UNIVERSITY OF NAIROBI

MARKERS OF MICROBIAL TRANSLOCATION IN HIV-INFECTED AND HIV-NEGATIVE ADULTS IN A KENYAN URBAN CENTRE: A CROSS-SECTIONAL COMPARATIVE STUDY

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APPROVAL BY SUPERVISORS

This dissertation for Master of Medicine in Internal Medicine has been submitted to the Department of Clinical Medicine and Therapeutics with our approval as university supervisors.

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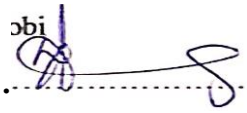
Signature: …….  **…….. Date:** 5th December2022

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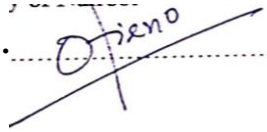
Signature: …  **….. Date:** 5th December 2022

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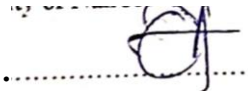
Signatuer: ….  **.. Date:** 5th December 2022

DECLARATION OF ORIGINALITY

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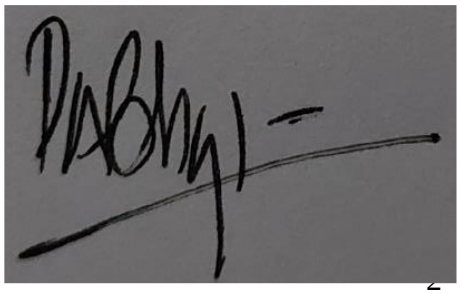
Signature:  **Date:** 5th December2022

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LIST OF ABBREVIATIONS

ADC	AIDS-defining Cancer
AIDS	Acquired Immunodeficiency Syndrome
AKI	Acute Kidney Injury
ART	Antiretroviral Therapy
BMD	Bone Mineral Density
CCC	Comprehensive Care Center
CD	Cluster of Differentiation
CKD	Chronic Kidney Disease
CMV	Cytomegalovirus
CNS	Central Nervous System
CRP	C-reactive Protein
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-linked Immunosorbent Assay
EndoCAb	Endotoxin Core Antibody
ESRD	End-stage Renal Disease
GIT	Gastrointestinal Tract
GVHD	Graft-versus-host Disease
HAART	Highly Active Antiretroviral Therapy
HAD	HIV-associated Dementia
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HDL	High-density Lipoproteins
HIV	Human Immunodeficiency Virus
HIVAN	HIV-associated Nephropathy
HIVICK	HIV Immune Complex Kidney Disease
IBD	Inflammatory Bowel Disease
Ig	Immunoglobulin
IL	Interleukin

IRF	Interferon Regulatory Factor
KAIS	Kenya AIDS Indicator Survey
KAVI-ICR	Kenya AIDS Vaccine Initiative Institute of Clinical Research
KNH	Kenyatta National Hospital
LBP	Lipopolysaccharide-binding Protein
LPS	Lipopolysaccharide
NADC	Non-AIDS-defining Cancer
NCD	Non-communicable Disease
NTD	Neglected Tropical Disease
RANK	Receptor Activator of NF-κβ
RANKL	Receptor Activator of NF-κβ Ligand
RNA	Ribonucleic Acid
sCD14	Soluble CD14
SHIV	Simian Human Immunodeficiency Virus
SSA	Sub-Saharan Africa
TLR	Toll-like receptor
TNF	Tumour Necrosis Factor
WHO	World Health Organisation

ABSTRACT

BACKGROUND

The incidence of non-communicable diseases in persons infected with HIV has been on the increase, due to persistent chronic immune activation and inflammation despite effective HAART. Passage of microbes from the gastrointestinal system across a damaged epithelium, termed microbial translocation (MT), is believed to be a key factor in the continued inflammation. The effect of MT on the immune system is influenced by environmental and genetic factors, and, if present, is a potentially modifiable driver of the chronic immune activation. This study sought to determine the presence of MT using indirect markers lipopolysaccharide (LPS) and soluble CD14 (sCD14) in HIV-infected adults, with HIV-uninfected age-matched participants as a comparative population.

METHODS

This was a 1:1 age-matched cross-sectional comparative survey conducted at the Mbagathi District Hospital Comprehensive Care (CCC) and Voluntary Testing and Counselling (VCT) centres, and the Kenyatta National Hospital Voluntary Counselling Centre. HIV-infected persons were drawn from adults attending the CCC, while HIV-uninfected persons were matched in age to within five years of the test population, and drawn from attending the VCT centres.

Presence of MT was assessed using LPS and sCD14 levels in plasma. Focused medical history that included CD4 and plasma viral load levels, and drug history for the HIV-infected population was obtained from the patients' medical charts.

Statistical analysis was done on IBM® SPSS® Statistics v23.

Mean levels of LPS and sCD14 from the two groups were compared using the Wilcox signed-rank test. A p value of ≤ 0.05 was considered significant.

RESULTS

Ninety-four participants were included in the primary analysis, 47 HIV-infected and 47 HIV-uninfected. The age ranged from 21–61 years for all participants, with a mean age of 47.5±9.0 years for the HIV-infected group and 47.7±9.8 years in the HIV-uninfected group. Female participants were 51.1% in the HIV-infected group and 66% in the HIV-uninfected group. All the HIV-infected participants were on HAART, with a mean 12.9±3.6 year duration of use, with 91% (n=46) of them having an undetectable HIV viral load.

Mean plasma LPS for the HIV-infected group was 0.95±0.43 EU/mL compared to 0.98±0.42 EU/mL for the HIV-uninfected. There was no statistically significant difference between the two groups (p=0.38). Mean plasma sCD14 values were 447.74±360.22 pg/mL (median 316 pg/mL; IQR 250.40–441.83) and 797.19±364.27 pg/mL (median 742 pg/mL; IQR 541.53–981.65) for the HIV-infected and HIV-uninfected, respectively. The HIV-uninfected group had significantly higher sCD14 levels than the HIV-infected group (p=0.00). The HIV-uninfected group showed greater variability than the HIV-infected group for both LPS and sCD14 levels. There was no correlation between LPS and sCD14 levels in either group.

CONCLUSION

While this study demonstrated detectable indirect markers of microbial translocation in both HIV-infected and HIV-uninfected groups, HIV infection was not associated with higher levels of either LPS or sCD14.

CHAPTER ONE: INTRODUCTION

Remarkable gains have been made in the global fight against human immunodeficiency virus (HIV) infection. Increased access to antiretroviral therapy (ART) has led to reduction in new HIV infections and increased survival while reducing AIDS-related deaths in general (1). However, differences in mortality rates exist within the different age groups, being markedly higher in the >50 years olds (2).

Data from sub-Saharan Africa (SSA) show a higher mortality rate in the >50 years old HIV-infected patients than their younger counterparts. This higher mortality rate is set down to an increased risk of non-AIDS defining illnesses such as cardiovascular and kidney disease. In populations with a high prevalence of HIV infection and high AIDS-related mortality rates, the full burden of non-communicable diseases (NCDs) is not clear because a large number of the population pass away too early to manifest those diseases. With increasing life expectancy in persons living with HIV the relative burden of other diseases in SSA, notably NCDs, is also likely increase, due to a combination of factors including chronic immune activation, side effects from HAART and adjunct medication, co-infections, and the ageing process itself (2).

Immune system abnormalities in HIV infection persist despite effective suppression of viral replication. These abnormalities are similar to age-associated changes seen in the immune system, especially in the adaptive arm (immunosenescence). They include changes in T-cell number and function and increased levels of pro-inflammatory cytokines. These changes are thought to be related, in part, to chronic immune activation. Chronic exposure to HIV antigens leads to expansion of pro-inflammatory cells which contribute to heightened systemic inflammation (3). Other viral and bacterial antigens also play a role in the systemic inflammation. In HIV infection, entry of bacterial products from the gut lumen to the bloodstream through a compromised mucosal layer has been shown to play an important part in continued immune activation and inflammation seen (4).

Persistent activation of the immune system with inflammation due to HIV infection and HIV-associated premature immunosenescence are central to HIV pathogenesis and are associated with development of end organ diseases (5). HIV-associated inflammation and immunosenescence have a causal relationship to earlier-than-expected onset of NCDs seen in HIV-infected individuals, with associated increase in morbidity and mortality (3). Prevention or attenuation of this systemic inflammation can lead to a decrease in the incidence of NCDs. Interventions to reduce microbial translocation, and subsequently systemic inflammation, have been developed (6). Data on the

prevalence of microbial translocation in our population is necessary before such interventions can be put in place.

CHAPTER TWO: LITERATURE REVIEW

2.1 HIV: THE CHANGING FACE OF THE EPIDEMIC

There were an estimated 37.7 million people living with HIV (PLHIV) worldwide in 2020, an increase from past years as more people receive life-saving anti-retroviral therapy (ART). Globally, there was ~50% fall in the number of new infections since 2001, from 3.4 million to 1.5 million in 2020. The number of annual AIDS-related mortalities has been decreasing since the global treatment target was set in 2003, falling by 47% since 2010 (1, 7, 8).

Sub-Saharan Africa bears the worst burden of the epidemic, with 60% of new global infections occurring in the region in 2020. However, the region has witnessed the largest decline in annual new adult HIV infection rates worldwide and a significant fall in AIDS-related deaths by 36% since 2010 (1, 8). The epidemic in Kenya is following the global and regional trends. HIV prevalence in the country fell from 7.1% in 2007 to 5.9% in 2016. The number of new infections dropped by 19% between 2013 and 2015, with the number of persons living with HIV on treatment increasing by 40% between the same time period. Annual AIDS-related deaths decreased to 30,817 persons in 2015 from 120,000 persons in 2001 (7, 9, 10).

Of note in the global HIV epidemic is the growing cohort of older PLHIV aged 50 years and above. This is not only due to increased access to ART, but also increasing new infection rates in the older age group, estimated at 8% in 2020 (8, 11). In 2017 there were an estimated 6.7 million PLHIV aged 50 years and over, representing ~15% of the PLWHIV worldwide (12). Locally, the Kenya AIDS Indicator Survey (KAIS) carried out in 2012 found a HIV prevalence rate of 5% among people aged 50-64 years (13). The rates were higher the more recent 2018 KENPHIA survey: the prevalence rate was 9.2% among 50–54 years, 7.5% for 55–59 years, and 8.5% among the 60–64 year age group (14).

Mortality among HIV-positive persons aged ≥50 years was higher compared to those aged <50 in SSA (2). Increased mortality in this age group in higher income countries is put down to an increased risk of non-AIDS defining illnesses such as cardiovascular and kidney disease (2), and similar trends are being observed in SSA (15). Although there is little data from low- and middle-income countries (LMICs) on the relative burden of non-AIDS chronic conditions in HIV-positive patients, evidence suggests that cardiovascular and chronic respiratory diseases are increasing in this group of patients (16). Life expectancy has increased as a result of increased access to ART and is likely to increase the relative burdens of NCDs in SSA (2).

Long-term outcomes in HIV-positive people will largely be determined by the associated chronic non-AIDS conditions. Action taken now to identify these conditions, optimise their early and prompt recognition and diagnosis, and put in place effective prevention strategies and/or disease intervention is likely to have the greatest effect on limiting NCD comorbidity and improving population health among HIV-infected patients in LMICs (16). Identifying the factors that contribute to chronic immune activation in our HIV-infected population will be useful in helping us identify potentially modifiable risk factors.

2.2 CHRONIC INFLAMMATION AND IMMUNE ACTIVATION IN HIV INFECTION

Since its discovery and characterisation in 1983/84 (17), much of the focus in HIV infection has been on inhibiting viral replication and preventing AIDS (6). There have been significant gains in this regard, both globally and locally (7). Despite a remarkable decline in the number of AIDS-related deaths, opportunistic infections and non-AIDS related events (7), the risk of developing non-AIDS related comorbidities such as cardiovascular disease, bone disease, and non-AIDS related cancers remains high (6). HIV-associated inflammation has been correlated to the premature onset of these end-organ diseases (3).

Chronic activation of the immune system is one of the hallmarks of HIV infection. It serves to provide the virus with activated CD4+ T-cells. Immune activation predicts disease progression better than peripheral blood CD4+ T-cells or the viral load in plasma (4). The role of immune activation in the pathogenesis of non-AIDS clinical events such as cardiovascular, kidney, liver and bone diseases and neurocognitive decline (3), which are a major cause of morbidity and mortality in HIV-infected people on ART, is receiving increased recognition (18).

High levels of inflammation as persist in untreated HIV infection, as defined by elevated levels of inflammatory cytokines such as interleukin (IL) 1-β, IL-6, tumour necrosis factor alpha (TNF-α) and other acute phase reactants (C-reactive protein (CRP), cystatin-C, D-dimers) (3). The levels of these and other biomarkers decline during ART but remain higher when compared to HIV-negative cohorts (19).

Multiple factors are believed to be responsible for the persistent inflammation seen in HIV infection even during ART. These factors include HIV production and replication, co-pathogens such as cytomegalovirus (CMV), hepatitis B and C viruses (HBV and HCV, respectively), loss of immune regulatory cells, microbial translocation, and ART toxicity and lipodystrophy. Traditional metabolic risk factors are also implicated. These factors, either singly or in combination, cause

increased monocyte activation, T-cell activation and endothelial adhesion, dyslipidaemia and hypercoagulation (6).

2.3 MICROBIAL TRANSLOCATION AS A CONTRIBUTOR TO CHRONIC IMMUNE ACTIVATION IN HIV INFECTION

Chronic immune activation in HIV-infected persons is thought to be due to, among other mechanisms, the translocation of lipopolysaccharide (LPS) across damaged gut mucosa. LPS is a major cell wall component of Gram-negative bacteria (4).

2.3.1 Gut microbiota

The gastrointestinal lumen is a complex ecosystem; a direct consequence of the mutualism (i.e. both parties benefit) between the host and the microbiota (20). It involves interaction between members of the microbiome, the mucosal layer, the immune system (local, innate, and adaptive arms), and the enterocytes (21). A normal gut microsystem is fundamental for the maintenance of a healthy individual. Microbial colonisation of the human gut begins at birth, with the diversity and richness of the microbiota reaching the levels seen in adulthood in early childhood. The composition of the microbiota is influenced by maternal colonisation, diet, environmental exposures, and antimicrobial therapies (22).

Data suggest that the actual composition of the microbiota is essential to the health of the host. Altered microbiota (dysbiosis) has been reported in several disease states, including inflammatory bowel disease, type 2 diabetes, and HIV infection (23).

2.3.2 Prevention of gut microbiota from accessing systemic circulation

The intestinal lumen has a high load of bacterial antigens. These include LPS which has been shown to trigger septic shock. It is therefore imperative that the luminal antigens be excluded from tissues and the systemic circulation (23). In health, the epithelial layer and immune system function to reduce the risk of excessive translocation of microbes from the lumen into the systemic circulation (21).

Gut translocation of microbes is defined as the non-physiological passage of the gastrointestinal microbes/microbial products through the intestinal epithelial barrier and lamina propria and eventually to the local mesenteric lymph nodes and ultimately to extranodal sites, without overt

bacteraemia. It causes both local inflammation of the GIT microenvironment and systemic immune activation. It has been described in graft-versus-host-disease (GVHD), inflammatory bowel disease (IBD), and after invasive gastrointestinal surgery (4). Translocation of the microbes causes systemic immune activation and establishment of a pro-inflammatory state that has adverse effects on the immune system and health of the infected individuals (5).

There are several lines of defence against microbial translocation. At the GI surface, macromolecules such as the mucus layer, luminal immunoglobulin A (IgA) and antimicrobial defensins prevent attachment of, inhibit, and kill bacteria. The epithelial surface has intercellular tight junctions that prevent intercellular movement of microbes/microbial products.

There are specialised resident macrophages in the lamina propria which are able to recognise and phagocytose bacterial antigens without inducing an inflammatory response; they do not produce pro-inflammatory cytokines. If microbes/microbial products do enter the enteroportal circulation into the liver, the liver sinusoidal endothelial cells and the Kupffer cells are also able to clear the antigens without inducing an inflammatory response.

In the systemic circulation, there are immunoglobulin antibodies collectively named EndoCAb which target the LPS core antigen and neutralise LPS activity. EndoCAb levels are increased in chronic microbial translocation. Other soluble factors include soluble CD14, an LPS co-receptor, and LPS-binding protein (LBP), an acute phase reactant synthesised by the liver. CD14 is expressed by peripheral blood monocytes and tissue macrophages. On LPS stimulation, these cells secrete soluble CD14 and shed their surface CD14. Both CD14 and LBP bind LPS in the circulation and transfer it either to high density lipoproteins (HDL) to reduce its bioavailability, or to a receptor complex on monocytes/macrophages which causes LPS-mediated immune stimulation. These soluble factors circulate at high concentrations in healthy humans (4).

2.3.3 LPS and soluble CD14 in inflammation

Lipopolysaccharide is a potent biologic response modifier (24). LPS is a major component of the outer cell membrane of Gram-negative bacteria and it induces a host response which includes expression of pro-inflammatory cytokines and proteins (25).

CD14 is a multifunctional glycoprotein receptor. It is constitutively expressed on mature monocytes, macrophages, and neutrophils. It is important as a homing receptor for LPS, playing a key role in its neutralisation. Soluble CD14 (sCD14) is a marker of monocyte activation and is

abundant in serum. It is derived from both from the secretion of CD14 and enzymatic cleavage of anchored tissue CD14 (26, 27).

The lipid A moiety of Gram-negative bacteria has potent biologic properties. In the circulation, the lipid A moiety binds with high affinity to LBP and subsequently forms a ternary complex with sCD14. The binding with sCD14 enables LPS to be transferred to the LPS receptor complex comprising Toll-like receptor 4 (TLR4) and MD-2. Subsequently, TLR4 transduces signals for cellular activation. Among those is the nuclear translocation of nuclear factor-kappa beta (NF-κβ), which regulates production of pro-inflammatory cytokines (25). The sCD14/LPS/LPB complex binds to a variety of cell types, and may be internalised to initiate cellular responses, causing systemic inflammation (28).

2.3.4 Microbial translocation in HIV infection

The GIT is a major site of HIV replication. HIV-associated GIT pathology includes pronounced damage to enterocytes, with elevated levels of inflammation and decreased levels of mucosal repair and regeneration. This disrupts the immunological mechanisms that make up the mucosal barrier that prevent translocation of normal gut flora into the systemic immune system (29).

Microbial translocation in HIV infection results from several factors. There is early and severe mucosal depletion of CD4+ T cells, preferentially T helper-17 cells. Mucosal immune hyper-activation and persistent inflammation leads to injury to the intestinal epithelium integrity, with apoptosis of the enterocytes and disruption of the tight junctions. The composition of the gut microbiota is also disrupted, causing a predominance of opportunistic infectious agents (29).

In HIV infection, there is also decreased clearance of microbes/microbial products that find their way into circulation due to failure of GI macrophages to phagocytose all translocated bacterial products, decreased numbers of Kupffer cells, and decreased EndoCAb levels (21).

Luminal microbial products that could translocate include ribonucleic acid (RNA), deoxyribonucleic acid (DNA), peptidoglycan, flagellin, and LPS, all of which are potent immunostimulants. LPS can be quantitatively measured in plasma, and LPS levels are commonly used to assess the magnitude of microbial translocation in GVHD and IBD (4).

Other sources of LPS in the circulation could be other commensal and pathogenic bacteria from other mucosal surfaces and subclinical opportunistic infections. However, the GIT is the main source of microbial products because of it carried the largest bacterial load compared to other body

sites. Data from the landmark study by Brenchley *et al* showed that the GIT is the major source of plasma LPS in chronic HIV infection (4).

2.3.5 Clinical implications of microbial translocation in HIV infection

Data available strongly suggest that dysfunction of the GI mucosa and microbial translocation negatively impact progression of disease in HIV infection (30). Microbial translocation has been shown be an important contributor to the chronic immune activation now believed to the cause of non-AIDS co-morbidities seen in chronic HIV infection (4).

2.3.5.1 Clinical progression of HIV disease and poor response to antiretroviral therapy

Immune activation in HIV infection has been shown to be a major determinant of CD4 cell depletion. It is an important predictor of disease progression. Microbial products have emerged in recent times as one of the drivers of this continued immune activation (31). HIV positive persons with lower CD4+ cells counts, ART-experienced or not, have higher levels of LPS and sCD14 (18). Those whose CD+ T cells levels fail to recover during ART (immunologic non-responders) have higher levels of LPS which are linked with an increased frequency of activated CD4+ and CD8+ cells. High levels of sCD14 correlate with higher plasma viral loads, more activated CD4+ cells and lower nadir CD4+ cell counts (32). Lower nadir CD4+ cell count has been associated with faster progression to AIDS in HIV infected persons.

Data also suggest that LPS and sCD14 are independent predictors of disease progression and mortality (30).

2.3.5.2 Role of microbial translocation in non-AIDS co-morbidities

Persons with HIV infection are at increased risk of developing non-infectious co-morbidities, even while on effective ART (18). This is due to the enhanced immune activation and inflammation (5).

2.3.5.2.1.Cardiovascular disease

HIV infected persons are at higher risk for cardiovascular disease compared to their non-infected counterparts (33). Many studies in higher income countries have found higher rates of cardiovascular disease (CVD) in HIV-infected populations than in age-matched HIV-uninfected

populations (3). Evidence suggests that this is true even for low- and middle-income countries (16). A retrospective analysis of medical records of a large HIV treatment program in western Kenya carried out between 2006 and 2009 found a high prevalence of hypertension and overweight/obesity among HIV-infected patients, both well recognised cardiovascular disease risk factors. The prevalence of hypertension among was 11.2% among men and 7.4% among women and that of obesity was 10.6% and 22.6% among men and women, respectively (34). A study by Ngare in Kenyatta National Hospital (KNH) in 2009 found a period prevalence of hypertension of 12.9% in HIV-infected patients who were on ART and 14.3% in those not on ART. Among the patients with hypertension, dyslipidaemia was the most common cardiovascular risk factor at 71.3% (35).

The higher rates of CVD may be explained by, among other factors, the increased systemic inflammation seen in HIV infection (36). While several risk factors, either singly or in combination, determine individual cardiovascular risk, the chronic pro-inflammatory state seen in HIV infection may lead to vascular damage through various mechanisms (6). A growing body of evidence suggests that the chronic inflammation and immune activation and immunosenescence may contribute to development and progression of arteriosclerosis in HIV positive persons (3).

LPS and other gut microbial antigens are important drivers of this chronic immune activation (3). LPS and flagellin increase the expression of tissue factor on monocyte surfaces, causing a procoagulant effect which may initiate thrombosis (34). HIV-infected individuals with more arterial plaque deposition have been shown to have higher levels of sCD14 than those with normal carotid intima thickness (18).

2.3.5.2.2 Kidney disease

At the advent of the HIV epidemic, it appeared there were no major complications from the virus on the kidney. However, with rising numbers of HIV-infected and AIDS patients presenting with renal complications, early histopathological studies showed a variety of kidney lesions. HIV-associated nephropathy (HIVAN) was also described, with a predominance in black HIV positive persons and rapid progression to end-stage renal disease (ESRD) in those who were not on ART (37).

While the wide coverage of ART has modified the natural history of HIVAN, a wide spectrum of kidney disorders are still seen in HIV infection, causing both acute kidney injury (AKI) and chronic kidney disease (CKD) (37). This is due to a combination of:

- direct effects of the virus on the kidney;

- host genetic factors;
- comorbidities such as diabetes, hypertension;
- opportunistic and co-infections, e.g. HCV;
- medication toxicities: ART (tenofovir, indinavir), drugs used to treat opportunistic infections;
- immune dysregulation, and
- increasingly older population of HIV-infected patients (38).

The common causes of AKI in HIV infection are acute infections and drug-related nephrotoxicity. Chronic kidney disease is commonly caused by HIVAN, HIV immune complex kidney disease (HIVICK), and non-HIV causes such as diabetes, hypertension and vascular disease (39), incidences of which are increasing in the HIV infected population (40). CKD in HIV disease is associated with an increased progression to AIDS, greater risk for cardiovascular disease, and increased mortality, even in the post-HAART era (39).

In HIV infection, chronic immune activation results in production and increased levels of pro-inflammatory cytokines regulated by NF-kB (IL-6, TNF-alpha) and interferon regulatory factor (IRF)-7 (IFN-alpha), both in the kidney microenvironment and the systemic circulation (38). Renal tubular epithelial cells express Toll-like receptors (TLRs), including TRL-4, which binds LPS. These TRLs trigger immune responses within the kidney, and they are likely involved in many, if not all, types of renal inflammation (41). HIVICK causes immune-mediated glomerulonephritis. While data are inconclusive whether development of HIVAN is dependent on direct infection of renal cells (42), the systemic and local immune responses are thought to be important contributors to disease severity and/or progression (38).

ART mitigates but does not completely eliminate the risk of CKD in HIV infected persons (38), supporting the role of other causes of kidney injury other than direct viral effects.

2.3.5.2.3. Liver disease

Liver disease is emerging as an important cause of mortality in HIV positive persons, mostly from chronic viral hepatitis (43). In health, the liver detoxifies LPS from the circulation. LPS from the intestinal lumen translocated into the portal circulation is sensed and cleared by liver macrophages, Kupffer cells, through interaction with circulating LPS-binding protein (LBP) and cell surface CD14. In liver dysfunction LPS enters into the peripheral circulation to a greater extent. This in turn activates the Kupffer cells through Toll-like receptor-4 (TLR-4) signalling through the binding of LPS to LBP and CD14. Activation of TLR-4 results in generation of reactive oxygen species and both proinflammatory and profibrogenic cytokines. Through this interaction, LPS has been

shown to speed up liver fibrosis. Microbial translocation has been implicated in liver disease associated with alcohol, GVHD, and celiac sprue (43).

HIV co-infection with hepatitis viruses accelerates progression of liver disease (43). Microbial translocation has been linked to HIV infection, and is believed to exert an important role in the worsening of liver disease in HIV-infected individuals (4, 43-45).

2.3.5.2.4. Metabolic bone disease: osteopenia and osteoporosis

With an ageing HIV-infected cohort due to more effective ART, bone complications may worsen due to increasing age and longer HIV infection/disease. People living with HIV are susceptible to reduced bone mineral density (BMD) (osteopenia and osteoporosis), osteomalacia (impaired mineralization on the bone matrix), and osteonecrosis (bone death due to poor blood supply) (46). In HIV infection, multiple factors play a role in bone disease: low vitamin D levels, lipoatrophy, ART-related factors (47), and consequences of chronic HIV infection, including undernutrition and chronic inflammation (48).

Changes in BMD are frequently associated with pathologic conditions characterised by immune dysregulation and chronic inflammation such as rheumatoid arthritis, inflammatory bowel disease and systemic lupus erythematosus (49). Osteoclasts are responsible for bone resorption, are of myeloid origin. They express the receptor activator of NF-κβ (RANK). Association of RANK with its ligand (RANKL) on osteoclast precursors induces their differentiation into pre-osteoclasts and ultimately mature osteoclasts. Pro-inflammatory cytokines including TNF-α, IL-1, -6, -7, -17, IFN-γ are able to up-regulate osteoclastogenesis through mechanisms involving RANK and RANKL (49). These cytokines are increased in chronic HIV infection (3). LPS itself stimulates osteoclastogenesis by promoting osteoblasts to produce RANKL, IL-1 and TNF-α (49).

While bone disease in HIV is multifactorial, immune dysregulation appears to be play a pivotal role. It is therefore plausible that controlling the chronic immune activation may improve bone health in persons living with HIV.

2.3.5.2.5. Non-AIDS related cancers

Cancers are an important cause of morbidity and mortality in HIV-infected persons (50). They may be either AIDS-defining (Kaposi's sarcoma, cervical cancer, non-Hodgkin lymphoma) or non-AIDS defining (51). ART has led to declining incidence of AIDS-defining cancers (ADCs), but that of non-AIDS defining cancers (NADCs) has been increasing, a trend observed in both the developed and developing countries**.** NADCs whose incidence is higher in the HIV-infected

population include Hodgkin lymphoma, leukaemia, colorectal, lung and renal malignancies (52). The chronic inflammatory state in HIV-infection, driven in part by microbial translocation, is believed to be a contributing risk factor to development of NADCs (53).

Chronic inflammation, whether due to infectious or noninfectious causes, is now linked to development of several cancers, including colorectal and liver cancers (54). The inflammatory cytokines TNF-α, IL-1β, and IL-6, which have shown convincing tumour-promoting activity in various animal models (55), are elevated in HIV infection even during effective ART (19). These cytokines are part of the TNF superfamily, and are mainly regulated by NF-κβ (56). LPS activates NF-κβ through its binding to TLR-4.

The risk factors contributing to the increased incidence of NADCs are complex and multifactorial, and warrant further investigation. However, it is becoming apparent that chronic inflammation plays a key role, and might be a potentially modifiable risk factor in HIV infection.

2.3.5.2.6. Neurological Disease

Increased trafficking of monocytes into the brain is associated with development of HIV-associated neurological disease in the absence of other infectious causes. Activated monocytes contribute to the pathogenesis of HIV-associated dementia (HAD) and minor cognitive motor disorder by various mechanisms:

- carrying the virus into the central nervous system (CNS),
- supporting productive infection (production of complete viral particles capable of infecting other cells) upon differentiation into macrophages, and
- production of neurotoxic factors (57).

An increased frequency of activated and primed CD16+/CD69+ monocytes was associated with HAD in the pre-ART era. However, neurocognitive impairment still occurs in 10-20% of AIDS patients despite effective ART reducing both the frequency of activated monocytes and levels of HIV in the brain (57).

It has been hypothesised that immune activation continues to activate monocytes despite ART. Elevated plasma LPS secondary to microbial translocation triggers monocyte activation via CD14 and TRL-4-mediated signalling pathways. A study looking at LPS levels in patients who had AIDS found that LPS levels were higher in patients who had HAD compared to controls, and it was associated with HAD irrespective of CD4 counts or plasma viral load (58).

2.4 MICROBIAL TRANSLOCATION IN HIV-INFECTED AFRICAN POPULATIONS

Data from Africa show that immune activation is present in HIV-infected individuals, and is associated with disease progression (59). However, data on the contribution of microbial translocation to this chronic immune activation are conflicting, as summarised in Table 1 below:

Table 1. Studies on microbial translocation in HIV-infected populations in SSA

Year	Author	Study design	Study population	Main findings
2009	Redd, A. D., *et al* (56)	Longitudinal	- HIV-1 positive ART-naive adults (107) from a community cohort in Rakai, Uganda - HIV-1 negative American controls (24) from Johns Hopkins Emergency Department	- Levels of LPS, EndoCAb, and sCD14 in HIV-infected Africans remain relatively stable throughout disease progression
2009	Lester, R. T., *et al* (57)	Cross-sectional	- HIV uninfected (31) women - HIV-infected (57) women on ART-experienced (19) and ART-naive (38) - All subjects from the Pumwani sex-worker cohort in Nairobi,Kenya	- Significantly increased LPS levels were associated with chronic HIV-1 infection both treated and untreated - LPS levels were not associated with other acute or semi-acute conditions reported
2010	Cassol, E., *et al* (58)	Cross-sectional	- HIV-1 infected ART-naive Africans with and without OIs (60) - HIV-1-infected ART-experienced adults (20) - HIV-uninfected healthy adults (10) f - All subjects from Tshwane District Hospital, Pretoria, South Africa	- sCD14 and TNF levels correlated with LPS levels before and during ART, respectively, and, like LPS, remained persistently elevated even after >1 year of successful therapy

ART: antiretroviral therapy; EndoCAb: endotoxin core antibody; HIV: Human Immunodeficiency virus; LPS: lipopolysaccharide; sCD14: soluble CD14; TNF: tumour necrosis factor

(60-62)

2.4.1. Potential role of parasitic infections in Africa

Helminth infestation is extremely common worldwide and carries the greatest burden of neglected tropical diseases (NTD) in SSA (63). Given the high prevalence of HIV/AIDS in SSA, and that helminth infection has a broad impact on the immune system, the link between the two is being explored . The contribution of an impaired intestinal mucosal barrier to bacterial translocation and immune activation has already been extensively explored in HIV infection. There is a clear possible connection between this phenomenon and helminths which infect the intestines (63, 64).

Helminth infestation is associated with chronic immune activation which may facilitate and influence the course of other infections, including HIV. Chronic helminth infection is associated with immune responses that may suppress anti-HIV immune responses (65, 66). The associated chronic immune activation may also cause a more rapid decrease in CD4+ T-cells in HIV infection (67).

Primate studies show that infection with both *Schistosoma mansoni* and simian human immunodeficiency virus (SHIV) results in lower CD4 counts and higher viral loads than infection with SHIV alone (68). Studies in humans have however had varying results. Some studies have found significant increases in CD4 counts and reduction in HIV RNA on deworming the subjects. Other studies have found no effect or even an increase in HIV RNA and decrease in CD4 count (67).

There are insufficient data to conclude how helminth infection affects our HIV epidemic. It is plausible that chronic helminth infection may lead to higher levels of endotoxin core antibody which neutralises LPS (61).

2.5 THERAPEUTIC OPTIONS FOR PREVENTION OR ATTENUATION OF MICROBIAL TRANSLOCATION

Focus in HIV treatment directed at developing therapies that stem the excessive immune activation may eventually modify the current course of HIV infection (68). Strategies that target microbial translocation are currently being evaluated.

2.5.1 Antiretroviral therapy

HIV-infected persons on ART have lower levels of LPS and sCD14 than their counterparts who are not on treatment, albeit higher when compared to their non-infected counterparts (62). ART leads to almost complete reconstitution of intestinal CD4+ cells when initiated during primary HIV infection but only partial reconstitution when started during the chronic phase of infection. However, ART still decreases microbial translocation, possibly by the partial recovery of T-h17 cells and improvement of LPS clearance mechanisms (e.g. increased Kupffer cells) (18).

2.5.2 Pro- and Prebiotics

Gut commensals have been shown to have a role in protecting the intestinal barrier and in the health of the individual (23). Therapies that correct the dysbiosis seen in HIV infection may therefore have possibly reduce microbial translocation. These include administration of either pre- or probiotics.

A *probiotic* is defined as a preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonisation) in a compartment of the host and by that exert beneficial health effects in this host (69).

A *prebiotic* is a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon (69).

In the "COPA" trial, a prebiotic oligosaccharide mixture given to ART-naive HIV-infected patients significantly improved microbiota. Bifidobacteria, which has no LPS and has been shown to have anti-inflammatory activity (18), was increased, and there were decreased numbers of pathogenic groups of bacteria. Lower levels of sCD14 and activated CD4+ T-cells, and improved natural killer cell activity were also demonstrated (70).

Klatt demonstrated less inflammation-induced scarring and more CD4+ T cells and antigen presenting cells in the gut of SIV-infected pigtails macaque monkeys given both probiotics and HAART than those given HAART alone (71). HIV infected women in Nigeria who were ART-naive and had moderate diarrhoea given probiotic-treated yoghurt reported improved quality of life and no decline or rise in peripheral blood CD4 counts compared to their counterparts who got non-supplemented yoghurt (72). Safety of probiotics has been evaluated and they are generally well tolerated, in addition to being inexpensive (73).

Further investigation is warranted, but these data suggest that pre- and probiotics may be promising as adjunctive treatments for HIV infection.

2.5.3 Agents under investigation

Sevelamer is a drug used in CKD to lower serum phosphorus. It has also been found to decrease LPS levels by up to 80% in subjects undergoing haemodialysis for ESKD (18). Its proposed mechanism of action is by binding chylomicron-LPS complexes and preventing their reabsorption (74). In this population, sevelamer also lowers level of sCD14, IL-6, and LDL cholesterol. However, a recent prospective clinical trial (ACTG A5296) found that sevelamer had no effect on either LPS or sCD14 levels in HIV infected individuals who were not on ART. It however resulted in decreases in LDL cholesterol, oxidised LDL cholesterol and serum tissue factor levels. These findings suggest a net cardiovascular benefit (74).

Mesalamine (5-aminosalicylic acid) reduces mucosal inflammation in mild to moderate ulcerative colitis. Initial trials however show no significant reduction in microbial translocation in HIV infected subjects (75).

Rifaximin is an orally administered antibiotic with little systemic absorption that targets Gram positive, Gram negative and anaerobic bacteria. It reduces LPS levels significantly in patients with liver cirrhosis (18). The drug minimally affected microbial translocation in HIV infected subjects in a recently published trial (76).

Agents that target downstream effects of LPS are also under investigation. *Chloroquine and hydroxychloroquine* block NF-κβ, thus inhibiting signalling through several TLRs, including TLR4. Data on the effect of these antimalarials in HIV infection have been varied. Pilot studies showed positive results on reduction of inflammatory markers. More recent clinical data show non-remarkable effect (18, 77). *Monoclonal antibodies* directed against cytokines and cytokine signalling could reduce the immune activation driven by microbial translocation by interfering with downstream microbial product signalling (18).

Many of these studies have had small patient numbers, and the results may therefore not be generalisable. Further investigation is therefore warranted, and is ongoing.

CHAPTER THREE: STUDY JUSTIFICATION

More HIV infected patients are living longer. These patients are developing and succumbing to non-AIDS related illnesses. These diseases are occurring in younger persons than would typically be expected. They are contributing to an increase in morbidity and mortality in the HIV-infected population, as well as increasing health-related costs in low- and middle-income countries. This increased incidence of non-HIV related NCD is thought to be due to, among other factors, the chronic immune activation that is a hallmark of HIV infection. Gut pathology causing translocation of microbes into the peripheral blood stream is one of the important drivers of this chronic systemic activation of the immune system.

Genetic factors influence the development and composition of the gut microbiome and its interaction with the immune system. Regional differences also exist in the composition of gut microbiota and endemic gut parasites. Thus, the contribution of microbial translocation to chronic immune activation in different populations and regions may differ. Local studies to determine the prevalence of microbial translocation in our population are therefore needed. If present, various interventions are available to prevent or reduce microbial translocation in HIV-infected patients. These, in turn, would reduce the chronic inflammation and ultimately, the incidence of non-HIV related NCDs. In addition to informing on the burden of chronic inflammation from microbial translocation, data collected from these local studies can be helpful in efforts to develop novel monitoring and treatment modalities for HIV-infected persons in the region.

3.1 RESEARCH QUESTION

How does occurrence of microbial translocation compare between HIV-infected versus HIV-uninfected persons?

3.1.1 HYPOTHESIS

There is no difference in the occurrence of microbial translocation between HIV-infected versus HIV-uninfected persons.

3.2 OBJECTIVES

3.2.1 Broad objective

To determine the occurrence of microbial translocation using LPS and sCD14 in HIV-infected patients attending the Comprehensive Care Clinic at Mbagathi District Hospital compared to age-matched HIV-uninfected adults attending the voluntary counselling and testing centres at Mbagathi District and Kenyatta National hospitals.

3.2.2 Specific objectives

1. To determine levels of LPS and soluble CD14 in HIV-infected and uninfected participants.
2. To compare the levels of LPS and soluble CD14 in HIV-infected and uninfected participants.

CHAPTER FOUR: STUDY METHODOLOGY

4.1 STUDY DESIGN

The study design was a 1:1 age-matched cross-sectional comparative survey. The outcome, which was to compare the prevalence of microbial translocation between ambulatory HIV-infected patients attending Mbagathi District Hospital Comprehensive Care Clinic and age-matched HIV negative respondents attending the Voluntary Counselling and Testing Centres at both Mbagathi and Kenyatta National Hospitals was assessed at the same time.

4.2 STUDY SITE

Mbagathi District Hospital (MDH) is a level V public hospital operated by the Ministry of Health located in the metropolitan city of Nairobi, Kenya. MDH has a large catchment population. The hospital offers both inpatient and outpatient services. The Comprehensive Care Clinic (CCC), established in 1997, is part of the outpatient service and offers, among other services, HIV counselling and testing, and treatment and follow-up of HIV-infected persons.

The MDH CCC has over 4,000 active patients on ART, most of whom are adults. An average of 70 new patients per month are enrolled. The CCC runs on Monday to Friday from 0800h to 1700h, with an average daily attendance of 60-80 patients. Though the official opening time is 0800 hours, clients can be attended to from as early as 0630h.

The Voluntary Counselling and Testing (VCT) centre at MDH is open Monday to Friday from 8.00am to 5.00am. On average 14 patients are tested every day, with a HIV positivity rate of approximately 9%.

Kenyatta National Hospital is a parastatal and one of two national teaching and referral hospitals in Kenya; offering both inpatient and outpatient services. It is located 2 km away from MDH in Nairobi. It serves the same catchment population as MDH, but also caters to referrals from the entire country. The VCT centre at KNH runs from Monday to Friday from 8.00am to 5.00pm. An average of 50 clients are tested per week, with 2–3 clients testing positive for HIV per week. The VCT centre has a separate department for youth (10–24 years) which does more robust testing: 25–40 clients per day. Seropositivity in this department is rare; about 1 in 100 clients.

4.3 STUDY POPULATION

All patients enrolled in the MDH CCC or attending the VCT centres at MDH and KNH:

- HIV-infected participants were drawn from HIV infected persons attending the MDH CCC.
- HIV-uninfected participants were drawn from HIV uninfected persons attending MDH and KNH VCT centres.

4.4 PATIENT SELECTION

4.4.1 Case definition

- HIV-infected: Adult out-patient (≥18 years) attending MDH CCC with a positive HIV test (rapid or ELISA) on record.
- HIV-infected on ART: Adult out-patient ≥18 years attending MDH CCC with a positive HIV test (rapid or ELISA) on record, and using combined antiretroviral drugs.
- HIV-uninfected: Adult out-patient (≥18 years) attending the KNH/MDH VCT with a negative rapid HIV test on record; age matched to HIV-infected participants to within 5 years.

4.4.2 Inclusion and exclusion criteria

Table 2: Inclusion and exclusion criteria for study subjects

Inclusion Criteria	Exclusion Criteria
Age ≥18 years	Indeterminate or missing HIV test results
Documented positive HIV results (rapid test or ELISA) for HIV-infected participants	Known liver disease[1]
Documented negative HIV results (rapid test or ELISA) for HIV-uninfected participants	
Written informed consent	

[1] *LPS is detoxified in the liver. Hepatic dysfunction leads to an increase in LPS levels which may be a result of the hepatic dysfunction rather than a cause of it (39).*

4.5 SAMPLE SIZE ESTIMATION

The formula for comparing means between groups was used to compute the sample size (Epidemiology: Study Design and Data Analysis by Mark Woodward):

$$n = \{(r + 1)^2 (zalpha + zbeta)^2\} \sigma^2 / rD^2\} \pi r^2$$

Where

- r = ratio of sample in each group =1
- zalpha= 1.959964
- zbeta = 1.281552
- σ (pg/mL) = 6
- D (difference between HIV+ and HIV- groups) = 4

Giving

n = 94.56681 which is **47** patients per group.

Values from Sandler NG, Wand H, Roque A, Law M, Nason MC, Nixon DE, et al. Plasma levels of soluble CD14 independently predict mortality in HIV infection. J Infect Dis. 2011;203(6):780-90 (32). (See Table 1 from the study in Appendix 1.)

4.6 SAMPLING METHOD

Simple random sampling was utilised to identify the HIV-infected participants up till the required sample size was met. Convenience sampling was then used to recruit HIV-uninfected participants (to within five years of age of the recruited HIV-infected participants) until the desired number was reached.

4.7 PARTICIPANT RECRUITMENT

HIV-infected participants

Clinicians working at the MDH CCC were alerted to the study and they helped refer suitable participants to the Principal Investigator (PI) after their clinic visit was over. The PI and research assistant also alerted the clients in the waiting room of the on-going study and encouraged them to come determine if they were eligible. Participants who were eligible were given all the relevant information on the study in their preferred language (English or Kiswahili) and any queries raised were addressed to the participants' satisfaction. Those who gave the written informed consent were recruited into the study.

HIV-uninfected participants

On attaining the required number of HIV-infected-participants, their ages were reviewed and the appropriate ages of the participants were determined. Staff at the VCT centres at both MDH and KNH were alerted to the study and requested to refer eligible clients to the PI for recruitment into the study.

4.8 DATA COLLECTION

4.8.1 Clinical methods

Once written consent was given, the first part of the study proforma was filled by the PI and or RA (Appendix 3). The medical records of the HIV-infected participants were reviewed to obtain information on duration of illness, duration the patient has been in care, WHO stage, latest CD4 counts, ART use and duration, and latest plasma viral load levels. This information was recorded on the study pro forma.

The PI and RA used an aseptic technique to draw blood samples for evaluation (sCD14 and LPS).

4.8.2 Laboratory methods

4.8.2.1 Specimen collection, transportation, storage

Blood was collected from each participant from the antecubital fossa or the dorsal surface of the hand using the BD Vacutainer® Eclipse™ blood collection set to minimise contamination. For HIV-infected participants who needed blood drawn as part of their clinic visit, both samples were collected at the same sitting to the same reason and to minimise participant discomfort. For study purposes, a total of 8 mL of blood was drawn into clearly labelled vacutainers: 4 mL of blood into a

sterile plain one and 4 mL into a sterile EDTA-coated vacutainer. Cooler boxes with ice packs at approximately 4^0C (2 - 8^0C) were used to temporarily store the samples and facilitate transport to the laboratory. Samples were transported to the laboratory no more than 4 hours after collection.

All samples were centrifuged using a Rotana 460R centrifuge at 1800RPM for ten minutes to separate the serum from the packed cells in the plain collection tubes and to obtain plasma from the EDTA-treated collection tubes.

Each serum and plasma sample was split into two serum vials and were stored frozen at -80 degrees Centigrade awaiting assaying as a batch.

Aseptic techniques were strictly observed at all times.

4.8.2.2 Specimen Analysis

sCD14

Soluble CD14 levels were determined using the quantitative Thermo Scientific™ Pierce™ Human CD14 ELISA Kit. Using the principal of the enzyme-linked immunosorbent assay, an enzyme (Streptavidin-HRP Reagent©) was linked to the known antigen (Biotinylated Antibody Reagent©). The enzyme-linked substrate was then reacted. The enzyme's substrate (TMB Substrate©) was then added and the enzyme activity is then assayed as a colour change detected by a spectrophotometer and the absorbency was recorded. The test was done on the Tecan Infinite 200 ELISA Reader ™ at KAVI-ICR laboratory. A standard curve was generated and results were calculated using Microsoft® Office Excel™ software for Windows® 10. For the comprehensive laboratory protocols and interpretation of results, please refer to Appendix 5.

LPS

LPS was determined using the quantitative Thermo Scientific™ Pierce™ LAL Chromogenic Endotoxin Quantitation Kit. The test uses modified Limulus Amoebocyte Lysate (LAL). The bacterial endotoxin catalyses the activation of a proenzyme in the LAL which in turn induces the splitting of the yellow p-Nitroaniline from a colourless compound. The chromogenic pNA is then measured, with the colour intensity being proportional to the amount of endotoxin in the test sample. The ELISA reader Boitek X 808™ was used at the ConnectAfya Medlinks Laboratories under supervision by KAVI-ICR technicians.[1] A standard curve was then generated from which the

[1]The LPS test needed a 37ºC incubator bath for approximately thirty minutes which the Biotec X 808™ at Medlinks has the capability to do, unlike the Tecan Infinite 200 ELISA Reader™ at KAVI-ICR.

the results were calculated using Biotek Gen 5™ software. For the complete laboratory protocols and interpretation of results, please refer to Appendix 4.

4.9 QUALITY ASSURANCE

The study protocols were adhered to at every step.

Only eligible participants were recruited into the study.

HIV-infected participant patient data was retrieved from patient charts by both the PI and RA and double-checked before recording onto the study pro-forma and again when entering it into the database. Any inconsistencies were immediately clarified and corrected.

The standard recommended procedure for specimen collection was followed. Specimens were clearly and properly labelled with timely delivery to the laboratory in a cool box with ice packs at 4-8^{o}C where they were immediately processed and frozen to minimise pre-analytical errors.

The manufacturers' guidelines were adhered to when running the tests. The KAVI-ICR and Medlynks laboratories run daily quality controls on their equipment. Tests done at Medlynks were overseen by technicians from KAVI-ICR who were conversant with the study protocol. Controls were run on every batch of samples before generation of the required standard curves, and these were also counterchecked before publishing of the final results.

The PI's supervisors offered guidance throughout the entire process.

4.10 STUDY VARIABLES

4.10.1 Independent variables

Age: this was recorded in years according to participants' self report.

Sex: noted as male or female as determined from the participant's phenotypical secondary sexual characteristics

HIV status: was recorded as positive or negative based on a rapid test or ELISA test in the participant's chart for the HIV-infected participants and VCT records for the HIV-uninfected.

WHO HIV stage: the clinical disease staging (1–4) according to WHO guidelines was determined from the HIV-infected participant's chart as the latest recorded staging.

Antiretroviral drugs: the drugs and their duration of use in years was retrieved from the patient's file. Any change of drugs was noted as well as the reason for the change. The total duration of ARV drug use (all regimens) was also recorded.

CD4 cell count: number of CD4+ positive T-cells per micrometer in peripheral blood. Where available, two values were taken—the initial CD4 count at enrolment, and the most recently recorded in the participants's file.

Plasma viral load: number of copies of the virus per microlitre in peripheral blood; the latest recorded in the patient's file.

Marital status: was self reported as married, single, separated/divorced, or widowed.

Level of education: self-reported highest level of education achieved--primary, secondary, or post-secondary level.

4.10.2 Test variables

1. **Plasma LPS:** was determined using a chemiluminescent assay (Thermo Scientific™ Pierce™ LAL Chromogenic Endotoxin Quantitation Kit) in endotoxin units per millilitre (Refer to appendix 4 for the laboratory protocols).
2. **Soluble CD14**: was determined from serum using a commercially available ELISA kit (Thermo Scientific™ Pierce™ Human CD14 ELISA Kit) in picograms per millilitre (refer to appendix 5 for the laboratory protocols).

The above markers are currently only used in research settings and not for clinical purposes. There are therefore no established cut off or normal value ranges for either of the two.

4.11 ETHICAL CONSIDERATIONS

The study was undertaken only after approval by the Department of Clinical Medicine & Therapeutics, University of Nairobi and the KNH/UoN Scientific and Ethical Review Committee (ERC approval number P314/05/2018) and Mbagathi District Hospital. Authorisation from the hospital administration as well as the heads of the respective units (CCC and VCT) at both Mbagathi District and Kenyatta National Hospitals was obtained prior to commencement of the study.

The objectives and purposes of the study were clearly explained to eligible participants in the language that was preferable to them-English or Kiswahili. Those who agreed to participate in the study were provided with a written copy of the study details to keep for continued reference. They were also given a written consent form which they signed in duplicate (signature or thumbprint for those who were not able to write) with one copy given to the participants and one retained for the

PI's records. Only participants who give written informed consent were enrolled. Participants were informed that they were free to withdraw consent during the study period without discrimination.

There was no inducement or reimbursement offered for participating in the study.

The study posed minimal risk to the participants. The drawing of blood did not pose any more risk than that encountered in routine medical care. It was explained that there may be slight pain and minimal bleeding from the puncture site which a few participants experienced but with spontaneous and prompt resolution.

Only blood samples intended for study were drawn and were processed and thereafter discarded after analysis according to the KAVI-ICR standard operating procedures.

Participant confidentiality was maintained at all times. The data collection form and specimen labels had unique study numbers and no participant identifiable data, which was only available to the PI, stored in a password protected database. The statistician was provided with de-identified data.

On completion of the study, each participant will be provided with a copy of the their respective sCD14 and LPS results accompanied by a simplified report to explain the results. A copy of the results will be provided to the participants who request for the same. A copy of this complete study will also be provided to the relevant UoN, KNH, and MDH management.

All costs pertaining to the study were met by the PI.

4.12 DATA MANAGEMENT

4.12.1 Data collection, entry, and validation

Only data relevant to the study was collected, with strict adherence to the study protocol. Data was collected on a paper-based form. The PI and RA then confirmed that the forms were correctly and completely filled before entry into a password protected iWork Numbers™ file. The electronic data was then compared with the paper-form data to ascertain accuracy. For analysis, data was entered into a password-protected electronic database managed by the statistician. Inconsistencies in the data were detected by use of simple frequencies and correlations. And were corrected prior to commencement of analysis of the data.

4.12.2 Data handling

All collected data was kept confidential and was only accessible to the PI and research assistant, statistician, and the PI's supervisors. Only the PI had access to the participant identifying data.Data

capture forms were kept in a sturdy box file which was with the PI at all times and was be stored in a locked cabinet in the PI's residence at the end of the day to which only the PI had access. Electronic data was stored in a password-protected iWork Numbers™ file. The PI took responsibility for the restriction of access to these data.The statistician was only provided de-identified data for analysis.The data capture forms will continue to be stored safely as per the department's specifications for a period of 5 years, and shall remain available to the department for scrutiny if deemed necessary.

4.12.3 Data analysis

Data was statistically analysed using IBM® SPSS® Statistics v23 for Windows™.

Continuous data-LPS and sCD14 levels-were summarised using measures of central tendency (mean with standard deviation (SD), median, interquartile range (IQR)). Participants' age, duration of ARV use, and CD4 counts were summarised using mean±SD. Nominal variables-participants' sex, level of education, martial status, type of ARV regimen and combination therapies, as well as category of plasma viral load (undetectable, <1000 copies/mL, ≥1000 copies/mL)-were summarised using counts and proportions (percentages).

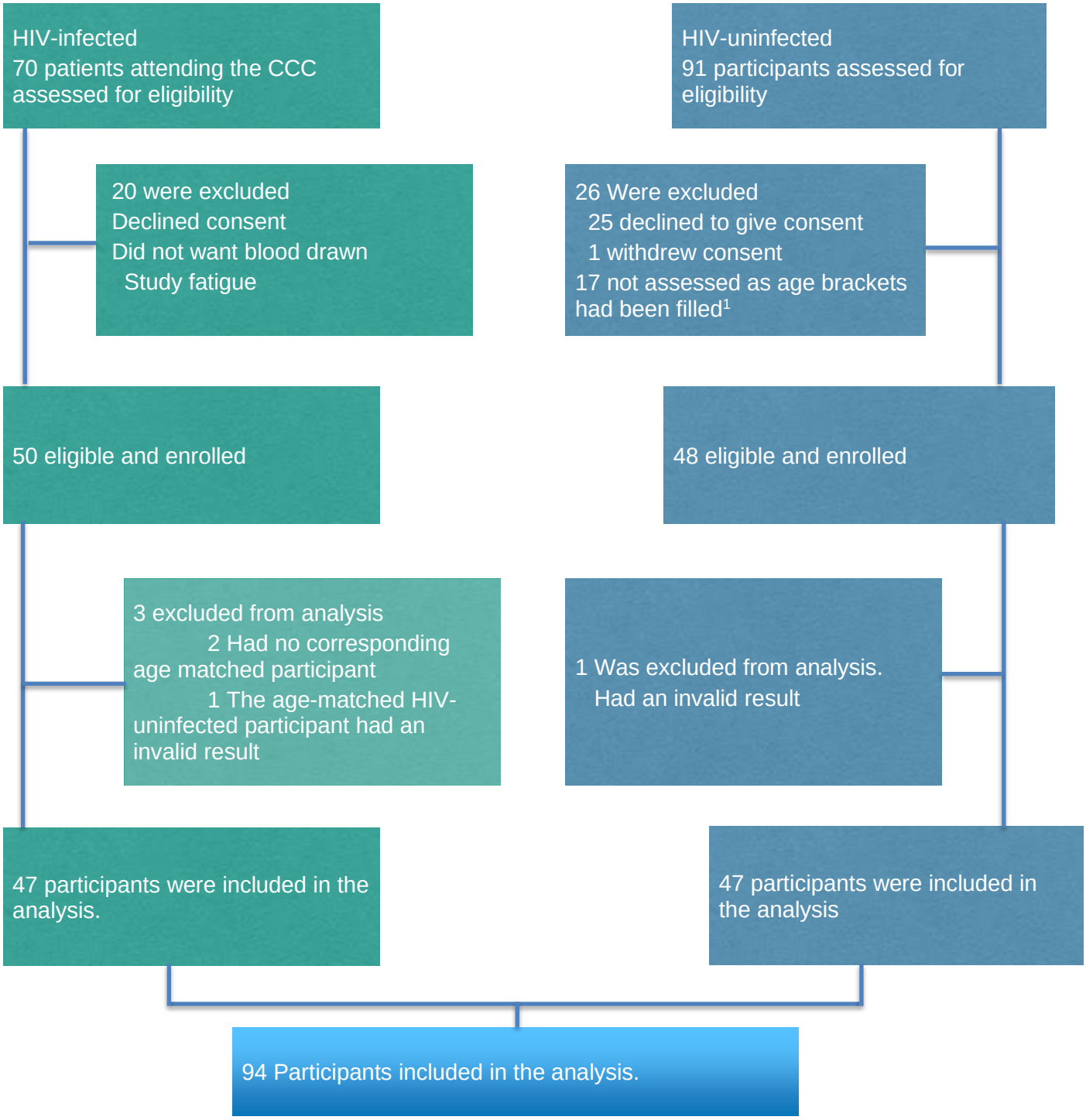
The Wilcox signed-rank paired difference test was performed on for each of the test variables to compare their means between the two participant groups.

Logistic regression was employed to determine the correlation between LPS and sCD14 levels, but the results were inconclusive.

CHAPTER FIVE: RESULTS

This was a 1:1 age-matched cross-sectional comparative study. HIV-infected persons were enrolled form the HIV-infected adults seeking care at the Mbagathi District Hospital CCC while HIV-uninfected were selected from the VCT centres at both Mbagathi District and Kenyatta National hospitals.

Figure 1: Enrolment of Study Participants



[1]Since the age-match was to within five years, the staff at the VCT centres were given ranges of eligible ages of HIV-uninfected participants; all interested participants in the specified age range would be referred to the PI. Once the predetermined number of participants for a specific age range was met, the remaining eligible participants in that age range were not assessed further.

Table 3: Demographic Characteristics of Study Participants

	HIV-infected (N = 47)	**HIV-uninfected (N = 47)**
Characteristic		
Age (years, mean ± SD)	47.5 ± 9.0	47.7 ± 9.8
Sex (N, %)		
Female	24 (51.1)	31 (66)
Male	23 (48.9)	16 (34)
Marital Status (N, %)		
Single	8 (17)	9 (19.1)
Married	30 (63.8)	32 (68.1)
Separated/Divorced	4 (8.5)	4 (8.5)
Widowed	5 (10.6)	2 (4.3)
Education (N, %)		
Primary	18 (38.3)	0
Secondary	23 (48.9)	24 (51.1)
Post-secondary	6 (12.8)	23 (48.9)

There was a significantly higher number of female respondents in the HIV-uninfected group compared to the HIV-infected group. This was due to more male participants declining the HIV test.

The clinical characteristics of the HIV-infected participants were as below.

Table 4: Clinical Characteristics of HIV-infected Participants

Characteristic		
Duration of ARV use (years, mean ± SD)*	***N = 47***	12.9 ± 3.6
ARV regimen (N, %)**	***N = 47***	
First line		29 (62)
Second line		18 (38)
Combination therapies***	***N = 47***	
2NRTI+1INSTI		34
2NRTI+1PI		6
2NRTI+1NNRTI		7
Plasma viral load (copies/mL, N, %)§	***N=46***	
Undetectable		42 (91)
<1000		3 (7)
≥1000		1 (2)
CD4 count (copies/µL)¶		
At time of diagnosis (mean ±SD)	***N = 42***	172.89 ± 150.45
Latest (mean ± SD)	***N = 41***	481.17 ± 201.95

*This was the total duration of ARV use since initiation. All participants had been on their current regimens for over two (2) years except for 2 (<6 months; <1 year)

**85% (40) of the participants had had their drug regimens changed since initiation, but only 43% (17) of these were switched to second line regimens following virologic failure of their first line regimen. The rest had single drug switches following drug toxicity and/or optimisation of their first line regimens according to the current evidence based regimens.

***All participants were on standard regimes as recommended by the Ministry of Health (78)

§Plasma viral loads are done at least every 6 months; one participant had been on ARVs for <6 months therefore had no viral load done yet.

¶ CD4 counts are not done as part of routine evaluation following a change in the guidelines in monitoring of patients while on ARVs. Patients enrolled in care after 2015 did not have CD4 counts on file.

ARV: anti-retroviral; INSTI: integrate strand transfer inhibitor; NNRTI: non-nucleoside reverse transcriptase inhibitor; NRTI: nucleoside/nucleotide reverse transcriptase inhibitor; PI: protease inhibitor.

Markers of microbial translocation

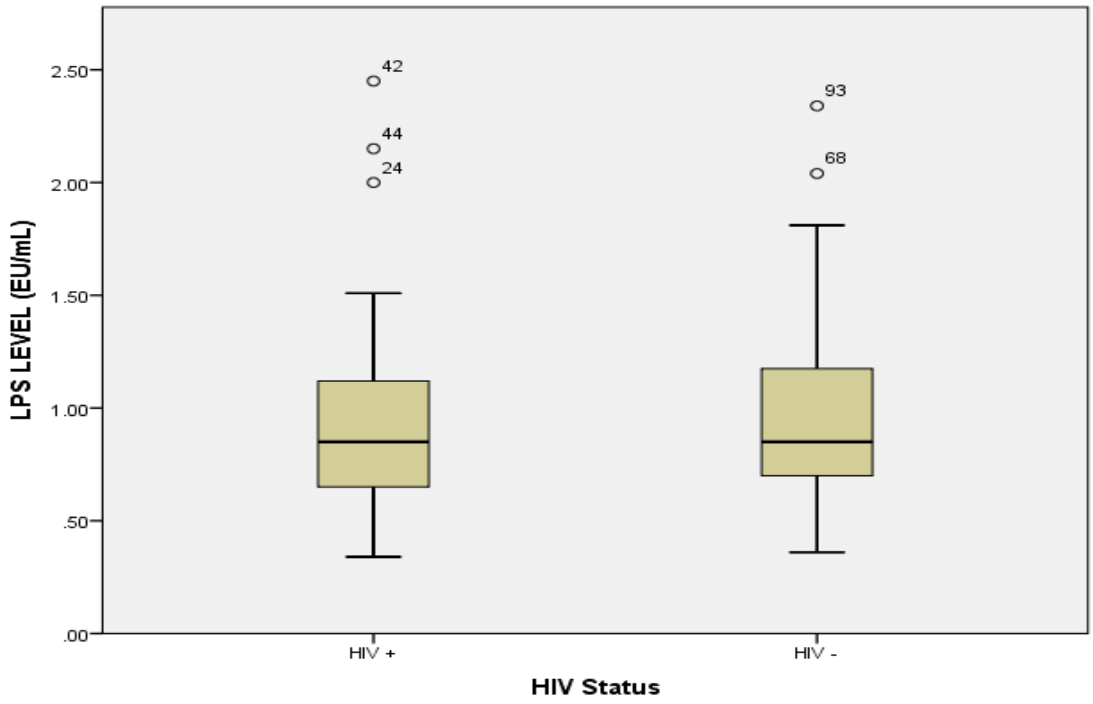


Figure 2: LPS levels in Study Participants

There are no established reference ranges for LPS in health. It can be measured qualitatively (present or absent) or quantitatively. This study did a quantitative assessment of the plasma LPS in study participants.

As demonstrated in the box plot above, the median LPS levels were similar across both groups: 0.85 EU/mL, with IQR 0.65–1.12 EU/mL for the HIV-infected and 0.7–1.18 EU/mL for the HIV-uninfected group. The mean LPS levels were 0.95±0.43 EU/mL for the HIV-infected and 0.98±0.42 EU/mL for the HIV-uninfected group. LPS levels showed more variability in the HIV-uninfected group.

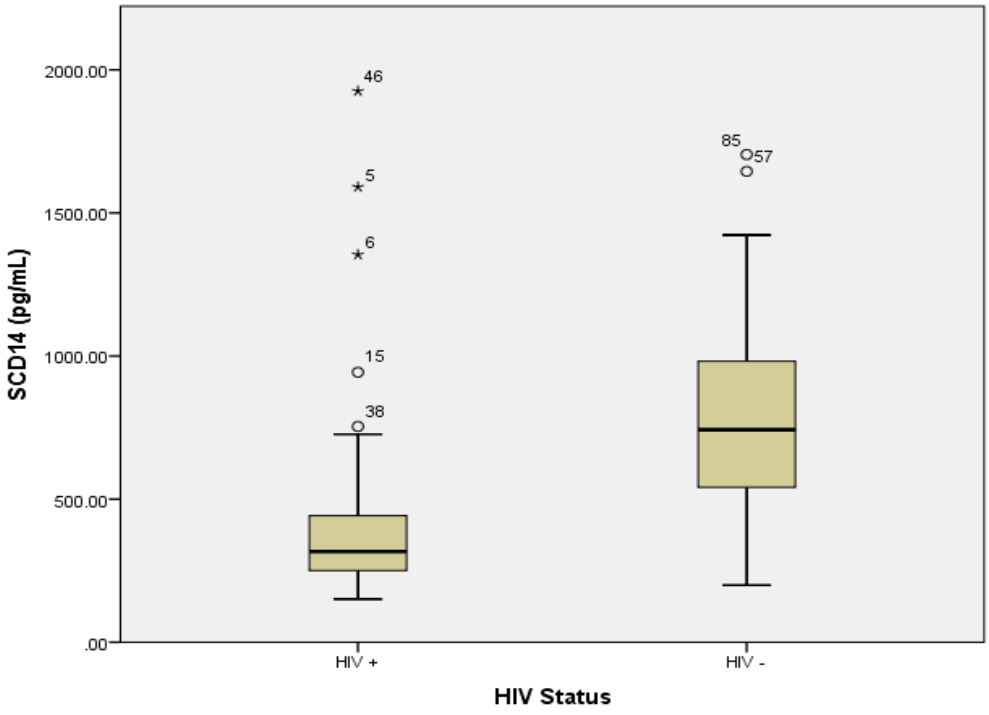


Figure 3: Soluble CD14 levels in Study Participants

There are no established reference values for sCD14 in health, with no established normal values in our population. This study utilised a quantitative method to measure the sCD14 levels.

The median sCD14 levels of HIV-infected participants was lower (316 pg/mL; IQR 250.40–441.83 pg/mL) than that of the HIV-uninfected participantss (742 pg/mL; IQR 541.53–981.65 pg/mL).

The mean sCD14 level was 447.74±360.22 pg/mL for the HIV-infected group and 797.19±364.27 pg/mL for the HIV-uninfected group. There was more variability noted in sCD14 levels in the HIV-uninfected group.

To test for normal distribution of both LPS and sCD14 levels, histograms were utilised.

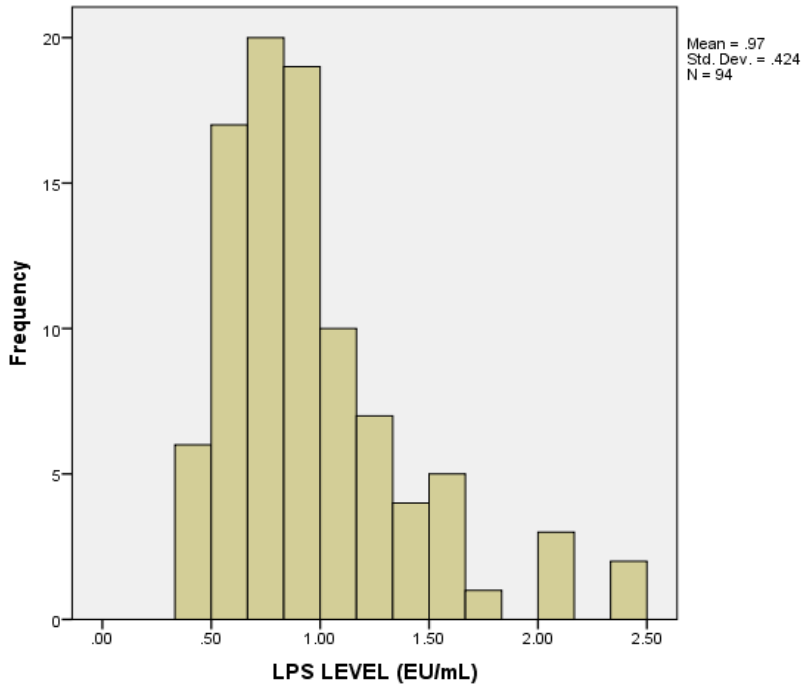


Figure 4: Distribution of LPS Levels in Study Participants

The data series is skewed to the right. Log transformation was attempted on the data but the skew remained. The paired difference was therefore tested on the original (non-transformed) data using the Wilcoxon signed-rank test. The test was done at a 95% confidence level and a 5% significance level. The results are as displayed below.

Table 5: LPS paired sample test statistics

	Mean	SD	Ranks		Z	Df	Assymp. Sig (2-tailed)
			Negative	Positive			
HIV +	0.95	0.43	20	27	-0.878	46	**0.380**
HIV -	0.98	0.42					

The test showed that there was no statistically significant difference between the mean LPS levels in HIV-infected participants compared to the HIV-uninfected ones (Z = -0.878, p = 0.380).

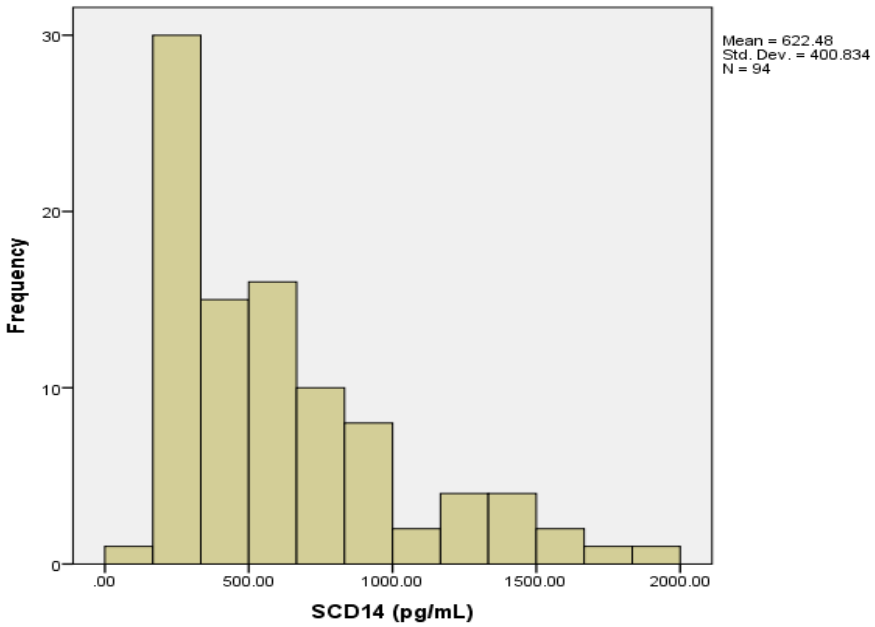


Figure 5: Distribution of sCD14 Levels in Study Participants

The data series is also skewed to the right. A log transformation was attempted on the data but the skew remained, therefore the Wilcoxon signed-rank test was used on the non-transformed data.

Table 6: Soluble CD14 paired sample test statistics

	Mean	SD	Ranks		Z	Df	Assymp. Sig (2-tailed)
			Negative	Positive			
HIV +	447.74	360.22	7	40	-4.148	46	**0.000**
HIV -	797.19	364.27					

As tabulated above, the Wilcoxon signed-rank test showed that the levels of sCD14 in the HIV-uninfected participants were statistically higher than those of the HIV-infected participants ($Z = -4.148$, $p = 0.000$).

CHAPTER SIX: DISCUSSION

This study sought to determine the occurrence of microbial translocation using the indirect markers LPS and soluble CD14 in HIV-infected participants attending the Mbagathi Comprehensive Care Clinic. Age-matched HIV-uninfected participants recruited from the Voluntary Counselling and Testing centres at Mbagathi District Hospital and Kenyatta National Hospital served as a comparative population.

The median age of HIV-infected participants was 50 years old. This is in keeping with the global trend of an ageing HIV-population, in part due to increased access to medication (11, 79). All of HIV-positive study subjects were on ARV medication (mean duration in years ± SD, 12.9 ± 3.6). Kenya adopted the test-and-treat protocol in its 2018 treatment guidelines, which bridges gaps previously noted in ART coverage (78, 80). Majority of the HIV-infected participants had had their

drug regimens optimised in line with existing guidelines. While the participants in both groups were age-matched to within five years, there was a significantly higher number of female participants in the HIV-uninfected compared to the HIV-infected arm (66% vs 51%). Of the eligible HIV-uninfected participants identified, a disproportionate number of men declined to have the HIV rapid test done. Men are less likely to get tested at health care facilities than women, with fear of testing as one of the reasons cited (81).

Endotoxin levels may or may not be detected in human plasma. LPS levels are however frequently detected in the plasma of both HIV-infected and HIV uninfected-persons, with higher levels reported in HIV-infected persons (3, 4, 60). All study participants had detectable LPS levels which were quantified. The minimal detectable threshold of the assay used was 0.1 EU/mL. Samples were tested in batches with the same lot of reagents to minimise assay variability. The LPS levels were fairly homogenous in the HIV-infected group, with more variation noted in the HIV-uninfected group. All the HIV-infected persons were in WHO stage 1 of disease and 91% (42 of 46) of patients who had recorded viral loads were virologically suppressed, which may explain the homogeneity of the LPS levels. There was no statistically significant difference in the levels of lipopolysaccharide between the HIV-infected and the HIV-uninfected participants.

Our data demonstrating similar levels of circulating LPS in HIV-infected African Kenyans compared with uninfected persons are consistent with a study from Uganda, but differ from data from another Kenyan study and from studies in South Africa and North America. In the longitudinal study done in Rakai, Uganda, looking at markers of microbial translocation and the innate cytokine response in HIV-infected African individuals over a period of time, levels of LPS and other inflammatory markers were taken at baseline before HIV-1 seroconversion, and yearly after HIV-1 seroconversion for 7+ years or until demise. There was no difference in LPS levels between the baseline levels of LPS from the then HIV-negative African cohort and HIV-negative US adults used as controls. After HIV-1 seroconversion, it was found that plasma LPS levels remained relatively stable throughout disease progression.

In contrast, several other studies that have shown that HIV-infected persons continue to have higher circulating LPS levels than healthy controls, even after effective treatment (3, 4, 18). Cross-sectional studies done in Pumwani, Kenya, and Pretoria, South Africa both looking at subclinical endotoxaemia and the immune response and found significantly higher levels of LPS in HIV-

infected individuals (60, 62). The cross-sectional nature of this study could not determine if or how circulating endotoxin levels had changed in the HIV-positive participants over time, especially in response to effective treatment.

Subclinical endotoxaemia can occur in otherwise healthy individuals with no acute or chronic viral infections and has been described in HIV-negative individuals. It has been associated with a wide range of immune effects which are yet to be fully described (82). The effect of endemic intestinal infestations on mucosal health and subsequent microbial translocation the African population is unknown but cannot be discounted. LPS levels may be also higher in Africans: In Rakai, Uganda, higher levels of LPS were found in the HIV-uninfected African subjects than in their American counterparts, though the difference was not statistically significant. In the Rakai study, baseline EndoCAb levels in the uninfected Africans were found to be higher than the uninfected American controls, and they remained consistently high throughout seroconversion and disease progression, which might have partly explained why LPS levels did not rise (61). This hypothesis might hold true for our population as well, but needs further study.

Elevated levels of sCD14 have been described in HIV-infected patients and been associated with poor prognosis of HIV-1 infection and other non-communicable diseases (30, 32). There are no established reference ranges for sCD14 levels in healthy individuals. All participants in this study had quantifiable levels of sCD14. The minimal detectable threshold of the assay used was 6 pg/mL. The HIV-infected group had a lower median sCD14 level (median, IQR (pg/mL) 316; 250.40–441.83) than the HIV-uninfected group (median, IQR (pg/mL) 742; 541.53–981.65). The study found sCD14 levels more homogeneously distributed among the HIV-infected than the HIV-uninfected participants. Unexpectedly, the HIV-uninfected participants had statistically significant higher levels of sCD14 than the HIV-infected participants.

Increased serum levels of sCD14 are evidence of an activated status of macrophages and monocytes (83). While levels are thought to be reflective of LPS exposure, CD14 is not exclusively activated by LPS (84). CD14 is a pattern recognition receptor for a wide variety of ligands, including Gram positive cell wall components and endogenous lipids (27, 84). A number of non-myeloid cells have also been shown to express what is believed to be biologically functional CD14. These include, among others, vascular endothelial cells, smooth muscle cells, and epithelial cells lining the gingiva and respiratory tract (27).

Increased plasma soluble CD14 concentrations have been associated with cardiovascular disease, insulin-resistance, and asthma in HIV-negative persons as well (84-86). In the Cardiovascular Health Study cohort comprising of >5000 European-American and Black adults ≥65 years who had been assessed for atherosclerotic risk factors and subclinical vascular disease, baseline sCD14 was shown to be positively correlated with presence of traditional cardiometabolic risk factors such as smoking, diabetes and hypertension, as well as presence of markers of subclinical cardiovascular disease independent of traditional risk factors (86).

Presence of chronic NCDs in the study participants was beyond the scope of this study and these data were not collected. However, it was noted that none of the HIV-infected participants had any other chronic diseases on file, nor were they on any other medication except ARVs and/or prophylactic antimicrobials (sulfamethoxazole/trimethoprim, isoniazid). Similar information on presence of chronic disease was not available for the HIV-uninfected participants. Studies have shown an increasing prevalence of cardiovascular disease risk factors in Kenyan urban populations (87, 88), and it is plausible that the HIV-uninfected participants may have had these conditions, leading to elevated sCD14 levels.

The HIV-infected participants had been on follow-up and were stable, coming in for routine care, while the HIV-negative participants might have had acute or chronic symptoms of disease that prompted them to seek HIV testing. These reasons may explain why our findings are contradictory to several others from Europe and Africa where HIV-infected persons have had higher sCD14 levels than their HIV-uninfected counterparts (4, 58, 60, 62, 89).

Despite the higher number of female participants in the HIV-uninfected arm, there are no conclusive data available on if or how sex and sex hormones may influence the immune system response or subsequent levels of immune response markers.

There was no correlation found between plasma LPS and soluble CD14 levels. Studies have had inconsistent data on the relationship between LPS and sCD14, with positive, negative, and no correlation all reported. This underlies the complexity of nature of the human immune system activation and response.

CONCLUSION

Indirect markers of microbial translocation-LPS and sCD14 were measurable in both the HIV-positive and HIV-negative participants. There was no significant different in levels of circulating

LPS between HIV-positive and HIV-negative age-matched study participants. The HIV-uninfected group had significantly higher levels of plasma soluble CD14 that the HIV-infected group. HIV-infection was therefore not associated with higher levels of either LPS to sCD14. This study underlies the complex nature of the interaction between the microbiome and the immune system in different populations and warrants further investigation.

RECOMMENDATIONS

1. This serves as a gateway study into further exploration of the nature of the microbiome and its interaction with the immune system in our population. Determination of the composition of the microbiome in different populations in both health and disease states would establish a useful baseline for further studies on this interaction.
2. This study highlights the need to further explore the prevalence of non-communicable disease and their mechanisms in morbidity and mortality in the HIV-infected population.

LIMITATIONS

1. The small sample size did not allow for exploration of confounding factors that could have contributed to the results that we got. These include the presence of chronic disease in the study participants, especially in the HIV-uninfected comparative arm.
2. At the time of the study, all the HIV-infected participants were in WHO stage 1 and had stable disease. They might have been over-represented in the ambulatory clinic.
3. LPS levels can be detectable even in health, therefore its presence does not necessarily indicate an acute or chronic illness. It has however been widely studied and accepted as a marker of microbial translocation in several disease states.

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APPENDIX I: DATA USED IN SAMPLE SIZE ESTIMATION

Plasma Levels of Soluble CD14 Independently Predict Mortality in HIV Infection

Netanya G. Sandler,1 Handan Wand,10 Annelys Roque,1 Matthew Law,10 Martha C. Nason,3 Daniel E. Nixon,5 Court Pedersen,8 Kiat Ruxrungtham,9 Sharon R. Lewin,11,12,13 Sean Emery,10 James D. Neaton,6 Jason M. Brenchley,2 Steven G. Deeks,7 Irini Sereti,4 and Daniel C. Douek,1 for the INSIGHT SMART Study Group

Table 1.

Baseline Characteristics of Study Subjects

Category	Individuals who died (*n*=74)	Control subjects (*n*=148)	*P*	CVD events (*n*=120)	Control subjects (*n*=238)	*P*	AIDS events (*n*=81)	Control subjects (*n*=162)	*P*
Demographic characteristic									
Age, years (25^{th}, 75^{th} percentile)	50 (42, 55)	48 (43, 55)	.009	49 (44, 56)	48 (42, 55)	<.0001	46 (40, 53)	45 (39, 52)	.04
Female sex, %	21.6	21.6	N/A	19.2	19.3	N/A	28.4	28.4	N/A
White/other race, %	50	60	(Ref)	58.3	61.8	(Ref)	69.1	63.6	(Ref)
Black race, %	50	40	.16	41.7	38.2	.51	30.9	36.4	.36

$CD4^+$ cell count, cells/mm^3									
Nadir (25^{th}, 75^{th} percentile)	249 (152, 360)	245 (118, 351)	.90	209 (107, 328)	241 (133, 350)	.44	225 (140, 368)	243 (135, 350)	.97
Baseline (25^{th}, 75^{th} percentile)	551 (410, 713)	619 (476, 838)	.051	607 (463, 841)	638 (496, 816)	.88	588 (465, 729)	577 (488, 764)	.88
Prior AIDS, %	28.4	27.7	.91	39.2	26.1	.02	37.4	19.1	.001
United States, %	96.0	96.0	N/A	90.0	90.8	N/A	85.2	85.2	N/A
ART/HIV RNA level									
No ART	21.6	17.8	(Ref)	14.2	13.9	(Ref)	27.2	20.3	(Ref)
ART, HIV RNA level ≤400 copies/mL, %	54.1	64.2	.32	65.8	58.8	.76	50.6	61.7	.13
ART, HIV RNA level >400 copies/mL, %	24.3	18.2	.87	20.0	27.3	.38	22.2	18.0	.81
Hepatitis B/C, %	46.0	23.0	.001	25.8	18.9	.12	19.8	21.0	.81
Other characteristics									

Current smoker, %	56.8	33.8	.002	55.0	37.4	.002	45.7	37.0	.18
BMI (25th, 75th percentile)	24.8 (21.6, 29.9)	25.6 (23.2, 29.4)	.45	25.2 (22.4, 28.7)	25.6 (23.2, 29.6)	.15	25.3 (22.1, 28.3)	25.4 (23.0, 28.8)	.67
Diabetes, %	23.0	13.5	.08	19.2	9.2	.01	9.9	10.5	.88
Blood pressure–lowering drugs, %	37.8	24.3	.04	43.3	32.4	.04	28.4	20.4	.16
Lipid-lowering drugs, %	16.2	23.0	.22	27.5	24.4	.50	21.0	16.7	.42
Prior CVD, %	14.9	3.4	.006	13.3	5.5	.01	8.6	3.1	.08
Total cholesterol (mg/dL)/HDL cholesterol (mg/dL) (25th, 75th percentile)	4.5 (3.5, 6.1)	4.8 (3.6, 5.8)	.58	5.1 (3.9, 6.8)	4.5 (3.5, 5.6)	.01	4.9 (3.7, 6.6)	4.7 (3.5, 5.9)	.10
LDL cholesterol (mg/dL) (25th, 75th percentile)	100 (72, 132)	107 (88, 139)	.04	108 (83, 150)	111 (93, 136)	.73	106 (88, 129)	112 (90, 137)	.56
Triglycerides (mg/dL) (25th, 75th percentile)	169 (110, 305)	200 (120, 300)	.51	193 (140, 305)	180 (124, 289)	.68	196 (128, 272)	165 (128, 262)	.19
Drug conservation arm, %	62.2	58.8	.63	62.5	47.5	.01	79.0	47.5	<.001

Biomarkers									
LPS, pg/mL (25th, 75th percentile)	32.7 (24.7, 42.9)	32.6 (24.2, 47.9)	.76	32.7 (23.7, 47.4)	34.0 (25.8, 45.0)	.62	35.9 (22.4, 52.3)	31.2 (23.3, 43.6)	.40
16S rDNA level, copies/μL (25th, 75th percentile)	7.70 (2.6, 34.3)	7.62 (3.9, 12.7)	.56	7.45 (2.65, 14.15)	8.00 (4.06, 13.88)	.33	9.72 (3.97, 16.91)	7.85 (3.45, 14.0)	.15
sCD14 level, ×10⁶ pg/mL (25th, 75th percentile)	2.47 (2.19, 2.91)	2.23 (2.01, 2.63)	<.001	2.44 (2.10, 2.79)	2.33 (2.01, 2.67)	.11	2.38 (2.14, 2.70)	2.31 (2.05, 2.68)	.43
EndoCAb level, MMU/mL (25th, 75th percentile)	128.1 (56.1, 177.2)	115.1 (54.0, 168.6)	.54	104.6 (37.0, 160.7)	118.9 (50.4, 171.2)	.19	112.5 (71.9, 180.7)	134.0 (79.6, 190.3)	.48
I-FABP level, pg/mL (25th, 75th percentile)	174.4 (20.0, 520.7)	72.3 (20.0, 345.4)	.10	149.7 (20.0, 447.2)	140.7 (20.0, 405.8)	.53	175.3 (20.0, 396.0)	113.5 (20.0, 478.0)	.55

NOTE. Median values are reported, unless otherwise indicated. ART, antiretroviral therapy; BMI, body mass index, calculated as weight in kilograms divided by the square of height in meters; CVD, cardiovascular disease; EndoCAb, endotoxin core antibody; HIV, human immunodeficiency virus; I-FABP, intestinal fatty acid binding protein; LDL, low-density lipoprotein; rDNA, ribosomal DNA; Ref, reference; sCD14, soluble CD14.

APPENDIX II: PARTICIPANT INFORMATION AND CONSENT FORM

Title of Study:

Markers of microbial translocation in HIV infected and HIV negative adults at Mbagathi District and Kenyatta National Hospitals.

Principal Investigator\and institutional affiliation:

Dr. Murage, Tabitha Wambaire,

Department of Medicine and Clinical Therapeutics,

University of Nairobi.

This participant information and consent form has two parts:

1. Information sheet: to give you information about the study.
2. Consent form: for your signature if you agree to participate in the study.

Part 1: Information Sheet

Introduction:

I would like to tell you about a study being conducted by the above listed researchers. The purpose of this consent form is to give you the information you will need to help you decide whether or not to be a participant in the study. Feel free to ask any questions about the purpose of the research, what happens if you participate in the study, the possible risks and benefits, your rights as a volunteer, and anything else about the research or this form that is not clear. You may take as much time as you need, as well as discuss the study with anyone you feel comfortable with. When we have answered all your questions to your satisfaction, you may decide to be in the study or not. This process is called 'informed consent'. Once you understand and agree to be in the study, I will request you to sign your name on this form. You should understand the general principles which apply to all participants in a medical research:

i) Your decision to participate is entirely voluntary

ii) You may withdraw from the study at any time without necessarily giving a reason for your withdrawal

iii) Refusal to participate in the research will not affect the services you are entitled to in this health facility or other facilities. We will give you a copy of this form for your records.

This study has approval by The Kenyatta National Hospital-University of Nairobi Ethics and Research Committee protocol No. P314/05/2018

What is this study about?

We are interviewing 2 groups of individuals: one group who are infected with HIV and the other group without HIV. The purpose of the study is to find out if bacteria from the gut of patients with HIV is entering their bloodstream, which may have negative effects on their overall health. We shall compare their results with the results of the people who are not infected with HIV to see if it is more, less, or the same Participants in this research study will be asked questions about their age and occupation, as well as questions related to their health, such as their HIV status, if they are taking medication, how long they have been taking medication, etc. Those who are not infected with HIV will have to undergo a HIV test before they qualify to be included in the study. Participants will also have the choice to undergo a blood test, to test for the bacteria mentioned above.

There will be approximately 100 participants in this study; they will be randomly selected. We are asking for your consent to consider participating in this study.

What will happen if you decide to take part in this research study?

If you agree to participate in this study, the following things will happen.

You will be interviewed by a trained interviewer in a private area where you feel comfortable answering the research questions. The interview will last approximately 30 minutes. The interview will cover topics such as how old you are, where you live, where you went to school, if you are married or not and what work you do. The interviewer will also get some information from your health chart if you are under care of the CCC, like when the diagnosis was made, the drugs you have been using, and your CD4 cell count (to check how the body's immune system is recovering) and your viral load, which looks if the multiplication of the virus has been adequately slowed.

After the interview is finished, the interviewer will get your permission to draw blood from the blood vessels in the crook of your arm (the front part of your elbow joint region). (S)he shall clean the area with antiseptic, and use the smallest needle possible to get about 2.5 teaspoonfuls of blood (8mLs). The area will then be cleaned again and a small elastic bandage will be placed over the area to keep it clean and prevent it from bleeding.

You can come back to this clinic to get your blood test results as well as the results of the study, should you wish to know them.

Are there any risks, harms, or discomfort associated with this study?

Effort has been made to ensure that the above mentioned risks are minimised.

One potential risk of being in the study is loss of privacy. We will keep everything you tell us as confidential as possible. We will use a code number to identify you in a password-protected computer database and we will keep all of our paper records in a locked file cabinet. These records will only be available to persons directly involved in the study. However, no system of protecting your confidentiality can be absolutely secure, so it is still possible that someone could find out you were in this study and could find out information about you.

If there are any questions you do not want to answer or that make you uncomfortable during the interview, you can skip them. You have the right to refuse the interview or any questions asked during the interview.

We will do everything we can to ensure that the interview and blood draw is done in private. Furthermore, all study staff and interviewers are professionals with special training in these procedures/interviews. You may feel some discomfort when blood is being drawn and you may have a small bruise or swelling in your arm where the needle prick will be. The blood draw is however no different from that which is done in hospitals for other reasons. The amount of blood drawn is minimal and will not negatively affect your health. In the highly unlikely event of an injury, illness or complications related to this study, contact the study staff right away at the number provided at the end of this document. The study staff will treat you for minor conditions or refer you when necessary.

Are there any benefits of being in the study?

You may benefit by receiving free counselling and health information. We will refer you to a hospital for care and support where necessary. Also, the information you provide will help us better understand this relatively new aspect of bacteria crossing the gut into the blood. This information is a contribution to science and the body of knowledge that we have about HIV infection. This knowledge may lead to improved management of patients.

Will being in this study cost you anything?

Being in this study will only take up a bit of your time; participation in the study and the laboratory tests will bear no costs to you. We shall do the interview and blood draw on the same day you have come to the clinic or centre so there are no additional costs to you.

Wil you get a refund for any money spent as part of this study?

You will not incur any extra cost by taking part in this study, therefore there are no refunds. There is also no monetary compensation for taking part in this study.

What if you have questions in the future?

If you have further questions or concerns about participating in this study, please call or send a text message to the study staff at the number provided at the bottom of this page.
For more information about your rights as a research participant you may contact:

Secretary/Chairperson,

Kenyatta National Hospital-University of Nairobi Ethics and Research Committee,

Telephone: +254 02 2726300 Ext. 44102

email uonknh_erc@uonbi.ac.ke.

The study staff will pay you back for your charges to these numbers if the call is for study-related communication.

Do you have other choices?

Your decision to participate in research is voluntary. You are free to decline participation in the study and you can withdraw from the study at *any time* without injustice or loss of any benefits.
Sharing of results

Please feel free to get in touch with the principal investigator should you wish to find out the results of this study. We intend to present this study as a dissertation in the University of Nairobi, Department of Clinical Medicine and Therapeutics; and later in a medical journal as a way of sharing this information with the larger medical community. Your identifying information *will not* be disclosed as part of the results.

PART 2: CONSENT FORM (STATEMENT OF CONSENT)

Participant's statement

I have read this consent form/have had the information read to me. I have had my questions answered in a language that I understand. The risks and benefits have been explained to me. I understand that my participation in this study is voluntary and that I may choose to withdraw any time. I freely agree to participate in this research study.

I understand that all efforts will be made to keep information regarding my personal identity confidential.

By signing this consent form, I have not given up any of the legal rights that I have as a participant in a research study.

Participant printed name:_____

Participant signature/Thumb print: _____

Date (day/month/year): _____

Researcher's statement

I, the undersigned, have fully explained the relevant details of this research study to the participant named above and believe that the participant has understood that the study is voluntary and carries minimal risk to the participant.

I confirm that the participant was given an opportunity to ask questions about the study and that all the questions asked have been accurately answered to the best of my ability.

I confirm that the participant has willingly and freely given his/her consent.

A copy of this informed consent form has been provided to the participant.

Researcher's printed name: _____

Signature: _____

Role in the study: _____

For more information, contact:

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APPENDIX III: FOMU YA MAELEZO YA WASHIRIKI NA HATI YA RUHUSA

Mplelezi Mkuu:

Dr. Murage, Tabitha Wambaire,

Department of Medicine and Clinical Therapeutics,

University of Nairobi.

Fomu hii ya ruhusa ina sehemu mbili:

1. Karatasi ya taarifa: habari kuhusu utafiti huu
2. Hati ya Ruhusa: ya kutia sahihi ikiwa umekubali kushiriki kwa utafiti huu

Utapewa nakala ya fomu hii ya ruhusa kwa rekodi zako.

SEHEMU YA 1: Karatasi Ya Taarifa

Utangulizi

Ningependa kukuambia kuhusu utafiti unaofanywa na watafiti waliotajwa hapo juu. Madhumuni ya fomu hii ya idhini ni kukupatia taarifa unayohitaji ili kukusaidia kuamua kama utashiriki katika utafiti huu au la. Tafadhali soma fomu hii ya idhini kwa makini na ujisikie huru kuuliza maswali yoyote unayo kuhuh madhumini ya utafiti, kinachotokea ikiwa unshiriki katika utafiti, hatari na faida ziwezekanavyo, haki zako kama unajitolea, na kitu kingine chochote kuhusu utafiti huu au fomu hii usichoelewa. Unaweza kuchukua muda mwingi unavyohitaji na pia kujadili masomo na mtu yeyote unyejisikia kabla ya kukubali kushiriki. Mara unapoelewa na kukubali kuwa katika utafiti huu, nitakuomba uweke sahihi kwenye fomu hii.

Unapaswa kuelewa kanuni za jumla zinazotumika kwa washiriki wote katika utafiti wa matibabu:

i) uamuzi wako wa kushiriki ni kwa hiari yako pekee

ii) Unaweza kujiondoa kwenywe utafiti wakati wowote bila kutoa sababu

iii) Kukataa kushiriki katika utafiti hakutaathriri huduma unzostahili kupata kwenye kituo hiki cha afya au vituo vingine.

Utafiti huu una kibali kutoka Kenyatta National Hospital/University of Nairobi Ethics and Review Committee (KNH/UON ERC) ambayo ina wajibu wa kuhakikisha kuwa miongozo ya kimaadili, heshima, haki, na usalama wa washiriki wa utafiti imezingatiwa.

Namba ya kibali ni: P314/05/2018

Kusudi la utafiti huu

Tutahoji vikundi viwili vya washiriki binafsi: kundi moja la wale walioambukizwa na virusi vya HIV, na kundi lingine la watu bila virusi hivyo. Madhumuni ya utafiti huu ni kuangalia kama bakteria kutoka kwa matumbo ya washiriki waliyo na virusi vya HIV zinaingia kwenye damu yao; hii inaweza kuwa na madhara mabaya kwa afya yao. Tutalinganisha matokeo yao na matokeo ya washiriki wasiokuwa na virusi ili tuone kama yanalingana au ni tofauti.

Washiriki katika utafiti wataulizwa maswali kuhusu umri wao na kazi wanayofanya, pamoja na maswali yanayohusiana na afya yao kama vile hali yao ya HIV, ikiwa wanatumia dawa na kwa muda gani, na kadhalika. Washiriki amabao hawana virusi vya HIV watalazimika kupima hali yao ya HIV kabla ya kustahili kuingizwa katika utafiti huu. Washiriki pia watakuwa na uchaguzi wa kupima damu ili kuangalia bakteria zilizotajwa hapo awali.

Kutakuwa na washririki wapatao 100 katiak utafiti huu watakaochaguliwa kwa nasibu. Tunaomba ukublai kushiriki katika utafiti huu.

Nini kitakachofanyika unapokubali kushiriki katika utafiti huu?

Mnfanyakazi mwenye ujuzi wa utafiti huu anakuhoji katika eneo la kibinafsi. Mahojiano itachukua kama nusu saa hivi. Maswali utakayoulizwa ni kama umri wako, mahali unpaoishi, ulipoenda shule, ikiwa umeoa/olewa au la, na kazi unayoifanya. Atakayekuhoji pia atapata maelezo zaidi kutoka kwa kwenywe rekodi zako za afya ikiwa unapata huduma kwa CCC, kama vile wakati wa utambuzi wa HIV ulipofanywa, madawa unayotumia na hesabu yako ya seli za CD4 na kiwango cha virusi vya HIV kwenywe damu. Hizi huangalia vile kinga ya mwili inaendelea kupona.

Baada ya mahojiano kukamilika, anayekuhoji atapata ruhusa kutoka kwako kutoa damu kutoka kwenywe mishipa ya damu mkononi mwako. Atasafisha eneo hili na ‘antiseptic’ ya kuondoa uchafu, na kisha kutumia sindano ndogo iwezekanavyo kupata takriban 2.5 ya vijiko vidogo vya damu (mililita 8). Eneo hilo litasafishwa tena na kuwekwa bandia ndogo ya ‘elastic’ ili kuliweka likiwa safi na kulizua kutoka damu.

Utaweza kupata majibu yako ya damu na matokeo ya utafiti huu kutoka kwa kliniki hii ukitaka kuyajua.

Kuna madhara yanaoyohusishwa na utafiti huu?

Jitihada imewekwa ili kupunguza madhara yoyote.

Usiri: tutaweka kila kitu unachotuambia kwenywe kompyuta iliyohifadhwa na nenosiri na tutahifadhi kumbukumbu zote za karatasi kwenye kabati itakayofungwa. Watu wanaoshiriki moja kwa moja katika utafiti ndio pekee wataweza kupata rekodi hizi. Hata hivyo hakuna mfumo wa kulinda siri ulio salama kabisa kwa hivyo bado inawezekana kwamba mtu aiyehusika na utafiti anaweza kujua kwamba wewe ulikuwa katika utafiti huu na anaweza kupata habari kukuhusu.

Ikiwa kuna maswali yanayokutia wasiwasi unaweza kosa kuyajibu. Unayo haki ya kukataa mahojiano au maswali yoyote uliyoulizwa wakati wa mahojiano.

Tutafanya kila kitu tunachoweza kuhakikisha kuwa mahojiano haya yafanyike kwa usiri. Aidha, wafanyikazi wote wa utafiti huu na wahojiwa ni wataalam wenywe mafunzo maalum katika taratibu hizi.

Unaweza kuhisi uchungu kiasi kidogo na uvimbe mdogo damu yako itakapotolewa. Hata hivyo, taratibu hii ya kutoa damu haina tofauti na ile inayofanywa kwa hospitali kwa sababu nyingine.

Hatutarajii maumivu yoyote yanayohusiana na utafiti huu, lakini yoyote yakitokea, wasiliana na watafiti kwa anwani au namba ya simu iliyotolewa mwanzoni mwa fomu hii.

Je, kuna faida yoyote kwako kuwa katika utafiti huu?

Unaweza kufaidika kwa kupata ushauri na habari za afya bila malipo. Tutakuelekeza kwenye hospitali kwa huduma na msaada ikiwa ni lazima. Pia, maelezo unayoyatoa yatatusaidia kuelewa vizuri zaidi kuhusu bakteria zinazovuka tumboni na kuingia ndani ya damu. Matokeo ya utafiti huu yataeneza ujuzi wa sayansi kuhusu maabukizi ya virusi vya ukimwi. Ujuzi huu huenda hatimaye kusababisha usimamizi bora ziadi wa wagonjwa.

Gharama ya kushiriki

Kuwa katika utafiti huu na vipimo vya maabara haitazidi gharma yoyte kwako. Tutafanya mahojiano na kutoa damu siku ile ile uliyokuja kwenye kliniki au kituo cha VCT kwa hivyo hakutakuwa na gharama ya ziada kwako.

Kuna malipo ya kifedha kwa ushiriki?

Kwa vile hutapata gharama yoyote ya ziada kwa kushiriki kwa utafiti huu kwa hivyo hakuna marejesho yoyote. Aidha, hakuna fidia ya fedha utakayopata kwa kushiriki.

Kushiriki matokeo

Tafadhali jisikie huru kuwasiliana na mpelelezi mkuu ukitaka kupata matokeo ya utafiti huu. Jina lako na utambulisho wako utabaki siri hata tunapotoa matokeo ya utafiti. Tuna nia ya kuchapisha utafiti kama 'dissertation' katika Chuo Kikuu cha Nairobi, na baadaye katika jarida za matibabu kama njia ya kushiriki na wenzetu katika nchi nyingine.

Kwa maswali zaidi:

Ikiwa una maswali zaidi au wasiwasi juu ya kushiriki katika utafiti huu, tafadhali piga simu au tuma ujumbe wa maandishi (SMS) kwa namba ya simu iliyotolewa mwaznoni mwa fomu hii.

Pia unaweza kuwasilisha mawsali kwa

Secretary/Chairperson,

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SEHEMU YA 2: Hati ya Ruhusa

Taarifa ya mshiriki

Nimesoma na kuelezwa maelezo iliyo hapo juu kuhusu utafiti. Nimekuwa na uwezo wa kuuliza maswali yangu na nikaridhika na majibu niliyopata. Ninakubali kwa hiari yangu kushiriki katika utafiti huu. Ninaelewa kwamba ninweza kujiondoa kwenye utafiti huu wakati wowote.

Jina la mshiriki: _____

Sahihi ya mshiriki/alama ya kidole: _____

Tarehe (siku/mwezi/mwaka): _____

Taarifa ya mtafiti

Nimeeleza sahihi mzazi wa mshiriki mambo yaliyo katika hii fomu, na kadri ya uwezo wangu nimehakikisha kwamba anaelewa kwamba ushiriki kwenye utafiti huu ni kwa hiari, na hatari kwa mshiriki ni nadra.

Ninathibitisha kuwa mshiriki alipewa nafasi ya kuuliza maswali kuhusu utafiti, na maswali yake yamejibiwa kwa usahihi kadri ninavyoweza. Ninathibitisha kwamba mshiriki huyu hakulazimishwa kutoa idhini, na ridhaa imetolewa kwa uhuru na kwa hiari.

Nakala ya ICF hii imetolewa kwa mshiriki.

Jina la mtafiti/anayechukua idhini:_____

Sahihi ya mtafiti / anayechukua idhini:_____

Tarehe (siku/mwezi/mwaka):_____

Kwa maelezo zaidi, wasiliana na

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APPENDIX IV: KNH-UON ETHICS REVIEW COMMITTEE APPROVAL

APPENDIX V: COUNTY HEALTH SERVICES-MBAGATHI HOSPITAL-RESEARCH AUTHORISATION

APPENDIX VI: DATA EXTRACTION TOOL

Study details

Study number:
Hospital number:

Biodata

Age *(years)*	
Sex *(check)*	☐Male ☐Female
Marital status *(check)*	☐Married ☐Single ☐Separated/divorced ☐Widowed
Level of education *(check)*	☐Primary ☐Secondary ☐Post-secondary

1.

2. HIV Serostatus

HIV status	☐Positive (continue below) ☐Negative (skip to Table 3)	
If HIV status positive		
Year of diagnosis		
WHO HIV stage (current)	☐ 1 ☐ 2 ☐3 ☐4	
ARV use	☐ No ☐ Yes	
If using ARVS:	Year when ARVS first started:	
	Regimen currently in use:	☐First line ☐Second line ☐Third line

	If regimen previously changed, reason for change	☐Failure ☐Toxixicty
	Medication currently in use	
	Duration of current current regimen:	☐ ≤6mo ☐ >6mo but <2yr ☐≥2 yr
	Total duration of ARV use:	
CD4 count	Initial : Date done:	Latest: Date done:
Plasma viral load	☐None on record If done: Latest done: Date:	

4. Laboratory results

LPS level (EU/mL)	
sCD14 level (ng/mL)	

ARV Abbreviations:

3TC Lamivudine
ABC Abacabir
ATV Atazanavir
ATV/r Atazanavir/ritonavir
AZT Zidovudine
DRV Darunavir
DRV/r Darunavir/ritonavir
DTG Dolutegravir
EFV Efavirenz
ETR Etravirine
FTC Emtricitabine
LPV/r Lopinavir/ritonavir
NVP Nevirapine
RAL Raltegravir
TDF Tenofovir disoproxil fumarate

APPENDIX VII: LPS LABORATORY PROTOCOL

APPENDIX VIII: SOLUBLE CD14 LABORATORY PROTOCOL