUSION AND EXCLUSION CRITERIA

HIV-infected patients attending the HIV treatment clinic on a regular basis were included.

ART-experienced patients were defined as patients who reported prior use of three antiretroviral drugs, whereas ART-naïve patients had never taken antiretroviral drugs. Male and female HIV-infected patients aged 18 years or older were included. Patients with documented history of diabetes, non-adherence, cardiovascular disease, hypertension, and dyslipidemia before being infected with HIV were excluded. Patients suffering from dental disease, sexually transmitted infections and concurrent co-morbidities which can also cause inflammation were also excluded.
3.3 STUDY DESIGN

There were three distinct groups in this study.

1. HIV unexposed, uninfected to control for traditional cardiovascular risks for corresponding gender and age mates. (These were HIV negative individuals)
2. HIV infected, but HAART naïve to account for effects of the virus alone
3. HIV infected on HAART for at least months possibly account for effects of HIV and the therapy

For the third group, there were samples for two time points, the baseline samples and six months follow up.

3.3.1 SAMPLE SIZE CALCULATION

Using \[ N_1 = \frac{[Z\alpha/2 \sqrt{\frac{1}{r} + \frac{1}{q^2}} + Z\beta \sqrt{\frac{r}{p_1 q_1 + p_2 q_2}}]^2}{r(p_1 - p_2)} \]

where \( p_1 \) (test group) = 43.4% and \( p_2 \) (control group) = 15.9%, \( q_1 = 56.6\% \) and \( q_2 = 84.1\% \); ratio of ART experienced: ART-naïve used in the study = 5:1, hence \( r = 5 \); \( N_1 = \) minimum sample size for the ART-naïve control group; \( N_2 = 5 \times N_1 \) is the minimum sample size for the ART-experienced test group; \( Z\alpha/2 = 1.96 \) (95% confidence interval); \( Z\beta = 0.84 \) (power of study).

The minimum sample size was 186, (31 ART-naïve, 155 ART-experienced).
3.3.2 ETHICAL CONSIDERATION
This study was approved by the Joint Parirenyatwa Hospital and College of Health Sciences Research Ethics Committee (JREC Ref: 72/13) and the Medical Research Council of Zimbabwe (Ref: MRCZ/A /1768). Individual written informed consent was obtained from all willing participants.

3.4 ASSAYS FOR CLINICAL PARAMETERS

All markers were measured in thawed EDTA plasma using commercially available kits according to the manufacturer’s instructions, below are the general outlines.

3.4.1 Lipids: Test principles for BS120 Mindray® machine (Chemistry Analyser)

Total Cholesterol was measured enzymatically in serum in a series of coupled reactions that hydrolyse cholesterol esters and oxidize the 3-OH group of cholesterol. One of the reaction by-products, H₂O₂, is measured quantitatively in a peroxidase-catalysed reaction that produces a color. Absorbance is measured at 500 nm. The color intensity is proportional to the cholesterol concentration (Bachorik and Albers, 1986). High Density Lipoprotein (HDL) concentration in the serum supernatant was determined by the same process after the precipitation of very low density lipoprotein (VLDL) cholesterol, LDL and chylomicrons. Results were calculated using the formula: TC (g/l) or HDL (mg/dl) concentrations = (Optical Density at 500nm of sample ÷ Optical Density at 500nm of standard) × Concentration of standard (essentially as recommended by the manufacturer in the kits with all units later converted to mg/dl were necessary). LDL concentration was determined using the formula of Friedewald et al. LDL-c (mg/dl) = TC (mg/dl)-[HDL-c (mg/dl)-Triglycerides (mg/dl)/5]( Friedewald et al., 1972).
3.4.2 Plasma glucose : Glucose oxidase test

Plasma glucose was determined using the glucose oxidase method, where the enzyme (glucose oxidase) catalyses the oxidation of glucose to gluconic acid. Glucose oxidase catalyse the oxidation of Beta D- glucose present in the plasma to D glucono -1 ,5 - lactone with the formation of hydrogen peroxide; the lactone is then slowly hydrolysed to D-gluconic acid. The hydrogen peroxide produced is then broken down to oxygen and water by a peroxidase enzyme. Oxygen then react with an oxygen acceptor such as ortho toluidine which itself converted to a coloured compound, the amount of which is measured colorimetrically at 500nm. The color intensity is proportional to the glucose concentration (Howanitz, Howanitz, 1984).

3.4.3 Quantitative determination of markers of inflammation (IL - 6 , TNF –alpha) and markers of endothelial damage (VCAM and ICAM)

Below is an outline of the sandwhich Enzyme Linked Immunosorbent Assay (ELISA) protocol to measure IL - 6 , TNF –alpha, VCAM and ICAM

The ELISA plate(s) would be always coated the night before assaying.
3.4.3.1 Plate Preparation

The Capture Antibody was diluted to the working concentration in PBS without carrier protein. Immediately a 96-well microplate was coated with 100 μL per well of the diluted Capture Antibody. The plate was then sealed and incubated overnight at room temperature. An autowasher was used to wash each well by repeated aspirating and then dispensing Wash Buffer (400 μL). After the last wash any remaining Wash Buffer was removed by blotting it against clean paper towels. Plates were blocked by adding 300 μL of 1 X Reagent Diluent to each well. The plate was incubated at room temperature for a minimum of 1 hour and thereafter washed in auto washer.

3.4.3.2 Assay Procedure

100 μL of sample or standards in Reagent Diluent, were added into wells and plate covered with an adhesive strip and incubated for 2 hours at room temperature. Plates were washed then 100 μL of the Detection Antibody, diluted in Reagent Diluent was added to each well.

Plates were covered with a new adhesive strip and incubated for 2 hours at room temperature thereafter washing was repeated in an autowasher. 100 μL of the working dilution of Streptavidin-HRP was added to each well, plate covered and incubated for 20 minutes at room temperature away from direct light. Plates were washed in autowasher, then 100 μL of Substrate Solution added to each well and incubated for 20 minutes at room temperature. 50 μL of Stop Solution was added to each well, gently tapping the plate to ensure thorough mixing. The optical density of each well was determined immediately, using a microplate reader set to 450 nm.
Reconstitution of reagents and preparation of working solutions was done by following manufacturer's instructions. Quantities of capture antibody, detection antibody and standards were different per analyte/kit so had to be calculated first.

### 3.4.3.3 Order of loading the plate

Row 1 and 2 had the standards in duplicate, starting from the least concentrated in 1A and 2A, increasing in concentration up to 1H and 2H. Row 3 and 4 had the blanks. Serum samples were loaded from row 5 up to 12 in duplicate. From the absorbances, a standard curve is generated then used to extrapolate concentrations of the selected analyte.

### 3.5 Cardiovascular risk values

Overall CHD risk was calculated using age, sex, TC, HDL, smoking history, and systolic BP on the US National Health Institute Framingham On-line calculator. The Framingham calculator applies National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) guidelines as follows: older age (being older than 55 years for women and older than 45 years for men); elevated TC, >200 mg/dL; decreased HDL, <40 mg/dL; and elevated systolic BP, >140 mmHg. Those on treatment for hypertension were categorized as having a slightly higher risk of CHD in the next 10 years than those of the same sex and age, by the Framingham risk calculator. Framingham risk scores were then used to define risk of coronary artery disease or heart attack as low (less than 10% risk), moderate (10%–20% risk), or high (more than 20% risk) (US Department of Health and Human Services, 2015).
3.6 Statistical analysis

Data were entered into and analysed using Statistical Package for Social Sciences version 21 (IBM Statistics, Armonk, NY, USA) and Stata version 13 (StataCorp, College Station, TX, USA) software. Shapiro-Wilk test ($P<0.05$) and visual inspection of histograms, normal Q-Q plots, and box-plots were used to check dependent variables for normality (Shapiro and Wilk, 1965). Continuous data were described as the mean ± standard deviation in descriptive statistics and analyzed for differences using $t$-tests, with the level of significance set at $P<0.05$. Skewed data were reported as medians (interquartile ranges) and compared using $k$ median tests. The relationships between the dependent variables and treatment group were adjusted for demographic factors (i.e., age and sex), known risk factors for CHD (i.e., history of stroke before age 50 years in first-degree relatives, personal history of stroke, body mass index [BMI, smoking status [current, ex-smoker, unknown], presence of physician-reported lipodystrophy, hypertension [systolic blood pressure $\geq 150$ mmHg and/or diastolic blood pressure $\geq 100$ mmHg] or use of antihypertensive agents, diabetes or use of antidiabetic agents, and use of lipid-lowering or antiplatelet agents), and HIV-related variables(CDC, JAMA 1993).
4. RESULTS

4.1 Demographics of participants

A total of 294 participants were approached for enrolment of which 226 had HIV infection and 68 were HIV negative. Seven did not satisfy the inclusion criteria for the HIV infected and three for the HIV uninfected. 284 were finally enrolled. The majority (85.4%) of the HIV infected were receiving ART while 14.6% were ART naïve. Most (91%) of the subjects on ART had received therapy for at least one year. Majority of them (90.9) were on first line triple combination therapy comprising of TDF, 3TC and Nevirapine (63.4%), TDF, 3TC and Efavirenz (17.2%) and ART combinations with Stavudine or zidovudine (10.2%). The remaining 9.1% had received ART including a PI. 72.3 % of the HIV infected had CD4 levels higher than 350 copies/mL. Both ART receiving and ART naïve had the same mean CD4 count of 481.4 and 421.5 respectively. a large proportion (80.1%) of the HIV infected subjects had suppressed viral load (≤20 copies/ mL), and the mean viral load was higher for ART naive (4514.2 sd23537) than for ART receiving subjects (685.4 sd 2052.5). Of the HIV infected, 46 (21%) self-reported a history or current morbidity in heart disease, stroke or hypertension. As compared to HIV uninfected a higher proportion of the HIV infected participants (40.1%) were hypertensive (40.1% vs 29.1%). The difference was however not statistically significant (x= 2.889; p=0.089). The proportion of hypertensives was the same for participants on ART and pre ART. With regard to SBP there was no difference across the strata. Table 2 on next page summarizes the demographics and clinical characteristics of the study subjects.
Table 2: Demographic, clinical, and biochemical parameters by history of antiretroviral therapy

<table>
<thead>
<tr>
<th>Demographic and clinical data</th>
<th>All (n=215)</th>
<th>HAART negative (n=30)</th>
<th>HAART positive (n=185)</th>
<th>Significance P &lt; 0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>40.3±10.14</td>
<td>37.3±10.60</td>
<td>40.9±9.28</td>
<td>46.3±13.38</td>
</tr>
<tr>
<td>Male/female ratio</td>
<td>0.23±0.77</td>
<td>0.3±0.69</td>
<td>0.22±0.78</td>
<td>0.19±0.81</td>
</tr>
<tr>
<td>Systolic BP mmHg</td>
<td>81.5±16.09</td>
<td>81.5±16.89</td>
<td>81.4±16.65</td>
<td>81.9±14.54</td>
</tr>
<tr>
<td>Diastolic BP mmHg</td>
<td>125.6±19.05</td>
<td>123.7±16.59</td>
<td>124.8±18.67</td>
<td>123.9±18.76</td>
</tr>
<tr>
<td>Smoking history</td>
<td>4.2% yes</td>
<td>4.5% yes</td>
<td>4.7% yes</td>
<td>4.5% yes</td>
</tr>
<tr>
<td>History of heart disease</td>
<td>7.0% yes</td>
<td>8.8% yes</td>
<td>8.2% yes</td>
<td>9.1% yes</td>
</tr>
<tr>
<td>Tuberculosis history</td>
<td>20.8% yes</td>
<td>13.3% yes</td>
<td>29.4% yes</td>
<td>43.8% yes</td>
</tr>
<tr>
<td>Median (IQR) TC mg/mL</td>
<td>173.3 (144.40–208.49)</td>
<td>149.0 (134.52–176.64)</td>
<td>175.87 (155.21–210.04)</td>
<td>179.54 (145.17–227.41)</td>
</tr>
<tr>
<td>Median (IQR) HDL mg/dL</td>
<td>45.6 (37.12–56.46)</td>
<td>38.28 (34.61–47.95)</td>
<td>46.40 (40.22–56.04)</td>
<td>48.72 (43.70–56.51)</td>
</tr>
<tr>
<td>Median (IQR) LDL mg/dL</td>
<td>93.0 (52.70–124.00)</td>
<td>89.13 (53.71–112.38)</td>
<td>94.55 (58.13–125.35)</td>
<td>105.40 (65.10–130.20)</td>
</tr>
<tr>
<td>Mean ± SD TC/HDL ratio</td>
<td>3.97±1.30</td>
<td>4.0±1.32</td>
<td>3.86±1.13</td>
<td>4.27±1.51</td>
</tr>
<tr>
<td>Median (IQR) BMI</td>
<td>23.30 (21.0–27.20)</td>
<td>22.65 (21.35–27.10)</td>
<td>23.40 (21.1–27.2)</td>
<td>23.60 (20.50–31.00)</td>
</tr>
<tr>
<td>Mean ± SD random BG mmol/L</td>
<td>5.25±1.81</td>
<td>5.09±1.84</td>
<td>5.32±1.81</td>
<td>5.00±1.80</td>
</tr>
</tbody>
</table>

Abbreviations: ART, antiretroviral therapy; BMI, body mass index; BP, blood pressure; CHD, coronary heart disease; CI, confidence interval; HDL, high-density lipoprotein; IQR, interquartile range; LDL, low-density lipoprotein; OR, odds ratio; SD, standard deviation; TC, total cholesterol.
The ART-experienced group as well as the ART-naïve group had normal average values for TC, HDL, and glucose based on NCEP ATP III guidelines. There was a significant difference between ART-experienced and ART-naïve participants for mean years since diagnosis, median TC, and median HDL, but no significant differences for distribution by sex, economic earnings, BMI, median systolic and diastolic BP.

When patients were stratified by different ART combinations and compared with ART-naïve patients, the distribution of BMI, random blood glucose, and TC/HDL ratio was all the same. Those on protease inhibitor-based second-line treatment had on average the highest levels of TC and LDL. Those on stavudine-based or zidovudine-based first line had the highest levels of HDL.

There was no significant difference across the groups for diastolic BP, systolic BP, BMI, or random blood glucose. From the observations made, it is clear that use of antiretroviral therapy (ART) in people living with human immunodeficiency virus (PLWHIV) is associated with disturbances in blood lipids which should be monitored. More data on such disturbances are needed in Zimbabwe to persuade the country program to institute the routine monitoring of the lipids. It is also important to follow up on these patients such that each time they attend clinics for routine check-ups, not monitor only the CD4 count and viral load but also these lipid profiles. In a study such as this due to the use of means, a smaller group may be over shadowed by a larger percent such that the general risk of the population for developing CVD might not be high, though not to say the relative individual risk is not there. Therefore it would benefit the HIV community and the country at large to measure lipid profiles for individuals with HIV both on HAART and HAART naïve.
4.2 PROVINCES OF ORIGIN OF PARTICIPANTS

The participants originated from different parts of Zimbabwe, coming mostly from provinces near Harare and Mutare, whilst 8.4% originated from neighbouring Sub-Saharan regional countries (mainly Malawi and Mozambique). This geographic heterogeneity is apparent across all urban centers of Zimbabwe, as people move across the country in search of opportunities and jobs.

Figure 6: Provinces of origin of participants.
4.3 DIAGNOSIS AND TREATMENT HISTORY

Participants had been diagnosed for a mean 5.5±4.1 years prior to the study, 38% had been diagnosed within 3–5 years, with 24% of them having been diagnosed less than 2 years or 6–10 years, respectively, before data collection; only 14% of participants had been diagnosed for more than 10 years before the study. ART-experienced participants had been on ART for a mean duration of 3.9±3.4 years. There was a positive correlation between years since diagnosis and years since beginning ART.
4.3 ANTIRETROVIRAL THERAPY REGIMENS

Figure 8: Distribution of different ART regimens and combinations amongst the participants.

Figure 8 shows the ART regimens that were used by the participants. The majority of participants (60.5%, n=130) in our study were on a triple combination of tenofovir, nevirapine, and lamivudine (GlaxoSmithKline, Brentford, London, UK), 23 (21.1%) were on a tenofovir, efavirenz (Bristol-Myers Squibb, New York City, NY, USA), and lamivudine regimen, and the rest were on either stavudine (Bristol-Myers Squibb) or zidovudine (ViiV Healthcare, Brentford, London, UK) in combination with nevirapine and lamivudine or a protease inhibitor-based second-line therapy.
4.4 Plasmatic Levels of Vascular Cell Adhesion Molecule (VCAM)

Figure 9: Effect of antiretroviral treatment on VCAM a marker of endothelial dysfunction

Key. Y axis shows mean concentration of VCAM, scale 1: 150 ng/ml

Testing significance difference between HAART Naïve and HAART, p value<0.001.

Figure 9 shows that levels of circulating vascular cell adhesion molecule 1 are significantly higher in HIV infected patients than in healthy age and gender based individuals. This shows that the HIV virus leads to a response to injury by the endothelial leading to accelerated secretion of adhesion molecules. There was a significant difference between levels of VCAM in HIV infected individuals compared to the negative controls. A smaller control group excluding smokers and a few other individuals with confounding traditional risk factors for CVD had way lower levels of VCAM. This shows that HIV-1 acquisition is associated with
endothelial activation, with sustained elevations of soluble VCAM-1 post infection. Soluble VCAM-1 may be an informative biomarker for predicting the risk of HIV-1 disease progression, morbidity, and mortality. In HAART naïve patients the plasmatic circulating levels are high on average up to 1100ng/ml but those on HAART have significantly lower concentrations. HAART initiation is associated with short-term improvement in HIV-mediated endothelial damage and dysfunction.

HIV individuals on HAART have significantly higher concentrations of VCAM than healthy individuals. HIV infection leads to increased levels of adhesion molecules and HAART partially corrects these.
4.5 PLASMATIC LEVELS OF ICAM

![Bar chart showing levels of ICAM]

**Figure 10: Effect of different ART combinations on levels of ICAM**

Key Y axis shows mean of the concentrations of ICAM. Scale 1: 100 ng /ml

Testing significance difference between other HAART regimens and those with PI Inhibitors, p value<0.001

Figure 10 shows that the levels of marker of endothelial activation ICAM, are significantly decreased in HIV-infected subjects receiving HAART as compared to HIV infected individuals who are not on treatment. When stratified by different regimens of treatment, protease inhibitors, there wasn’t much difference in levels of ICAM in HAART naïve and those on PI inhibitors. This shows that protease inhibitors mitigate endothelial dysfunction to a significantly lower extent as compared to other treatment regimens using non-nucleoside reverse-transcriptase inhibitors as second line therapy.
4.6 RESULTS FOR PLASMATIC LEVELS OF IL-6.

Figure 11: A measure of levels of IL-6 in the participants.

We also compared IL-6 levels by HIV status and found significantly higher levels in the HIV infected than HIV uninfected for both ART naïve (p=0.008) and ART receiving (p=0.006). Significant differences were also observed within the HIV infected group with the ART naïve having higher levels of IL-6 than those on ART (0.044).
4.6 RESULTS FOR PLASMATIC LEVELS OF TNF ALPHA

We did not find significant differences in TNF-\(\alpha\) levels by HIV or ART status.

In this light we proceeded to check for any associations between these levels and CD4 count as well as viral load.

Results for antibody assays were not ready by the time of thesis submission due to a delay in getting the reagents.
### 4.6.1 Association of Inflammation markers with CD4s and viral load.

<table>
<thead>
<tr>
<th>Clinical Marker</th>
<th>Biomarker</th>
<th>( r; p )</th>
<th>( r; p )</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4</td>
<td>TNF-( \alpha )</td>
<td>0.1401; 0.0668</td>
<td>-0.4143; 0.0284</td>
</tr>
<tr>
<td></td>
<td>IL6</td>
<td>0.008; 0.9917</td>
<td>-0.1208; 0.5405</td>
</tr>
<tr>
<td>Viral Load</td>
<td>TNF-( \alpha )</td>
<td>-0.0412; 0.6723</td>
<td>0.8263; 0.0032</td>
</tr>
<tr>
<td></td>
<td>IL-6</td>
<td>0.0391; 0.6875</td>
<td>0.4408; 0.2023</td>
</tr>
</tbody>
</table>

**Figure 13: Correlations between cytokines as markers of inflammation and clinical markers.**

Within the group of HIV infected participants, only in ART naïve did TNF-\( \alpha \) levels correlate with levels of both CD4 \( (r=0.41; p=0.0284) \) and HIV-1 RNA \( (r=0.83; p=0.0032) \), IL6 did not correlate with the neither CD4 nor viral load.
4.7 Laboratory results for the different ELISA tests.

Figure 14a and 14b: Some samples were clear stand outs with high concentrations of IL 6 and VC AM.
4.8 EVALUATING RISK OF CVD

The majority of patients (97%, n=208) had a low risk of CHD as indicated by Framingham risk scores less than 10%, and only four (1.9%) had moderate risk, ie, Framingham risk scores of 10%–20%. There was a 1.4% (n=3) prevalence of participants with a Framingham risk score higher than 20%, indicating a high risk of CHD.
5. DISCUSSION

This study described the lipid profile, biomarkers of inflammation, markers of endothelial damage and the calculated risk of future cardiovascular disease in HIV-positive Zimbabwean individuals treated with different ART regimens. Although our results indicate a low risk of long-term cardiovascular disease, ART is associated with some changes in lipid metabolism that should be taken notice of. When considering serum TC and LDL levels, lipid profiles seemed more atherogenic in the antiretroviral-treated participants compared with their therapy-naïve counterparts. Data Collection on Adverse Events of Anti-HIV Drugs (DAD) study group showed evidence of multiple risk factors for cardiovascular disease, including dyslipidemia among HIV-infected persons on NRTIs and protease inhibitors, with an increased risk in older patients; 1.4% of patients in that study had a previous history of cardiovascular disease and 51.5% were cigarette smokers (Friis-Møller et al., 2003).

In contrast to the DAAD study, less than 10% of our participants had been on a protease inhibitor-based regimen, while more than 80% were on an NNRTI-containing regimen with a mean exposure time of 3.5 years, indicating that even NNRTI-based ART regimens influence lipid metabolism unfavourably, as shown in previous cohorts and clinical trials (Fontas E, Van Leth F, Sabin CA, et al., 2004). Most studies have shown that the level of HDL cholesterol is lower in non-treated HIV patients than in ART-exposed patients, in accordance with our results. The DAD study showed a higher risk of cardiovascular disease risk in older patients, 25% of DAD patients were over 50 years of age.

Our study population was made up of relatively young adults, with a mean age of 40.3±10.1 years and only 17% of participants being older than 50 years. The low levels of HDL observed in antiretroviral-naïve patients seem to be a recurring theme in most studies,
including ours, but whether ART contributes to increased cardiovascular risk remains uncertain. The mechanisms of how HIV infection and ART induce these lipid abnormalities are still unknown. Chronic immune activation caused by HIV has been proposed, an activation that persists even after successful treatment with ART. Even if ART induces lipid abnormalities that are classic risk factors for the development of atherosclerosis, ART is of tremendous importance for survival and reduction of morbidities in HIV-positive patients. These patients have longer survival, but are more threatened by cardiovascular disease (Glass et al., 2006).

We showed that HIV-infected patients have increased levels of circulating endothelial inflammatory markers and that the short term use of HAART reduced some of these, showing that chronic infection itself, and not necessarily antiretroviral treatment, is responsible for endothelial damage. Those on a PI based second line regimen had a significantly lower ability to reduce these markers at P<0.05. Though concentrations of circulating ICAM improve, they persist when treated with PI based regimens and this may contribute to adverse cardiovascular events. There is need for measurements at more time points in follow up.

In the AIR study in HIV-positive patients treated with PI or NNRTI, serum levels of ICAM were significantly higher in treated than in naive HIV-infected subject, in contrast to our findings. In addition, endothelial derived markers are higher in HIV infected individuals as compared with healthy controls, suggesting that HIV can infect endothelial cells thus activating them.

Plasma levels of TNF-α and IL-6 are significantly higher in HIV-infected patients than in non-infected patients, and these increases are correlated with HIV viral load.
Our study found higher plasma levels of inflammation marker IL-6 in HIV infection regardless of treatment, an indication of higher risk for cardiovascular disease. Previous studies found elevated levels of inflammation markers including IL-6 to be associated with cardiovascular events (De L a et al., 2013). For instance, findings from the Strategies for Management of Anti-Retroviral Therapy (SMART) trial showed that elevated levels of inflammation markers IL-6 and hsCRP were associated with CVD outcomes including CVD death, non-fatal myocardial infarction (MI), non-fatal stroke and congestive heart failure (Duprez et al., 2012). In the current study, we observed significantly higher levels of IL-6 in infected subjects as compared to the HIV infected (p<0.001). The levels of IL-6 have been reported to fall after initiation of ART. From our results HIV treatment naïve had the higher levels of IL-6 indicating a higher degree of inflammation which seems to substantiates the claim. Untreated HIV infection is characterized by increased levels of pro-inflammatory cytokines such as IL-6 and hsCRP, and increased expression of adhesion molecules, factors identified to be important in the pathogenesis of atherosclerosis (De L a et al., 2013).

Compared to the ART naïve, ART receiving subjects had lower levels probably due to the effects of antiretrovirals, however, the levels remained significantly higher than the HIV uninfected. Currently there is conflicting data on whether the levels of IL-6 return to normal or remain elevated after initiation of ART. Our results seem to suggest that the levels do not normalize. Although the plasma levels of IL-6 were elevated, we did not find any relationship with traditional risk factors such as hypertension and history of morbidity. IL-6 did not show significant relationship with CD4 or viral load but an inverse relationship was hinted with CD4.

TNF-α was strongly positively correlated with viral load and negatively correlated with CD4, a result which is in agreement with findings by Aukrust and co-workers (1999). It is worth
noting that we observed the correlation in HIV ART naïve group but not in the ART treated group. The possible reason why correlation was not shown in ART experienced is the suppression of HIV by drugs while in ART naïve TNF-α would be produced in response to viral multiplication (19). These findings further support a role for enhanced TNF-α activity in the pathogenesis of HIV infection. Report by Aukrust (1999) also showed that TNF-α remain elevated in people exhibiting virological treatment failure.

We did not find significant differences in TNF-α levels by HIV or ART status. Some studies have reported an association between high serum concentration of TNF-α and hypertension (Vaidya et al., 2014), but many others have failed to find a relationship (Naya et al., 2007). Activation of the TNF-α system was reported to be linked with SBP and DBP. TNF-α was demonstrated to decrease the expression of endothelial nitric oxide synthase which in turn decreases the bioavailability of Nitric oxide leading to endothelial dysfunction and hypertension (Yoshizumi et al., 1993). Further studies need to be done to ascertain whether IL-6 could be useful tool in predicting future CVD risk in HIV infection.

Regardless of the source and initial stimulus, continued production of IL-6 and TNF-alpha may result in augmentation in an auto-feedback manner, accompanied by increases in Ig synthesis and, more importantly, HIV replication (Hypergammaglobulinemia).

In this study, the HIV-infected participants, who were HAART naive, had higher IL-6, ICAM-1 and VCAM-1 levels than their age-, gender and BMI matched controls. The higher levels of inflammatory markers could point to endothelial dysfunction, which is seen as the link between infection, inflammation and atherosclerosis (Andrade and Cotter, 2006). The contribution of HIV to endothelial dysfunction is difficult to distinguish from traditional
cardiovascular risk factors. Therefore we carefully matched the control participants’ gender, age and BMI to minimize the confounding effect of these conditions on the study findings.

It is important to note that seventy percent of the ART-positive patients under study were treated with lamivudine in combination with tenofovir and non-nucleoside antiretroviral drugs, which has not been associated with an increased risk of myocardial infarction, in contrast with some protease inhibitors and other NRTIs, yet there is a level of CVD risk. The participants in our study had a low risk of developing cardiovascular disease for the next 10 years when evaluated by Framingham risk score. The ranges are far lower than reported in Europe and developed countries, but is in agreement with studies done elsewhere in Africa (Jeemon et al., 2011)

Markers of immune activation IL-6, VCAM and ICAM are elevated in HIV infected individual, not on treatment. The significant difference between these elevated markers compared to healthy controls shows that HIV itself leads to chronic immune activation. Treatment with HAART dampens these markers but still they do not normalise. These results are for patients on HAART for not more than 3 years and thus a long term follow up would give more comprehensive results. The study was also influenced by selection bias with more females than males due to differences in the health-seeking behaviors of men and women in our setting and lack of locally generated reference ranges for comparison. The HAART naïve patient group was small and this increases the risk of type II statistical errors. It has been proposed that the Framingham risk score may underestimate the 10-year risk in some subgroups and overestimate this risk in others, depending on risk factors and geographic origin, which also posed another weakness (Mascolini M, 2015).
HAART has made HIV a chronic manageable disease, and increased longevity is continually troubled by abnormal lipid alterations, elevated markers of inflammation and immune activation, that may lead to complications due to non AIDS illnesses.

This study had limitations in that there were no endpoint events to associate with the biomarker levels but self-reported CVD morbidity. Data on history of CVD morbidity were not obtained from the HIV uninfected and these may have impact on interpretation of our results. In addition, a limited number of biomarkers from the platelet activation and inflammation pathways were studied. The strengths of the study were its high statistical power as well as the presence of HIV uninfected group to compare with the infected subjects.
6. CONCLUSIONS

HIV infection is posing serious consequences on CVD. Even though HIV/AIDS is now a manageable disease, the immune responses require monitoring because even when the viral load is controlled there is indication of extensive damage caused by the inflammatory agents. There is still a need to control plasma lipid levels and endothelial injury in HIV-positive patients in an attempt to reduce the long-term cardiovascular risk, which is upcoming in HIV-positive patients. There is a need for the country HIV program to institute laboratory monitoring of lipids, markers of inflammation and markers of endothelial damage for patients on HAART at least once a year. Our main finding is that as HIV progresses, the immune system is baffled by this chronic infection, and in response different cells secrete molecules that can be used as surrogate markers to predict the relative risk of CVD. The risk of future cardiovascular disease is low among outpatients at a Zimbabwean HIV treatment clinic, although there were the worrying aspects of high atherogenic lipid profiles and hypertension. This study substantiates the concept of persistent platelet abnormalities in HIV-infected individuals including those with good response to ART. The causes of this phenomenon and its implications in the cardiovascular pathology of this population will require further investigations.
6.1 RECOMMENDATIONS

Treatment regimens require to be investigated further with the provision of anti-inflammatory agents. When antibody profiles, cytokine profiles and markers of endothelial damage are known, necessary corrections to repair the weakened immune system can be done.

We may end up with anti–HIV therapy not sorely based on the reduction of plasma viral load, but also to restore the immune weakness due to disease progression. More work is warranted on antibodies in such an inflamed environment. Discrete signs of systemic and vascular inflammation persist even after use of HAART hence there is need for research on adjunctive therapies to cater for the virologic control as well as repair of weakened immune system.
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