University of Zambia
School of Medicine
Department of Pathology & Microbiology

Assessment of D-Dimer and IL-6 levels in HIV positive individuals at the University Teaching Hospital, Lusaka

A Dissertation Submitted to the University of Zambia, in Partial Fulfillment of the Requirements for the Master of Science Degree in Pathology (Haematology) by:

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DECLARATION

This work/dissertation in its present form has not been submitted or accepted previously for the award of a degree or diploma in this or any tertiary institution, and is not being submitted for a degree or diploma in any tertiary institution or for another degree or diploma at this institution. I declare that this Dissertation contains my own work except where specifically acknowledged. I further declare that I followed all the applicable ethical guidelines in the conduct of the research. This dissertation has been prepared in accordance with the Master of Science in Pathology (Haematology), University of Zambia guidelines.

Candidate's Name: Panji Nkhoma

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Supervisor's Signature:……. Date:…….

CERTIFCATE OF APPROVAL

The University of Zambia approves this Dissertation on *"**Assessment of D-Dimer and IL-6 levels in HIV positive individuals at the University Teaching Hospital, Lusaka**".*

Candidate's Signature..... **Date.....**

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ABSTRACT

Context – Chronic inflammation and activated coagulation are well known features of HIV infection and evidence has accrued indicating that both processes contribute to an increased risk of death. High levels of the inflammatory cytokine IL-6 and the thrombogenic marker d-dimer are strongly associated with a higher mortality risk in patients with HIV infection.

Aims – The aim of the study was to determine if D-Dimer levels in conjunction with IL 6 levels, as markers of predisposition to thrombosis, are higher in HIV positive than in HIV negative indigenous black Zambians.

Methods and Results – A prospective cross-sectional study was undertaken at the University Teaching Hospital in Lusaka, Zambia. D-dimers, IL-6, CD4, triglycerides and cholesterol were assessed in HIV positive on ART, HIV positive ART- naïve and HIV negative control participants. Our results showed that HIV ART naïve participants had higher D-dimer (794.71 ± 318.07 ng/ml) and IL-6 concentrations (2.83 ± 1.60 ng/ml) than those on ART (514.39 ± 187.19 ng/ml), (2.49 ± 1.21 ng/ml) p =0.004 and 0.020 respectively. They also had higher d-dimer concentration (794.71 ± 318.07 ng/ml) than the HIV negative control participants (375.08 ± 165.95 ng/ml) p = 0.001. HIV negative control participants however, had higher concentrations of IL-6 (3.24 ± 1.33 ng/ml) than HIV positive participants on ART (2.49 ± 1.21) p = 0.002. IL-6 correlated to d-dimer in HIV positive treatment naïve and HIV negative controls with statistical significance (r = 0.509; p < 0.001 and r = 0.396; p =0.008 respectively). HIV positive participants on ART had higher cholesterol levels (4.87 ± 0.36 mmol/l) than both HIV negative control (4.27 ± 0.32 mmol/l) and HIV positive ART naïve participants (3.78 ± 0.34 mmol/l) p = 0.022 and p < 0.001 respectively. Triglyceride levels correlated to d-dimer levels in HIV positive ART naïve participants with statistical significance (r = 0.332; p < 0.020). However, there was no statistically significant correlation between CD4 and D-dimer in both the HIV positive ART naïve and HIV positive on ART participants (r = 0.064; p = 0.744 and r = 0.019; p =0.904 respectively).

Conclusion – This study showed that the levels of IL-6 and d-dimers were higher in HIV positive ART naïve individuals. This in part explains why they are more at risk of cancers, thrombosis and cardiovascular diseases which contribute to a high mortality rate in this group of individuals.

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LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
AIDC	Adult Infectious Diseases Centre
ART	Antiretroviral Therapy
BMI	Body Mass Index
CD4	Cluster of Differentiation 4
CLSI	Clinical Laboratory Standards Institute
CRP	C-Reactive Protein
DM	Diabetes Mellitus
DNA	Deoxyribonucleic Acid
DVT	Deep Vein Thrombosis
EFV	Efavirenz
ELISA	Enzyme Linked Immunosorbent Assay
ETC	Evacuated Tube System
FTC	Emtricitabine
HAART	highly active antiretroviral therapy
HDL	High Density Lipoprotein
HIV	Human Immunodeficiency Virus
hsCRP	high sensitivity C - reactive protein
IFN-γ	Interferon Gamma
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-13	Interleukin 13
IL-1α	Interleukin 1 Alpha
IL-1β	Interleukin 1 Beta
IL-4	Interleukin 4
IL-6	Interleukin – 6
IL-8	Interleukin 8
LDL	Low Density Lipoprotein
LPS	Lipopolysaccharide
MI	Myocardial Infarction
mmol/l	Milimoles per litre
ng/ml	Nanogrammes per mililitre
NNRTI	Non – Nucleoside Reverse Transcriptase Inhibitor
NRTI	Nucleoside Reverse Transcriptase Inhibitor
OIs	Opportunistic Infections
PE	Pulmonary Embolism
RNA	Ribonucleic Acid
Rpm	Revolutions per minute
STI	Sexually Transmitted Infections

TDF	Tenofovir
TF	Tissue Factor
TG	Triglyceride
TNF-α	Tumor Necrosis Factor – alpha
UTH	University Teaching Hospital
VCT	Voluntary Counseling and Testing
VLDL	Very Low Density Lipoprotein

1.0 INTRODUCTION

1.1 BACKGROUND

Chronic inflammation and activated coagulation are well known features of HIV infection (Calmy, et al., 2009; Neuhaus, et al., 2010) and evidence has accrued indicating that both processes contribute to an increased risk of death (Ledwaba, et al., 2012; Boulware, et al., 2011) due to thrombotic events whose incidence in Human Immunodeficiency Virus (HIV)-infected patients is rising as suggested in recent retrospective cohort studies (1%- 2%, which is 10 times that expected among people without HIV) (Ahonkhai , et al., 2008). These two conditions in HIV infected persons are thought to be a consequence of viral replication or persistence, high levels of bacterial lipopolysaccharide (LPS), bacterial DNA and their associated immune activation (Funderburg, et al., 2010). High levels of the inflammatory cytokine interleukin-6 (IL-6) and the thrombotic marker D-dimer are strongly associated with a higher mortality risk in patients with HIV infection (Kuller, et al., 2008). It has actually been reported that these markers of inflammation and coagulation remain important predictors of death even at higher CD4+ cell counts (Tien , et al., 2010).

D-dimers, the fibrinogen degradation products of cross-linked fibrin, have emerged as the most useful of the procoagulant activity and ongoing fibrinolysis markers. During thrombus formation, fibrinogen is converted to fibrin monomers that are extensively cross-linked into a polymer network. This cross-linking of fibrin takes place in the region of the polymer termed the “D-domain.” Adjacent D-domains are covalently linked and constitute a fibrin specific feature of a thrombus, not found in fibrinogen or non-cross-linked fibrin degradation products (Dempfle, 2000). One of the terminal products of fibrinolysis is the covalently linked D-Domain called the D-Dimer fibrin fragment. Monoclonal antibodies to D-Dimer have been developed that can differentiate fibrin specific clot from non-cross- linked fibrin as well as fibrinogen. As opposed to other markers that only detect products of acute coagulation, D-Dimer assays expand the diagnostic window (Reber, 2000). A strong association between HIV replication and raised D-dimer levels has been demonstrated. Correlations of D-dimer with HIV viremia

and markers of endothelial dysfunction and microbial translocation (Baker, et al., 2010; Kuller, et al., 2008; Funderburg, et al., 2010) have also been reported. This favors the hypothesis that HIV replication and microbial translocation are among the main determinants of the hypercoagulable state seen in HIV-infected persons. HIV positive persons have increased levels of microbial products in their plasma (Marchetti , et al., 2008). Certain microbial toll like receptor ligands such as Lipopolysaccharides from these microbes can increase surface expression of the procoagulant tissue factor (TF also known as thromboplastin) on circulating monocytes (Drake , et al., 1989). This Tissue Factor expression on monocytes promotes coagulation (Saulius, 2009). This has been proved by the presence of dramatically higher frequencies of monocytes expressing TF in fresh blood samples from HIV-infected persons than in samples from uninfected controls (Funderburg, et al., 2010). Finally, the in vivo biologic activity of monocyte TF expression is suggested by a correlation with plasma levels of D-dimers. These findings suggest that a variety of microbial products, including those derived from HIV itself, and perhaps bacterial products translocated from the damaged gut in chronic HIV infection, may contribute to a heightened risk for clotting and cardiovascular disease in HIV infection by increasing cell surface expression of the procoagulant TF (Funderburg, et al., 2010).

Interleukin 6 (IL-6) is an interleukin that acts as both a pro-inflammatory cytokine and an anti-inflammatory cytokine. In humans, it is encoded by the *IL6* gene (Ferguson, et al., 1988) and it is secreted by T cells and macrophages to stimulate immune response, e.g. during infection and after trauma, especially burns or other tissue damage leading to inflammation. It also plays a role in fighting infection, as it has been shown in mice to be required for resistance against bacterium *Streptococcus pneumoniae* (Van der Poll , et al., 1997). It is an important mediator of fever and of the acute phase response. Among its several functions, IL-6 induces terminal differentiation of lymphocytes to plasma cells (Muraguchi, et al., 1981) and proliferation of precursor and mature T cells (Lotz, et al., 1988), and acts as an autocrine growth factor for multiple myelomas/ plasmacytomas (Nordan & Potter, 1986). The release of pro-inflammatory cytokines which include IL-6 is caused by IL-2 (Sereti , et al., 2001; Fortis, et al., 2002), these

cytokines are known to increase acute phase reactants such as C-reactive protein (CRP) and activate pro-thrombotic pathways (Baars , et al., 1992; Deehan , et al., 1994). Multiple studies have established associations between levels of CRP and the fibrinogen breakdown product D-dimer with both cardiovascular and thrombotic disease (Lowe , 2005; Roumen-Klappe , et al., 2002). With regard to the relationship of this cytokine to HIV, in vitro infection of normal monocyte/macrophages with HIV-1 has been found to induce gene expression and secretion of IL-6 (Nakajima, et al., 1989). In addition, increased levels of IL6 have been recently reported both in the serum (Breen, et al., 1990) and in the cerebrospinal fluid (Gallo, et al., 1989) of HIV-infected patients. It has been demonstrated that IL-6 directly stimulates HIV replication in primary human macrophages acutely infected in vitro (Poli , et al., 1990). IL-6 also induces HIV expression in chronically infected promonocytic cells and synergizes with TNF-α in this effect (Poli , et al., 1990). Furthermore, at the molecular level, it is evident that IL-6 induction of HIV expression involves multiple and diverse mechanisms (Poli , et al., 1990). Proinflammatory cytokines are important mediators of activation of coagulation. Infusion of tumour necrosis factor (TNF)-α into healthy humans induces not only signs of a systemic inflammatory response, but also activation of coagulation as indicated by an increase in plasma concentrations of the prothrombin fragment F1+2 (Van Der Poll, et al., 1990). However, blocking TNF-α with monoclonal antibodies does not neutralise coagulation activation during endotoxaemia in chimpanzees (van der Poll, et al., 1994). Rather, blocking interleukin (IL)-6 attenuated activation of coagulation in the same model of endotoxaemia, both systemically and locally in the bronchoalveolar compartment (van der Poll, et al., 1994). This suggests that IL-6 is the most important mediator in inflammation-induced coagulation. Hence, the proinflammatory cytokines IL-6 and TNF-α establish a procoagulant shift in the haemostatic balance, promoting fibrin generation in severe inflammatory states, both systemically and locally.

Elevated triglyceride (TG) levels are common in HIV positive persons for several reasons. Firstly, conditions that traditionally result in elevated TG, such as insulin resistance, diabetes mellitus (DM) and fatty liver are prevalent in the HIV-positive population (De, et al., 2008; Brown, et al., 2005). Secondly, the physiological distress

that results from untreated HIV infection may cause lipid perturbations, in particular elevated TG (Grunfeld , et al., 1992; Phillips, et al., 2008). It has been suggested that HIV-positive patients may experience increased postprandial TG levels (Stein, et al., 2005), this being one potential explanation for the increased risk of myocardial infarction (Breen, et al., 1990) that is seen in HIV-positive persons. The presence of increased levels of atherogenic remnant lipoproteins (chylomicron remnants and very low-density lipoprotein remnants) might be associated with increased levels of non-fasting triglycerides. These smaller triglyceride-rich lipoproteins may penetrate the endothelial cell layer where they can contribute to the formation of foam cells, involved in early stages of atherosclerosis (Shafran, et al., 2005; Van, et al., 2004). Elevated TG is a frequent side-effect of antiretroviral therapy (Shafran, et al., 2005; Van, et al., 2004). Different antiretroviral drugs have different propensities to cause elevated TG, with drugs from the protease inhibitor (Spira, et al., 1998) class, but also the non-nucleoside reverse transcriptase inhibitor (NNRTI) efavirenz and some nucleoside reverse transcriptase inhibitor (NRTI) drugs (Shafran, et al., 2005; Van, et al., 2004) all being reported to cause elevated TG. Some of these drugs have been associated with an increased risk of myocardial Infarction in HIV positive patients (Sabin, et al., 2008; Worm, et al., 2010).

Hyperlipidemia elicits a profound enrichment of a pro-inflammatory subset of monocytes. These pro-inflammatory monocytes home to atherosclerotic lesions, where they propagate the innate immune response by expressing high levels of pro-inflammatory cytokines and other macrophage mediators. Many links exist between lipids and innate immunity. Modified lipoproteins interact with scavenger receptors and may thus send pro-inflammatory signals. Oxidized phospholipids derived from modified lipoproteins may also drive inflammation (Libby, et al., 2009). A lipoprotein-associated phospholipase A2 (Lp-PLA2), may generate pro-inflammatory derivatives of oxidatively modified lipoproteins (Serruys, et al., 2008). Lipid protein interactions play a pivotal role in blood coagulation. Assembly of blood clotting enzyme complexes on appropriate phospholipid membranes provides a design in which a small initiating stimulus incites an amplified reaction that culminates in a near explosive generation of thrombin. This highlights the important role lipids play in blood coagulation

1.2 STATEMENT OF THE PROBLEM

As of 2011 Zambia had a total population of 13.47 million people. Out of these 980,000 were living with HIV/ AIDS and 45,000 deaths from HIV were recorded (Central Intelligence Agecy, 2011). In Zambia we have 980,000 HIV positive patients both on treatment and not on treatment and are all at risk of having elevated levels of IL-6 and D-Dimers which are markers of inflammation and coagulation respectively.

Studies have been done which have concluded that among patients with advanced HIV disease, elevated pre-ART levels of IL-6 and D-dimer are strongly associated with early mortality after commencing ART (Ledwaba, et al., 2012; Kuller, et al., 2008). Another study which was carried out in Gaborone- Botswana revealed that persistently elevated serum interleukin-6 predicts mortality among adults receiving combination antiretroviral therapy (McDonald, et al., 2013). These markers of inflammation and coagulation are not being assessed and monitored in Zambian patients. With a large pool of people living with HIV infection, the chances of having increased cardiovascular associated mortality risk would be high.

1.3 STUDY JUSTIFICATION

This study would provide knowledge on levels of inflammation and coagulation biomarkers in HIV individuals. Knowing that elevated levels of these biomarkers are predictors of mortality in these individuals, those with high levels of these markers would benefit from treatment to reduce their levels and reduce mortality. This study would also contribute to awareness of whether HIV positive individuals in Zambia have high or normal levels of inflammation and coagulation biomarkers so that if they were high, the government through the Ministry of Health could introduce and fund tests to monitor their levels and this could lead to a reduced mortality after treatment.

1.4 LITERATURE REVIEW

A number of studies have been carried out to determine the outcomes of elevated levels of inflammation (IL-6) and coagulation markers (D-Dimers) in HIV infection. Below are some of the studies that have been carried out in this regard.

In a study which was done in South Africa it was found that pre-ART levels of inflammation and coagulation markers are strong predictors of death in a cohort with advanced HIV disease. Over a median follow-up of 24.7 months 208 deaths occurred. Prior to starting ART, median hsCRP, D-dimer, and IL-6 levels were all significantly elevated in those who died in the ART group compared to controls and strong risk gradients for all-cause mortality were evident for higher levels of all three biomarkers. Baseline levels of the three markers of inflammation and coagulation were directly correlated (Brown, et al., 2005).

However, McDonald B et al in another study which was a retrospective nested case-control analysis of clinical trial data in Gaborone Botswana, it was found that D-Dimers were not significant predictors of all-cause mortality in HIV individuals. To confirm the findings of the study in South Africa above, it was found that persistently elevated serum interleukin-6 predicts mortality among adults receiving combination antiretroviral therapy. Pretreatment serum levels of interleukin-6 (IL-6), high sensitivity C-reactive protein, and D-dimer in stored plasma samples from 32 deceased participants (cases) and 64 survivors (controls), matched for age, sex, baseline CD4(+) cell count, and plasma HIV-1 RNA were measured. Of 37 (86%) study participants who died on study 32 were included in this analysis. Causes of death (n=32) included non-AIDS-defining events. Median time to death was 31 weeks. Median baseline levels of all three biomarkers were higher in cases compared to matched controls. After adjusting for BMI and the presence of Opportunistic Infections, only baseline and most recent (near time of event) levels of IL-6 remained as significant predictors of all-cause mortality [adjusted OR (aOR)=1.25, 95% CI (1.05-1.48); p=0.012; and aOR=1.48 (1.05-2.09); p=0.027, respectively] (McDonald, et al., 2013).

Lewis H Kuller et al conducted a study in Minnesota to determine if inflammatory and coagulation biomarkers are associated with mortality in patients with HIV Infection. IL-6

and D-dimers were strongly related to all-cause mortality. It was also concluded that interrupting ART may further increase the risk of death by raising IL-6 and D-dimer levels. Therapies that reduce the inflammatory response to HIV and decrease IL-6 and D-dimer levels may warrant investigation (Kuller, et al., 2008).

To determine the association of inflammatory markers, fibrinogen, and C-reactive protein (CRP), a study was carried out in the United States of America in 2010. In this study, Fibrinogen and CRP were found to be strong and independent predictors of mortality in HIV-infected adults. The study also concluded that HIV-infected participants with fibrinogen levels in the highest tertile (>406 mg/dL) had 2.6-fold higher adjusted odds of death than those with fibrinogen in the lowest tertile (<319 mg/dL) (Correll, et al., 1998).

To determine Lipid Profile of HIV-Positive Patients a study was conducted at the University of Calabar Teaching Hospital in Calabar – Nigeria. The study showed that some lipid profiles were altered in HIV subjects compared to the controls. The fasting total cholesterol did not differ compared to the HIV negative controls ($P>0.05$). While the VLDL-Cholesterol, LDL-Cholesterol and Triglyceride levels of HIV positive subjects were significantly higher compared to the HIV negative controls ($P<0.050$). Interestingly, the HDL-cholesterol levels of HIV-positive subjects were significantly lower than those of the HIV-negative controls ($P<0.05$). A significant positive correlation was found between HDL-Cholesterol levels and the CD4+ T- lymphocyte levels of HIV-positive subjects. A significant negative association was found between TG and CD4+T-lymphocyte levels of HIV positive subjects; a similar association also exists between VLDL-C levels and CD4 T-lymphocyte levels (Iffen, et al., 2010).

1.5 RESEARCH QUESTION

Do HIV infected persons seeking medical attention at the University Teaching Hospital (UTH) have elevated levels of IL-6 and d-dimers which are significant predictors of mortality in HIV infection?

2.0 OBJECTIVES

2.1. GENERAL OBJECTIVE

To assess D-Dimer and IL 6 plasma levels, as biomarkers of predisposition to thrombosis in HIV positive individuals.

2.2 SPECIFIC OBJECTIVES

2.2.1 To compare plasma levels of D-dimer and IL-6 in HIV positive and HIV negative individuals.

2.2.2 To compare plasma levels of triglycerides and cholesterol in HIV positive and HIV negative individuals.

2.2.3 To correlate CD4 count with levels of D-dimer and IL-6 in HIV positive individuals.

3.0 METHODOLOGY

3.1 STUDY DESIGN

The study was a cross sectional study

3.2 STUDY SITE

The study was conducted at the University Teaching Hospital Department of Pathology and Microbiology and the ART clinic.

3.3 TARGET POPULATION

HIV positive adult aged 18 years and above participants without any clinical evidence of acute or opportunistic infections were included in the study

3.4 STUDY POPULATION

All persons who met the inclusion criteria (indicated below) were enrolled into the study. A study control group of persons who were HIV negative was constituted. Study participants consisted of the following two groups:

- HIV positive persons on ART.
- HIV positive persons not yet on ART.
- HIV negative controls.

3.4.1 INCLUSION CRITERIA

- HIV positive adult individuals aged 18 and above were included in the study
- HIV positive persons with no known bleeding or clotting disorders were included in the study.
- Persons who read and understood the rationale for participating in the study and given written consent without being forced to were included in the study.

3.4.2 EXCLUSION CRITERIA

- All HIV positive individuals on second or third line ART because of the assumption that they have switched treatment from first line because of treatment failure or drug reactions which would act as confounders to this study
- Smokers because smoking influences blood coagulation which may result in elevation of D-dimer levels and also causes elevation of cholesterol and triglyceride levels.
- Pregnant women
- Patients with malignancies because malignancies are predictors of a D-dimer positive result
- Patients with diabetes because hyperglycemia exerts a procoagulant effect which can cause a rise in D-dimer levels
- All persons with a platelet count less than $150x10^9$/l or greater than $450x10^9$/l
- Participants currently taking any of the following platelet inhibiting drugs: aspirin, clopidogrel, etc.

3.4.3 SELECTION OF CONTROLS

Controls were selected from the Voluntary Counseling and Testing (VCT) department. Information sheets were designed which were issued to individuals going for VCT. After counseling and testing, it was totally up to those with a negative HIV test result to decide whether to participate in the study or not. No individual was forced to do an HIV test in order to participate in the study. The controls were matched for age and sex.

3.5 SAMPLE SIZE

To compare mean levels of d dimers between HIV positive and HIV negative participants, the following equation was used to calculate the sample size.

$$N = (u + v)^2 (\sigma 1^2 + \sigma 2^2) / (\mu 1 - \mu 2)^2$$

The study had an 80% power at a significance level (alpha or p value) of 0.05. Therefore, U=0.84 and V=1.96.

The estimated mean and standard deviation for the HIV positive participants group were 150.7 and 12.34 respectively. For HIV negative participants (controls) the estimated mean and standard deviation were 144.3 and 15.23 respectively.

Using the values above, the sample size was calculated as follows:

$$N= (0.84+1.96)^2(12.34^2+15.23^2)/ (150.7-144.3)^2$$

$$=73.5$$

Therefore, N=74

This meant that HIV positive participants would be 74 and HIV negative participants will also be 74. Therefore, the study will be composed of a **total of 148 participants**

3.6 SAMPLING METHOD

Convenience sampling in which consecutive individuals reporting to ART clinic and found to meet the inclusion criteria (given below) were included in the study.

3.7. DATA COLLECTION

3.7.1. Clinical and Demographic Data Collection

Participants were recruited at Adult Infectious Diseases Centre (AIDC) for the test group and at Clinic 3 for the controls during normal clinic hours from 08:00 AM to 16:00 PM, from Monday to Friday. As researchers met the participants they were informed and explained to about the study. The researchers also provided the participants with the study information sheet. If patients autonomously agreed to participate in the study, they were required to sign the consent form and assigned a serial number.

Thereafter information on the participant's demographic data and medical history was collected and compiled using a questionnaire. The demographic data included the participants' age and sex. The medical history data included the year and month in which the participant did an HIV test and the results, HIV medication list for those on HIV medication, and the presence of medical condition that may confound the research finding; included here were malignancies, clotting/bleeding disorders, diabetes and pregnancy for women. The patients' files were also reviewed for any further relevant data to the research and also to confirm the accuracy of information provided by the participant and recorded in the questionnaire. Only participants on first line HIV treatment and without any complications were selected for the study.

3.7.2 Specimen Collection

Blood samples were collected from research participants via venipuncture using the Evacuated Tube System (ETS). The Clinical Laboratory Standards Institute (CLSI) order of draw was followed and the tubes were collected in the following order

I. Plain container for IL 6, cholesterol and triglycerides
II. Sodium Citrate for D-Dimers.

A total volume of 8 ml of blood was collected from every participant, 4 ml in the sodium citrate container and 4 ml in the plain container. Upon collection, specimen tubes were labelled with the participants' study ID corresponding to the one on the questionnaire. Within 3 hours of collection, blood was transported to the Haematology laboratory for processing.

3.7.3. Specimen Preparation and Storage

In the laboratory, each specimen serial number was recorded on to a compilation summary sheet. Thereafter the blood specimens in the sodium citrate tubes were centrifuged at 3000 revolutions per minute (3000 rpm) in order to separate the plasma from the blood cellular component. Plain tubes were centrifuged at 1500 rpm for 15 minutes to separate serum from the blood cellular components. Only serum and plasma were meticulously collected from the vacutainers using pasture pipettes and transferred to 2ml cryovials with sealable screw caps, which were stored in a freezer at -80^{o}C until the specimens were required for analysis. Four cryovials (2 for serum and 2 for plasma) were stored for every participant.

3.8. QUALITY CONTROL

To ensure reliable results, quality control was performed on all the analytical instruments and analysers used for any purpose during specimen analysis according to the UTH quality control guidelines. Quality control included equipment calibrations and analytical control runs on every analyser before each test analysis.

3.9. SPECIMEN ANALYSIS

3.9.1. Interleukin-6 (IL-6) ELISA Test Protocol

Serum IL-6 concentration was assessed using the Abcam IL-6 High Sensitivity Human ELISA Kit (manufactured and supplied by Abcam plc, United Kingdom); a quantitative immunoassay for measurement of Human IL-6 in supernatants, buffered solutions, serum, plasma and other body fluids. This assay employed an antibody specific for Human IL-6 coated on a 96-well plate. ELISA plates were read using the VersaMaxPLUS Rom v1.23 ELISA plate reader.

3.9.1.1. Reagent Preparation

All kit components and samples were brought to room temperature before use. The microtiter plate was brought to room temperature before opening. The WASH BUFFER concentrate (200×) was diluted with 1990 mL of distilled water. The Standard Diluent Buffer concentrate 10X was diluted 10 fold in distilled water.

3.9.1.2. Assay Procedure

Prior to use, all reagents were thoroughly mixed taking care not to create any foam within the vials. 100 µL of SAMPLE, CONTROL solution and STANDARD (1 to 6) were added in duplicate to the appropriate wells in the supplied microtiter plate. Then 50 µL of 1X Biotinylated anti-IL-6 was added to each well and mixed well. The plate was then covered and Incubated for 3 hours at room temperature (18-25^{0}C). After incubation, each well was washed 3 times with 300 µL 1X WASH BUFFER per well. After the last wash, the plate was inverted and blotted dry by tapping on absorbent paper to completely remove the liquid at each step. Then 100 µL of 1x Streptavidin-HRP was added to each well including the blank wells and incubated at room temperature for 30 minutes. After incubation each well was washed 3 times with 300 µL 1X WASH BUFFER per well. After the last wash, the plate was inverted and blotted dry by tapping on absorbent paper to completely remove the liquid at each step. 100 µL of Chromogen TMB substrate solution was then added into each well and incubated in the dark for 12-15 minutes at room temperature avoiding direct exposure to light. After incubation, 100

µL of STOP REAGENT were added to each well and the contents mixed well. The optical density (O.D.) was immediately read at 450 nm.

3.9.1.3. Data Processing

The O.D. of other non-zero standards were divided by that of the zero standard, and then multiplied by 100 (used as X variables). Then, the base 10 logarithm of other standard concentration was calculated (taken as Y variables). A standard curve was generated from these variables in Microsoft Excel 2011 for Mac.

FIGURE 1: IL-6 Calibration Curve

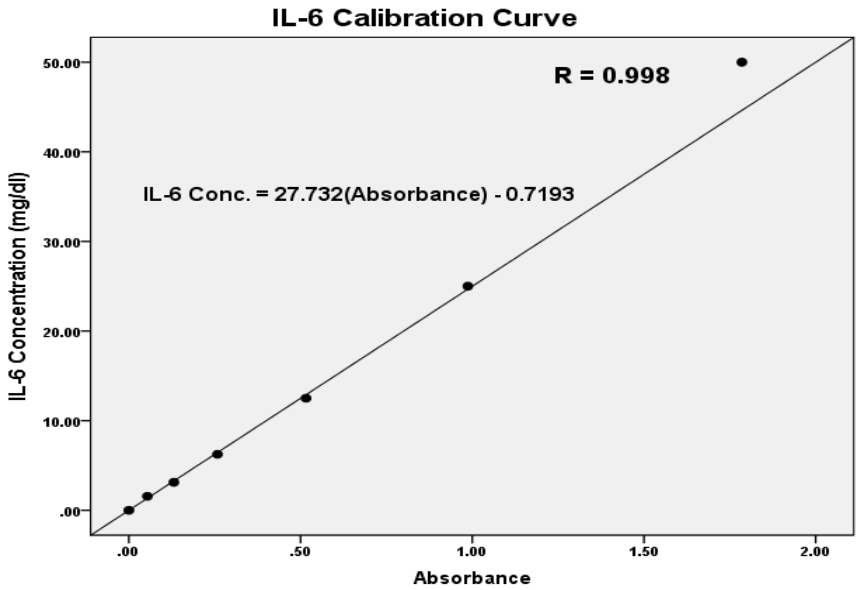


Fig. 1: IL-6 calibration curve plotted from standard absorbances (O.Ds) against concentrations. The regression equation was used to calculate sample concentration from their respective O.Ds.

To calculate results: the sample O.D. was processed as follows: O.D. of sample divided by that of standard 0, then multiplied by 100, to get Y values using the formulation y = -0.023x + 2.9743. To get the concentration of samples: 10 was powered to Y (10^{Y}).

3.9.1.4. Sensitivity And Specificity

The sensitivity, minimum detectable dose of IL-6 using this Abcam IL-6 ELISA kit is less than 0.81pg/mL. The assay has high sensitivity and specificity for detection of both natural and recombinant Human IL-6. No cross-reactivity or interference has been observed between IL-6 and any proteins tested such as IL-1α, IL-1β, IL-10, IL-12, IFN-

γ,IL-4, TNF-α, IL-8 and IL-13 . Species cross reactivity has not been specifically determined.

3.9.2 D-Dimer Test Protocol

D-Dimer levels were assessed using the ichroma™ D-Dimer along with the ichroma™ Reader (manufactured by Boditech - South Korea and supplied by Onyx Technologies – Lusaka, Zambia) which is a fluorescence immunoassay that quantifies the total D-Dimer concentration in plasma.

3.9.2.1 Reagent Preparation

The specimens, test cartridges and the detection buffer were all allowed to attain room temperature prior to performing the test. Lot numbers of all the test components (test cartridges, ID chip and detection buffer) were matched with each other and not mixed up during the procedure.

3.9.2.2 Assay Procedure

10 µl of plasma sample was transferred to a tube containing the detection buffer using a transfer pipette. The lid of the detection buffer tube was then closed and the sample mixed thoroughly by shaking it about 10 times. From the mixture, 75 µl was pipetted out and dispensed into the sample well on the test cartridge which was left to incubate at room temperature for 12 minutes. After incubation, the sample loaded test cartridge was inserted into the test cartridge holder in the ichroma™ reader pushing it all the way inside the test cartridge holder. The “select” button on the ichroma™ reader was pressed to start the scanning process and the test results were read on the display screen of the ichroma™ reader.

3.9.2.3 Sensitivity and Specificity

Working range of ichroma™ D-Dimer is 50 – 10,000 ng/mL. There is no significance interference and cross-reactivity in the D-Dimer measurement with other bio-molecules such as Hb, CEA, AFP, ALP, CRP, Troponin I, CK-MB, Myoglobin, Albumin and hyper lipid.

3.9.3. Triglyceride Test Protocol

Triglycerides levels were determined using the ABX Pentra 400 Clinical Chemistry Analyser. ABX Pentra Triglycerides CP reagent is intended for the quantitative *in vitro* diagnostic determination of triglycerides in human serum and plasma based on an enzymatic colorimetric assay.

3.9.3.1. Handling

The cap of the reagent cassette was removed and all foam removed using a Pasteur pipette before placing the cassette into the refrigerated ABX Pentra 400 reagent compartment. The analyser was then calibrated using ABX Pentra Multical after which ABX Pentra N Control and ABX Pentra P Control were run and passed before running the specimens.

3.9.3.2 Sensitivity and Specificity

The assay has a measuring range from 0.08 to 16.39 mmol/L (7 to 1434.1 mg/dL), with an automatic post-dilution up to 67.20 mmol/L (5880 mg/dL). It has linearity up to16.8 mmol/L (1470.0 mg/dL). No cross-reactivity or interference has been observed between triglycerides and haemoglobin and bilirubin.

3.9.4. Cholesterol Test Protocol

Cholesterol levels were determined using the ABX Pentra 400 Clinical Chemistry Analyser. ABX Pentra Cholesterol CP is used for determination of cholesterol after enzymatic hydrolysis and oxidation. The colorimetric indicator is quinoneimine which is generated from 4-aminoantipyrine and phenol by hydrogen peroxide under the catalytic action of peroxidase (Trinder's reaction).

3.9.4.1. Handling

The cap of the reagent cassette was removed and all foam removed using a Pasteur pipette before placing the cassette into the refrigerated ABX Pentra 400 reagent compartment. The analyser was then calibrated using ABX Pentra Multical after which ABX Pentra N Control and ABX Pentra P Control were run and passed before running the specimens.

3.9.4.2. Sensitivity and Specificity

The assay has a low and high linearity of 0.09 mmol/l and 15 mmol/l respectively. No cross-reactivity or interference has been observed between cholesterol and haemoglobin, bilirubin and triglycerides.

3.10. ETHICAL CONSIDERATIONS AND PERMISSIONS

Patient information and results were kept confidential and access to this information was restricted to the researcher and supervisors only. The questionnaire had captured the participants' file number, which was assigned a serial number hence specimen containers were identified by serial numbers. The file number was obtained for the purpose of retrieving the CD4 results.

The study participants were provided with an information sheet and given a thorough explanation of intent and rationale of the research after which the participants had given written informed consent without duress, thus ensure a true meeting of minds between the researcher and the participants. All the above mentioned was done in private on a one to one basis to avoid undue influence that may have affected or substituted the patient's will for that of any other persons.

The research proposal for this study was submitted for ethical approval and was approved by **ERES CONVERGE** I.R.B No. 00005948, F.W.A No. 00011697, Ref. No. 2014-May-002

Permission to conduct the study was obtained from the: UTH medical superintendent, the consultants in the Department of Medicine and the Directorate of Research and Graduate Studies (DRGS) through the Assistant Dean, Postgraduate.

Permission to use equipment and facilities in the Department of Pathology and Microbiology in the UTH was obtained from the Head of the Department of Pathology and Microbiology at the University Teaching Hospital, and permission to use the ELISA equipment and laboratory facilities in the KS Research Laboratory was obtained from the KS Research Laboratory Head of Department.

4.0 DATA PROCESSING AND STATISTICAL ANALYSIS

Data were expressed as mean ± SEM for normally distributed continuous variables or median (interquartile range) for non-normally distributed variables. Normality was assessed using the Shapiro and Wilk statistic and the normality plots. Skewed variables were log – transformed prior to analysis.

The one way Anova and Tukey post hoc test was used to compare mean values of plasma D-Dimer concentration, Serum IL-6, cholesterol and triglycerides concentrations, between the three groups (HIV+ ART naïve, HIV+ on ART and HIV- control). The data was cleaned and thereafter showed no violation of normality as assessed by use of the Shapiro and Wilk statistic,

Bivariate linear regression and correlation coefficients were used to assess correlation between D-Dimer and IL-6, D-Dimer and cholesterol, D-dimer and triglyceride and D-Dimer and CD4 in the HIV+ ART naïve and HIV+ on ART groups. Bivariate linear regression data of IL-6 vs. D-Dimer and D-Dimer vs. triglycerides were plotted and presented on scattergraphs. When D-dimer was the dependent variable, the independent variables were IL-6, CD4, cholesterol and triglycerides. When IL-6 was the dependent variable, the independent variables were cholesterol and triglycerides.

Data were analysed in IBM SPSS Statistics version 21 for Windows and Microsoft Excel 2010 for Windows. Results were summarised on to tables and graphs as given below. All statistical tests were performed at 5% significance level or 95% confidence interval and differences were considered significant if $p<0.05$.

5.0 RESULTS

5.1. Anthropometric Characteristics

A total of 150 participants took part in this study, 50 were HIV positive on ART, 50 HIV positive ART naive and 50 HIV negative controls. The age for HIV+ participants on ART was 39.16 ± 2.67 years, for the HIV+ ART naive participants 37.48 ± 3.14 years and for the HIV- control participants 33.2 ± 3.12 years (Table 1). The age for HIV+ ART naive participants was not significantly different from the HIV+ on ART and HIV- control participants p = 0.703 and p = 0.107 respectively. However, the HIV+ on ART participants were stattistically older than the HIV – control participants with p = 0.014.
The minimum ages for the HIV + ART, HIV + ART naive and HIV- control participants were 19, 18 and 18 respectively. The maximum ages for the HIV + ART, HIV + ART naive and HIV- control participants were 58, 57 and 61 respectively. All the three groups consisted of 60% female and 40% males (Table 1).

TABLE 1: Anthropometric Characteristics of Participants

	HIV - Control	HIV + on ART	HIV + ART Naive
N	50	50	50
Age (Yrs.)	33.2 ± 3.12	39.16 ± 2.67	37.48 ± 3.14
Minimum Age (Yrs.)	18	19	18
Maximum Age (Yrs.)	61	58	57
Sex (% Female)	60	60	60
Sex (% Male)	40	40	40

Table 1: The mean age for HIV+ participants on ART was 39.16 ± 2.67 years, for the HIV+ ART naive participants 37.48 ± 3.14 years and for the HIV- control participants 33.2 ± 3.12 years.

5.2. D-Dimer Mean Concentration Differences

The mean d-dimer concentration for HIV+ ART naive participants was 794.71 ± 318.07 ng/ml, for the HIV+ on ART 514.39 ± 187.19 ng/ml and HIV- control participants 375.08 ± 165.95 ng/ml. HIV+ ART naive participants had a statistically significant higher mean d-dimer concentration than the HIV positive participants on ART and the HIV- control participants with p = 0.004 and 0.001 respectively. However, despite the HIV+ participants on ART having a higher mean d-dimer concentration than the HIV- control participants, the difference was not statistically significant with p = 0.865. Fig 2 and Table 2

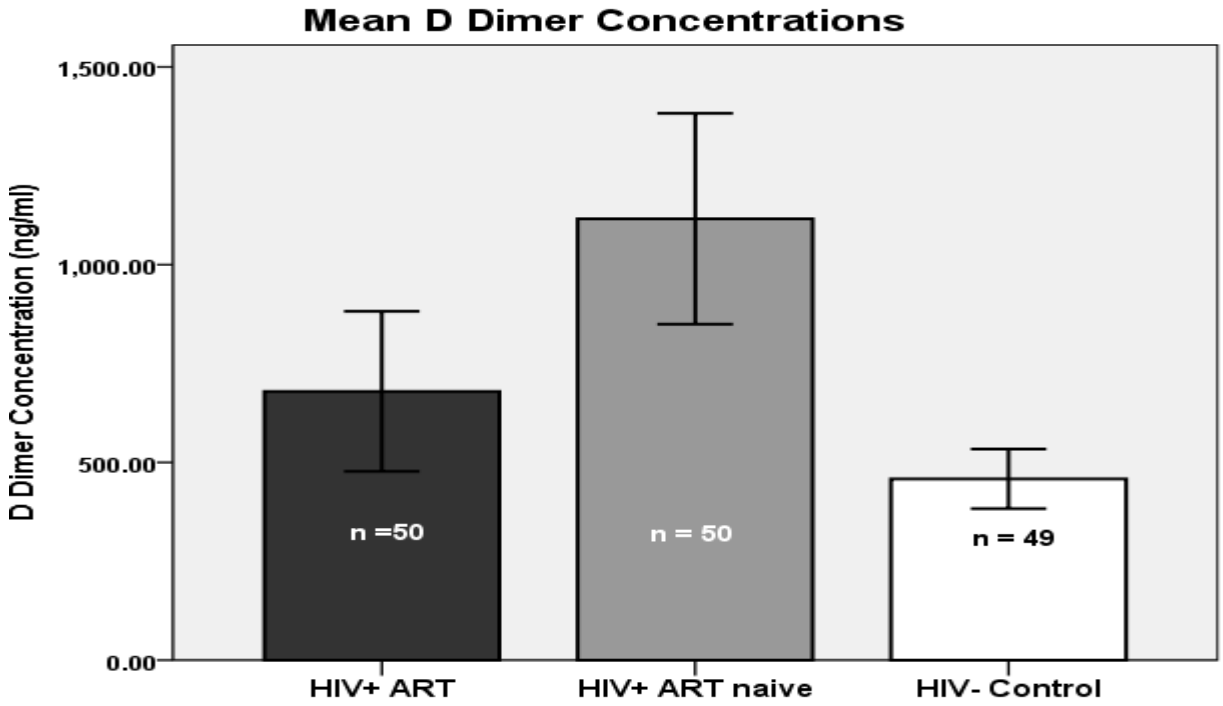


Fig 2: Mean d-dimer concentration for HIV+ ART naive (794.71 ± 318.07 ng/ml) was higher than for the HIV+ on ART (514.39 ± 187.19 ng/ml) and HIV- control participants (375.08 ± 165.95 ng/ml).

TABLE 2: Multiple Comparisons for D-Dimer Between groups

Variable	HIV+ ART	HIV+ ART Naive	HIV- Control	P-Value
D-Dimer	514.39 ± 187.19	794.71 ± 318.07		0.004
	514.39 ± 187.19		375.08 ± 165.95	0.865
		794.71 ± 318.07	375.08 ± 165.95	0.001

Table 2: Multiple comparisons of p-values for D-Dimer Concentrations between participants

5.3. IL-6 Mean Differences

The mean IL-6 concentration for HIV+ ART naïve participants was 3.24 ± 1.33 ng/ml, for the HIV+ on ART 2.49 ± 1.21 ng/ml and for the HIV- control participants 2.83 ± 1.60 ng/ml. HIV+ ART naive participants had a statistically significant higher mean IL-6 concentration than the HIV positive participants on ART p = 0.020. HIV- control participants had a statistically significant higher mean IL-6 concentration than the HIV positive participants on ART p = 0.020. However, despite the HIV+ ART naive participants having a higher mean IL-6 concentration than the HIV- control participants, the difference was not statistically different with p = 0.721. Figure 3 & Table 3

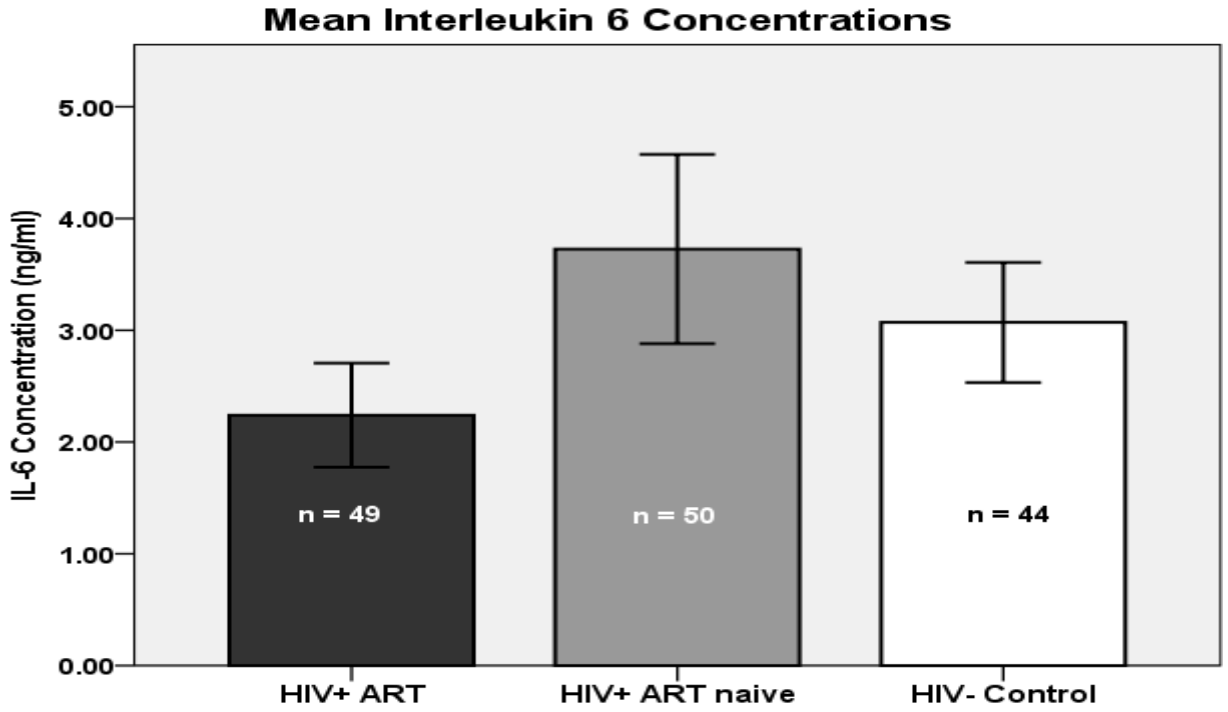


Fig 3: Mean IL-6 concentration for HIV+ ART naïve (3.24 ± 1.33 ng/ml) was higher than for the HIV+ on ART (2.49 ± 1.21 ng/ml) and HIV- control participants (2.83 ± 1.60 ng/ml).

TABLE 3: Multiple Comparisons for IL-6 Between groups

Variable	HIV+ ART	HIV+ ART Naive	HIV- Control	P-Value
IL-6	2.49 ± 1.21	3.24 ± 1.33		0.020
	2.49 ± 1.21		2.83 ± 1.60	0.002
		3.24 ± 1.33	2.83 ± 1.60	0.721

Table 3: Multiple comparisons of p-values for IL-6 Concentrations between participants

5.4. Mean CD4 Counts

The mean CD4 count for HIV+ on ART was 339.57 ± 89.45 cells/µl and for the HIV+ ART naive participants 311.83 ± 95.35 cells/µl. There was no statistical difference in CD4 count between the two groups P = 0.501. Figure 4

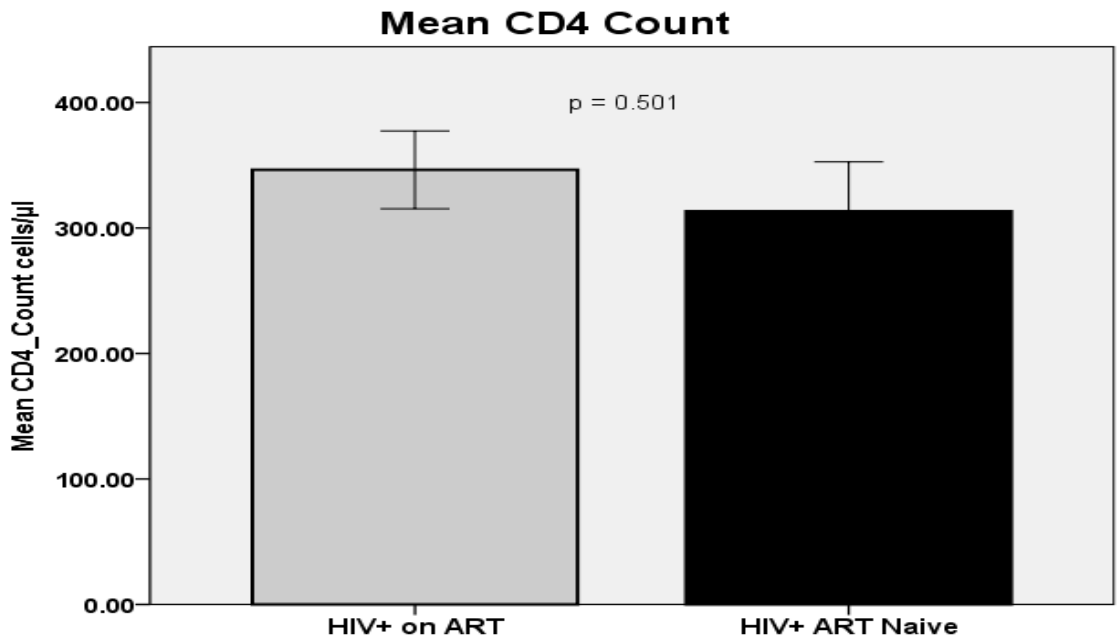


Fig 2C: Mean CD4 count for HIV+ on ART (339.57 ± 89.45 cells/µl) was higher than for the HIV+ ART naive participants (311.83 ± 95.35).

5.5. Mean Cholesterol Concentrations

The mean Cholesterol concentrations for HIV+ participants on ART was 4.87 ± 0.36 mmol/l, for the HIV+ ART naive participants 3.78 ± 0.34 mmol/l and for the HIV- control participants 4.27 ± 0.32 mmol/l**.** The mean cholesterol concentration for HIV+ participants on ART was significantly higher than for the HIV+ ART naive and HIV- control participants $p < 0.001$ and $p = 0.022$ respectively. However, despite the HIV- control participants having a higher mean cholesterol concentration than the HIV+ ART naive participants, the difference was not statistically significant with $p = 0.131$. Figure 5 and Table 4

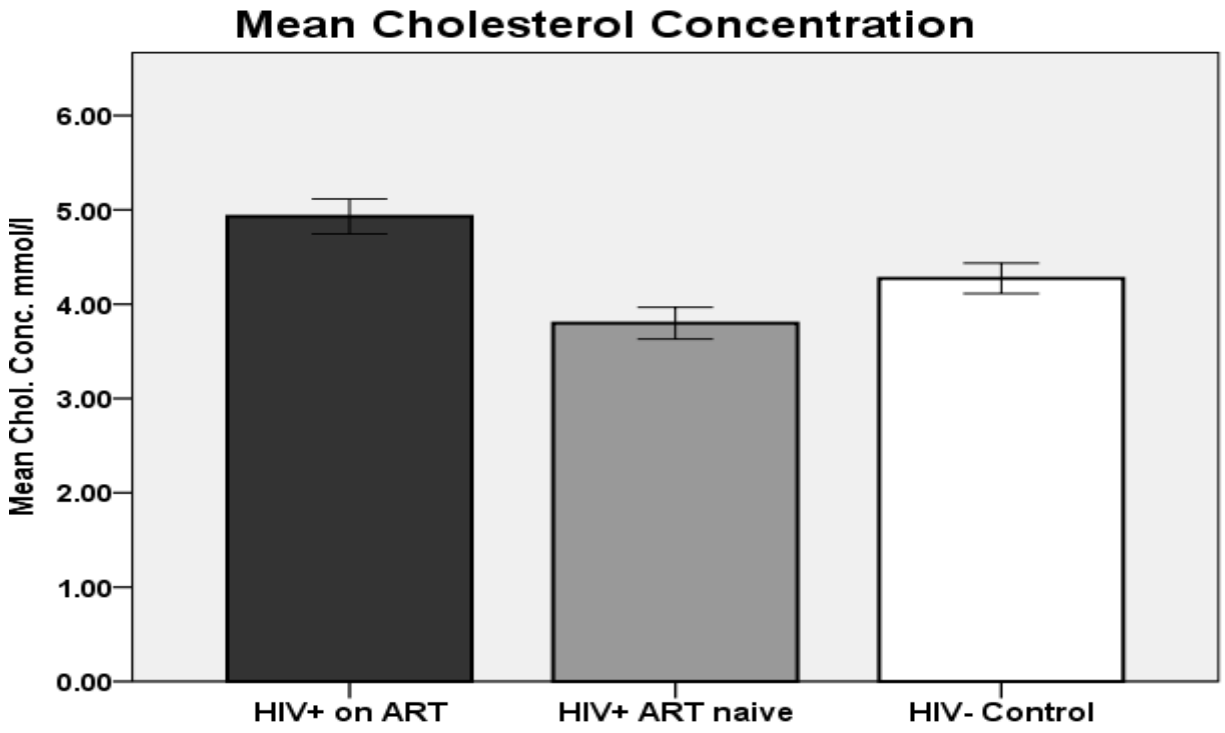


Fig 5: Mean Cholesterol concentrations for HIV+ on ART (4.87 ± 0.36) were higher than HIV+ ART naive (3.78 ± 0.34) and HIV- control participants (4.27 ± 0.32)

TABLE 4: Multiple Comparisons for Cholesterol

Variable	HIV+ ART	HIV+ ART Naïve	HIV- Control	P-Value
Cholesterol	4.87 ± 0.36	3.78 ± 0.34		< 0.0001
	4.87 ± 0.36		4.27 ± 0.32	0.022
		3.78 ± 0.34	4.27 ± 0.32	0.131

Table 4: Multiple comparisons of p-values for cholesterol Concentrations

5.6. Mean Triglycerides Concentrations

The mean Triglycerides concentration for HIV+ participants on ART was 1.24 ± 0.21 mmol/l, for HIV+ ART naive participants 1.17 ± 0.23 mmol/l and HIV- control participants 1.01 ± 0.21 mmol/l. There was a statistical difference between the HIV+ participants on ART and the HIV- control participants p = 0.037. However, there was no statistical difference between the HIV+ ART and the HIV+ ART naive participants and between HIV+ ART naive and the HIV- control participants p = 0.438 and 0.421 respectively. Figure 6 and Table 5

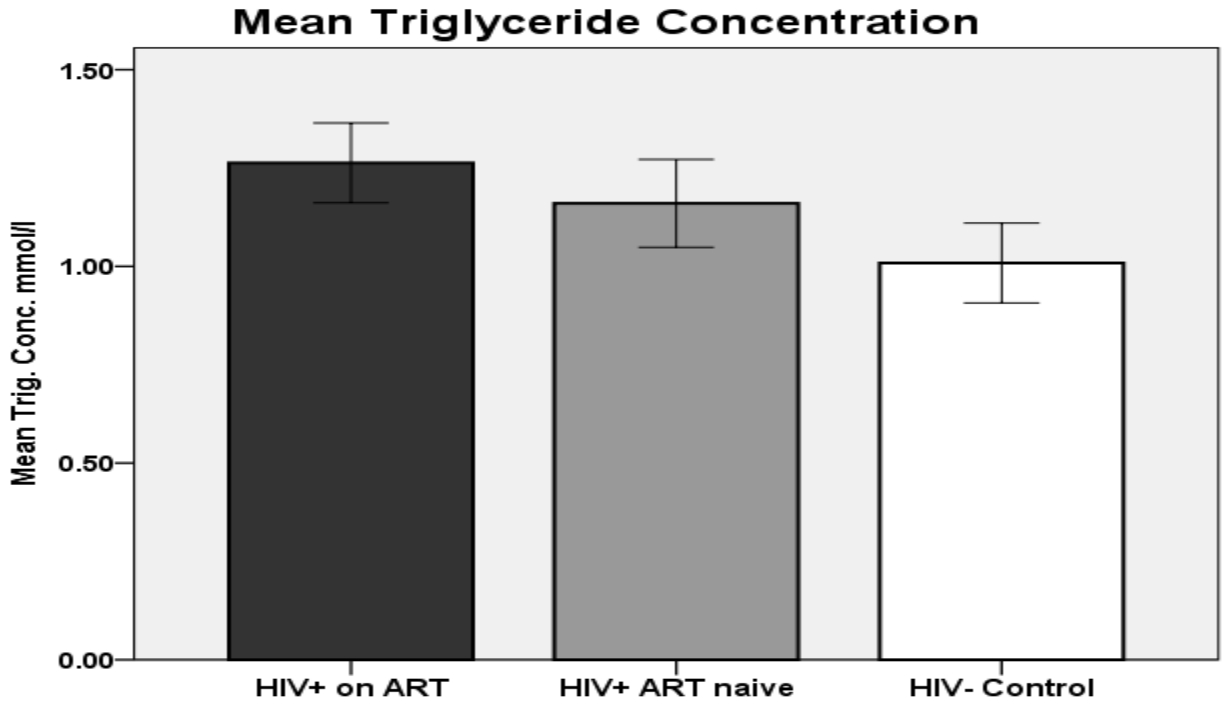


Fig 6: Mean Triglycerides concentrations for HIV+ on ART (1.24 ± 0.21)were higher than in HIV+ ART naive (1.17 ± 0.23) and HIV- control participants (1.01 ± 0.21).

TABLE 5: Multiple Comparisons for Triglycerides

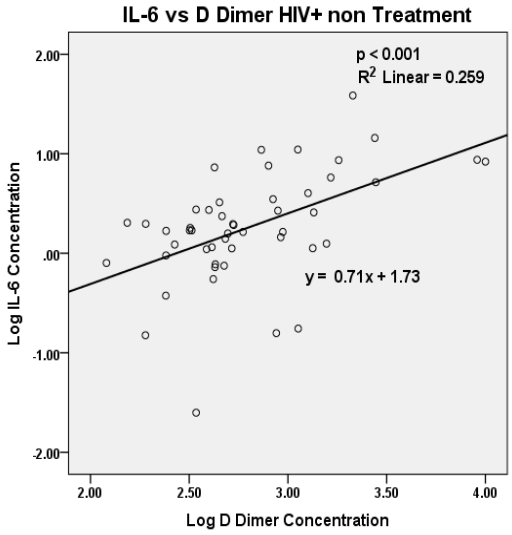
Variable	HIV+ ART	HIV+ ART Naïve	HIV- Control	P-Value
Triglycerides	1.24 ± 0.21	1.17 ± 0.23		0.438
	1.24 ± 0.21		1.01 ± 0.21	0.037
		1.17 ± 0.23	1.01 ± 0.21	0.421

Table 5: Multiple comparisons of p-values for Triglycerides Concentrations

5.7. Linear Regression of IL-6 vs. D-Dimer and CD4 vs. D-Dimer

Simple linear regression analysis of IL-6 vs. D-Dimer showed moderate positive correlation in the HIV positive participants not on treatment and statistically significant ($r^2 = 0.259$, $p < 0.001$) (Fig. 7A). IL-6 vs. D-Dimer showed a moderate correlation in HIV negative Control participants with statistical significance ($r^2 = 0.157$, $p = 0.008$) (Fig. 7B). IL-6 vs. D-Dimer showed a weak correlation in HIV positive participants on treatment and statistically not significant ($r^2 = 0.036$, p = 0.194) (Fig. 7C). There was no correlation between CD4 and D-Dimer concentration in both the HIV positive non treatment and HIV positive treatment groups with ($r^2 =0.004$, p= 0.744) and ($r^2 = 0.00$, p= 0.904) respectively. There was a significant correlation between high triglyceride and D-Dimer concentrations in the HIV+ ART naïve group Figure 7D

A



B

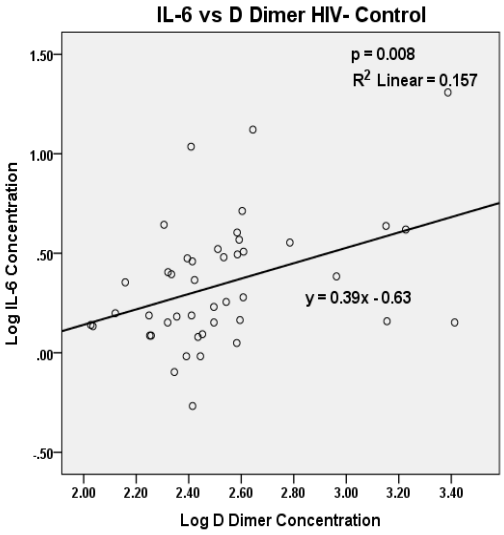
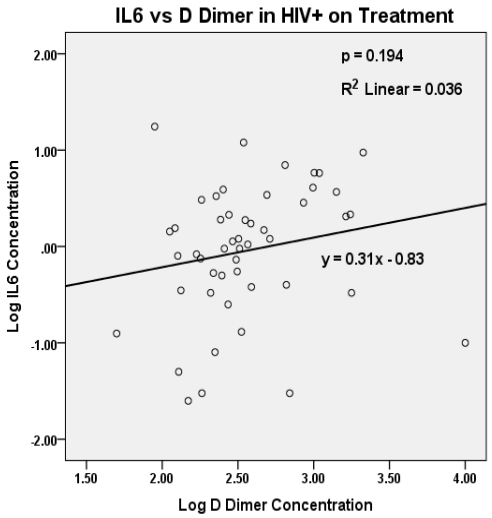


Fig. 7A: There was a significant correlation between a high IL-6 concentration and a high D-Dimer concentration in the HIV+ ART naïve group. **Fig. 7B**: There was a significant correlation between a high IL-6 concentration and a high D-Dimer concentration in the HIV- control.

C



D

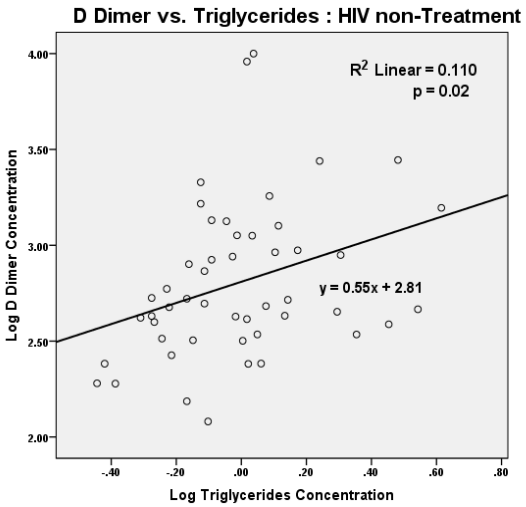


Fig. 7C: There was no significant correlation between a high IL-6 concentration and a high D-Dimer concentration in the HIV+ group on ART. **Fig 7D**: There was a significant correlation between high triglyceride and D-Dimer concentrations in the HIV+ ART naïve group.

5.8. Linear correlation between D-dimers, IL-6, Cholesterol, Triglycerides and CD4

Linear association between D-dimers, IL-6, Cholesterol, Triglycerides and CD4 in HIV+ on ART, HIV+ ART naïve and HIV- control groups. There was a correlation between high IL-6 and D-Dimer concentrations in the HIV+ ART naïve $r^2 = 0.259$, $p < 0.001$. There was also a correlation between triglycerides and D-Dimer concentrations in the HIV+ ART naïve participants $r^2 = 0.110$, $p = 0.020$. Table 6

Table 6: Linear correlation between D-dimers, IL-6, Cholesterol, Triglycerides and CD4

INDEPENDENT VARIABLE	DEPENDENT VARIABLE	R	R^2	P-VALUE
Log d-dimer HIV+ on ART	CD4 count HIV+ on ART	0.019	0.000	0.904
Log d-dimer HIV+ ART naïve	CD4 count HIV+ ART naïve	0.64	0.004	0.744
Log d-dimer HIV+ on ART	Log IL-6 HIV+ on ART	0.189	0.036	0.194
Log d-dimer HIV+ ART naïve	Log IL-6 HIV+ ART naïve	0.509	0.259	0.000
Log d-dimer HIV- control	Log IL-6 HIV- control	0.396	0.157	0.008
Log d-dimer HIV+ on ART	Cholesterol HIV+ on ART	0.027	0.001	0.853
Log d-dimer HIV+ ART naïve	Cholesterol HIV+ ART naïve	0.174	0.030	0.232
Log d-dimer HIV- control	Cholesterol HIV- control	0.236	0.056	0.106
Log d-dimer HIV+ on ART	Triglycerides HIV+ on ART	0.030	0.001	0.838
Log d-dimer HIV+ ART naïve	Triglycerides HIV+ ART naïve	0.332	0.110	0.020
Log d-dimer HIV- control	Triglycerides HIV- control	0.101	0.010	0.487

Table 6: Linear association between D-dimers, IL-6, Cholesterol, Triglycerides and CD4 in HIV+ on ART, HIV+ ART naïve and HIV- control groups. P-value +/- 0.05 shows an association between the two variables. There was a correlation between high IL-6 and D-Dimer concentrations in the HIV+ ART naïve and the HIV- control participants. There was also a correlation between triglycerides and D-Dimer concentrations in the HIV+ ART naïve participants.

6.0 DISCUSSION

IL-6

IL-6 is a typical pleiotropic cytokine that acts on various cells. IL-6 production has been detected in many cell types; however, the primary sources of the cytokine are monocytes and macrophages at sites of inflammation during acute inflammation, as well as T cells in chronic inflammation (Naugler & Karin, 2007). Accumulating evidence indicates pathological roles for IL-6 in various disease conditions, such as inflammatory, autoimmune and malignant diseases (Kishimoto and Ishizaka, 1971). It is released in almost all situations of homeostatic perturbation, which include endotoxaemia, trauma, and acute infection. In addition, circulating IL-6, together with tumour necrosis factor α (TNF- α) and interleukin 1 (IL-1) is required for induction of the acute phase response which comprises fever, corticosteroid release, and hepatic production of acute phase proteins which are mostly protease inhibitors.

Results from this study showed that IL-6 levels in the HIV positive ART naive individuals were significantly higher than in the HIV positive individuals on ART. Our findings agreed with those of (Klein, et al., 2012) who found that IL-6 dropped by 32% over the first 24 weeks on ART $p<0.0001$. IL-6 is an interleukin that acts as a pro-inflammatory cytokine and is therefore used as an inflammatory biomarker. The results therefore, indicated that HIV positive ART naive individuals underwent a significant inflammatory process than their counterparts on ART. As revealed by most researchers, this cytokine when elevated has negative implications especially on the health of HIV positive individuals. Persistent systemic inflammation as denoted by elevated levels of IL-6 has been associated with an increased risk of serious non-AIDS defining events such as cardiovascular disease (CVD), in HIV-positive adults despite effective treatment with antiretroviral therapy (Worm & Hsue , 2010; Tien , et al., 2010). IL-6 is a major mediator of the acute-phase response that is expressed by antigen-presenting cells and non-hematopoietic cells. It is an important growth factor for B cells while also promoting CD4 T cell proliferation and survival (Dienz & Rincon , 2009). During the acute stage of an infection, relatively high levels of IL-6 are produced and this, together with other cytokines, help to activate T cells, increase the number of antibody producing B cells

and stimulate the release of hormones. Such a response during an acute infection is useful. However, prolonged production of relatively high levels of IL-6 may weaken the immune system over the long- term. This weakness can occur because higher than normal levels of IL-6 could cause the premature death of immune cells, increase the susceptibility of the liver to injury and raise the risk of cardiovascular disease. IL-6 production has been shown to be an important component of autocrine (Dougan , et al., 2011) and paracrine (Stone, et al., 2012) circuits that fuel the growth of solid tumours. A review of data from 5023 HIV positive individuals statistically found that participants with elevated IL-6 levels in their blood had the strongest risk of developing cancer (Álvaro , et al., 2013). Our study showed that HIV positive participants on ART had significantly lower IL-6 mean concentrations than HIV positive ART naive participants. This was because the initiation of ART improved the immune system which caused a reduction in the inflammatory processes caused by many organisms such as bacteria. This agreed with a study that was conducted which found that at week 12 after commencement of ART, IL-6 levels were significantly lower compared to pre-ART levels in the same group of individuals (Hamlyn, et al., 2015) thereby leading to a reduced risk of associated injurious effects. However, HIV negative controls had a significantly higher mean IL-6 concentration than the HIV positive participants on ART. This could have been due to various factors to do with participant recruitment. All HIV negative control participants were recruited from the skin clinic at UTH and some of them despite being HIV negative, had skin conditions. Studies have actually proved that IL-6 is essential in the skin wound healing process as demonstrated by delayed wound healing in IL-6 deficient mice (Lin, et al., 2003). Wound healing in participants with skin conditions could be a result of the sores caused by scratching of the skin which caused inflammation and wounds both of which predisposed them to an elevation in IL-6 levels. These participants were also not screened for many other ailments that could also activate the inflammatory process compared to HIV positive on ART who report to the clinic for periodic reviews and receive treatment not only for HIV but also for many other ailments.

D-dimers

Normal haemostasis comprises a series of regulated processes that maintain blood in a fluid, clot-free state in normal vessels while rapidly forming a localized haemostatic plug at the site of vascular injury (Kumar, et al., 2013). The pathologic counterpart of haemostasis is thrombosis, the formation of blood clot (thrombus) within intact vessels. D-dimers, the fibrinolytic degradation products of cross-linked fibrin, have emerged as the most useful of the procoagulant activity and ongoing fibrinolysis markers. Evidence has accumulated confirming the effectiveness of incorporating a D-Dimer assay into the diagnostic algorithm of deep vein thrombosis (DVT) and pulmonary embolism (PE) and has proved to be a definitive non-invasive diagnosis to be reached in 94% of patients, and appears to be safe (Arnaud, et al., 1999).

In this study, it was observed that D-dimers measured in HIV positive ART-naive patients prior to ART initiation were significantly raised compared to HIV negative controls. Results from other studies have suggested that D-dimer and IL-6 could be useful in identifying ART-naive patients at higher risk of AIDS or death after ART initiation (Boulware, et al., 2011). An elevated D-dimer concentration is thought to be a consequence of viral replication or persistence, high levels of bacterial lipopolysaccharides (LPS), bacterial DNA and their associated immune activation in HIV infected persons (Funderburg, et al., 2010). It has also been demonstrated that plasma samples from HIV-infected patients contain higher levels of bioactive Tissue Factor (TF) the major in vivo activator of coagulation than do samples from HIV negative controls (Funderburg, et al., 2010). Increased TF expression in HIV infection is underscored by the correlation between TF expression and D-dimer levels (Funderburg, et al., 2010). Thus, high levels of TF probably contribute to an increased coagulation tendency in chronic HIV infection. This could lead to a state of hypercoagulability which predispose HIV positive individuals to an increased risk of both arterial and venous thrombosis (Matta , et al., 2008).

The study also found that HIV positive ART naive participants had significantly higher mean d-dimer concentration than HIV positive participants on treatment. This probably showed that the commencement of ART improved the immune system leading to

reduced viral replication, low levels of bacterial lipopolysaccharides (LPS), bacterial DNA and all mechanisms which lead to an increased expression of Tissue Factor. This agreed with a study by (Hamlyn, et al., 2015) which found that at week 12 after commencement of ART, D-dimer levels were significantly lower compared to pre-ART levels in the same group of individuals. This also agreed with the results obtained from our study which showed no significant difference in D-dimer levels between HIV positive participants on treatment and the HIV negative control participants. HIV positive on ART still had higher mean D-dimer levels than the HIV negative controls despite the difference not being statistically significant.

Linear regression results showed that triglycerides had some moderate correlation with the elevated levels of D-dimers in the treatment naive HIV positive population. This meant that the concentrations of D-dimers were enhanced positively by triglyceride levels in combination with other factors which may include viral replication or persistence, high levels of bacterial lipopolysaccharides (LPS) or Tissue factor to induce intravascular thrombosis. However, cholesterol levels were not associated with high D-dimer levels.

Cholesterol

The study revealed that HIV positive participants on treatment had a significantly higher serum concentration of Cholesterol compared to HIV negative control participants. However, this did not correspond to high D-dimer levels. The pathogenesis of HAART-related dyslipidaemia is complex and involves various drug induced effects in association with hormonal and immunological influences superimposed upon genetic predisposition (Fisher , et al., 2006; Guardiola , et al., 2006). All the HIV positive participants on ART who took part in this research were on first line HIV treatment taking a combination of Nucleoside Reverse – Transcriptase Inhibitors (NRTIs) and Nonnucleoside Reverse – Transcriptase Inhibitors (NNRTIs), both of which could cause increases in cholesterol concentration. This is consistent with a study carried out by (Fontas, et al., 2004) in which they found that for total cholesterol levels, the prevalence

of dyslipidemia increased in patients receiving an NNRTI containing regimen compared to ART naïve individuals

There was no significant difference in mean total cholesterol concentration between the HIV negative control group and the HIV positive treatment naïve. However, the treatment naive group had a higher mean total cholesterol concentration.

Relationship between IL-6 and D-dimers

The study demonstrated that there was a close link between inflammation and coagulation. Linear regression analysis between IL-6 and D-dimers showed a correlation between the two biomarkers when participant's results were matched. This meant that high IL-6 levels corresponded to high D-dimer levels when the participants' results were matched. We could therefore infer that inflammation predisposed the participants to hyper-coagulation. However, the link was only in the HIV positive ART naïve participants and the HIV negative control participants. The link between inflammation and coagulation was even much stronger in the HIV positive ART naïve participants than in the control group. This demonstrated that in the HIV positive ART naïve individuals, inflammation contributed more to coagulation than it did in the HIV negatives. Inflammatory cytokines are fundamental mediators of the immune system, and among them, IL-6 has been shown to stimulate platelet aggregation and adhesion (Peng, et al., 1996). Inflammation induced activation of platelets can further perpetuate the inflammatory response by two means. First the activated platelets aggregated to provide the negatively charged phospholipid surface necessary for secondary haemostasis to occur. The end result of secondary haemostasis is the formation of thrombin which is the catalyst for the conversion of fibrinogen to fibrin; however, thrombin is itself a strong platelet agonist and inflammatory mediator (Esmon, et al., 1999). Second, activated platelets interact with underlying endothelial cells to stimulate the adhesion and recruitment of inflammatory leucocytes. Several mediators and products of inflammation produce a pro-coagulant effect on secondary haemostasis, including tumor necrosis factor – α (TNF- α) and other inflammatory cytokines, lipoproteins, C-reactive protein (CRP), bacterial endotoxin, as does complement activation (Boos , et al., 2006; Levi, et al., 2003). For the HIV positive on ART group,

there was no relationship between D-dimers and IL-6 results obtained**.** This demonstrated that inflammation in these individuals was under control due to treatment and the cause of a raised d-dimer (coagulation) was something else other than IL-6 (inflammation).

6.1 CONCLUSION

This study showed that the levels of IL-6 and d-dimers were higher in HIV positive ART naïve individuals. The results also showed that ART lowers inflammation in HIV and this may explain why ART reduces the risk of developing opportunistic tumors and that of thrombosis. This in part explains why ART naïve individuals are more at risk of cancers, thrombosis and cardiovascular disease which contribute to a high mortality rate in these individuals.

6.2 IMPLICATIONS AND RECOMMENDATIONS

Antiretroviral therapy (ART) has substantially reduced morbidity and mortality in human immunodeficiency virus (HIV)-infected persons. Yet even with adequate HIV virologic suppression, substantial morbidity and mortality still occurs during the first year of ART (Castelnuovo , et al., 2009). Although many studies have confirmed persistently high morbidity and mortality during the first year and particularly the first 6 months of ART, identifying patients prior to starting ART who are at high subsequent risk remains a clinical challenge. Certain clinical variables, such as hemoglobin level, preexisting AIDS-defining illness, CD4 T-cell count, and injection drug use, have been identified as predictors of morbidity or mortality in the first months after ART initiation (Mocroft , et al., 2004).

Biomarkers such as IL-6 and d-dimers could also be important in guiding intense monitoring of selected subgroups of high-risk patients and could be used to elucidate pathogenesis pathways amenable to targeted therapeutic interventions.

6.3 LIMITATIONS / WEAKNESSSES AND ASSUMPTIONS

- The study did not have a follow up programme to assess the implications of high levels of IL-6 and D-dimer concentrations in HIV positive individuals. A follow up programme could have provided morbidity and mortality data for our participants.
- HIV negative control participants were recruited from the skin/ STI clinic and this could have been the cause of a higher IL-6 concentration in this group than in the HIV positive on treatment group as some skin conditions could have activated the inflammatory process and wound healing both of which could increase IL-6 levels.
- With the IL-6 result obtained, it could have been better to run a profile of inflammatory biomarkers such as C-reactive protein (CRP), IL-1β, Interferon γ (IFN γ), Tumor Necrosis Factor α (TNF α) in order to compare the results and see if they correspond with each other.
- The study could have included a budget for CD4 reagents because not all the CD4 results for our participants were obtained from the database as others were lost despite the blood being collected and sent to the lab for analysis.
- Viral load which is an important test for determining or assessing the level of immunosuppression was not done. If done, it could have added more value to the study

6.4 FUTURE DIRECTION

Follow up studies may be warranted to determine whether for our population pre ART levels of IL-6 and D-dimers are really important as far as mortality and prognosis is concerned in HIV positive individuals.

Trials of interventions may also be warranted to assess whether mortality or cancer risks can be reduced by lowering D-dimer and IL-6 levels in HIV-positive individuals before and after the initiation of ART.

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8.0 APPENDICES

Appendix 1: INCLUSION CRITERIA EXPLAINED

- HIV adult individuals aged above 18 years old were included in the study
- HIV persons with no known bleeding or clotting disorders were included in the study.
- Persons who read and understood the rationale for participating in the study and given written consent without being forced to were included in the study.
- Smokers because smoking influences blood coagulation which may result in elevation of D-dimer levels and also causes elevation of cholesterol and triglyceride levels were excluded from the study
- Pregnant women were excluded because pregnancy can raise IL-6 due to underlying inflammation.
- Patients with malignancies were excluded because malignancies are predictors of a D-dimer positive result
- Patients with diabetes were excluded because hyperglycemia exerts a procoagulant effect which can cause a rise in D-dimer levels
- Participants currently taking any of the following platelet inhibiting drugs: aspirin, clopidogrel were excluded.

Selection of Controls

HIV negative controls were recruited from the Voluntary Counseling and Testing (VCT) centre at skin and STI Clinic of UTH and their inclusion criteria was as above. After counseling and testing, it was totally up to those with a negative HIV test result to decide whether to participate in the study or not. No individual was forced to do an HIV test in order to participate in the study. The controls were matched for age and sex.

Appendix 2.1: Table 7: HIV Negative Participants' Raw Data

NUMBER	AGE	D-DIMER ng/mL	IL-6 ng/ml	CHOLESTEROL mmo/l	TRIGLYCERIDES mmol/l
01C	24	313.12	1.7	2.93	0.46
02C	30	257.49	1.54	3.93	0.43
03C	28	391.03	3.7	3.72	0.83
04C	43	2437.68	20.34	3.58	0.69
05C	36	209.51	2.54	3.62	4.28
06C	36	440.87	13.22	3.88	0.45
07C	24	384.89	3.12	1.54	0.35
08C	26	259.8	0.54	2.6	0.58
09C	32	256.35	10.86	4.66	0.5
10C	61	609.89	3.58	6.25	1.05
11C	23	278.3	0.96	4.06	0.49
12C	18	202.2	4.4	6.18	1.31
13C	45	221.47	0.8	5.9	1.38
14C	36	1684.25	4.16	4.44	1.1
15C	29	226.18	1.52	4.36	0.93
16C	42	259.24	2.88	3.74	1.72
17C	37	143.98	2.26	3.26	1.59
18C	46	177.39	1.54	4.51	2.05
19C	56	401.41	5.16	3.67	0.7
20C	43	348.88	1.8	3.43	0.92
21C	29	248.1	2.98	4.96	0.45
22C	23	106.46	1.38	3.88	0.6
23C	61	1416.52	4.34	2.94	0.82
24C	24	108.46	1.36	4.73	0.88
25C	31	215.93	2.48	4.56	0.61
26C	34	313.22	1.42	5.95	1.06
27C	22	272.42	1.2	4.31	0.42
28C	29	131.95	1.58	6.5	1
29C	35	917.43	2.42	3.65	0.9
30C	30	384.03	4.02	5.17	0.98
31C	32	2587.31	1.42	3.07	0.86
32C	23	324.22	3.32		1.03
33C	36	180.64	1.22		0.96
34C	45	246.02	0.96	5.26	0.59
35C	30	393.66	1.46	5.29	1.12
36C	18	406.19	3.22	5.06	1.47
37C	32	264.31	2.32	5.52	1.66
38C	32	1428.47	1.44	5.22	1.7
39C	26	382.77	1.12	2.99	0.48
40C	30	283.3	1.24	3.4	0.79
41C	27	341.43	3.02	4.96	0.8
42C	29	208.85	1.42	5.84	0.72
43C	33	178.53	1.22	6.09	0.85
44C	32	404.96	1.9	3.54	0.5
45C	32	601.43		2.74	0.62
46C	43	289.97		3.2	0.63
47C	19	252.06		4.52	0.57
48C	41	173.06		3.17	0.74
49C	26	130.6		4.35	3.52
50C	25	225.56		3.99	1.28

Appendix 2.2: Table 8: HIV Positive on ART Participants' Raw Data

#	AGE	MEDICATION	CD4 cells/µl	D-DIMER ng/mL	IL-6 ng/ml	CHOL mmol/l	TRIG mmol/l
01	37	TDF,NVP,FTC	808	307.48	0.73	4.57	0.75
02	33	TDF,EFV,FTC	376	694.06	0.03	4.6	0.91
03	37	TDF,EFV,FTC	8	312.77	0.55	2.47	1.61
04	43	TDF,EFV,FTC	488	469.34	1.48	5.19	0.73
05	30	TDF,EFV,FTC	416	247.57	0.5	4.58	1.18
06	47	TDF,EFV,FTC	295	181.79	3.05	4.63	0.83
07	46	AZT,NVP,3TC	476	323.88	0.95	6.19	1.22
08	21	TDF,EFV,FTC	517	1775.43	0.33	3.49	1.09
09	40	TDF,EFV,FTC	280	275.73	2.13	3.91	1.15
10	38	TDF,EFV,FTC		2120.69	9.45	3.5	1.52
11	46	TDF,NVP,3TC	2194	252.52	3.9	3.27	0.96
12	31	TDF,EFV,FTC	429	126.69	0.8	4.5	1.46
13	43	TDF,EFV,FTC	220	344.38	12	5.45	0.54
14	44	AZT,EFV,3TC	236	257.1	0.95	5.69	1.3
15	30	EFV,TDF,FTC		353.15	1.88	4.7	1.05
16	31	TDF,EFV,FTC	340	1411.66	3.68	4.82	0.56
17	32	ABC,EFV,3TC	311	178.98	0.75	7.84	1.14
18	26	TDF,EFV,FTC	1409	988.94	4.08	6.12	1.73
19	30	TDF,EFV,FTC		222.99	0.08	3.45	0.82
20	44	TDF,EFV,FTC	400	1088.91	5.78	2.58	0.64
21	52	TDF,EFV,FTC		319.15	1.2	5.29	1.07
22	48	TDF,EFV,FTC	454	367.23	1.05	4.16	0.46
23	36	TDF,EFV,FTC	650	227.08	3.33	6.69	1.41
24	53	TDF,EFV,FTC	142	384.43	1.73	4.27	1.38
25	25	TDF,EFV,FTC	32	647.16	7	4.74	2.5
26	35	TDF,NVP,FTC	14	856.41	2.85	4.96	2.45
27	55	TDF,NVP,FTC	634	272.2	0.25	5.32	1.55
28	52	TDF,EFV,FTC		217.61	0.53	3.14	1.01
29	38	TDF,EFV,FTC	466	291.57	1.13	6.21	1.69
30	50	TDF,NVP,FTC	209	106.24	0	3.76	0.56
31	44	TDF,EFV,FTC	253	1007.98	5.83	4.3	0.89
32	28	TDF,EFV,FTC	423	121.44	1.55	4.45	1.03
33	58	TDF,EFV,FTC		1630.17	2.05	8.14	2.44
34	51	TDF,NVP,FTC	565	388.01	0.38	4.01	0.52
35	43	TDF,EFV,FTC	111	112.14	1.43	6.43	1.58
36	37	TDF,EFV,FTC	342	168.36	0.83	5.63	1.03
37	37	TDF,EFV,FTC	280	10000	0.1	5.83	1.67
38	42	TDF,EFV,FTC	655	148.48	0.03	3.9	4.67
39	19	AZT,NVP,3TC	9	242.76	1.9	3.77	1.21
40	27	TDF,EFV,FTC	424	50	0.13	4.68	1.2
41	44	TDF,EFV,FTC	313	89.25	17.55	4.22	1.76
42	33	TDF,EFV,FTC	432	132.89	0.35	8.53	1.17
43	29	TDF,EFV,FTC	274	128.43	0.05	4.88	0.88
44	40	TDF,EFV,FTC	325	513.25	1.2	6.44	2.52
45	31	TDF,EFV,FTC	175	333.55	0.13	3.98	1.21
46	34	TDF,EFV,FTC	175	490.9	3.43	4.93	0.94
47	45	TDF,EFV,FTC		208.81	0.33	4.69	0.56
48	45	TDF,NVP,FTC	163	1743.83	2.15	5.33	0.43
49	58	TDF,EFV,FTC	623	659.6	0.4	6.89	1.5
50	40	TDF,EFV,FTC		182.81	0.03	5.37	0.67

Appendix 2.3: Table 9: HIV Positive ART Naive Participants' Raw Data

#	AGE	CD4 cells/µl	D-DIMER ng/mL	IL-6 ng/ml	CHOL mmol/l	TRIG mmol/l
01	32		449.9	3.25	3.67	1.97
02	42		481.65	1.4	3.92	1.19
03	40	62	190.48	1.98	3.14	0.36
04	30	529	266.91	1.23	4.93	0.61
05	52	52	1351.3	2.58	2.67	0.81
06	49	334	418.55	0.55	5.16	0.49
07	25		2786.12	5.18	3.9	3.03
08	56	164	1335.2	1.13	3.19	0.9
09	46	471	240.66	0.38	6.05	1.05
10	28	280	1264.78	4.03	3.79	1.3
11	36		733.19	10.95	3.02	0.77
12	20	166	428.88	0.78	2.85	1.36
13	46		2752.52	14.43	3.6	1.74
14	30	457	411.88	1.15	3.99	1.04
15	20	271	120.46	0.8	2.77	0.79
16	42		427.11	0.73	4.21	0.53
17	43		9086.31	8.7	2.75	1.04
18	40	350	342.71	0.03	5.19	1.12
19	38	684	1647.52	5.78	4.39	0.75
20	45	346	189.8	0.15	2.95	0.41
21	50	50	387.03	1.1	3.99	2.84
22	33		797.22	7.6	2.34	0.69
23	35	386	241.15	0.95	3.43	0.38
24	20	15	919.59	1.45	5.6	1.27
25	22		475.18	0.75	2.96	0.6
26	18	87	872.99	0.16	3.23	0.94
27	21			1.13	3.43	1.15
28	54	277	397.72	2.73	3.88	0.54
29	39	32	1567.9	1.25	6.1	4.12
30	49		342.52	2.75	5.24	2.26
31	41		531.11	1.93	1.34	0.53
32	25	690	1807.14	8.63	5.16	1.22
33	27	374	319.72	1.8	3.16	0.71
34	50		317.5	1.7	2.64	1.01
35	40		424.6	7.3	2.78	0.96
36	29		1122.23	11.03	2.58	1.08
37	24		153.57	2.03	5.9	0.68
38	24		1126.99	0.18	2.5	0.97
39	46		526.12	1.96	5.06	0.68
40	31		325.93	1.7	3.7	0.57
41	46	280	840.26	3.5	2.73	0.81
42	56	164	941.86	1.64	4.09	1.49
43	46	471	241.49	1.68	5.37	1.15
44	46	83	496.18	1.58	3.04	0.77
45	36		10000	8.34	3.31	1.09
46	48	700	463.69	2.36	7.04	3.49
47	40	523	592.42	1.64	2.89	0.59
48	25		519.55	1.12	3.65	1.39
49	36	475	889.32	2.68	4.28	2.02
50	57		2131.66	38.54	2.39	0.75

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01st April, 2014

Dr. Panji Nkhoma
Department of Pathology & Microbiology
School of Medicine
UNZA
LUSAKA

Dear Mr Nkhoma,

RE: GRADUATE PROPOSAL PRESENTATION FORUM

Having assessed your dissertation entitled " **Determinations of D- Dimer, Fibrinogen and IL – 6 levels in HIV Positive Individuals at the University of Teaching Hospital, Lusaka",** we are satisfied that all the corrections to your research proposal have been done. The proposal meets the standard as laid down by the Board of Graduate Studies.

You can proceed and present to the Research Ethics.

Yours faithfully,

Dr. S.H. Nzala
ASSISTANT DEAN, POSTGRADUATE
CC: HOD, Pathology

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24th July, 2014

Ref. No. 2014-May-002

The Principal Investigator
Mr. Panji Nkhoma
C/o The University of Zambia- School of Medicine
Dept. of biomedical Sciences
P.O. Box 50110,
LUSAKA.

Dear Mr. Nkhoma,

RE: DETERMINATION OF D-DIMER, FIBRINOGEN AND IL-6 LEVELS IN HIV POSITIVE PATIENTS AT THE UNIVERSITY TEACHING HOSPITAL, LUSAKA.

Reference is made to your corrections submitted on 21st July, 2014. The IRB resolved to approve this study and your participation as principal investigator for a period of one year.

Review Type	Ordinary	Approval No. **2014-May-002**
Approval and Expiry Date	Approval Date: 24th July, 2014	Expiry Date: 23rd July, 2015
Protocol Version and Date	Version-Nil	23rd July, 2015
Information Sheet, Consent Forms and Dates	• English, Nyanja	23rd July, 2015
Consent form ID and Date	Version-Nil	23rd July, 2015
Recruitment Materials	Nil	23rd July, 2015
Other Study Documents	Questionnaire, Checklist,	23rd July, 2015
Number of participants approved for study	148	23rd July, 2015

Specific conditions will apply to this approval. As Principal Investigator it is your responsibility to ensure that the contents of this letter are adhered to. If these are not adhered to, the approval may be suspended. Should the study be suspended, study sponsors and other regulatory authorities will be informed.

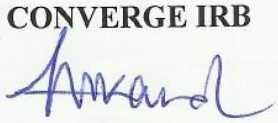
Conditions of Approval

- No participant may be involved in any study procedure prior to the study approval or after the expiration date.
- All unanticipated or Serious Adverse Events (SAEs) must be reported to the IRB within 5 days.
- All protocol modifications must be IRB approved prior to implementation unless they are intended to reduce risk (but must still be reported for approval). Modifications will include any change of investigator/s or site address.
- All protocol deviations must be reported to the IRB within 5 working days.
- All recruitment materials must be approved by the IRB prior to being used.
- Principal investigators are responsible for initiating Continuing Review proccedings. Documents must be received by the IRB at least 30 days before the expiry date. This is for the purpose of facilitating the review process. Any documents received less than 30 days before expiry will be labelled "late submissions" and will incur a penalty.
- Every 6 (six) months a progress report form supplied by ERES IRB must be filled in and submitted to us.
- ERES Converge IRB does not "stamp" approval letters, consent forms or study documents unless requested for in writing. This is because the approval letter clearly indicates the documents approved by the IRB as well as other elements and conditions of approval.

Should you have any questions regarding anything indicated in this letter, please do not hesitate to get in touch with us at the above indicated address.

On behalf of ERES Converge IRB, we would like to wish you all the success as you carry out your study.

Yours faithfully,
ERES CONVERGE IRB



Dr. E. Munalula-Nkandu
BSc (Hons), MSc, MA Bioethics, PgD R/Ethics, PhD
CHAIRPERSON