PLASMA INTERFERON-GAMMA, INTERLEUKIN-10 AND ADIPONECTIN LEVELS IN HIV-1 AND TUBERCULOSIS CO-INFECTED INJECTION DRUG USERS AT BOMU HOSPITAL, MOMBASA, KENYA

KIBOI NATHAN
Reg. No. 156/23554/2013

A Thesis Submitted in Partial Fulfilment of the Requirements for the Award of the Degree of Master of Science (Medical Biochemistry) in the School of Pure and Applied Sciences, Kenyatta University

June, 2016
DECLARATION

This thesis is my original work and has not been submitted for a degree award in any other University

Kiboi Nathan
Reg. No: 156/23554/2013

Signature ………………….. Date……………………

This thesis has been submitted for examination with our approval as Supervisors.

Supervisors

1. Dr. David Mburu
   Department of Biochemistry and Biotechnology
   Kenyatta University

   Signature ……………………….. Date ………………………

2. Dr. Tom Were
   Department of Medical Laboratory Sciences
   Masinde Muliro University of Science and Technology

   Signature ………………………..Date ………………………

3. Dr. Gerald Juma
   Department of Biochemistry
   University of Nairobi

   Signature ……………………….. Date ………………………
DEDICATION

This work is dedicated to my family and friends whose continued support and encouragement gave me the motivation and strength to complete this study.
ACKNOWLEDGEMENTS

This thesis is a product of co-operative efforts from several key individuals and institutions. First and foremost, I am greatly indebted to my supervisors Dr. David Mburu, Dr. Tom Were and Dr. Gerald Juma who devoted a lot of time to enable me complete this study. I thank them for their timely pieces of advice, critical suggestions and invaluable support without which this work could not be accomplished. Special thanks to Dr. Tom Were and Valentine Bundambula for their expertise in designing and executing this study. I extend my appreciation to the study participants who consented into the study, the management, laboratory and other support staff of Bomu Hospital where the study was conducted. I thank the Kenya National Commission for Science, Technology and Innovation [NCST/5/003/065], and Partnership for Advanced Clinical Education (PACE) project in Kenya (NIH 1R24TW008889) grants to Dr. Tom Were and Valentine Budambula for funding this study. Above all, I acknowledge the hand of Almighty God throughout this study period.
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## ABBREVIATIONS AND ACRONYMS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Acrp30</td>
<td>Adiponectin (adipocyte complement-related protein)</td>
</tr>
<tr>
<td>AFBs</td>
<td>Acid fast bacilli</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>ART</td>
<td>Antiretroviral therapy</td>
</tr>
<tr>
<td>ARVs</td>
<td>Antiretroviral drugs</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CMI</td>
<td>Cell mediated immunity</td>
</tr>
<tr>
<td>DLTLD</td>
<td>Division of leprosy, TB, and lung disease</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra acetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>HAART</td>
<td>Highly active antiretroviral therapy</td>
</tr>
<tr>
<td>HIV-1</td>
<td>Human immunodeficiency virus-1</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse-radish peroxidase</td>
</tr>
<tr>
<td>IDUs</td>
<td>Injection drug users</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>IL-10</td>
<td>Interleukin 10</td>
</tr>
<tr>
<td>IRIS</td>
<td>Immune reconstitution inflammatory syndrome</td>
</tr>
<tr>
<td>KAIS</td>
<td>Kenya AIDS indicator survey</td>
</tr>
<tr>
<td>KNBS</td>
<td>Kenya National Bureau of Statistics</td>
</tr>
<tr>
<td>MARPs</td>
<td>Most at risk populations</td>
</tr>
<tr>
<td>MOH</td>
<td>Ministry of Health</td>
</tr>
<tr>
<td>MTB</td>
<td>Mycobacterium tuberculosis</td>
</tr>
<tr>
<td>NACC</td>
<td>National AIDS control council</td>
</tr>
<tr>
<td>NASCOP</td>
<td>National AIDS/STI control programme</td>
</tr>
<tr>
<td>NK cells</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>PBMC’s</td>
<td>Peripheral blood mononuclear cell suspensions</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PLWHAs</td>
<td>People living with HIV/AIDS</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>Th-1</td>
<td>T helper-1 cells</td>
</tr>
<tr>
<td>Th-2</td>
<td>T helper-2 cells</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethylbenzidine</td>
</tr>
<tr>
<td>TNF-γ</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>UNODC</td>
<td>United Nations Office on Drugs and Crime</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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ABSTRACT

Sub-Saharan Africa accounts for high tuberculosis cases that result from widespread HIV infections, which is exacerbated by injection substance use. Immunologically, HIV critically impairs cell-mediated host responses to *Mycobacterium tuberculosis*. IFN-γ, IL-10 and Acrp30 are key mediators of systemic inflammation. Although circulating IFN-γ and IL-10 levels are increased, Acrp30 levels are lowered and associated with disease severity among HIV and TB co-infected non-substance users. In contrast, circulating IFN-γ and Acrp30 levels are decreased while IL-10 levels are upregulated among injecting heroin addicts. However, no studies to date have reported on these cytokine profiles among Kenyan HIV-1 and TB co-infected injection drug users. This study, therefore, investigated plasma IFN-γ, IL-10 and Acrp30 levels among IDUs, and their association with CD4+ T cell counts, HIV-1 viral load and BMI. A cross-sectional study was conducted from August, 2012-November, 2013 using 138 participants recruited at Bomu hospital; a major centre for rehabilitation of drug and substance users in Mombasa County. Following informed consent, IDUs were enrolled through respondent driven sampling, snowball and makeshift methods while convenience and purposive sampling were used for recruiting the control group. IDUs and controls were screened for HIV and TB respectively through Determine™ and Bioline™ rapid tests, and Ziehl Neelsen stained sputum smears. Subsequently, the study participants were categorised into: HIV-1/TB co-infected ART-naive (n=9) and -experienced (n=27); HIV-1 mono-infected ART-naive (n=26) and -experienced (n=13); TB mono-infected (n=21), HIV-1 negative and TB uninfected (n=25) IDUs and controls (n=17). Demographic, drug use information and physical measurements were recorded using assisted interviews. EDTA venous blood samples were collected and used for preparing plasma and enumerating CD4+ T cell counts. Frozen plasma samples were used for determining cytokine concentrations, and HIV-1 viral load. CD4+ T cell counts were enumerated using flow cytometry; cytokine levels were measured using a sandwich ELISA technique, while HIV-1 viral load was determined by RT-PCR, respectively. Across-group comparisons in continuous data were performed using Kruskal Wallis followed by post-hoc Dunn’s tests. Plasma IFN-γ (P<0.0001), IL-10 (P<0.0001) and Acrp30 (P=0.006) levels differed significantly across groups. IFN-γ levels were high in co-infected ART-naive (P<0.001) and -experienced (P<0.001), and HIV-1 mono-infected ART-experienced (P<0.001) IDUs relative to healthy controls. IL-10 levels were elevated in uninfected IDUs (P<0.001) compared to healthy controls. Acrp30 levels were lower in TB mono-infected (P<0.01) relative to controls. IFN-γ/IL-10 ratio varied across-groups (P<0.0001) and higher in co-infected ART-naive (P<0.001) and -experienced (P<0.001), and HIV-1 mono-infected ART-experienced (P<0.001) compared to uninfected IDUs. The IFN-γ/Acrp30 ratio also differed across groups (P<0.0001) with HIV-1 mono-infected ART-experienced (P<0.001), and co-infected ART-naive...
(P<0.001) and -experienced (P<0.001) IDUs exhibiting higher ratio relative to uninfected IDUs. CD4+ T cells correlated inversely with Acrp30 (ρ=-0.717, P=0.030) levels in TB mono-infected IDUs whereas BMI correlated positively with Acrp30 (ρ=0.523, P=0.022) among co-infected ART-naive IDUs, respectively. Altogether, circulating IFN-γ, IL-10 and Acrp30 production is altered in ART-naive and -experienced HIV-1 and TB co-infected IDUs, suggesting a role as disease markers in HIV and TB co-infection among IDUs.
CHAPTER ONE
INTRODUCTION

1.1 Background information

Human immunodeficiency virus (HIV) and *Mycobacterium tuberculosis* (MTB) are among the major pathogens responsible for high infectious disease burden worldwide. Infection with HIV results in acquired immune deficiency syndrome (AIDS), while *tuberculosis* (TB) is an infectious disease caused by MTB. A synergistic association has been reported to exist between HIV and TB infections (Mayer and Hamilton, 2010), with co-infections by both infectious agents having been implicated in increased morbidity and mortality worldwide (WHO, 2011). It is estimated that approximately, 14 million individuals globally are co-infected with HIV and MTB (UNAIDS 2012; WHO, 2012b). In Sub-Saharan Africa, Kenya has been ranked among the countries with the highest HIV and TB prevalence rates (NACC, 2012), with the latter being reported as the most common opportunistic infection among most of the HIV-AIDS patients (Corbett *et al.*, 2003). For example approximately, 5.6% of the population aged between 15-64 years in the year 2012 were living with HIV/AIDS (KAIS, 2013). Similarly, among all the new TB cases reported in 2010 in Kenya, 41% of the total were HIV infected suggesting a close association between HIV and TB infections (NASCOP, 2011).
Drug and substance use has been implicated as one of the major factors that increase HIV and TB mono and co-infections (Altice et al., 2010). It is reported that an estimated 12.7 million individuals inject drugs yearly (UNODC, 2013). Previous clinical reports indicate that HIV-1 drug user patients have a high risk of over 13-fold of acquiring TB infection (Keizer et al., 2000), implying a great role of drugs in HIV/TB infections. As a result, injection drug users (IDUs) are reported to be a highly vulnerable population in regards to HIV/TB infections (Weber et al., 2009), due to the common practice of sharing unsterilized needles and syringes (Beckerleg et al., 2005). Equally, due to constant influence of drugs, injectable drug users are often careless about their social lives with concomitant high degree of exposure risks through unprotected sex (Dehne et al., 2000).

Although HIV-1 prevalence among people who inject drugs is estimated at 13.1% globally (UNODC, 2014) and at 18.3% in Kenya (UNAIDS, 2015), an approximation of such prevalence among the HIV-TB co-infected IDUs worldwide remains unknown (WHO, 2010), since this risk group are often an isolated and difficult to reach population (Heckathorn, 1997).

Immunologically, infection by HIV-1 has been reported to lead towards a dysregulated production of a repertoire of cytokines (Agarwal et al., 2001), most of which have been used as markers of disease progression as well as in the assessment of patient’s response to antiretroviral therapy (ART) (Tateyama et al., 1994). For example, interferon-gamma (IFN-γ), a pro-inflammatory cytokine
mainly produced by CD4+ T cells and exerting both antiviral and immunostimulatory functions (Schroder et al., 2004) is highly expressed during HIV-1 disease pathogenesis (Breen, 2002). The plasma IFN-γ levels are raised following HIV-1 and TB co-infection and these levels are reported to remain significantly high during the advancement of disease (Benjamin et al., 2013; Mihret et al., 2014). Hence, elevated plasma levels of IFN-γ have been positively associated with reduced CD4+ T cells, high viral loads and disease severity (Subramanyam et al., 2004). Similarly, a milieu of pro-inflammatory cytokines has been implicated in active TB infection (Sahiratmadja et al., 2006). Plasma IFN-γ levels are reported to be highly elevated in TB-infected individuals (Riou et al., 2012), which initially serves as an immuno-protective mechanism.

On the other hand, anti-inflammatory cytokines such as interleukin-10 (IL-10) and adiponectin (Acrp30) are cytokines generally secreted within the body in response to excessive inflammation and consequent tissue damage that may partially be mediated by the pro-inflammatory cytokines (IFN-γ) towards various pathogenic micro-organisms including HIV-1 and MTB (Sunder et al., 2012). Their production functions to down-regulate Th-1 associated responses thus regulating immune balance. Although IL-10 is usually elevated during advanced HIV-1 and TB co-infection (Geldmacher et al., 2010; Elliott et al., 1999), this elevation has been suggested not to be directly associated with protection against TB infection (Zhang, et al., 1995a; Fauci et al., 1996).
Injection drugs and polysubstance abuse has been reported to cause a dysregulation in cytokine production (Weiss et al., 2006; Finley et al., 2008). For instance, heroin use has been implicated in the suppression of plasma IFN-γ levels (Zaki et al., 2005), while up-regulating IL-10 production (Azarang et al., 2007). Additionally, serum Acrp30 levels have been demonstrated to be reduced in frequently injecting heroin addicts (Housova et al., 2005; Shahouzehi et al., 2013). On the other hand, cannabinoids, the active compounds in bhang are reported to stimulate production of IL-10 an anti-inflammatory cytokine (Weiss et al., 2006). Similarly, khat chewing has been demonstrated to induce IL-10 as well as IFN-γ secretion (Murdock et al., 2011). Hence, these observations collectively depict that illicit drugs and polysubstance use significantly contribute to altered cytokine expression patterns independent of pathogenic infections.

With the growing understanding of their roles during infections and disease progression, cytokines including IFN-γ, IL-10 and Acrp30 have been assayed in plasma to assess the efficacy of ART during HIV-1 infection (Kosmiski et al., 2008; Brockman et al., 2009; Roff et al., 2013). For example, highly active antiretroviral therapy (HAART) markedly increases plasma IFN-γ levels (Reuben et al., 2002; Watanabe et al., 2010) and considerably lowers IL-10 levels during HIV and TB co-infection (Stylianou et al., 1999). Similarly, the interaction between injection drugs and HAART has been associated with poor clinical
outcomes in many HIV-1 positive IDUs (MOH, 2012). For instance continued drug injection has been identified as an obstacle to both ART success and HIV-1 virological suppression (Weber et al., 2009). Furthermore, injection drugs are reported to impair both short and long term CD4+ T cell recovery (Dronda et al., 2004; Meijerink et al., 2014), which consequently accelerates disease progression among IDUs.

On the whole, concurrent HIV-1 and TB infections, substance use and antiretroviral therapy subject IDUs to various immunological, biochemical and metabolic derangements (Meng et al., 2002; Solomon et al., 2008; Tang et al., 2011; Estrella et al., 2012), however, the cytokine profiles as probable markers of disease progression among the HIV-TB co-infected IDUs either exposed to ART or naive for treatment remains less defined. Similarly, the relative concentration of both anti- and pro-inflammatory cytokines in plasma samples is not yet fully determined. Thus, the present study sought to determine levels of circulating plasma IFN-γ, IL-10 and Acrp30 among HIV-1/TB mono- and co-infected HAART-naive and -experienced IDUs from Bomu Hospital; a drug and substance rehabilitation facility in Mombasa Kenya, and relate the plasma levels of these cytokines to CD4+ T cell counts, HIV-1 viral load and BMI as the commonly used biomarkers of either HIV-1 or TB disease progression among IDUs.
1.2 Problem statement

Clinical outcomes including CD4+ T cell counts, HIV-1 viral load, and BMI comprise integral components of patient evaluation prior to and after initiation of HAART (CDC, 2006; NASCOP, 2012b) in HIV and TB co-infected patients. These clinical measures have hence been used as immunological baseline predictors of disease progression and successful therapeutic intervention (Thompson et al., 2010; Rutherford et al., 2014). On the other hand, the pro-inflammatory cytokines such as IFN-γ, and anti-inflammatory cytokines such as IL-10 and Acrp30 are reported as key markers of systemic inflammation and immunologic derangements in HIV-1 and TB infected individuals (Meng et al., 2002; Ouchi et al., 2011; Tang et al., 2011; Estrella et al., 2012). These cytokines therefore have been used to determine the extent of disease progression and host immune response in a number of HIV and TB infected subjects (Romagnani and Maggi, 1994; Klein et al., 1997; Elliot et al., 1999).

Injection drug use has been reported to be highly prevalent among HIV and TB-infected population (Friedman et al., 1996; Keizer et al., 2000; Nieburg and Carty, 2011). However its impact on HIV-1/TB disease pathogenesis remains less well characterised (Kapadia et al., 2005; Lucas et al., 2006; Deiss et al., 2009). Similarly, drug and substance abuse is believed to cause a disregulation in plasma cytokines during concurrent HIV-1 and TB co-infection and ongoing ART. Hence, the current hospital-based cross-sectional study sought to quantify plasma
IFN-γ, IL-10 and Acrp30 levels as possible markers of disease progression among the ART-naive and -experienced HIV-1 and TB mono- and co-infected IDUs, while also examining their association with the established HIV-1/TB clinical disease biomarkers including CD4+ T cell counts, HIV-1 viral load and BMI.

1.3 Hypotheses

1) Plasma IFN-γ, IL-10 and Acrp30 levels are not different in both HIV-1/TB mono-infected and HIV-1/TB co-infected injection drug users from Mombasa County.

2) Highly active antiretroviral therapy (HAART) has no effect on IFN-γ, IL-10 and Acrp30 levels in HIV-1 infected injection drug users from Mombasa County.

3) The CD4+ T cell counts, HIV-1 viral load and BMI are not significantly associated with changes in IFN-γ, IL-10 and Acrp30 levels among the HIV-1/TB mono- and co-infected injection drug users.

1.4 Objectives

1.4.1 General objective

To determine the circulating plasma levels of IFN-γ, IL-10 and Acrp30 and their association with CD4+ T cells, HIV-1 viral load and BMI in both ART-naive and
experienced HIV-1 and TB mono- and co-infected injection drug users from Mombasa County.

1.4.2 Specific objectives

1) To determine the plasma concentrations of IFN-\(\gamma\), IL-10 and Acrp30 among the ART-naive and -experienced HIV-1 and TB mono- and co-infected injection drug users.

2) To evaluate the impact of antiretroviral treatment (HAART) on cytokine production among the ART-experienced HIV-1 infected injection drug users.

3) To determine the association between plasma IFN-\(\gamma\), IL-10 and Acrp30 levels with CD4+ T cell counts, HIV-1 viral load and BMI among the ART-naive and -experienced HIV-1 and TB mono- and co-infected injection drug users.

1.5 Significance of the study

Epidemiologically, HIV-1 and TB are identified as common infections among the population of injection drug users (Friedman et al., 1996; Keizer et al., 2000). However the immunological profiles that underlie disease progression among the HIV-1 and TB co-infected IDUs have not been critically assessed. Additionally, injection drug and substance use has been reported to complicate ART and causes immune modifying effects among ART-experienced IDUs (Hsier et al., 2000;
Palepu et al., 2003). Hence, taken together, this information defines the need for measurement of the pro- and anti-inflammatory cytokines including IFN-γ, IL-10 and Acrp30 levels as potential correlates of clinical disease progression among the ART-naive and -experienced HIV-1 and TB mono- and co-infected IDUs.

Mombasa town has a strategic geographical location along the Kenyan coastline, therefore creating avenues for drug trafficking (Beckerleg et al., 2005). As a consequence, Mombasa harbours among the highest number of IDUs (UNAIDS, 2015), thus making it a suitable site to conduct the study. On the other hand, Bomu hospital (Appendix I) is a social enterprise facility that offers drug and substance rehabilitation services, hence presents as an ideal setting for our study population. On the whole, measurement of plasma IFN-γ, IL-10 and Acrp30 levels may be helpful in defining the course of disease progression, evaluation of treatment and highlighting benefits of timely antiretroviral therapy amongst the population of injection drug users. Furthermore, the ability to visualise profile of cytokine expression during disease or treatment is critical to advance the understanding of the immune response to these pathogens.
CHAPTER TWO
LITERATURE REVIEW

2.1 HIV infection

HIV is a virally transmitted disease pathogen that affects the host immune system to result in acquired immune deficiency syndrome (AIDS). Two related, but antigenically and genetically distinct viruses that cause AIDS, namely HIV-1 and HIV-2 have been described (Heeney et al., 2006). HIV-1 is the most common and widely distributed sub-type of HIV disease causing pathogen, while HIV-2 is mainly confined to parts of West Africa and India (Heeney et al., 2006; de Silva et al., 2008). Persons infected with HIV-2 have a longer asymptomatic phase and are clinically characterized by higher CD4+ cell counts, lower viral RNA load, and slower progression to AIDS compared to their HIV-1 infected counterparts (Murdoch and Finn, 2000).

2.1.1 Transmission of HIV

HIV is present primarily in the blood, semen, vaginal secretions, and breast milk of infected individuals. Transmission of the virus occurs mainly through unprotected sex, contaminated needles/infected blood products, occupational exposure to infected blood or its components, or by vertical transmission, for instance from infected mothers to infants (Beyrer, 2007). The most at risk and vulnerable populations of HIV disease acquisition and transmission comprise the sex workers and their clients, men who have sex with men, prisoners, injection
drug users and truck drivers (NACC, 2012; NASCOP, 2012a). Needle sharing and blood flashing practices are highly correlated with the risk of HIV transmission and are reported to account for the majority of HIV positive cases among injection drug users (NASCOP, 2012a; Weber et al., 2015).

2.1.2 HIV-1 disease pathogenesis

The pathogenesis of HIV infection is a function of the virus life cycle, host cellular environment, and quantity of virus in the infected individual (Centlivre et al., 2007). On the other hand, the probability of infection is a function of both the number of infective HIV virions in the body fluid which contacts the host as well as the number of cells available at the site of contact that have appropriate CD4 receptors (Sundquist and Krausslich, 2012). HIV pathogens target CD4+ T cells, macrophages and some dendritic cells (DC’s), with CD4 being the major cell-surface receptor molecule affected by HIV (Mellors et al., 1997). In addition, during cell infections the virus also requires additional binding through co-receptor, especially the chemokine co-receptors CXCR5 and CCR4 (Lusso, 2006). Hence, the presence of mutations in the chemokine co-receptor may explain the phenomenon of resistance to HIV infection in some individuals (O’Brien and Moore, 2000).
The CCR5 tropic virus is predominant during early HIV infection because it readily infects antigen presenting cells such as dendritic cells and macrophages, has a high rate of replication, and is also less visible to cytotoxic T-lymphocytes (Levy, 2009). About 60% of all the CD4 and CCR5 memory T cells are located in the gut-associated lymphoid tissue (GALT) which presents as an early target of infection (Veazey et al., 2003; Chun et al., 2008). Enhanced viral replication and CD4+ T cell depletion during acute HIV infection is accompanied by a massive depletion of CD4 memory T cells, primarily in mucosal tissue (Brenchley et al., 2004). The resultant destruction of the CD4+ T-cell population within the gastrointestinal tract has been shown to contribute to the chronic inflammation and immune exhaustion associated with HIV/AIDS (Douek et al., 2009).

HIV infected cells are carried first to draining lymphoid tissue which allows infection of an activated CD4+ T cell or macrophage thereby inducing cytokine release and recruitment of more activated cells (Moir et al., 2006). Further immune activation allows the virus to replicate and disseminate to secondary lymphoid tissue throughout the organism before being spread systemically (Brenchley et al., 2004). Intense immune activity causes lymph node hyperplasia and lymphadenopathy that eventually causes destruction of lymphoid architecture with the inability to initiate new immune responses. Within 14 to 21 days after acute HIV infection, the viral load begins to fall, partially due to effective adaptive immune response and HIV specific antibodies, particularly CD8+
cytotoxic T cells (CTLs) which have been shown to inhibit HIV replication in \textit{in vitro} cell lines through cytolysis or by the release of chemokines and other associated cytokines (Yang \textit{et al.}, 1997).

During the clinical latency period of HIV infection, there is little detectable virus in peripheral blood, although viral replication actively continues in the lymphoid tissues (Pantaleo \textit{et al.}, 1993a). Unlike the CD4 T cell moiety that gets depleted over time with continued HIV associated viraemia, the CD8+ T cell expansions persist until far advanced stages of HIV disease, when all T-cell numbers tend to fall (Margolick \textit{et al.}, 2006). This loss is largely irreversible and has immunological consequences that eventually manifest as failure of the host immune defenses and subsequent progression to AIDS later during infection (Picker \textit{et al.}, 2003).

\textbf{2.1.3 Diagnosis of HIV infection}

The antigenicity of the HIV virus proteins, which include p24 (core antigen) and gp41 (envelope antigen) provides a means for detection of HIV stimulated antibodies, the basis for most HIV testing (Fauci \textit{et al.}, 1996). Following primary HIV infection there is always a delay “window period” before HIV induced antibodies become detectable in the blood (CDC, 2006). The antibody responses vary according to the virus load and immune competence of the host (Walker and Burton, 2008). However, within two to twelve weeks antibodies formed against
the core and surface protein can easily be detected by Enzyme immunoassay (EIA) and confirmed by both polymerase chain reaction and Western-blot techniques (Martin and Sim, 2000). Hence, ELISA is among the most widely used assay techniques for the detection of HIV antibodies, with a very high (98-99%) sensitivity and specificity (Greenwald et al., 2006). However, a small number of individuals infected with HIV do not develop antibodies to the virus, a phenomenon that has been attributed to the dysfunction of an individual’s immune response (Ellenberger et al., 1999).

2.1.4 Treatment of HIV infection
At present, there is no defined drug for the cure of HIV infection. The aim of current treatment is to lower the plasma concentration of viral load (Thompson et al., 2010). Therapy for adults is based upon CD4+ cell counts, which are initially low prior to treatment but progressively, rise upon institution of ART (Thompson et al., 2010). The current HIV-1 management guidelines suggest the initiation of ART when peripheral blood CD4 T cells decrease to below 350 cells per microlitre (Hammer et al., 2008). Standard ART regimen for the management of HIV comprises of at least three antiretroviral drugs that suppress HIV replication. Typical combinations include 2 Nucleoside Reverse Transcriptase Inhibitors (NRTIs) + 1 Non-Nucleoside Reverse Transcriptase Inhibitor (NNRTI) (Gazzard and Moyle, 1998). For instance, Tenofovir + Lamivudine + Efavirenz or
Nevirapine with Zidovudine replacing Tenofovir in pregnant women and those unable to tolerate Tenofovir (NASCOP, 2012b).

2.2 *Mycobacterium tuberculosis* infection

*Mycobacterium tuberculosis* is an obligate intracellular bacterial pathogen and a major cause of TB infection in humans (Cole *et al.*, 1998). Tuberculosis is the most common opportunistic infection associated with HIV infection (Shafer and Edlin, 1996) and the risk of progression of latent TB to active TB has been reported to increase with ongoing immune suppression (Sonnenberg, *et al.*, 2001). Transmission of MTB primarily occurs through air during coughing and sneezing of aerosolized respiratory droplets or dust particles containing the infective acid-fast bacilli (Riley, 1957). The risk of TB infection is dependent on several factors including, the degree of infectiousness of the source, the closeness of contact, the bacillary load inhaled, and the immune status of the potential host (Frieden, 2003; Hill *et al.*, 2004).

2.2.1 Pathogenesis of TB infection

Several potential mechanisms exist by which HIV infection may increase susceptibility to respiratory symptoms and diseases (Drummond *et al.*, 2010). Profound symptoms observed in TB infection are either due to the presence of a focal TB disease process or as a result of cytokine mediated immune process (Chan and Flynn, 2004). The course of TB disease pathogenesis is usually
influenced by factors such as dose, virulence, age and immune status of the host which has led to the disease to manifest itself as either pulmonary or non-pulmonary TB (Frieden, 2003; Hill et al., 2004). The major route of TB infection involves the lungs. Hence during primary infection, inhaled droplet nuclei containing infective bacilli lodge in the pulmonary alveoli where they are engulfed by phagocytic immune cells such as macrophages and dendritic cells (Bermudez and Goodman, 1996). Later, they cross the alveolar barrier and disseminate into the surrounding lymph glands (Chackerian et al., 2002) which results in lesions that are characterised by acute inflammatory reaction with accumulation of fluid and white blood cells around the bacilli (Doenhoff, 1998).

Depending on the host immune status, lesions may heal by resolution or disease may advance to a productive stage which results in liquefied destruction and caseation of infected lung tissue (Young et al., 2009). The caseation material containing large number of bacilli is coughed out in sputum, creating cavities in the lung and subsequently making breathing rather difficult (Dannenberg and Bloom, 1994). In majority of infected individuals, MTB are killed in these caseating granulomas, and disease progression is arrested (Hingley-Wilson et al., 2003; Skeiky and Sadoff, 2006).
2.2.2 Diagnosis of TB infection

In resource limited settings such as the Sub-Saharan Africa, TB diagnostic strategies rely mainly on sputum microscopy for the determination of the presence of acid-fast bacilli and clinician judgement (WHO, 2007). The patient’s sputum specimen is stained using either Ziehl-Neelsen stain or fluorescent auramine or rhodamine stains (Mendelson, 2007). Although, the more advanced fluorescent microscopy is approximately 10% more sensitive than the conventional staining methods that have routinely been applied (Steingart et al., 2006). Generally, sputum smear microscopy has low sensitivity for TB diagnosis than the TB culture which is considered as the gold-standard for TB diagnosis especially for patients manifesting pulmonary TB (Drobniewski et al., 2003). In all TB suspects with negative sputum smears, a chest radiograph is an essential investigation for TB confirmation (Lonnroth et al., 2010). Other tests such as Tuberculin skin testing (Mantoux test) is still considered to be part of routine care for TB infected persons despite the limited sensitivity and specificity of the test (CobeLens et al., 2006).

2.2.3 Treatment of TB

Prophylactically, inactive (latent) TB may be suppressed with an antibiotic such as isoniazid (INH), to prevent active disease manifesting itself (Bass et al., 1994). On the other hand, active TB is successfully treated with isoniazid (INH) in combination with one or more of several drugs, including rifampicin, ethambutol,
pyrazinamide and streptomycin (WHO, 2009). Persons with concurrent HIV and TB infections usually can be treated with standard anti-TB regimens with good results, although in some cases, prolonged therapy has been suggested (CDC, 1998).

2.3 Trend and impact of HIV and TB co-infection

Tuberculosis and HIV are global health problems and among the major causes of infectious disease burden. The number and proportion of individuals living with HIV and TB varies considerably both regionally and globally (UNAIDS, 2012). Approximately 14 million individuals worldwide have been diagnosed with HIV and TB co-infection (WHO, 2012b). Specifically, in 2010 alone, 39% of new TB cases were recorded among HIV co-infected persons worldwide (WHO, 2012b). In the Sub-Saharan Africa, Kenya is ranked as among the world’s largest HIV-1 and TB stricken countries (UNAIDS, 2012). For instance, approximately 105,781 new TB infection cases were reported in Kenya, of which 41% of them turned out to be HIV-1 infected (NASCOP, 2011; NACC, 2012). The major factor responsible for the large TB disease burden both in Kenya and the world as a whole is the concurrent HIV co-infection (International Medical Corps, 2010; NASCOP, 2011). Other than TB being the second leading cause of death in people living with HIV-AIDS (PLWHA’s), it also accounts for about 26% of acquired immune deficiency syndrome related deaths (UNAIDS, 2012; WHO, 2012b).
2.4 Epidemiology of drug use and HIV-1/TB co-infection

Drug use among the HIV-1 infected persons and associated co-morbidities is currently alarming (Altice et al., 2010; UNODC, 2014). The actual number of IDUs worldwide is not precisely known, although it has been estimated that about 12.7 million individuals inject drugs (UNODC, 2013), with majority of the affected residing in low- and middle-income countries (Aceijas et al., 2004). Drug injection behaviour is reported to be highly responsible for the high prevalence rate of HIV and TB co-infection (Friedman et al., 1996; Keizer et al., 2000). As a consequence, the injection drug user population contributes substantially to HIV-1 acquisition and associated morbidity and mortality globally (Nieburg and Carty, 2011; UNODC, 2012). For instance, there were an estimated 183,000 drug-related deaths occurring globally in the year 2012 (UNODC, 2013).

The notably high burden of disease and drug-related deaths among IDUs has been linked with the use of addictive opioid drugs, predominantly heroin and cocaine (UNODC, 2007). In Kenya, multiple drug abuse studies also demonstrate an extremely high HIV prevalence among IDUs that ranges from 43-49% with new HIV-1 infections occurring at rates of 17% (IRIN, 2012; UNODC, 2012). This phenomenon partly explains why Mombasa alone with more than 26,000 IDUs (Tun et al., 2011), has at least 1 in every 4 IDUs turning out to be HIV positive (MOH, 2012). However, there is paucity of information on HIV-1/TB co-
infection burden among IDUs in many African countries, partly because people who inject drugs are often hard to reach and difficult population to sample (WHO, 2012a). Moreover, very few IDUs actually report to the approved harm reduction centres to seek for medical interventions (Mathers et al., 2010).

2.4.1 Injecting drug use in Kenya

People who inject drugs are key in the spread of HIV-1 infection and are hence categorised as among the most at risk populations of HIV transmission in Kenya (Brodish et al., 2011). The HIV infection rates within risk groups such as IDUs, commercial sex workers and men who have sex with men (MSM) are extremely high (UNAIDS, 2010). Women who inject drugs have consistently higher HIV prevalence as compared to their male counterparts (MOH, 2012), arguably since they are more vulnerable in the drugs and sex trade. High prevalence of HIV among IDUs in Kenya is attributed to a notable rise in drug trafficking (Beckerleg et al., 2005), with Coastal and Nairobi regions identified as the major hot-spots (Brodish et al., 2011).

It is estimated that approximately 98% of IDUs inject themselves with heroin (Tun et al., 2011), although cocaine and amphetamines are also among the abused injection drugs (UNODC, 2013). Additionally, other than injectable drugs such as heroin and cocaine, studies conducted in Kenya also documented high use of bhang, khat, tobacco and alcohol among drug addicted youths in the country
(Ndetei et al., 2009). Needle sharing coupled with low HIV and TB disease awareness levels has been singled out as among the major risk factors for HIV transmission, as compared to other non-occupational exposures (Beckerleg et al., 2005; Nieburg and Carty, 2011). This has made IDUs a significant contributor to the HIV-1 menace in the country (NACC, 2009).

2.5 Mechanisms of HIV induced TB pathogenesis during concurrent HIV-TB co-infection

Infection with HIV-1 has been reported to increase the risk of acquiring primary MTB infection by approximately 2.2-5.5 times (Sonnenberg et al., 2001; Crampin, 2010). Likewise, HIV-1 infection raises the risk of reactivation of latent TB by about 20-fold (Pawlowski et al., 2012). Several potential mechanisms exist by which HIV-1 infection may increase susceptibility of the host to respiratory diseases (Drummond et al., 2010). It is reported that infection with HIV-1 critically impairs cell-mediated host responses thereby providing a suitable environment for infection by MTB (Pawlowski, et al., 2012).

Among the HIV-1 infected individuals, the risk of TB infection increases following HIV sero-conversion and remains elevated across the full spectrum of immunodeficiency, increasing steeply with time as the CD4+ cell count declines (Leeds et al., 2012). Moreover, with the progressive loss of CD4+ T cells, the dysfunction in the T cells compartment is also reflected by altered cytokine
expression levels and profile (Graziosi et al., 1996). Consequently, depressed levels of inflammatory cytokines such as IFN-γ become evident in the broncho-alveolar fluid of asymptomatic HIV-1 infected individuals (Hirsch et al., 1999b). On the other hand, increased IL-10 levels may be demonstrated in the lungs of pulmonary TB patients (Redford et al., 2011) which may contribute to progressive TB disease.

During HIV and TB pathogenesis, the lung resident CD4+ T cell population, alveolar macrophages and fibroblasts initially become infected by HIV. This reduces the lung immune response which becomes highly susceptible to opportunistic infections, such as TB (Meltzer et al., 1990; Dolei et al., 1992; Meduri et al., 1992). For example, the examination of broncho-alveolar lavage fluid from TB infected lungs of HIV-1 patients reveals failure of recruitment and activation of CD4+ lymphocytes. Additionally, lymph nodes and other tissues infected by MTB exhibit large number of neutrophils and necrosis (Smith et al., 2000). Numeric depletion, functional impairment, and disruption of CD4+ lymphocyte-macrophage interactions thus results in impaired granuloma formation and ultimately failure of TB restriction (Lawn et al., 2002; Diedrich and Flynn, 2011).

On the other hand, immune activation increases susceptibility of mononuclear cells to infection with HIV and causes accelerated HIV replication within infected
cells (Fauci et al., 1996). Other HIV induced immune defects that may facilitate infection with MTB include the suppression of cellular immune responses by regulatory T cells (Sarrazin et al., 2009), and the impairment of TNF-α mediated apoptotic responses to MTB (Patel et al., 2007). HIV-1 viral load is thus increased at sites of disease and within the systemic circulation. Additionally, it has also been demonstrated that TB infection induces nuclear factor kappa-B (NFkB), a cellular factor that binds to promoter regions of the HIV virus and enhances replication (Zhang et al., 1995b). Overall, these observations suggest that infection with HIV leads to systemic and local immunological abnormalities that promote the development of TB in HIV-1 infected patients.

2.6 Cytokines and associated immunopathology in HIV-1 and TB co-infection

The presence of bacterial and viral pathogens in patients’ bloodstream often stimulates the production of plasma cytokines and other immunomodulatory proteins (Koyama et al., 2008). Cytokines are peptides with hormone-like actions that function to regulate the immune system by being involved in both inflammatory and immune mediated processes (Poli, 1999). The pro- and anti-inflammatory cytokines such as IFN-γ and IL-10 respectively, and their signalling pathways are reported to play key roles in protection from and pathogenesis of both HIV-1 and TB infections (Poli, 1999; Sahiratmadja et al., 2006). Hence their
balance and dynamic changes may control or predict clinical outcome in HIV/TB infections.

Infection with the HIV-1 virus is characterized by dysfunction in T cells which results from the combined effects of changes in antigen specific lymphocytes, and soluble factors such as chemokines and cytokines (Barber et al., 2006). The lymphocyte subset known as Th-1 (T helper 1) is responsible for directing a cytotoxic CD8+ T-lymphocyte response, while the Th-2 (T helper 2) subset of CD4+ and CD8+ T-lymphocytes diminishes the cytotoxic lymphocyte response while increasing antibody production (Mosmann et al., 1986). During concurrent HIV-1 and TB infections, plasma levels of the Th-1 associated cytokine profile including IL-2, IFN-γ, IL-12 and TNF-α decline as disease progresses while the Th-2 based anti-inflammatory cytokine expression profile such as IL-4 and IL-10 become elevated (Klein et al., 1997; Hirsch et al., 1999b). The T helper 2 based cytokines are expressed in an attempt to partially control virus and bacterial replication by down regulating the expression of the pro-inflammatory cytokines, although it has been suggested that this action does not necessarily serve immuno protective purposes (Poli et al., 1990; Zhang et al., 1995a; Fauci et al., 1996).

Therefore, during the course of HIV and TB disease progression, the predominant cytokine response shifts from Th-1 to Th-2 feedback (Clerici and Shearer, 1994), consequently and positively modulating the ratio of anti-inflammatory to the pro-
inflammatory cytokines. Intensified Th-1 responses are associated with a slower rate of disease progression and increased lifespan (Breen, 2002; Verani et al., 2005). During early TB infection however, there is significant elevation in the levels of interleukin-12 which stimulates Th-1 differentiation leading to increased production of IFN-γ that is necessary for resistance to TB infection (Trinchieri, 1994). It has been thus proposed that failure of HIV-AIDS patients to produce high levels of IFN-γ in the lungs during co-infection with pulmonary TB most likely contributes to an enhanced MTB pathogenesis (Vassiliki et al., 2000).

Interestingly, the changes observed in the pattern of Th-1 and Th-2 type cytokine expression during the course of HIV-1 infection seem contradictory. Some studies have proposed that in early HIV-1 infection, Th-1 cells effectively control the level of plasma HIV viral load. However, during the course of time, the predominant cytokine response shifts from a Th-1 to Th-2 feedback (Clerici and Shearer, 1994). Contrastingingly, other studies have reported no changes in the ratio of Th-1 to Th-2 cytokine shift with HIV disease progression (Romagnani and Maggi, 1994; Estaquier et al., 1995). In addition, others have reported lack of correlations between HIV infectivity of Th-1 and Th-2 subjects (Mikovits et al., 1998). Moreover, a number of studies hypothesise that HIV-1 infection stimulates the production of both Th-1 and Th-2 cytokines but fails to induce a polarised Type 1 or 2 state (Graziosi et al., 1994; Fakoya et al., 1997). These observed conflicting findings have however been attributed to the different methods
employed in the assessment of cytokine expression (Klein et al., 1997). Moreover, the many reports supporting the theory of Th-1 cytokine shift towards the direction of Th-2 cytokine profile during HIV disease pathogenesis are based on production of cytokines under *in-vitro* conditions which may possibly fail to correspond with the *in-vivo* environment (Breen, 2002).

### 2.7 Interferon-γ, IL-10 and Acrp30 in HIV-TB infection

Interferon gamma, a pro-inflammatory cytokine, IL-10 and Acrp30 both anti-inflammatory cytokines, are all associated with profound immunologic, biochemical, metabolic and haematologic alterations in both HIV-1 and TB infections (Meng et al., 2002; Solomon et al., 2008; Tang et al., 2011; Estrella et al., 2012). Although, at least three types of interferons; α-, β- and γ-interferon are reported to exist, the IFN-γ is the hallmark cytokine of Th-1 cells that possess antiviral, immunoregulatory and anti-tumor properties (Schroder et al., 2004). Protective immunity to intracellular bacteria such as MTB infection is dependent on cell-mediated immunity, partially due to enhanced production of IFN-γ and TNF-α (Cowley and Elkins, 2003). Similarly, infection with HIV induces pro-inflammatory cytokine production hence the Th-1 arm of the immune system appears to be maximally stimulated particularly in individuals with advanced levels of HIV disease (Liu et al., 2011).
However, research shows that TB and HIV co-infected individuals display a different cytokine expression pattern when compared to those individuals with either disease alone. TB and HIV co-infection is reported to be characterized by elevated plasma concentration of IFN-γ, TNF-α, IL-4 and reduced levels of IL-12 and IL-17 compared to TB mono-infection (Riou et al., 2012). On the other hand, high levels of IFN-γ, IL-12 and IL-18 are highly expressed in plasma of patients dually infected with TB and HIV compared with those with mono infection (Subramanyam et al., 2004). It is hence hypothesized that co-infections with both HIV and TB infections leads to the interference with the normal TB cellular immune responses thereby causing a dysregulation in cytokine expression profile. Alternatively, it is also presumed that TB infection influence different types of cytokine response during HIV infection.

Monocytes have been reported to be the major producer of IL-10 in HIV infected individuals (Clerici et al., 1994). However numerous other cell types have as well been identified to express this anti-inflammatory cytokine (Moor et al., 2001; Couper et al., 2008). Interleukin-10 exerts an immunoregulatory role and is largely associated with suppression of host antiviral immune response, partly by down-regulating the expression of Th-1cytokines (Klein et al., 1997), for instance by inhibiting IFN-γ cytokine production by macrophages (Fiorentino et al., 1991). Findings from various studies have pointed out a pathogenic role of IL-10 during
HIV infection (Stylianou et al., 1999). This can be evidenced by the fact that HIV viral replication is stimulated by a variety of cytokines inclusive of interleukins and TNF-γ, which activate CD4 T lymphocytes thereby making them more susceptible to HIV infection (Pantaleo et al., 1993b; Fauci et al., 1996).

Human immunodeficiency virus -infected individuals have been shown to have elevated systemic IL-10 levels (Trabattoni et al., 2003; Norris et al., 2006), with the highest measures being observed in patients with advanced clinical disease and increased virus load (Srikanth et al., 2000). Similarly, much higher IL-10 production has been detected in plasma of HIV and TB co-infected patients compared to their HIV mono-infected counterparts (Geldmacher et al., 2010; Benjamin et al., 2013; Chetty et al., 2014). Since IL-10 is involved in the mediation of many anti-inflammatory responses, it’s possible that it may also be an essential mediator of inflammation in HIV and TB disease processes.

Among the many cytokines, Acrp30, also known as adipocyte complement related protein (Acrp30), is a 30 kDa protein that belongs to a group of biologically active factors termed adipokines (Robinson et al., 2011). Adiponectin is secreted exclusively from the adipose although some other tissues including the placenta are also involved in its secretion (Chen, et al., 2006). Even though no studies have been conducted specifically for establishing normal values among healthy
individuals from sub-Saharan Africa, previous studies among healthy adults from Cameroon found that plasma Acrp30 levels were 12.9 ± 5.8 µg/ml (Sobngwi et al., 2007).

The cytokine modulates a number of endocrine-like and metabolic functions (Ouchi et al., 2011; Mattu and Randeva, 2013). Hence, decreased production of adipokines by adipose tissue has been reported to predispose artherosclerosis and obesity-related pathological conditions including diabetes mellitus, endothelial dysfunction, cardiovascular disease and chronic kidney disease (Adamczak et al., 2009; Thiondras et al., 2010; Ntaios et al., 2013). These activities thus portray adipokines as important surrogate markers of many metabolic processes including glucose regulation and fatty acid metabolism (Diez and Iglesias, 2003).

Normally, the plasma levels of Acrp30 are reported to be inversely correlated with body fat composition (Ukkola and Santaniemi, 2002), with high Acrp30 concentrations being positively associated with weight loss in obese individuals (Arita et al., 1999). During co-infection with TB and HIV there is notable depletion of body fat mass (Mupere et al., 2010). Additionally, HIV infection causes dysregulation in serum Acrp30 levels and associated body fat redistribution effects (de Luis et al., 2012). For instance, HIV-1 lipodystrophic patients are reported to exhibit relatively low serum Acrp30 levels despite the low body fat mass (Kosinski et al., 2003; Zinn et al., 2013). This observation may
suggest that during HIV infection, Acrp30 fails to maintain its inverse relationship with body fat mass, possibly due to adipocyte dysfunction as a result of the effects of HIV virus (Kosminski et al., 2003). The mechanism through which HIV inhibits Acrp30 production is not clear, although studies suggest that HIV proteins, particularly protein R suppresses transcriptional activity of peroxisome proliferator-activated receptor gamma (PPAR-γ), which is associated with regulation of Acrp30 gene expression in human adipocytes (Fiorenza et al., 2011).

On the other hand, although the role of Acrp30 in some anti-inflammatory conditions like type-2 diabetes is clear (Ntaios et al., 2013); levels observed in TB infection have yielded contradicting findings. Some studies on plasma Acrp30 levels during pulmonary TB infection indicate low stimulation of the cytokine during the active phase of disease (Xu et al., 2007), while others have established high levels in the chronic phase of TB infection relative to healthy controls (Keicho et al., 2012). This discrepancy in Acrp30 levels has been related to varied individual differences in immune response to pathogens, or due to influence of other underlying infections other than MTB. However, data on circulating Acrp30 levels in HIV-1/TB co-infection is still scanty, although it has been reported that heroin addicts have reduced Acrp30 levels relative to healthy persons (Housova et al., 2005). All in all, the underlying interaction of Acrp30 in concurrent HIV and
TB infections among patients actively using illicit drugs and other harmful substances requires further assessment.

2.8 Clinical and metabolic correlates of HIV and TB infections in IDUs

Like other pathological disorders, HIV-1 and TB infections are characterised by various types of clinical biomarkers. Hence, HIV-1 viral load and CD4+ T cell counts have long been employed as diagnostic and prognostic markers that predict the presence, course and response of disease to therapeutic interventions (HAART) among HIV infected subjects (Fahey et al., 1990; H.S.M.C, 2000). Previous research has demonstrated that the presence of active TB attenuates HIV disease, indicating that HIV and TB co-infection may be positively associated with a higher HIV viral load that corresponds with low baseline CD4+ T cell counts (Geldmacher et al., 2008), that rapidly decline with HIV-TB disease progression (Whalen et al., 1995). However, upon successful institution of antiretroviral therapy immune reconstitution and CD4+ T cell recovery occurs (Thompson et al., 2010), which is preceded by a measurable reduction in systemic HIV viral load (Kranzer et al., 2013).

Recent studies have recommended that HIV viral load should be the preferred clinical parameter to monitor individuals on ART (Bonner et al., 2013; Rutherford et al., 2014), arguably because it accurately detects virological failure prior to the manifestation of either immunological or clinical deterioration. On the
other hand, it is well documented that CD4+ T cell counts influence both the frequency and severity of active TB disease (Jones et al., 1997). Clinical reports indicate that during various microbial infections including MTB, the plasma CD4+ T cells mount crucial adaptive immune responses against these microbial pathogens (Gallegos et al., 2008).

However, investigations determining the effects of TB treatment on plasma CD4+ T cell counts have yielded contradicting results. Some of these studies describe that TB therapy significantly increases the CD4 cell numbers hence positively influencing the CD4 lymphocyte count (Martin et al., 1995), whereas other reports indicate lack of significant increase in CD4 cell counts nor reduction in plasma HIV viral load during the treatment of TB in HIV-1 co-infected patients (Morris et al., 2003; Wolday et al., 2003). This conflicting outcome has been associated with a dysregulated systemic TNF-α expression as a consequence of HIV/TB infection. Tumor necrosis factor-alpha which is pivotal in the immune response to MTB infection causes tissue damage if secreted in unregulated amounts (Rook and Graham, 2007). However, the exact immunological mechanisms behind this activity are not well understood.

During the clinical management of individuals co-infected with HIV and TB, the combination of HAART and TB therapy approach may be complicated by factors including overlapping drug toxicities, drug-drug interactions and immune
reconstitution reactions (Burman and Jones, 2001; WHO, 2004). Therefore, to address these concerns, it has been suggested that HIV-TB co-infected patients should be started on TB treatment immediately and thereafter initiated on antiretroviral therapy (ART) (Blumberg et al., 2003).

Substance abuse is an important co-morbidity factor that affects the outcomes of both HIV and TB clinical management (Lucas et al., 2002). For instance, injection drug use has been associated with an impaired short and long-term CD4+T cell recovery in HIV positive IDU’s on HAART (Siddiqui et al., 1993; Dronda et al., 2004). Interaction between HAART and injecting drug use has been linked to poor clinical outcomes in HIV positive injection drug users (MOH, 2012; NASCOP, 2012). For example, the regular use of cocaine has been associated with a rapid decline in CD4 cell counts among HIV-1 infected injection drug users (Meijerink et al., 2014), possibly due to excessive systemic inflammation associated with injection drugs (Were et al., 2014). As a consequence, repeated drug injection eventually results in impaired lymphocyte reactivity among both HIV-positive and -negative drug users (Mientjes et al., 1991). Likewise, reduced suppression of HIV viral load has been reported in various clinical studies and is linked to recurrent illicit drug injection (Weber et al., 2009). Hence these findings provide a good indication that injection drug use triggers significant level of immune suppression while also accelerating disease progression among injection drug users.
Body mass index (BMI) has been identified as an indicator of changes in body mass composition and has been strongly correlated with both HIV and TB infections (Mupere et al., 2010). As such, body weight and BMI have been frequently used as markers of malnutrition for predicting poor health outcomes in HIV patients (Normen et al., 2005). Additionally, low BMI (<18.5 kg/m²) has been related to higher risk of TB and death (Hanrahan et al., 2010). Reduced body weights and BMI have been reported among injection drug users (Varela et al., 1990; McCombie et al., 1995; Forrester et al., 2004). This has been related to extensive poverty and food scarcity that is common to majority of IDUs (Anema et al., 2013). Moreover, it has been recently demonstrated that HIV-1 positive IDUs exhibit lower BMI and are more malnourished compared to HIV-1 negative IDUs (Quach et al., 2008; Tang et al., 2011). Similarly TB infected IDUs manifest extreme weight loss relative to TB infected non-drug user counterparts (Ferreira et al., 2003). It is possible that chronic inflammation associated with HIV-1 and TB infections and injection drug use alters the nutritional profile (BMI) among HIV-TB co-infected injection drug users.

2.9 Impact of highly active antiretroviral therapy on IFN-γ, IL-10 and Acrp30 in HIV-1 and TB infections amongst IDUs

Based on previous studies, it is evident that various plasma cytokines are expressed within the body during HIV/TB disease progression (Benjamin et al.,
2013; Mihret et al., 2014). However, the effect of ART on the circulating plasma cytokine levels has not been fully interrogated. Highly active antiretroviral therapy (HAART) is a combination of HIV treatment drugs composed of at least three antiviral drugs selected from Nucleoside Reverse Transcriptase Inhibitors (NRTIs), Non-nucleoside Reverse Transcriptase Inhibitors (NNRTIs) and Protease Inhibitors (PIs) (Gazzard and Moyle, 1998). It has been previously reported that HAART is effective in the suppression of HIV-1 viral load (Wei et al., 1995), and subsequently increases the CD4+ T cell count during both HIV and TB infections (Ho et al., 1995; Martin et al., 1995). Additionally, HAART significantly lowers the chance of activating latent TB (Tseng et al., 2009), thereby minimising incidences of HIV-TB co-infection.

Description of plasma circulating IFN-γ levels during ART has yielded discordant results. In acute HIV-1 infection there is usually a steady raise in IFN-γ levels, which remain markedly increased during antiretroviral therapy (Reuben et al., 2002; Watanabe et al., 2010). Contrastingly, more recent studies have documented a significant reduction of plasma IFN-γ levels following administration of HAART among previously untreated HIV-1 infected subjects (Malherbe et al., 2014). These contradictory findings have been attributed to distinct mechanisms of immune activation that are differentially affected by ART. However, the incongruous results require further assessment to determine kinetics
of IFN-γ production during HIV-1 viral infection with particular emphasis on contribution of each antiretroviral agent towards cytokine production.

Successful ART has previously been reported to lower plasma IL-10 levels (Brockman et al., 2009), which is paralleled by a reduction in viral load among HIV-1 infected individuals (Imami et al., 1999). Similarly, other studies have indicated that serum IL-10 levels are highly elevated in HIV/AIDS patients co-infected with Mycobacterium avium complex. However, the systemic levels of this cytokine gradually decrease upon commencement of HAART (Stylianou et al., 1999). On the contrary, plasma levels of IL-10 remain unaffected before and after the initiation of anti-TB therapy in HIV-1 and TB co-infected patients (Mihret et al., 2014). This indicates that high HIV-viral load may be the main driver of high plasma IL-10 levels, which significantly reduce upon effective treatment with ART. Furthermore, a lack of decline in plasma IL-10 levels following HAART administration has been associated with virologic treatment failure (Stylianou et al., 1999).

Adiponectin levels have been shown to inversely correlate with body fat mass; hence reduced Acrp30 levels are associated with obesity and cardiovascular disease (Robinson et al., 2011). However, HIV infected individuals on HAART are reported to exhibit markedly reduced Acrp30 levels compared to uninfected persons (Addy et al., 2003; Kosmiski et al., 2008). This has been attributed to
changes in adipocyte function associated with HIV lipodystrophy (de Waal et al., 2013). Additionally, other factors including certain antiretroviral medication have been reported to influence plasma circulating Acrp30 levels. In particular, exposure to stavudine (d4T) treatment has been associated with lower plasma Acrp30 levels (Lindegaard et al., 2004).

Recurrent drug injection comportment has been associated with impaired short and long-term CD4+ T cell recovery upon institution of HAART in HIV positive IDUs (Dronda et al., 2004). Generally, the interaction between HAART and injecting drug use has been linked to poor clinical outcomes in HIV positive IDUs (MOH, 2012; NASCOP, 2012b). Likewise, drug injection and illicit substance use has been observed to cause a dysregulation in innate molecular markers, notably the pro- and anti-inflammatory cytokines (Zaki et al., 2005; Weiss et al., 2006; Azarang et al., 2007), which serve as regulators of immune function during HIV and TB disease processes (Schroder et al., 2004; Geldmacher et al., 2010).

Unfortunately, the information that exploits drug interactions between antiretrovirals and various illicit injection drugs and observed plasma cytokine levels among IDUs with concurrent HIV-1 and TB co-infection is scanty. Perhaps, ethical and study design challenges have prevented this studies from being fully undertaken. Nonetheless, the current study attempted to determine the relative plasma concentrations of IFN-γ, IL-10 and Acrp30 in HAART-naive and
-experienced HIV-1 and TB mono- and co-infected drug users and associate these levels to the often used clinical markers of HIV-1 and or TB pathogenesis. Hence, findings obtained may provide valuable information on HIV-1/TB disease progression in IDUs and showcasing the influence of ART towards the observed plasma cytokine levels.
CHAPTER THREE
MATERIALS AND METHODS

3.1 Study site
The study was conducted in Mombasa County (Aug, 2012-Nov, 2013) and sampling performed at Bomu hospital, a drug and substance rehabilitation facility in the Coastal region of Kenya (Appendix I). Mombasa is the second largest city and covers an area of approximately 212.5 km² with a population of about 900,000 inhabitants as per the 2009 government census report (KNBS, 2010). The County serves as a major tourist attraction centre and trade hub (Valle and Yobesia, 2009). However, Mombasa County has the fifth highest HIV/AIDS burden among all the Counties in Kenya (NACC, 2013), and accounts for the largest number of illicit drug users (UNAIDS, 2015), which can possibly be attributed to tourism associated commercial sex work and overall injecting drug behaviour that is favoured by a strategic geographical location for illicit drug trafficking (Nieburg and Carty, 2011). Additionally, it was estimated that 37.6% residents of Mombasa were living below poverty line (KNBS, 2010) which further aggravates the already existing drug abuse situation.

3.2 Study design
This study employed a cross-sectional hospital-based experimental study among IDUs from Mombasa, Kenya. The study was part of a larger project that was undertaken to investigate the socio-economic trends and nutritional determinants
among HIV-1 and TB co-infected IDUs. The present investigation made use of plasma sample repository besides demographic and anthropometric data that was previously gathered during this study period.

3.3 Study population

The target population comprised of both adult male and female (≥ 18) years HIV-1/TB infected IDU’s and healthy controls, from Mombasa County. The participants were categorised into seven study groups as follows: Group A) HIV-1/TB co-infected ART-experienced IDUs; group B) HIV-1 mono-infected ART-experienced IDUs; group C) HIV-1/TB co-infected ART-naive IDUs; group D) HIV-1 mono-infected ART-naive IDUs; group E) TB mono-infected IDUs; group F) HIV-1 negative and TB uninfected IDUs; group G) healthy controls.

3.4 Inclusion and exclusion criteria

Standardised self-report questionnaires were used to capture information describing age, gender, drug type and duration of injection, HIV status, initiation and span of ART use for the HIV-experienced injection drug users (Appendix V). Presence of needle scars on the hand and individual history of injection drug use qualified for the enrolment of IDUs into the study. Additionally, all IDUs and healthy controls were classified based on UNODC (2013) drug definition criteria. The healthy controls were recruited from among HIV-1 negative and TB uninfected individuals without evidence of illness and history of injection drug
use. At enrolment, none of the study participants reported prior anti-tuberculosis therapy whereas all ART-experienced IDUs were on 1st line antiretroviral treatment as per the Kenyan ART algorithm (NASCOP, 2012b).

3.5 Sample size calculation

The study sample size was calculated based on the methods for studies analysed using independent “t” tests and related tests (Dallal, 2012). Since, type I error (α=1.96), the incorrect rejection of a true H₀ hypothesis and type II error [(power, (1-β)], the failure to reject a false H₀ hypothesis are conveniently applied in calculating sample size that gives 80% power at the 0.05 level of significance (two sided), the formulae: 

\[ n = \left[ \frac{16\sigma^2}{\Delta \mu^2} \right] + 1 \] (Dallal, 2012), was therefore employed in sample size calculation, based on previous studies showing average plasma IFN-γ levels of 7.5±1.5 (pg/ml) among healthy controls and HIV-TB co-infected subjects presenting with average plasma IFN-γ levels of 31.0±10.1 (pg/ml).

Where;

\( n \) = desired sample size,

16 = constant obtained from α and 1-β values (Dallal, 2012)

\( \sigma \) = standard deviation = 10.1

\( \Delta \mu \) = mean difference. For independent “t” tests; thus \( \Delta = \mu_1 - \mu_2 = 31.0 - 7.5 = 23.5 \)

\[ n = \left[ \frac{16\sigma^2}{\Delta \mu^2} \right] + 1 = \left[ \frac{16 (10.1)^2}{23.5^2} \right] + 1 = 4 \sim 9 \text{ (Minimum)} \]
NB: To increase power of our study, sample size was raised to a minimum of 9 sera per study group.

3.6 Sampling procedure

Recruitment of the study participants was done via respondent-driven sampling (RDS) and makeshift outreach sampling methods (Heckathorn, 1997). Sampling was only performed once at enrolment of study participants. Makeshift outreach made use of rehabilitated IDUs whom were identified using selected special cards. These IDUs in turn attracted the non-rehabilitated IDUs through the respondent-driven sampling approach. Only the non-rehabilitated IDUs and a group of healthy controls were incorpotated into the study. The methods utilised were appropriate and consistent with UNODC criteria of sampling among IDU groups (UNODC, 2012). Injection drug users are a hidden and hard to reach population with a highly interconnected network and therefore only known among themselves (Heckathorn, 1997). Convenience and purposive sampling were used for recruiting the healthy control group.

3.7 Ethical considerations

All testing was voluntary and clearly written informed consent (Appendix IV) was obtained from each participant (≥ 18 years) prior to enrolment into the study. Ethical approval and research permit were obtained from Kenyatta University Ethical Review Committee (record number: PKU 019/116 of 2012; Appendix II)
and the Ministry of Public Health and Sanitation, Kenya (ref:ADM.3/5/37/121; Appendix III). Confidentiality was ensured throughout the study by removing all personal identifiers and instead using codes for sample identification. Study participant records were archived in secure lockable cabinets that were only accessed by the research team and authorised hospital personnel.

### 3.8 Physical measurements

Anthropometric measurements including height and weight and were obtained from each of the study participants during enrolment. The height (m) of the study subjects was measured to the nearest 0.1 cm using the Health-o-meter PORTROD wall mounted height rod (Healthometer®, McCook, USA) while body weights (kg) were measured to the nearest 1.0 g using the Feet design standard electronic manual weighing scale (Richforth Electronics Co., Fuzhou, China). The BMI of each participant was calculated based on height and weight measurement (de Onis, 2006) using the formula: $\text{BMI} = \frac{\text{Weight (kg)}}{\text{Height (m)}^2}$.

### 3.9 Laboratory analysis

#### 3.9.1 Blood sample collection and processing

A total of 10 ml blood sample was collected by venipuncture from each of the study participants and carefully dispensed into ethylene-diamine tetra-acetic acid (EDTA) BD vacutainer® tubes (BD Biosciences, Franklin Lakes, USA). Five (5)
ml of the EDTA anticoagulated blood was used to assay for HIV antibody and CD4+ T lymphocyte enumeration. The remaining EDTA anticoagulated blood was centrifuged at 2500 r.p.m for 15 minutes. The plasma portion was isolated by carefully aspirating the upper layer and transferring into 250 ml aliquots in clearly labelled 1.5 ml eppendorf tubes. The tubes were transferred into freezer boxes and archived at -80°C until assayed for cytokine concentrations and HIV-1 viral load. Upon determination of plasma cytokine levels, respective samples were thawed once only from the frozen stocks.

3.9.2 HIV-1 antibody tests

EDTA anticoagulated whole blood was used to assay for HIV antibody using Determine™ HIV-1/2 (Abbot, Illinois, USA) and Unigold™ (Trinity Biotech, Ireland) immunochromatographic rapid diagnostic kits. Briefly, 0.1ml EDTA anticoagulated blood was separately introduced to the sample pad of both Determine™ HIV-1/2 and Unigold™ antibody test strips. A drop of test kit buffer was then immediately introduced to the rapid test strip by use of a dropper. The sample was allowed to migrate across the test strip and test results were read after a period of between 10 to 15 minutes. Interpretation of the test results was performed as per the manufacturer kit instructions. Study participants who were sero-positive for both Determine™ and Unigold™ rapid antibody tests were considered HIV infected based on the Kenyan national HIV testing algorithm (NASCOP, 2012b).
3.9.3 TB diagnosis

The presence of MTB in the study participants was diagnosed using both clinical presentations and the microscopic demonstration of the presence of acid-fast bacilli (AFBs) in a Ziehl-Neelsen stained sputum smear. The staining technique makes use of carbol fuschin as primary stain and malachite green as counterstain. Briefly, two early morning sputum samples from participants presenting with signs of tuberculosis were obtained and smeared on well labelled frosted slides, air dried and stained first for 15 minutes using the primary stain (carbol fuschin). The primary stain was then washed off. The slide was differentiated using acid alcohol and counterstained with malachite green for 5 minutes. The slide was thereafter examined microscopically for the presence of gram negative short rods that morphologically characterized acid fast MTB. Suspected TB infected participants with negative sputum smear results were subjected to a chest radiograph examination and further TB culture for confirmatory diagnosis.

3.9.4 Enumeration of baseline CD4+ lymphocytes

Determination of CD4+ T cell counts from EDTA anticoagulated whole blood was undertaken using a four colour BD FACSCalibur flow cytometer (Becton-Dickinson™, USA) equipped with an automated acquisition and analysis software. The whole blood lysis procedure and BD multi-test reagent (BD Biosciences, USA) were used in the test. Briefly, 20μl of multi-test reagent was
pipetted into TruCOUNT tubes (BD Biosciences, USA) labelled according to the samples. 50μl of each well-mixed whole blood sample was then pipetted into its corresponding tube after which the mixture was agitated gently on a vortex mixer. Following incubation of the tubes at room temperature in the dark for 15 minutes, 450μl of FACS lysing solution (BD Biosciences, San Jose, CA, USA) was added to each tube and the mixture again vortexed.

Before usage, 1 part of the 10x concentrate FACS lysing solution had been diluted with 10 parts of room temperature deionised water to form 1x concentrate FACS lysing solution. After a further incubation of tubes at room temperature in the dark for 15 minutes, they were vortexed thoroughly at low speed to reduce aggregation and then fed into the automated sample loader on the FACSCalibur system. Using the software on the FACStation computer, the samples were run to identify lymphocytes and lymphocyte subsets. The CD4 lymphocyte cell population within each sample was then recorded from the results obtained and expressed as cells/μL. Fluorescence tagged monoclonal antibodies that were used for the CD4+ T lymphocyte enumeration were the BD Tritest™ : Anti-CD3 fluorescein isothiocyanate (FITC), Anti-CD4 phycoerythrin (PE) and Anti-CD45 peri-dinin chlorophyll protein (PerCP) (BD Biosciences, USA).
3.9.5 Determination of HIV-1 viral load

HIV-1 viral load in the plasma samples was quantified using the Abbott m2000 real-time system with an automated sample extraction, amplification and detection system according to the manufacturer’s instructions (Abbott Molecular Inc., Illinois, U.S.A). Assay sensitivity using the Abbott m2000 is equal to 40 copies/ml with a linear measurement range of 40-10 million copies/ml. Briefly, RNA was extracted from 0.2 ml plasma samples using the 0.2 ml serum RNA extraction and master mix addition protocol of the Abbott m2000sp sample preparation system. The master mix containing the viral RNA was then transferred to the Abbott m2000 real time instrument for viral load detection using the program for 0.2 ml RNA amplification. The extracted RNA was reverse transcribed into cDNA and then amplified using HIV-1 specific and internal control primers. Real time PCR technology used in the Abbott real time (rt) detection system uses two probes: a fluorescent-tagged longer fragment complementary to the target sequence and a quencher molecule bound onto a shorter fragment. Fluorescence emission of the HIV-1 probe was proportional to the amount of HIV-1 target sequence in the sample. The fluorescence counts were converted into viral loads by the analyzer.
3.9.6 Determination of plasma IFN-γ, IL-10 and Acrp30 levels

3.9.6.1 Interferon-gamma ELISA

Quantitative determination of plasma IFN-γ levels in the respective study samples was performed using a commercial sandwich enzyme-linked immunosorbent assay (ELISA) kit (R & D Systems® Europe, Axon, UK) according to the manufacturer’s operation protocol. Briefly, 96-well ELISA microplates (Fisher Scientific®, Pittsburgh, PA, USA) were coated with 100µL/well of anti-human IFN-γ capture antibody (R & D Systems® Europe, Axon, UK) diluted in phosphate buffered saline (PBS, 137 mM sodium chloride, 2.7 mM potassium chloride, 8.1 mM sodium phosphate dibasic (disodium hydrogen phosphate) and 1.5 mM potassium dihydrogen phosphate ; pH 7.2-7.4). The plates were sealed with parafilm and incubated overnight at room temperature.

Following incubation, the plate wells were aspirated and washed three times manually using 250µL/well of wash buffer containing 0.05% Tween® 20 in PBS, pH 7.2-7.4 (R & D systems catalogue). The plates were then blocked by adding 300µL/well of block buffer containing (1% bovine serum albumin [BSA] in PBS with 0.05% NaN₃) followed by incubation at room temperature for a minimum of 1 hour. The plate wells were then aspirated and washed three times manually once more using wash buffer and a multi channel pipette. After the third wash, any remaining wash buffer was removed by inverting the plates and blotting dry against clean paper towels. The plates were now ready for sample addition.
The standards and samples were first diluted 1:1000 in reagent diluent (0.1% BSA, 0.05% Tween® 20 in Tris-buffered saline) while making two fold serial dilutions starting from the highest concentration to lowest concentration. Thereafter, standards were added first (100µL/well), and then quickly preceded by addition of the samples (100µL/well) according to the template set up. After addition of the standards and the samples, plates were covered with parafilm and incubated at room temperature for 2 hours with gentle shaking. This was succeeded by another manual wash step three times using wash buffer. After the last wash, 100µL/well aliquot of detection antibody (biotinylated anti-human IFN-γ) reconstituted with reagent diluent (0.1% BSA, 0.05% Tween 20 in Tris-buffered saline pH 7.4) was added, plates sealed and incubated at room temperature for 2 hours with gentle shaking. Once again, the plate wells were manually washed three times then blotted dry on clean paper towels before 100µL of detecting enzyme (streptavidin conjugated to horseradish-peroxidase [HRP]) diluted in reagent diluent (0.1% BSA, 0.05% Tween 20 in Tris-buffered saline pH 7.4) was added to each plate well and the plates incubated at room temperature for 20 minutes with gentle shaking. The aspiration/wash steps were appropriately repeated using wash buffer and a multi channel pipette. Thereafter, 100 µL of tetramethylbenzidine (TMB): hydrogen peroxide (H₂O₂) prepared substrate solution in the ratio of 1:1 was added using a multi channel pipette to each plate well and incubated for 20 minutes at room temperature in the dark with gentle
shaking. During the incubation period, the plates were allowed to develop until a deep blue colour appeared in the wells. Following the incubation, 50µL of stop solution (2N H$_2$SO$_4$) was added to each plate well to stop the reaction.

The absorbance (optical density, OD) of each plate well was determined immediately using a Dynatech MR5000 microplate reader (Dynex Technologies Inc., Sullyfield, USA) set at 450nm. The wavelength correction was set at 540nm. Each plasma sample and standard was assayed in duplicate, and cytokine standards supplied by the manufacturer used to calculate the concentrations of the samples. Seven point standard calibration curve (1,000 – 500 – 250 – 125 – 62.5 – 31.25 – 15.62 pg/ml) of the corresponding recombinant human IFN-gamma protein included in each assay plate.

3.9.6.2 Interleukin-10 ELISA

The circulating plasma levels of IL-10 in the study samples was determined using a quantitative sandwich Elisa kit (R & D Systems® Europe, Axon, UK) as per the manufacturer’s operation protocol. Briefly, 96-well microplates (Fisher Scientific®, Pittsburgh, PA, USA) were coated with 100µl/well of anti-human IL-10 capture antibody (R & D Systems® Europe, Axon, UK) reconstituted with PBS (137 mM sodium chloride, 2.7 mM potassium chloride, 8.1 mM sodium phosphate dibasic and 1.5 mM potassium dihydrogen phosphate ; pH 7.4). The plates were sealed with parafilm and incubated overnight at room temperature.
Following incubation, the plate wells were aspirated and washed three times manually using 250µL/well of wash buffer containing 0.05% Tween® 20 in PBS, pH 7.2-7.4. The plates were then blocked by adding 300µL/well of reagent diluent containing (1% BSA in PBS pH 7.2-7.4) followed by incubation at room temperature for a minimum of 1 hour. The plate wells were aspirated and washed three times manually once more using wash buffer and a multi channel pipette. After the third wash, any remaining wash buffer was removed by inverting the plates and blotting dry against clean paper towels awaiting sample addition.

The standards and samples were first diluted 1:2000 in assay diluent (1% BSA in PBS, pH 7.2-7.4). Serial dilutions of the human plasma samples and the standards in assay diluent were made. Standards were added first (100µl/well), and then quickly preceded by addition of the samples (100µl/well) according to the template set up. After addition of standards and the samples, plates were covered with parafilm and incubated at room temperature for 2 hours with gentle shaking. This was succeeded by another manual wash step three times manually using wash buffer. After the last wash, 100µl/well of detection antibody (biotinylated anti-human IL-10) reconstituted with reagent diluent (1% BSA in PBS, pH 7.2-7.4) was added, plates sealed and incubated at room temperature for 2 hours with gentle shaking. Once again, the plate wells were manually washed three times then blotted dry on clean paper towels before 100µl of working ratio of detecting
enzyme (streptavidin-horseradish peroxidase [HRP]) was added to each plate well and the plates incubated at room temperature for 20 minutes with gentle shaking. The aspiration/wash steps were appropriately repeated using wash buffer and a multi channel pipette. Thereafter, 100 μl of tetramethylbenzidine (TMB): hydrogen peroxide (H₂O₂) substrate solution in the ratio of 1:1 was added using a multi channel pipette to each plate well and incubated for 20 minutes at room temperature in the dark with gentle shaking. Following the incubation, 50μl of stop solution (2N H₂SO₄) was added to each plate well to stop the reaction.

The absorbance (optical density, OD) of each plate well was determined immediately using a Dynatech MR5000 Microplate Reader (Dynex Technologies Inc., Sullyfield, USA) set at 450nm. The wavelength correction was also set at 540nm. Plasma cytokine concentrations were calculated according to the standard calibration curves (2,000 – 1,000 – 500 – 250 – 125 – 62.5 – 31.25 pg/ml) of the corresponding recombinant human IL-10 protein included in each assay plate.

3.9.6.3 Adiponectin ELISA

The circulating plasma levels of Acrp30 in the study samples was determined using a quantitative sandwich enzyme immuno assay kit (R &D Systems Europe, Axon, UK) as per the manufacturer’s instructions. Briefly, 96-well microplates (Fisher Scientific®, Pittsburgh, PA, USA) were coated with 100μl/well of anti-human Acrp30 capture antibody (R & D Systems® Europe, Axon, UK) diluted in
PBS (137 mM sodium chloride, 2.7 mM potassium chloride, 8.1 mM sodium phosphate dibasic and 1.5 mM potassium dihydrogen phosphate; pH 7.4). The plates were sealed with parafilm and incubated overnight at room temperature.

Following incubation, the plate wells were aspirated and washed three times manually using 250µL/well of wash buffer containing 0.05% Tween® 20 in PBS, pH 7.2-7.4. Blocking of plate was performed by adding 300µL/well of reagent diluent containing (0.05% BSA, 0.05% Tween® 20 in PBS pH 7.2-7.4) followed by incubation at room temperature for a minimum of 1 hour. The plate wells were aspirated and washed three times manually once more using wash buffer and a multi channel pipette. After the third wash, any remaining wash buffer was removed by inverting the plates and blotting dry against clean paper towels awaiting sample addition.

The standards and samples were first diluted 1:18,000 in assay diluent (0.05% BSA, 0.05% Tween® 20 in PBS pH 7.2-7.4). Thereafter, standards were added first (100µl/well), and then quickly preceded by addition of the samples (100µl/well) according to the template set up. After adding the standards and the samples, plates were covered with parafilm and incubated at room temperature for 2 hours with gentle shaking. This was succeeded by another manual wash step three times using wash buffer. After the last wash, 100µl/well of detection antibody (Biotin conjugated secondary antibody) diluted in reagent diluent
(0.05% BSA, 0.05% Tween® 20 in PBS pH 7.2-7.4) was added, plates sealed and incubated at room temperature for 2 hours with gentle shaking. Once again, the plate wells were manually washed three times then blotted dry on clean paper towels before 100µl of working ratio of detecting enzyme (streptavidin-horse radish peroxidase [HRP]) was added to each plate well and the plates incubated at room temperature for 20 minutes with gentle shaking. The aspiration/wash steps were appropriately repeated using wash buffer and a multi channel pipette. Thereafter, 100 µl of tetramethylbenzidine (TMB): hydrogen peroxide (H₂O₂) substrate solution in the ratio of 1:1 was added using a multi channel pipette to each plate well and incubated for 20 minutes at room temperature in the dark with gentle shaking. Following the incubation, 50µl of stop solution (2N H₂SO₄) was added to each plate well to stop the reaction.

The absorbance (optical density, OD) of each plate well was determined immediately using a Dynatech MR5000 Microplate Reader (Dynex Technologies Inc., Sullyfield, USA) set at 450nm. The wavelength correction was also set at 540nm. Sample cytokine concentrations were calculated based on the appropriate standard calibration curves (18,000 – 6,000 – 2,000 – 666.7 – 222.2 – 74.1 – 24.7 and 0.0 pg/ml) of the corresponding recombinant human Acrp30 protein was included in each assay plate.
3.10 Data processing and statistical analyses

The data generated was entered, cleaned and coded in Excel spreadsheets (MS®, Office). All non-parametric statistical analyses were performed using Statistical Product and Services Solutions package (SPSS) version 19.0 software (IBM®, Chicago, USA) and GraphPad Prism version 5.00 software for Windows (Graphpad, California). Continuous variables including; age, weight, height, BMI, CD4+ T cell counts, HIV-1 viral load, IFN-γ, IL-10 and Acrp30 levels were expressed as medians (interquartile range, IQR), in conformity with previous studies (Reuben et al., 2002; Rahman et al., 2011; Kang et al., 2012). On the other hand, categorical data including; gender, duration of drug injection, and ART use were presented as proportions (%). Differences in the distribution of proportions amongst the study groups were compared using the chi-square tests ($\chi^2$).

The plasma levels of IFN-γ, IL-10 and Acrp30 were presented as box plots. Statistical comparisons across study groups in continuous data such as IFN-γ, IL-10, Acrp30 levels, hematologic and anthropometric measures were performed using the Kruskal Wallis non-parametric analysis of variance (ANOVA). Subsequent post-hoc between study groups comparison in cytokine concentrations was performed using the Dunn’s post-hoc test for multiple comparisons, while associations between measured cytokine levels and CD4+ T cell counts, BMI and HIV-1 viral load were performed using the Spearman’s rank sum test. Hypothesis
testing was based on the ANOVA and correlation tests. All tests were two-tailed and $P$-values less than 5% (<0.05) were considered significant.
CHAPTER FOUR

RESULTS

4.1 Demographic and clinical characteristics of the study participants

The total study population comprised of 138 adult participants, of which 68 were males and 70 were females sampled from among HIV-1 and TB co-infected ART-naive (n=9) and -experienced (n=27); HIV-1 mono-infected ART-naive (n=26) and -experienced (n=13); TB mono-infected (n=21), HIV-1 negative and TB uninfected (n=25) IDUs and healthy controls (n=17; Table 4.1). The median age of the study participants ranged from 26.7 to 32.9 years with no significant difference across-groups (P=0.068). Similarly, height was comparable across the study groups ranging between 1.6 and 1.7 metres (P=0.350). On the other hand, body weights of study participants were found to range from 49.0 to 61.0 kg and showed significant difference across the study groups (P<0.0001) with the HIV-1/TB co-infected ART-naive (median, 49.0 kg; P<0.001) and -experienced (median, 50.0 kg; P<0.001) IDUs presenting with lower weights relative to healthy controls (median, 61.0 kg).

Likewise, BMI levels ranged between 17.3 and 23.0 and varied significantly across the study groups (P<0.0001) with the HIV-1 mono-infected ART-naive (median, 18.7 kg/m²; P<0.01), HIV-1/TB co-infected ART-naive (median, 17.3 kg/m²; P<0.001) and -experienced (median, 18.0 kg/m²; P<0.001), and uninfected (median, 19.0kg/m²; P<0.05) IDUs demonstrating significantly reduced BMI
measurements against healthy controls (median, 23.0 kg/m$^2$). Additionally, the CD4+ T cell counts ranged from 344 to 986 cells/µl, with significantly lowered cell counts observed among the TB mono-infected (median, 432 cells/µl; $P<0.001$) and HIV-1 mono-infected ART-naive (median, 344 cells/µl; $P<0.001$) relative to HIV-1 negative and TB uninfected IDUs (median; 986 cells/µl); HIV-1 mono-infected ART-naive (median, 344 cells/µl; $P<0.05$) versus HIV-1 mono-infected ART-experienced (median,722 cells/µl; $P<0.05$) and healthy controls (median, 755 cells/µl), respectively. However, among the HIV-1 infected IDUs, HIV-1 viral load showed no significant difference across groups ($P=0.063$).
Table 4.1 Demographic and clinical characteristics of the study participants

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Age, yrs.</td>
<td>26.7 (21.1-34.3)</td>
<td>29.2 (23.2-51.1)</td>
<td>30.6 (24.1-55.2)</td>
<td>32.9 (22.7-47.2)</td>
<td>28.1 (23.2-39.0)</td>
<td>29.2 (22.9-44.6)</td>
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<td>Male/female, %</td>
<td>58.8/41.2</td>
<td>32.0/68.0</td>
<td>52.4/47.6</td>
<td>61.5/38.5</td>
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</tr>
<tr>
<td>Height, m</td>
<td>1.6 (1.5-1.9)</td>
<td>1.7 (1.5-1.9)</td>
<td>1.7 (1.6-1.8)</td>
<td>1.7 (1.6-1.8)</td>
<td>1.7 (1.6-1.8)</td>
<td>1.7 (1.6-1.9)</td>
<td>1.7 (1.6-1.9)</td>
<td>0.350</td>
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<tr>
<td>Weight, kg</td>
<td>61.0 (44.0-85.0)</td>
<td>54.0 (45.0-69.0)</td>
<td>53.0 (39.0-74.0)</td>
<td>53.0 (46.0-62.0)</td>
<td>49.0 (44.0-52.0)^a</td>
<td>53.0 (46.0-67.0)</td>
<td>53.0 (39.0-71.0)^a</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>BMI, kg/m^2</td>
<td>23.0 (17.2-34.7)</td>
<td>19.0 (13.9-23.1)^f</td>
<td>19.4 (15.2-23.5)</td>
<td>18.7 (15.9-21.5)^d</td>
<td>17.3 (16.3-21.3)^a</td>
<td>18.8 (14.1-22.7)</td>
<td>18.0 (13.6-21.9)^a</td>
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</tr>
<tr>
<td>CD4+ T cells/µl</td>
<td>755 (354-1218)</td>
<td>986 (92-1845)</td>
<td>432 (41-1063)^b</td>
<td>344 (14-1078)^f,b</td>
<td>607 (162-1400)</td>
<td>722 (285-1409)^c</td>
<td>522 (39-1199)^g</td>
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<td>Log_{10} HIV-1 RNA, copies/ml</td>
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<td>...</td>
<td>...</td>
<td>...</td>
<td>2.2 (2.2-5.7)</td>
<td>4.1 (2.2-5.7)</td>
<td>2.2 (2.2-5.7)</td>
<td>3.6 (2.2-5.8)</td>
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Data are presented as medians (range) or as indicated. Abbreviations: ART, antiretroviral treatment. IDUs, injection drug users. HIV-1, human immunodeficiency virus type-1. BMI, body mass index. TB, tuberculosis. HC, healthy controls. HIV-TB-, HIV-1 negative and TB uninfected IDUs. HIV-TB+, TB mono-infected IDUs. HIV+ART-TB-, HIV-1 mono-infected ART-naive IDUs. HIV+ART-TB+, HIV-1 and TB co-infected ART-naive IDUs. HIV+ART+TB-, HIV-1 mono-infected ART-experienced IDUs. HIV+ART+TB+, HIV-1 and TB co-infected ART-experienced IDUs. Data analysis was performed using Kruskal Wallis tests for continuous data; and chi-square test χ^2 for proportions. Following the Kruskal Wallis tests, the Dunns post-hoc for multiple comparisons was performed. a. \( P<0.001 \) difference when compared to healthy controls; b. \( P<0.001 \) difference when compared to HIV-/TB- IDUs; c. \( P<0.05 \) difference when compared to HIV+ART-TB- IDUs; d. \( P<0.01 \) difference when compared to healthy controls; e. \( P<0.01 \) difference when compared to HIV-TB- IDUs; and f. \( P<0.05 \) difference when compared to healthy control. Values in bold indicate significant \( P \)-values.
4.2 Profile of substance use among the study participants

Among the study participants, a total of 121 individuals used six injection drugs including heroin, cocaine, a mixture of heroin and cocaine, diazepam, rohypnol and analgesics (Table 4.2). The use of heroin (n=93) as an injection drug was most common in all IDUs at proportions of 88.9%, 74.1%, 76.9%, 7.7%, 90.5% and 100.0%, respectively for HIV-1/TB co-infected ART-naive and -experienced, HIV-1 mono-infected ART-naive and -experienced, TB mono-infected and HIV-1 negative and TB uninfected IDUs. On the other hand, the proportion of IDUs reporting injecting cocaine (n=10) was 11.1% in the HIV-1/TB co-infected ART-naive, 18.5% in the HIV-1/TB co-infected ART-experienced, 11.5% in the HIV-1 mono-infected ART-naive and 4.8% in the TB mono-infected IDUs. Additionally, some IDUs reported using heroin-cocaine mixture (n=2), however the number was much lower than individual substance abuse and was only reported in 7.4% of the HIV-1/TB co-infected ART-experienced IDUs. Abuse of diazepam, rohypnol and analgesics was also prevalent in various categories of IDUs at proportions of 7.7% and 3.8% in the HIV-1 mono-infected ART-naive and 92.3% in the HIV-1 mono-infected ART-experienced IDUs respectively.

Participants in the study group also abused five non-injection substances namely; khat, alcohol, cigarettes, bhang and cocktail (cigarettes and bhang mixture). Cigarettes smoking (n=78) showed highest frequency and was reported in all study groups [HIV-1/TB co-infected ART-naive (55.6%) and -experienced
(66.7\%), HIV-1 mono-infected ART-naive (76.9\%) and -experienced (7.7\%), TB mono-infected (66.7\%) and HIV-1 negative and TB uninfected (80.0\%) IDUs, with the only exception of healthy controls. This was closely followed by alcohol (n=52) use at proportions of 61.5\%, 30.8\%, 11.1\%, 44.4\%, 52.9\%, and 32.0\%, respectively for HIV-1 mono-infected ART-naive and -experienced, HIV-1/TB co-infected ART-naive and -experienced, TB mono-infected and HIV-1 negative and TB uninfected IDUs. Likewise, use of bhang was encountered in about 40.0\% of the HIV-1 negative and TB uninfected, 42.9\% of the TB mono-infected, 53.8\% of the HIV-1 mono-infected ART-naive, 44.4\% of HIV-1/TB co-infected ART-naive and 51.9\% of the HIV-1/TB co-infected ART-experienced injection IDUs. On the other hand, chewing of khat showed least frequency and was reported by 34.6\% of the HIV-1 mono-infected ART-naive, 7.7\% of the HIV-1 mono-infected ART-experienced, 20.0\% of the HIV-1 negative and TB uninfected and 44.4\% of both the HIV-1/TB co-infected ART-naive and-experienced IDUs.
Table 4.2 Profile of substance use among the study participants

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<tbody>
<tr>
<td><strong>Types of injection drugs used, n (%)</strong></td>
<td></td>
<td></td>
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<tr>
<td>Heroin</td>
<td>0 (0.0)</td>
<td>25 (100.0)</td>
<td>19 (90.5)</td>
<td>20 (76.9)</td>
<td>8 (88.9)</td>
<td>1 (7.7)</td>
<td>20 (74.1)</td>
</tr>
<tr>
<td>Cocaine</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>1 (4.8)</td>
<td>3 (11.5)</td>
<td>1 (11.1)</td>
<td>0 (0.0)</td>
<td>5 (18.5)</td>
</tr>
<tr>
<td>Heroin/Cocaine</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>2 (7.4)</td>
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<tr>
<td>Diazepam</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>2 (7.7)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
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<tr>
<td>Rohypnol</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>1 (3.8)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
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<tr>
<td>Analgesics</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>1 (4.8)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>12 (92.3)</td>
<td>0 (0.0)</td>
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<tr>
<td><strong>Non-injection substances used, n (%)</strong></td>
<td></td>
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<tr>
<td>Alcohol</td>
<td>0 (0.0)</td>
<td>8 (32.0)</td>
<td>11 (52.9)</td>
<td>16 (61.5)</td>
<td>1 (11.1)</td>
<td>4 (30.8)</td>
<td>12 (44.4)</td>
</tr>
<tr>
<td>Cigarettes</td>
<td>0 (0.0)</td>
<td>20 (80.0)</td>
<td>14 (66.7)</td>
<td>20 (76.9)</td>
<td>5 (55.6)</td>
<td>1 (7.7)</td>
<td>18 (66.7)</td>
</tr>
<tr>
<td>Bhang</td>
<td>0 (0.0)</td>
<td>10 (40.0)</td>
<td>9 (42.9)</td>
<td>14 (53.8)</td>
<td>4 (44.4)</td>
<td>0 (0.0)</td>
<td>14 (51.9)</td>
</tr>
<tr>
<td>Cocktail</td>
<td>0 (0.0)</td>
<td>6 (24.0)</td>
<td>8 (38.1)</td>
<td>13 (50.0)</td>
<td>3 (33.3)</td>
<td>0 (0.0)</td>
<td>10 (37.0)</td>
</tr>
<tr>
<td>Khat</td>
<td>0 (0.0)</td>
<td>5 (20.0)</td>
<td>5 (23.8)</td>
<td>9 (34.6)</td>
<td>4 (44.4)</td>
<td>1 (7.7)</td>
<td>12 (44.4)</td>
</tr>
</tbody>
</table>

Data analysis was performed using chi-square test $\chi^2$ for proportions. Cocktail, cigarettes and bhang mixture. IDUs, injection drug users. HIV-1, human immunodeficiency virus type-1. TB, tuberculosis. ART, antiretroviral treatment. HC, healthy controls. HC, healthy controls. HIV-TB-, HIV-1 negative and TB uninfected IDUs. HIV-TB+, TB mono-infected IDUs. HIV+ART-TB-, HIV-1 mono-infected ART-naive IDUs. HIV+ART-TB+, HIV-1 and TB co-infected ART-naive IDUs. HIV+ART+TB-, HIV-1 mono-infected ART-experienced IDUs. HIV+ART+TB+, HIV-1 and TB co-infected ART-experienced IDUs.
4.3 Duration of drug injection and ART use of the study participants

Duration of drug injection varied among the population of IDUs selected for the study, with IDUs recruited into the study reporting drug injection for a duration categorised into: less than one year (<1 yr); one to three years (1-3 yrs); and greater than three years (>3 yrs; Table 4.3). The proportion of individuals reporting injecting drugs for less than one year was 48.0 % among the HIV-1 negative and TB uninfected, 28.6% in the TB mono-infected, 15.4% in the HIV-1 mono-infected ART-naive, 22.2% in the HIV-1/TB co-infected ART-naive and 11.1% in the HIV-1/TB co-infected ART-experienced IDUs. Likewise, the proportion of individuals reporting injecting drugs for one to three years was 28.0%, 47.6%, 30.8%, 44.4%, and 29.6% respectively for HIV-1 negative and TB uninfected, TB mono-infected, HIV-1 mono-infected ART-naive, and HIV-1/TB co-infected ART-naive and -experienced IDUs. Additionally, drug injection episodes lasting greater than three years was reported by 59.3% of the HIV-1/TB co-infected ART-experienced, 33.3% of the HIV-1/TB co-infected ART-naive, 53.8% of the HIV-1 mono-infected ART-naive, 23.8% of the TB mono-infected and 24.0% of the HIV-1 negative and TB uninfected IDUs.

During the study, a number of study participants reported having previously enrolled in comprehensive care centres for antiretroviral treatment. Among the antiretroviral treatment exposed IDUs, the treatment duration presented as
follows: less than one year (<1 yr) and greater than or equal to one year (≥ 1 yr) respectively. The proportion of IDUs reporting having enrolled for ART for less than one year was represented by 48.1% in the HIV-1/TB co-infected ART-experienced and 38.5% in the HIV-1 mono-infected ART-experienced IDUs. Likewise, 51.9% of the HIV-1/TB co-infected ART-experienced and 61.5% of the HIV-1 mono-infected ART-experienced IDUs reported ART enrolment for a period exceeding one year.
Table 4.3 Duration of drug injection and ART use of the study participants

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<tr>
<td><strong>Drug injection, n (%)</strong></td>
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<tr>
<td>&lt;1 yr</td>
<td>0 (0.0)</td>
<td>12 (48.0)</td>
<td>6 (28.6)</td>
<td>4 (15.4)</td>
<td>2 (22.2)</td>
<td>3 (23.1)</td>
<td>3 (11.1)</td>
</tr>
<tr>
<td>1-3 yrs</td>
<td>0 (0.0)</td>
<td>7 (28.0)</td>
<td>10 (47.6)</td>
<td>8 (30.8)</td>
<td>4 (44.4)</td>
<td>6 (46.2)</td>
<td>8 (29.6)</td>
</tr>
<tr>
<td>≥3 yrs</td>
<td>0 (0.0)</td>
<td>6 (24.0)</td>
<td>5 (23.8)</td>
<td>14 (53.8)</td>
<td>3 (33.3)</td>
<td>4 (30.8)</td>
<td>16 (59.3)</td>
</tr>
<tr>
<td><strong>ART use, n (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>&lt;1 yr</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>5 (38.5)</td>
<td>13 (48.1)</td>
</tr>
<tr>
<td>≥1 yr</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>8 (61.5)</td>
<td>14 (51.9)</td>
</tr>
</tbody>
</table>

Data presented as proportions (%) or as indicated. Analysis was conducted using chi-square test $\chi^2$ for proportions and Kruskal Wallis tests for continuous data. ART, antiretroviral treatment. HIV-1, human immunodeficiency virus type-1. TB, tuberculosis. IDUs, injection drug users. HC, healthy controls. HIV-TB-, HIV-1 negative and TB uninfected IDUs. HIV-TB+, TB mono-infected IDUs. HIV+ART-TB-, HIV-1 mono-infected ART-naive IDUs. HIV+ART-TB+, HIV-1 and TB co-infected ART-naive IDUs. HIV+ART+TB-, HIV-1 mono-infected ART-experienced IDUs. HIV+ART+TB+, HIV-1 and TB co-infected ART-experienced IDUs.
4.4.1 Plasma IFN-γ levels of the study participants

Circulating plasma IFN-γ levels of the study participants ranged from (median, 34.6 pg/ml) in healthy controls to (median, 72.5 pg/ml) among the HIV-1/TB co-infected ART-naive IDUs respectively (Table 4.4). Statistical analysis using Kruskal Wallis test demonstrated significant difference in IFN-γ levels across study groups ($P<0.0001$), with subsequent Dunn’s post-hoc revealing high IFN-γ levels in HIV-1/TB co-infected ART-naive (median, 72.5 pg/ml; $P<0.001$) and -experienced (median, 71.2 pg/ml; $P<0.001$), and HIV-1 mono-infected ART-experienced (67.1 pg/ml; $P<0.001$) IDUs relative to healthy controls (median, 34.6 pg/ml). In addition, the HIV-1/TB co-infected ART-naive (median, 72.5 pg/ml; $P<0.001$) and -experienced (median, 71.2 pg/ml; $P<0.001$), and HIV-1 mono-infected ART-experienced (median, 67.1 pg/ml; $P<0.001$) IDUs showed elevated plasma IFN-γ levels compared to HIV-1 mono-infected ART-naive (median, 35.7 pg/ml) IDUs (Figure 4.1).

On the other hand, the HIV-1 negative and TB uninfected IDUs (median, 38.4 pg/ml; $P<0.01$) elicited higher IFN-γ levels relative to healthy controls (median, 34.6 pg/ml), while the HIV-1/TB co-infected ART-naive (median, 72.5 pg/ml; $P<0.001$) and -experienced (median, 71.2 pg/ml; $P<0.001$) demonstrated raised IFN-γ cytokine levels compared to TB mono-infected (median, 35.5 pg/ml) IDUs. Furthermore, study findings illustrated increased plasma IFN-γ levels among HIV-1/TB co-infected ART-naive (median, 72.5 pg/ml; $P<0.05$) compared to
HIV-1 negative and TB uninfected IDUs (median, 38.4 pg/ml). Similarly, the levels were also elevated in HIV-1 mono-infected ART-experienced (median, 67.1 pg/ml; $P<0.05$) relative to TB mono-infected (median, 35.5 pg/ml) IDUs. *Post-hoc* analysis however illustrated no significant difference between-groups in IFN-γ levels observed in HIV-1/TB co-infected ART-experienced (median, 71.2 pg/ml; $P>0.05$) versus HIV-1/TB co-infected ART-naive (median, 72.5 pg/ml) IDUs nor the HIV-1 negative and TB uninfected IDUs (median, 38.4 pg/ml; $P>0.05$) against TB mono-infected (median, 35.5 pg/ml) IDUs.
Figure 4.1 Plasma levels of IFN-γ in HIV-1/TB mono- and co-infected, ART-experienced and ART-naive injection drug users, uninfected injection drug users and healthy controls.

Data are presented as box plots, where the line through the box represents the median, the lower and upper edge of each box indicate the 25th and 75th percentiles, the whiskers show the 10th and 90th percentiles, and the dots represent outliers. Statistical analysis was performed using the Kruskal Wallis tests (P<0.0001) for across group comparisons followed by Dunn’s multiple comparison test for between-group comparisons; where ** P<0.01 vs. HC, *** P<0.001 vs. HC; + P<0.05 vs. HIV-1-/TB- IDUs; # P<0.5 vs. HIV-/TB+ and ### P<0.001 vs. HIV-/TB+ IDUs; $$$ P<0.001 vs. HIV+/ART-/TB- IDUs. IFN-γ, interferon-gamma. ART, antiretroviral treatment. HIV-1, human immunodeficiency virus type-1. TB, tuberculosis. IDUs, injection drug users. HC, healthy controls. HIV-1-/TB-, HIV-1 negative and TB uninfected IDUs. HIV-1-/TB+, TB mono-infected IDUs. HIV-1+/ART-/TB-, HIV-1 mono-infected ART-naive IDUs. HIV-1+/ART-/TB+, HIV-1 and TB co-infected ART-naive IDUs. HIV-1+/ART+/TB-, HIV-1 mono-infected ART-experienced IDUs. HIV-1+/ART+/TB+, HIV-1 and TB co-infected ART-experienced IDUs.
4.4.2 Plasma IL-10 levels of the study participants

Plasma IL-10 levels of the study participants were highest in the HIV-1 negative and TB uninfected (median, 142.2 pg/ml) IDUs, whereas the HIV-1/TB co-infected ART-naive IDUs manifested the least levels (median, 67.2 pg/ml; Table 4.4). Kruskal Wallis test illustrated a statistically significant difference in IL-10 levels across study groups ($P<0.0001$), with post-hoc analysis indicating reduced levels in the TB mono-infected (median, 76.1 pg/ml; $P<0.001$), HIV-1 mono-infected ART-naive (median, 74.7 pg/ml; $P<0.001$), HIV-1/TB co-infected ART-naive (median, 67.2 pg/ml; $P<0.001$) and -experienced (median, 76.5 pg/ml; $P<0.01$) relative to the HIV-1 negative and TB uninfected (median, 142.2 pg/ml) IDUs (Figure 4.2).

On the other hand, the IL-10 levels were remarkably high in the HIV-1 negative and TB uninfected (median, 142.2 pg/ml; $P<0.001$) IDUs compared to healthy controls (median, 69.7 pg/ml). However, no significant between-groups differences in plasma IL-10 levels were observed between HIV-1/TB co-infected ART-naive (median, 67.2 pg/ml) and -experienced (median, 76.5 pg/ml; $P>0.05$). Nonetheless, HIV-1 mono-infected ART-experienced (median, 79.4 pg/ml; $P>0.05$) and HIV-1 negative and TB uninfected (median, 142.2 pg/ml) IDUs showed no statistical difference between groups.
Figure 4.2 Plasma IL-10 levels in HIV-1/TB mono- and co-infected, ART-naive and -experienced injection drug users, uninfected injection drug users and healthy controls.

Data are presented as box plots, where the line through the box represents the median, the lower and upper edge of each box indicate the 25th and 75th percentiles, the whiskers show the 10th and 90th percentiles, and the dots represent outliers. Statistical analysis was performed using the Kruskal Wallis tests ($P<0.0001$) for across group comparisons followed by Dunn’s post-hoc test for between-groups comparison; where *** $P<0.001$ vs. HC; ++ $P<0.01$ vs. HIV-1-/TB-; +++ $P<0.001$ vs. HIV-1-/TB- IDUs respectively. IL-10, interleukin-10. ART, antiretroviral treatment. HIV-1, human immunodeficiency virus type-1. TB, tuberculosis. IDUs, injection drug users. HC, healthy controls. HIV-1-/TB-, HIV-1 negative and TB uninfected IDUs. HIV-1-/TB+, TB mono-infected IDUs. HIV-1+/ART-/TB-, HIV-1 mono-infected ART-naive IDUs. HIV-1+/ART-/TB+, HIV-1 and TB co-infected ART-naive IDUs. HIV-1+/ART+/TB-, HIV-1 mono-infected ART-experienced IDUs. HIV-1+/ART+/TB+, HIV-1 and TB co-infected ART-experienced IDUs.
4.4.3 Plasma IFN-γ to IL-10 ratio of study participants

The HIV-1/TB co-infected ART-naive presented with the highest IFN-γ to IL-10 ratio (median, 1.1), while the HIV-1 negative and TB uninfected IDUs displayed the least ratio (median, 0.3; Table 4.4). Across group comparison established a significantly different IFN-γ to IL-10 ratio among study groups ($P<0.0001$). Subsequent *post-hoc* analysis revealed a higher IFN-γ to IL-10 ratio in the ART-naive (median, 1.1; $P<0.001$) and -experienced (median, 0.9; $P<0.001$) HIV-1/TB co-infected as well as HIV-1 mono-infected ART-experienced (median, 0.8; $P<0.001$) compared to HIV-1 negative and TB uninfected IDUs (median, 0.3; Figure 4.3) IDUs.

Likewise, the IFN-γ to IL-10 ratio was elevated in HIV-1/TB co-infected ART-naive (median, 1.1; $P<0.001$) relative to HIV-1 mono-infected ART-naive (median, 0.5) IDUs. In addition, the HIV-1/TB co-infected ART-naive (median, 1.1; $P<0.01$) IDUs presented with a higher IFN-γ to IL-10 ratio compared to healthy controls (median, 0.5). On the other hand, TB mono-infected (median, 0.5; $P<0.05$) presented a higher IFN-γ to IL-10 ratio compared to HIV-1 negative and TB uninfected (median, 0.3) IDUs but in turn elicited a significantly lower ratio (median, 0.5; $P<0.05$) compared to HIV-1/TB co-infected ART-naive (median, 1.1) IDUs. However, Dunn’s multiple comparison test yielded no significant between-groups difference among HIV-1 negative and TB uninfected (median, 0.3; $P>0.05$) IDUs against healthy controls (median, 0.5).
Figure 4.3 Plasma IFN-γ to IL-10 ratios of HIV-1/TB mono- and co-infected, ART-experienced and ART-naive injection drug users, uninfected injection drug users and healthy controls.

Data are presented as box plots, where the line through the box represents the median, the lower and upper edge of each box indicate the 25th and 75th percentiles, whiskers indicate the 10th and 90th percentiles, and the dots represent outliers. Statistical analysis was performed using the Kruskal Wallis test ($P<0.0001$) for across group comparisons followed by Dunn’s multiple between-groups comparison; where + $P<0.05$ vs. HIV-1-/TB-, +++ $P<0.001$ vs. HIV-1-/TB- IDUs; $$$ $P<0.001$ vs. HIV-1+/ART-/TB- IDUs; ** $P<0.01$ vs. HC; # $P<0.05$ vs. HIV-1-/TB+ IDUs. ART, antiretroviral treatment. HIV-1, human immunodeficiency virus type-1. TB, tuberculosis. IDUs, injection drug users. HC, healthy controls. HIV-1-/TB-, HIV-1 negative and TB uninfected IDUs. HIV-1-/TB+, TB mono-infected IDUs. HIV-1+/ART-/TB-, HIV-1 mono-infected ART-naive IDUs. HIV-1+/ART-/TB+, HIV-1 and TB co-infected ART-naive IDUs. HIV-1+/ART+/TB-, HIV-1 mono-infected ART-experienced IDUs. HIV-1+/ART+/TB+, HIV-1 and TB co-infected ART-experienced IDUs.
4.4.4 Plasma Acrp30 levels of the study participants

Among the study participants, plasma Acrp30 levels ranged from (median, 16.0 to 25.3 ng/ml) respectively, for TB mono-infected IDUs and healthy controls (Table 4.4). Additionally, Acrp30 cytokine varied significantly across the study groups (P=0.006), with TB mono-infected (median, 16.0 ng/ml; P<0.01) IDUs exhibiting significantly lower Acrp30 levels compared to healthy controls (median, 25.3 ng/ml; Figure 4.4). In addition, post-hoc analysis also indicated that HIV-1/TB co-infected ART-experienced (median 19.5 ng/ml; P<0.05) IDUs expressed lower Acrp30 levels relative to healthy controls (median, 25.3 ng/ml). Between-groups comparison of the circulating plasma Acrp30 levels however established no significant difference in the HIV-1 negative and TB uninfected (median, 21.9 ng/ml; P>0.05) IDUs compared to healthy controls (median, 25.3 ng/ml). Additionally, no statistical differences were found between the HIV-1/TB co-infected ART-experienced (median, 19.5 ng/ml; P>0.05) relative to HIV-1/TB co-infected ART-naive (median, 23.0 ng/ml) IDUs. The plasma Acrp30 levels were also comparable between the HIV-1 mono-infected ART-experienced (median, 19.1 ng/ml; P>0.05) versus HIV-1 mono-infected ART-naive (median, 21.0 ng/ml) IDUs.
Figure 4.4 Plasma levels of Acrp30 in HIV-1/TB mono- and co-infected, ART-experienced and ART-naive injection drug users, uninfected injection drug users and healthy controls.

Data are presented as box plots, where the line through the box represents the median, the lower and upper edge of each box indicate the 25th and 75th percentiles, whiskers indicate the 10th and 90th percentiles, and the dots represent outliers. Statistical analysis was performed using the Kruskal Wallis tests (P=0.006) for across group comparisons followed by Dunn’s multiple comparison test for between-groups comparison; where ** P<0.01 vs. HC and * P<0.05 vs. HC respectively. Acrp30, adiponectin. ART, antiretroviral treatment. HIV-1, human immunodeficiency virus type-1. TB, tuberculosis. IDUs, injection drug users. HC, healthy controls. HIV-1-/TB-, HIV-1 negative and TB uninfected IDUs. HIV-1-/TB+, TB mono-infected IDUs. HIV-1+/ART-/TB-, HIV-1 mono-infected ART-naive IDUs. HIV-1+/ART-/TB+, HIV-1 and TB co-infected ART-naive IDUs. HIV-1+/ART+/TB-, HIV-1 mono-infected ART-experienced IDUs. HIV-1+/ART+/TB+, HIV-1 and TB co-infected ART-experienced IDUs.
4.4.5 Plasma IFN-γ to Acrp30 ratio of the study participants

The IFN-γ to Acrp30 ratio of the study participants ranged from median, 1.4×10⁻³ to median, 3.7×10⁻³, respectively, for the healthy controls and HIV-1/TB co-infected ART-experienced IDUs (Table 4.4). Overall comparison revealed a strong statistical difference in IFN-γ to Acrp30 ratio across the study groups ($P<0.0001$; Figure 4.5). Additionally, *post-hoc* analysis showed IFN-γ to Acrp30 ratio was significantly higher in the HIV-1 mono-infected ART-experienced (median, 3.5×10⁻³; $P<0.001$) and HIV-1/TB co-infected ART-naive (median, 3.2×10⁻³; $P<0.001$) and -experienced (median, 3.7×10⁻³; $P<0.001$) IDUs relative to healthy controls (median, 1.4×10⁻³).

Similarly, the IFN-γ to Acrp30 ratio was elevated in the HIV-1 mono-infected ART-experienced (median, 3.5×10⁻³; $P<0.05$) compared to HIV-1 negative and TB uninfected (median, 1.8×10⁻³) IDUs. However, the ratio did not vary between the HIV-1 negative and TB uninfected IDUs (median, 1.8×10⁻³; $P>0.05$) and healthy controls (median, 1.4×10⁻³). Likewise no significant between-group differences in the IFN-γ to Acrp30 ratio was noted between the HIV-1 mono-infected ART-naive (median, 1.7×10⁻³; $P>0.05$) and HIV-1 mono-infected ART-experienced (median, 3.5×10⁻³) IDUs.
Figura 4.5 Niveles plasmáticos de IFN-γ a Acrp30 ratio entre infectados con VIH-1 y TB mono- y co-infestados, ART-experimentados y ART-naive usuarios de drogas inyectadas, usuarios no infectados de drogas inyectadas y controles sanos.

Los datos se presentan como gráficos de caja, donde la línea del centro del caja representa la mediana, el límite inferior y superior de cada caja indican el 25° y 75° percentiles, los alargadores indican el 10° y 90° percentiles, y los puntos representan outliers. El análisis estadístico se realizó utilizando los test de Kruskal Wallis ($P<0.0001$) para comparaciones entre grupos y test Dunn’s post-hoc para comparaciones entre grupos; donde *** $P<0.001$ vs. HC y + $P<0.05$ vs. HIV-1-/TB- IDUs respectivamente. IFN-γ, interferon-gamma. Acrp30, adiponectina. ART, antirretroviral tratamiento. HIV-1, virus de inmunodeficiencia humana tipo 1. TB, tuberculosis. IDUs, usuarios de drogas inyectadas. HIV-1-/TB-, HIV-1 negativo y TB no infectado. HIV-1-/TB+, TB mono-infectado. HIV-1+/ART-/TB-, HIV-1 mono-infectada ART-naive IDUs. HIV-1+/ART-/TB+, HIV-1 y TB co-infectado ART-naive IDUs. HIV-1+/ART+/TB-, HIV-1 mono-infectado ART-experimentado IDUs. HIV-1+/ART+/TB+, HIV-1 y TB co-infectado ART-experimentado IDUs.
Table 4.4 Plasma cytokine concentrations among the study participants

<table>
<thead>
<tr>
<th></th>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ, pg/ml</td>
<td>34.6 (31.5-38.0)</td>
<td>38.4 (34.6-74.2)</td>
<td>35.5 (30.1-74.3)</td>
<td>35.7 (32.2-64.6)</td>
<td>72.5 (70.9-75.5)</td>
<td>67.1 (63.8-71.1)</td>
<td>71.2 (34.8-76.6)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IL-10, pg/ml</td>
<td>69.7 (38.6-99.2)</td>
<td>142.2 (72.0-174.4)</td>
<td>76.1 (44.7-128.5)</td>
<td>74.7 (46.4-132.1)</td>
<td>67.2 (52.3-92.9)</td>
<td>79.4 (65.4-102.9)</td>
<td>76.5 (55.8-168.0)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IFN-γ/IL-10 ratio</td>
<td>0.5 (0.2-1.0)</td>
<td>0.3 (0.2-0.9)</td>
<td>0.5 (0.3-1.1)</td>
<td>0.5 (0.3-0.9)</td>
<td>1.1 (0.8-1.4)</td>
<td>0.8 (0.2-1.0)</td>
<td>0.9 (0.2-1.3)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Acrp30, ng/ml</td>
<td>25.3 (22.0-29.5)</td>
<td>21.9 (11.1-31.4)</td>
<td>16.0 (2.9-30.2)</td>
<td>21.0 (3.7-28.7)</td>
<td>23.0 (10.9-30.8)</td>
<td>19.1 (11.3-26.5)</td>
<td>19.5 (5.9-26.8)</td>
<td>0.006</td>
</tr>
<tr>
<td>IFN-γ/Acrp30 ratio (x10^3)</td>
<td>1.4 (1.2-1.7)</td>
<td>1.8 (1.2-6.7)</td>
<td>2.2 (1.4-10.8)</td>
<td>1.7 (1.2-9.9)</td>
<td>3.2 (2.4-6.7)</td>
<td>3.5 (2.6-5.7)</td>
<td>3.7 (1.1-12.7)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Cytokine levels expressed as medians (range). Statistical analysis of cytokine concentrations across study groups was conducted using Kruskal Wallis test followed by Dunn’s post-hoc test. IFN-γ, interferon-gamma. IL-10, interleukin-10. Acrp30, adiponectin. ART, antiretroviral treatment. HIV-1, human immunodeficiency virus type-1. TB, tuberculosis. IDUs, injection drug users. HC, healthy controls. HIV-TB-, HIV-1 negative and TB uninfected IDUs. HIV-TB+, TB mono-infected IDUs. HIV+ART-TB-, HIV-1 mono-infected ART-naive IDUs. HIV+ART-TB+, HIV-1 and TB co-infected ART-naive IDUs. HIV+ART+TB-, HIV-1 mono-infected ART-experienced IDUs. HIV+ART+TB+, HIV-1 and TB co-infected ART-experienced IDUs.
4.5.1 Associations between plasma cytokines and clinical outcomes in HIV-1/TB co-infected ART-experienced injection drug users.

Neither of the clinical outcomes including CD4+ T cell counts, HIV-1 viral load nor BMI was significantly correlated with either of the cytokines examined among the HIV-1/TB co-infected ART-experienced IDUs (Table 4.5). However, amongst the cytokines, the levels of IL-10 were significant and positively correlated with Acrp30 (ρ=0.502; P=0.015) and inversely with IFN-γ (ρ=-0.571; P=0.002). On the other hand, levels of IFN-γ were significantly inversely correlated with Acrp30 levels (ρ=-0.542; P=0.008). Additionally, the IFN-γ to IL-10 ratio was positively correlated with IFN-γ (ρ=0.647; P<0.0001) and inversely with IL-10 (ρ=-0.984; P<0.0001) and Acrp30 (ρ=-0.559; P=0.006) levels respectively. Likewise, the IFN-γ to Acrp30 ratio was correlated positively with both IFN-γ (ρ=0.747; P<0.0001) and IFN-γ to IL-10 ratio (ρ=0.703; P<0.0001), while significantly correlated inversely with IL-10 (ρ=-0.645; P=0.001) and Acrp30 (ρ=-0.926; P<0.0001) levels, respectively.
Table 4.5 Associations of cytokines and clinical parameters in HIV-1 and TB co-infected ART-experienced IDUs

<table>
<thead>
<tr>
<th>Marker</th>
<th>IFN-γ</th>
<th>IL-10</th>
<th>Acrp30</th>
<th>IFN-γ/IL-10 ratio</th>
<th>IFNγ/Acrp30 ratio</th>
<th>BMI</th>
<th>CD4+ T cells/μl</th>
<th>HIV-1 RNA, copies/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ρ</td>
<td>P</td>
<td>ρ</td>
<td>P</td>
<td></td>
<td>ρ</td>
<td>P</td>
<td>ρ</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>1.000</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>-0.571</td>
<td>0.002</td>
<td>1.000</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acrp30</td>
<td>-0.542</td>
<td>0.008</td>
<td>0.502</td>
<td>0.015</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ/IL-10 ratio</td>
<td>0.647</td>
<td>&lt;0.0001</td>
<td>-0.984</td>
<td>&lt;0.0001</td>
<td>-0.559</td>
<td>0.006</td>
<td>1.000</td>
<td>-</td>
</tr>
<tr>
<td>IFNγ/Acrp30 ratio</td>
<td>0.747</td>
<td>&lt;0.0001</td>
<td>-0.645</td>
<td>0.001</td>
<td>-0.926</td>
<td>&lt;0.0001</td>
<td>0.703</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>BMI</td>
<td>-0.254</td>
<td>0.200</td>
<td>0.171</td>
<td>0.404</td>
<td>0.041</td>
<td>0.854</td>
<td>-0.159</td>
<td>0.439</td>
</tr>
<tr>
<td>CD4+ T cells/μl</td>
<td>0.095</td>
<td>0.636</td>
<td>0.204</td>
<td>0.318</td>
<td>-0.410</td>
<td>0.052</td>
<td>-0.182</td>
<td>0.375</td>
</tr>
<tr>
<td>HIV-1 RNA, copies/ml</td>
<td>0.081</td>
<td>0.696</td>
<td>0.045</td>
<td>0.832</td>
<td>0.042</td>
<td>0.852</td>
<td>-0.013</td>
<td>0.950</td>
</tr>
</tbody>
</table>

Data presented are Spearman rank correlation coefficients (rho, ρ) and associated P-values with significant correlation shown in bold. IFN-γ, interferon-gamma. IL-10, interleukin-10. Acrp30, adiponectin. HIV-1, human immunodeficiency virus type-1. TB, tuberculosis. BMI, body mass index. ART, antiretroviral therapy. IDUs, injection drug users.
4.5.2 Associations between plasma cytokines and clinical outcomes in HIV-1 mono-infected ART-experienced injection drug users.

The Spearman’s correlation test established that none of the clinical parameters examined including CD4+T cell counts, HIV-1 viral load and BMI demonstrated any level of significant correlation with cytokine markers under study (Table 4.6). However, correlation tests between cytokine markers revealed that IFN-γ to IL-10 ratio was inversely correlated with IL-10 (\(\rho=-0.932; \ P<0.0001\)). Similarly, the IFN-γ to Acrp30 ratio was also inversely correlated with Acrp30 (\(\rho=-1.000; \ P<0.0001\)) levels.
### Table 4.6 Associations of cytokines and clinical parameters in HIV-1 mono-infected ART-experienced IDUs

<table>
<thead>
<tr>
<th>Marker</th>
<th>IFN-γ</th>
<th>IL-10</th>
<th>Acrp30</th>
<th>IFN-γ/IL-10 ratio</th>
<th>IFNγ/Acrp30 ratio</th>
<th>BMI</th>
<th>CD4+ T cells/μl</th>
<th>HIV-1 RNA, copies/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ρ</td>
<td>P</td>
<td>ρ</td>
<td>P</td>
<td>ρ</td>
<td>P</td>
<td>ρ</td>
<td>P</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>1.000</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>-0.060</td>
<td>0.854</td>
<td>1.000</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acrp30</td>
<td>0.333</td>
<td>0.347</td>
<td>-0.213</td>
<td>0.555</td>
<td>1.000</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ/IL-10 ratio</td>
<td>0.315</td>
<td>0.318</td>
<td>-0.932</td>
<td>&lt;0.0001</td>
<td>0.224</td>
<td>0.533</td>
<td>1.000</td>
<td>-</td>
</tr>
<tr>
<td>IFNγ/Acrp30 ratio</td>
<td>-0.333</td>
<td>0.347</td>
<td>0.213</td>
<td>0.555</td>
<td>-1.000</td>
<td>-</td>
<td>-0.224</td>
<td>0.533</td>
</tr>
<tr>
<td>BMI</td>
<td>-0.424</td>
<td>0.149</td>
<td>0.312</td>
<td>0.324</td>
<td>0.067</td>
<td>0.855</td>
<td>-0.357</td>
<td>0.255</td>
</tr>
<tr>
<td>CD4+T cells/μl</td>
<td>0.333</td>
<td>0.266</td>
<td>0.249</td>
<td>0.436</td>
<td>-0.479</td>
<td>0.162</td>
<td>-0.049</td>
<td>0.880</td>
</tr>
<tr>
<td>HIV-1RNA, copies/ml</td>
<td>-0.048</td>
<td>0.881</td>
<td>0.284</td>
<td>0.398</td>
<td>0.342</td>
<td>0.367</td>
<td>-0.310</td>
<td>0.353</td>
</tr>
</tbody>
</table>

Data presented are Spearman rank correlation coefficients (rho, ρ) and associated P-values with significant correlation shown in bold. IFN-γ, interferon-gamma. IL-10, interleukin-10. Acrp30, adiponectin. HIV-1, human immunodeficiency virus type-1. BMI, body mass index. ART, antiretroviral therapy. IDUs, injection drug users.
4.5.3 Associations between plasma cytokines and clinical outcomes in HIV-1/TB co-infected ART-naive injection drug users.

Among the clinical parameters, the BMI levels were correlated positively with Acrp30 ($\rho=0.523; P=0.022$) levels and inversely with IFN-$\gamma$ to Acrp30 ratio ($\rho=-0.549; P=0.015$) respectively (Table 4.7). Likewise, the cytokine marker IL-10 was positively correlated with IFN-$\gamma$ ($\rho=0.544; P=0.013$), whereas the IFN-$\gamma$ to IL-10 ratio and IFN-$\gamma$ to Acrp30 ratio were inversely correlated with IL-10 ($\rho=-0.488; P=0.029$) and Acrp30 ($\rho=-0.911; P=0.576$) levels, respectively.
Table 4.7 Associations of cytokines and clinical parameters in HIV-1/TB co-infected ART-naive IDUs

<table>
<thead>
<tr>
<th>Marker</th>
<th>IFN-γ</th>
<th>IL-10</th>
<th>Acrp30</th>
<th>IFN-γ/IL-10 ratio</th>
<th>IFNγ/Acrp30 ratio</th>
<th>BMI</th>
<th>CD4+ T cells/µl</th>
<th>HIV-1 RNA, copies/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ρ</td>
<td>P</td>
<td>ρ</td>
<td>P</td>
<td></td>
<td>ρ</td>
<td>P</td>
<td>ρ</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>1.000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.00</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.544</td>
<td>0.013</td>
<td>1.000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.000</td>
<td>-</td>
</tr>
<tr>
<td>Acrp30</td>
<td>-0.454</td>
<td>0.051</td>
<td>-0.264</td>
<td>0.290</td>
<td>1.000</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IFN-γ/IL-10 ratio</td>
<td>-0.341</td>
<td>0.142</td>
<td>-0.488</td>
<td>-0.090</td>
<td>0.723</td>
<td>1.00</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IFNγ/Acrp30 ratio</td>
<td>0.712</td>
<td>0.001</td>
<td>0.344</td>
<td>0.163</td>
<td>-0.911</td>
<td>0.576</td>
<td>0.255</td>
<td>0.307</td>
</tr>
<tr>
<td>BMI</td>
<td>-0.094</td>
<td>0.684</td>
<td>-0.325</td>
<td>0.162</td>
<td>0.523</td>
<td>0.022</td>
<td>0.243</td>
<td>0.302</td>
</tr>
<tr>
<td>CD4+T cells/µl</td>
<td>0.021</td>
<td>0.927</td>
<td>0.175</td>
<td>0.461</td>
<td>-0.128</td>
<td>0.601</td>
<td>0.090</td>
<td>0.705</td>
</tr>
<tr>
<td>HIV-1 RNA, copies/ml</td>
<td>-0.001</td>
<td>0.997</td>
<td>0.004</td>
<td>0.989</td>
<td>-0.010</td>
<td>0.970</td>
<td>0.052</td>
<td>0.844</td>
</tr>
</tbody>
</table>

Data presented are Spearman rank correlation coefficients (ρ, P) and associated P-values with significant correlation shown in bold. IFN-γ, interferon-gamma. IL-10, interleukin-10. Acrp30, adiponectin. HIV-1, human immunodeficiency virus type-1. TB, tuberculosis. BMI, body mass index. ART, antiretroviral therapy. IDUs, injection drug users.
4.5.4 Associations between plasma cytokines and clinical outcomes in HIV-1 mono-infected ART-naive injection drug users

In the current study, the Spearman’s correlation test indicated that BMI, CD4+ T cell counts, and HIV-1 viral load were not significantly correlated with Acrp30, IFN-γ and IL-10 levels (Table 4.8). However, significant correlations were observed within cytokine markers. The IL-10 levels were significantly correlated positively with IFN-γ (ρ=0.649; P<0.0001) levels. Similarly, the IFN-γ to Acrp30 ratio was positively correlated with IFN-γ (ρ=0.597; P=0.002) levels in HIV-1 mono-infected ART-naive IDUs. On the other hand, the IFN-γ to IL-10 ratio and IFN-γ to Acrp30 ratio were both inversely correlated with IL-10 (ρ=-0.876; P<0.0001) and Acrp30 (ρ=-0.958; P<0.0001) levels, respectively.
Table 4.8 Associations of cytokines and clinical parameters in HIV-1 mono-infected ART-naive IDUs

<table>
<thead>
<tr>
<th>Marker</th>
<th>IFN-γ</th>
<th>IL-10</th>
<th>Acrp30</th>
<th>IFN-γ/IL-10 ratio</th>
<th>IFN-γ/Acrp30 ratio</th>
<th>BMI</th>
<th>CD4+ T cells/µl</th>
<th>HIV-1 RNA, copies/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ρ</td>
<td>P</td>
<td>ρ</td>
<td>P</td>
<td>P</td>
<td>ρ</td>
<td>P</td>
<td>ρ</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>1.000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.649</td>
<td>&lt;0.0001</td>
<td>1.000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Acrp30</td>
<td>-0.385</td>
<td>0.057</td>
<td>-0.089</td>
<td>0.674</td>
<td>1.000</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IFN-γ/IL-10 ratio</td>
<td>-0.286</td>
<td>0.157</td>
<td>-0.876</td>
<td>&lt;0.0001</td>
<td>-0.129</td>
<td>0.538</td>
<td>1.000</td>
<td>-</td>
</tr>
<tr>
<td>IFN-γ/Acrp30 ratio</td>
<td>0.579</td>
<td>0.002</td>
<td>0.271</td>
<td>0.191</td>
<td>-0.958</td>
<td>&lt;0.0001</td>
<td>-0.004</td>
<td>0.985</td>
</tr>
<tr>
<td>BMI</td>
<td>-0.126</td>
<td>0.539</td>
<td>0.092</td>
<td>0.654</td>
<td>0.111</td>
<td>0.597</td>
<td>-0.164</td>
<td>0.424</td>
</tr>
<tr>
<td>CD4+ T cells/µl</td>
<td>-0.020</td>
<td>0.923</td>
<td>0.161</td>
<td>0.433</td>
<td>-0.034</td>
<td>0.872</td>
<td>-0.311</td>
<td>0.122</td>
</tr>
<tr>
<td>HIV-1 RNA, copies/ml</td>
<td>-0.044</td>
<td>0.842</td>
<td>-0.146</td>
<td>0.506</td>
<td>-0.332</td>
<td>0.131</td>
<td>-0.109</td>
<td>0.621</td>
</tr>
</tbody>
</table>

Data presented are Spearman rank correlation coefficients (rho, ρ) and associated P-values with significant correlation shown in bold. IFN-γ, interferon-gamma. IL-10, interleukin-10. Acrp30, adiponectin. HIV-1, human immunodeficiency virus type-1. BMI, body mass index. ART, antiretroviral therapy. IDUs, injection drug users.
4.5.5 Associations between plasma cytokine levels and clinical outcomes in TB mono-infected injection drug users.

Results from the spearman’s correlation test established a significant inverse correlation between Acrp30 ($\rho=-0.717; P=0.030$) levels and the CD4+ T cell counts. Additionally, IL-10 ($\rho=-0.983; P<0.0001$) and Acrp30 ($\rho=-0.983; P<0.0001$) levels both expressed inverse correlations with the IFN-γ to IL-10 ratio and the IFN-γ to Acrp30 ratio respectively (Table 4.9).
Table 4.9 Associations of cytokines and clinical parameters in TB mono-infected IDUs

<table>
<thead>
<tr>
<th>Marker</th>
<th>IFN-γ</th>
<th>IL-10</th>
<th>Acrp30</th>
<th>IFN-γ/IL-10 ratio</th>
<th>IFNγ/Acrp30 ratio</th>
<th>BMI</th>
<th>CD4+ T cells/µl</th>
<th>HIV-1 RNA, copies/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ρ</td>
<td>P</td>
<td>ρ</td>
<td>P</td>
<td>ρ</td>
<td>P</td>
<td>ρ</td>
<td>P</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>1.000</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>-0.450</td>
<td>0.224</td>
<td>1.000</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acrp30</td>
<td>0.167</td>
<td>0.668</td>
<td>0.250</td>
<td>0.516</td>
<td>1.000</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ/IL-10 ratio</td>
<td>0.567</td>
<td>0.112</td>
<td>-0.983</td>
<td>&lt;0.0001</td>
<td>-0.267</td>
<td>0.488</td>
<td>1.000</td>
<td>-</td>
</tr>
<tr>
<td>IFNγ/Acrp30 ratio</td>
<td>-0.100</td>
<td>0.798</td>
<td>-0.217</td>
<td>0.576</td>
<td>-0.983</td>
<td>&lt;0.0001</td>
<td>0.250</td>
<td>0.516</td>
</tr>
<tr>
<td>BMI</td>
<td>-0.102</td>
<td>0.795</td>
<td>0.153</td>
<td>0.695</td>
<td>0.203</td>
<td>0.600</td>
<td>-0.136</td>
<td>0.728</td>
</tr>
<tr>
<td>CD4+ T cells/µl</td>
<td>-0.300</td>
<td>0.433</td>
<td>-0.150</td>
<td>0.700</td>
<td>-0.717</td>
<td>0.030</td>
<td>0.167</td>
<td>0.668</td>
</tr>
<tr>
<td>HIV-1 RNA, copies/ml</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Data presented are Spearman rank correlation coefficients (rho, ρ) and associated P-values with significant correlation shown in bold. IFN-γ, interferon-gamma. IL-10, interleukin-10. Acrp30, adiponectin. HIV-1, human immunodeficiency virus type-1. TB, tuberculosis. BMI, body mass index. IDUs, injection drug users.
4.5.6 Associations between plasma cytokine levels and clinical outcomes in HIV-1 negative and TB uninfected injection drug users.

Among the cytokine markers evaluated, the Acrp30 ($\rho=0.980; \ P<0.0001$) and IFN-γ ($\rho=0.618; \ P=0.004$) levels were both positively correlated with the IFN-γ to Acrp30 ratio. On the other hand, the IFN-γ ($\rho=-0.498; \ P=0.025$) and IL-10 ($\rho=-0.930; \ P<0.0001$) levels were both correlated inversely with the Acrp30 levels and IFN-γ to IL-10 ratio respectively. However, none of the cytokine markers examined expressed a significant correlation with the clinical parameters comprising of CD4+ T cell counts, HIV-1 viral load and BMI in the HIV-1 negative and TB uninfected IDUs (Table 4.10).
Table 4.10 Associations of cytokines and clinical parameters in HIV-1 negative and TB uninfected IDUs

<table>
<thead>
<tr>
<th>Marker</th>
<th>IFN-γ</th>
<th>IL-10</th>
<th>Acrp30</th>
<th>IFN-γ/IL-10 ratio</th>
<th>IFNγ/Acrp30 ratio</th>
<th>BMI</th>
<th>CD4+ T cells/μl</th>
<th>HIV-1 RNA, copies/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ρ</td>
<td>P</td>
<td>ρ</td>
<td>P</td>
<td>ρ</td>
<td>P</td>
<td>ρ</td>
<td>P</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>1.000</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>0.076</td>
<td>0.732</td>
<td>1.000</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acrp30</td>
<td>-0.498</td>
<td>0.025</td>
<td>-0.169</td>
<td>0.490</td>
<td>1.000</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ/IL-10 ratio</td>
<td>0.116</td>
<td>0.597</td>
<td>-0.930</td>
<td>&lt;0.0001</td>
<td>-0.012</td>
<td>0.960</td>
<td>1.000</td>
<td>-</td>
</tr>
<tr>
<td>IFNγ/Acrp30 ratio</td>
<td><strong>0.618</strong></td>
<td><strong>0.004</strong></td>
<td>0.280</td>
<td>0.245</td>
<td><strong>0.980</strong></td>
<td>&lt;0.0001</td>
<td>-0.032</td>
<td>0.898</td>
</tr>
<tr>
<td>BMI</td>
<td>-0.238</td>
<td>0.252</td>
<td>-0.315</td>
<td>0.143</td>
<td>0.121</td>
<td>0.611</td>
<td>0.193</td>
<td>-0.222</td>
</tr>
<tr>
<td>CD4+ T cells/μl</td>
<td>-0.223</td>
<td>0.285</td>
<td>0.066</td>
<td>0.766</td>
<td>0.183</td>
<td>0.439</td>
<td>-0.062</td>
<td>0.778</td>
</tr>
<tr>
<td>HIV-1 RNA, copies/ml</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Data presented are Spearman rank correlation coefficients (rho, ρ) and associated P-values with significant correlation shown in bold. IFN-γ, interferon-gamma. IL-10, interleukin-10. Acrp30, adiponectin. HIV-1, human immunodeficiency virus type-1. TB, tuberculosis. BMI, body mass index. IDUs, injection drug users.
4.5.7 Associations between plasma cytokine levels and clinical outcomes in healthy controls.

While neither the CD4+ T cell counts, HIV-1 viral load levels nor BMI were associated with any of the cytokine markers examined among the healthy controls, the IL-10 ($\rho=0.826; P<0.0001$) and IFN-$\gamma$ to Acrp30 ratio ($\rho=0.663; P=0.037$) were both correlated positively with the IFN-$\gamma$ levels (Table 4.11). On the other hand, the IFN-$\gamma$ to IL-10 ratio ($\rho=-0.686; P=0.002$) and the IFN-$\gamma$ to Acrp30 ratio ($\rho=-0.853; P=0.002$) were both inversely correlated with IL-10 and Acrp30 levels, respectively.
Table 4.11 Associations of cytokines and clinical parameters in healthy controls

<table>
<thead>
<tr>
<th>Marker</th>
<th>IFN-γ</th>
<th>IL-10</th>
<th>Acrp30</th>
<th>IFN-γ/IL-10 ratio</th>
<th>IFNγ/Acrp30 ratio</th>
<th>BMI</th>
<th>CD4+ T cells/µl</th>
<th>HIV-1 RNA, copies/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>𝜌</td>
<td>P</td>
<td>𝜌</td>
<td>P</td>
<td>𝜌</td>
<td>P</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>1.000</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>0.826</td>
<td>&lt;0.0001</td>
<td>1.000</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acrp30</td>
<td>-0.240</td>
<td>0.504</td>
<td>-0.190</td>
<td>0.599</td>
<td>1.000</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ/IL-10 ratio</td>
<td>-0.451</td>
<td>0.069</td>
<td>-0.686</td>
<td>0.002</td>
<td>0.104</td>
<td>0.774</td>
<td>1.000</td>
<td>-</td>
</tr>
<tr>
<td>IFNγ/Acrp30 ratio</td>
<td>0.663</td>
<td>0.037</td>
<td>0.522</td>
<td>0.098</td>
<td>-0.853</td>
<td>0.002</td>
<td>-0.491</td>
<td>0.150</td>
</tr>
<tr>
<td>BMI</td>
<td>-0.361</td>
<td>0.155</td>
<td>-0.365</td>
<td>0.155</td>
<td>0.546</td>
<td>0.102</td>
<td>0.043</td>
<td>0.870</td>
</tr>
<tr>
<td>CD4+ T cells/µl</td>
<td>-0.062</td>
<td>0.814</td>
<td>-0.157</td>
<td>0.547</td>
<td>0.202</td>
<td>0.575</td>
<td>0.056</td>
<td>0.830</td>
</tr>
<tr>
<td>HIV-1 RNA, copies/ml</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Data presented are Spearman rank correlation coefficients (rho, ρ) and associated P-values with significant correlation shown in bold. IFN-γ, interferon-gamma. IL-10, interleukin-10. Acrp30, adiponectin. HIV-1, human immunodeficiency virus type-1. BMI, body mass index.
CHAPTER FIVE
DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

The present study determined plasma IFN-γ, IL-10 and Acrp30 levels as potential markers of disease progression among ART-naive and -experienced HIV-1 and TB mono- and co-infected Kenyan IDUs, and subsequently examined the association of the cytokine levels with the commonly employed HIV-1 and TB disease correlates including CD4+ T cell counts, HIV-1 viral load and BMI.

The study revealed lower body weights and overall reduced BMI among the HIV-1/TB co-infected ART-naive and -exposed, TB mono-infected, HIV-1 mono-infected ART-naive and -exposed and HIV-1 negative and TB uninfected IDUs relative to healthy controls, suggestive of the high HIV/TB disease burden and malnutrition often experienced among the population of IDUs. These findings are consistent with previous reports that have indicated lower body weights and BMI among the HIV-1 sero-negative and sero-positive injection drug users (Luder et al., 1995; Quach et al., 2008). The underlying causes of high incidence of malnutrition among the HIV-1 sero-negative and sero-positive IDUs, partly stems from the widespread poverty and food insecurity that is frequently encountered by both the HIV-infected and -uninfected IDUs (Weiser et al., 2009; Anema et al., 2013; Kalichman et al., 2014).
Moreover, low body weights could also be partially attributed to decreased nutrient absorption and/or altered metabolism as a result of increased cytokine-induced inflammatory response (Grunfeld et al., 1992). Furthermore, majority of IDUs frequently use non-injection substances such as khat, alcohol, bhang and other cocktail drugs which may heighten nutrient loss or interfere with utilisation in the gastro intestinal tract as a result of gastritis associated inflammation or duodenal ulcerations (Rajendram and Preedy, 2005; Roelandt et al., 2011).

On the other hand, reduced body weights among the HIV-1/TB co-infected ART-experienced IDUs despite initiation of ART, is in part, consistent with previous studies that illustrated reduced body weights, alcohol use, low adherence to ART and decreased suppression of HIV viral load among addicts initiated on ART programme (Weber et al., 2009; Cohn et al., 2011; Milloy et al., 2012). These findings may reflect delayed reconstitution of the T cell compartment among the IDUs on ART resulting from poor adherence to treatment (Vlahov et al., 2006; Gonzalez et al., 2013) possibly due to high drug burden and/or drug associated interactions between antiretroviral and the recreational drugs (Tseng and Antoniou, 2002).

The lack of ART adherence heightens development of virological treatment failure and consequent drug resistance (Vlahov et al., 2006). This results in the
failure to counteract HIV-1 viral replication while at the same time accelerating development of AIDS and associated weight loss effects (Gardner et al., 2008; Robbins et al., 2010). Additionally, it is possible that injection drug use may have manifested an adherence-independent mechanism of harm thereby aggravating weight loss and consequently leading to poor treatment outcome among the ART exposed population of IDUs. This hypothesis is corroborated by previous studies that have identified alcohol and illicit drug use as key confounders in the medical management of HIV infection (Altice et al., 2010).

The gender distribution and median ages of participants in the current study failed to establish statistical difference across and between-study groups. This observation contrasts with previous clinical studies that have reported significant gender differences in the practice of illicit substance use among middle aged drug users with or at risk of HIV infection (Hartel et al., 2006). In the previous study, men were described as being more likely to use drugs compared to women of similar age, while age at initiation of heroin and cocaine use occurred later for women, compared to men (Hartel et al., 2006). Reasons for similar drug use patterns between males and females in the present study may be related to the fact that age of debut for injection drug use in Kenya is reported to be almost equal between genders (Nacada, 2012). All in all, the demographics of illicit drug and substance use vary among populations and are confounded by multiple factors.
The CD4+ T cell counts which are often used as markers of HIV-1 disease progression were significantly reduced among the TB mono-infected, HIV-1 mono-infected ART-naive and HIV-1/TB co-infected ART-experienced IDUs relative to HIV-1 negative and TB uninfected IDUs. Likewise, the CD4+ T cell counts declined in the HIV-1 mono-infected ART-naive IDUs as compared to healthy controls. The drop in CD4+ T cell counts observed among the HIV-1/TB co-infected IDUs against HIV-1 negative and TB uninfected IDUs in the present study mimics past findings from various studies that reported higher depression in the CD4+ T cell counts among HIV sero-positive compared to sero-negative IDUs (Mientjes et al., 1991; Alcabes et al., 1993; Siddiqui et al., 1993). These marked reductions may be related to the fact that substance consumption such as active cocaine use leads to a rapid decline in CD4+ T cell counts among HIV-1 infected IDUs and non-IDUs (Baum et al., 2009; Meijerink et al., 2014).

Additionally, substance abuse contributes to rapid HIV replication. For example, systemic cocaine administration has been linked to an accelerated HIV-1 infection, with subsequent dramatic rise in circulating virus load and decreased CD4+ cell counts (Roth et al., 2002). Enhanced immune activation and rapid HIV viral replication causes apoptosis, massive depletion and exhaustion of the CD4+ memory T cells (McMichael et al., 2010). Consequently, when the production of new CD4+ cells cannot match the rate of destruction, then failure of the immune
system leads to the appearance of clinical AIDS (Green, 1993; Sierra et al., 2005). This finding highlights the merit of CD4+ T cell count as surrogate marker in assessing the functionality and integrity of the cellular immunity component among the population of both HIV-1/TB infected and uninfected IDUs.

On the contrary, the HIV-1 mono-infected ART-experienced IDUs expressed significantly higher CD4+ T cell counts compared to their HIV-1 mono-infected ART-naive counterparts. This observation is consistent with past clinical studies that showed increased CD4+ T cell counts following institution of ART among a cohort of HIV-1 infected IDUs (Palepu et al., 2006; Thompson et al., 2010). The significant rise in CD4+ T cell counts among the HIV-1 mono-infected ART-experienced IDUs can be explained by the phenomenon of immune reconstitution and CD4+ T cell recovery following reduction in HIV viral load with commencement of ART (Schacker et al., 2010).

The hypothesis can be further supported by the fact that CD4+ T cell loss correlates negatively with HIV viral load (Koot et al., 1996; Mellors et al., 1996), hence the CD4 cell numbers relentlessly decline with persistent HIV viraemia (McMichael et al., 2010). Although it has been suggested that the rise in CD4+ T cell counts may be attributed to production of newly synthesised/naive CD4+ cells (Imami and Gotch, 2002; Sarol et al., 2002; Schacker et al., 2010), other studies suggest that the increase in CD4+ T cell counts occurs as a result of
redistribution of memory CD4+ T cells (Nadeem et al., 2002). In general, CD4+ T cell count serves as significant prognostic indicator for treatment outcome and response to ART (Egger et al., 2002; Florence et al., 2003).

Interestingly, though not statistically significant, the present investigation showed that the CD4+ cell counts were higher in the HIV-1 negative and TB uninfected IDUs against healthy controls. This finding contrasts with the normal clinical expectations, however, the difference in CD4+ T cell counts may be attributed to distinct immunological differences of the study participants. Additionally, previous experimental animal studies using rhesus monkeys showed an increase in CD4+ T cell population following administration of morphine regularly (Carr and France, 1993). Hence, opioid use may stimulate CD4+ T cell production, though the underlying mechanisms remain unclear.

A good number of prospective studies involving IDUs have shown that greater CD4+ T cell loss directly corresponds with higher HIV-1 viral RNA levels among population of injecting drug users (Cook et al., 2008). However, in this study, HIV-1 viral load, a profound marker of both HIV/AIDS disease and immunologic derangement did not demonstrate significant differences both across and between study groups. In support of these findings, previous clinical case reports have also indicated that HIV-1 viral load did not differ significantly among people with and without a history of injection drug use (Meijerink et al., 2014).
In the present study, the IFN-γ levels were higher in the HIV-1 mono-infected ART-experienced compared to the HIV-1 mono-infected ART-naive IDUs. These results are similar to previous clinical case studies showing that HAART initiation leads to increased plasma IFN-γ levels among HIV-1 infected individuals (Imami et al., 1999). Likewise, increased plasma IFN-γ levels have been observed in HIV infected children receiving HAART (Reuben et al., 2002). While the sources for the increase in circulating IFN-γ production were not examined in the current investigation, previous studies indicate that both CD4+ and CD8+ T cells are the primary sources of IFN-γ (Wang et al., 1999). Comparatively however, CD4+ T cells mount a much stronger IFN-γ response and thus account for a greater IFN-γ secretion capacity (Smith and Dockrell, 2000; Fruch et al., 2001). HIV-1 pathogen primarily targets the CD4+ T cells (Mellors et al., 1997), hence infection with the HIV-1 virus results in immune activation promoting IFN-γ production (Verani et al., 2005). However, following successful ART institution, HIV-1 viral load is suppressed resulting in production of new CD4+ T cells (Hengel et al., 1999; Schacker et al., 2010). This phenomenon consequently leads to the rise in CD4+ T cell counts that is directly proportional to circulating plasma IFN-γ levels as observed in the HIV-1 mono-infected ART-naive IDUs in this case.
The HIV-1 negative and TB uninfected IDUs elicited higher IFN-γ levels compared to the healthy controls. This notably higher IFN-γ levels in this population may be linked to the observation that since a large number of IDUs in the present study were concomitantly consuming alcohol, cocktail (cigarettes and bhang mixture) and khat besides injecting hard drugs, it’s possible that these non-injection substances coupled with illicit drugs trigger immune hyperactivity and also synergistically promote alteration of the plasma cytokine levels which ultimately dampen normal host immune responses. This hypothesis parallels previous findings showing that opioid drugs modulate several aspects of the immune function including inflammatory responses which are dependent on cytokines (Roy et al., 2011). Likewise, frequent heroin use has been implicated in causing suppression of the cellular immune system (Zaki et al., 2005). Similarly, marijuana and cocaine are risk factors for rapid HIV progression due to their potential to alter host immunity and accelerate viral replication (Donahoe and Vlahov, 1998; Baldwin and Roth, 2005). Nonetheless, opioid substances including cocaine and heroin have been found to regulate cytokine and cytokine receptor expression (Finley et al., 2008). Hence, these actions cooperatively, may help explain the dysregulation in cytokine expression pattern observed among the uninfected IDUs against the healthy controls.

The plasma IFN-γ levels were also elevated in the HIV-1 mono-infected ART-experienced IDUs relative to healthy controls. This finding fails to concur with
previous studies which hypothesise that ART administration leads to a decrease in all cytokines to levels close in range to those observed in HIV uninfected individuals (Haissman et al., 2009). The persistently high IFN-γ levels in the plasma of HIV-1 mono-infected ART-experienced IDUs in the current study may be due to lack of ART adherence secondary to HIV-1 associated lipodystrophy syndrome (Carr et al., 1998). Disturbances in lipid and glucose metabolism, along with body shape abnormalities and alteration in fat redistribution mostly occur as a consequence of ART use. Hence, HIV associated lipodystrophy syndrome is a condition that involves body alterations which are usually associated with decrease in self esteem, that often leads to problems with adherence towards ART and ultimately treatment failure (Alves et al., 2014). Therefore, side effects associated with various ART regimens might have discouraged adherence among the HIV-1 mono-infected ART-experienced IDUs which is reflected by the constantly high IFN-γ levels as comparable to healthy controls.

The IFN-γ levels were also shown to be higher among the HIV-1/TB co-infected ART-naive relative to TB mono-infected injection drug users. This finding corroborates previous studies showing elevated IFN-γ levels in HIV-1/TB co-infected individuals compared to either infection alone (Subramanyam et al., 2004; Benjamin et al., 2013). The observation may suggest that in dually-infected participants, the HIV related changes dominate the overall immunological
response and leads to dysregulated cytokine production. Additionally, IFN-γ cytokine is pivotal in regulation of the host immune response against viral and intracellular bacterial pathogens (Roff et al., 2013). As a result, circulating IFN-γ levels are essentially influenced by the extent of host disease burden, and since this was a co-infection, the disease burden in co-infected individuals was assumptively high.

In a similar fashion, the plasma levels of IFN-γ were elevated in the HIV-1/TB co-infected ART-experienced compared to TB mono-infected IDUs. This result is similar to previous studies indicating higher IFN-γ expression in HIV-1 and TB co-infected patients compared to their TB mono-infected counterparts (Riou et al., 2012). The elevated levels of IFN-γ among the ART-experienced HIV and TB co-infected individuals is attributable to the fact that administration of HAART increases the numbers of MTB-specific CD4+ T cells among co-infected individuals (Hsieh et al., 2000). The mechanisms underlying this observation are, however, uncertain, but it appears immune reconstitution characterised by increases in CD4+ and CD8+ precursor cells (the main cellular sources of IFN-γ) promote increased production of circulating IFN-γ in co-infected patients (Schaker et al., 2010). In support of this hypothesis, previous studies have shown that immune reconstitution in TB and HIV co-infected patients is associated with increases in the number of circulating CD4+ and CD8+ T cells, including plasma IFN-γ levels (Bourgarit et al., 2009).
The increases in IFN-γ levels among the HIV-1/TB co-infected ART-naive relative to HIV-1 negative and TB uninfected IDUs may be an indication of derangements in host cell mediated immunity arising from the synergistic interaction between long term illicit substance use and on-going HIV-1 and TB co-burden. These assertions are, in part, supported by previous studies showing that cocaine use promotes HIV-1 disease progression largely by impairing macrophage and CD4+ T cell function as well as activating HIV-1 gene expression (Dhillon et al., 2007), while morphine induce HIV-1 replication in peripheral blood mononuclear cells (Banerjee et al., 2011). Since, IFN-γ induces immune activation, pro-inflammatory response and immune modulation (Agarwal et al., 2001; Koyama et al., 2008), in order to counteract the accelerated HIV-1 viral replication, host immunity induces and maintains antiviral responses through the massive production of pro-inflammatory cytokines such as IFN-γ as this cytokine is key in the primary immune clearance of HIV-1 infection (Reuben et al., 2002).

The higher IFN-γ levels in the HIV-1/TB co-infected ART-naive relative to HIV-1 mono-infected ART naive IDUs may be influenced by higher scale of disease severity and chronic immune activation owing to the multivariate HIV-1 and TB co-infection cascade. Co-infection with HIV-1 and TB triggers a more pronounced biological response to both viral and bacterial proliferation (Hertoghe
et al., 2000; Geldmacher et al., 2010), with consequent alteration in cytokine expression profile. Nonetheless, another possible mechanism behind the sustained IFN-γ levels among the HIV-1/TB co-infected ART-naive IDUs may be that IFN-γ production by HIV-1 specific CD8+ T lymphocytes is driven by HIV-1 viraemia which could be induced by rapid HIV-1 viral replication during HIV and TB co-infection. This premise can be supported by previous studies that described hyperactivation of T cells particularly CD8+ T cells in response to chronic HIV-1 infection and high virus load (Benito et al., 2004; Streeck et al., 2008).

The elevated IFN-γ levels in the HIV-1/TB co-infected ART-experienced compared to HIV-1 mono-infected ART-naive IDUs in spite of ART administration may be attributed either to non-adherence to therapy (Arnsten et al., 2002; Tucker et al., 2003), or alternatively due to development of resistance to antiretroviral agents which may ultimately result in failure to achieve HIV-1 viral suppression (Lucas et al., 2001; Palepu et al., 2003). Additionally, the poor HIV-1 treatment outcomes may be exacerbated by the complex drug-drug interactions involving antiretroviral and illicit drugs (Tseng and Antoniou, 2002). Altogether, HIV and drug abuse are essential interacting factors on the reactivity of T-cells during HIV-1/TB co-infection, and hence contribute substantially towards the alterations in IFN-γ levels as evidenced among the HIV-1/TB co-infected IDUs in this study.
The IFN-γ levels were also elevated among the HIV-1/TB co-infected ART-naive IDUs relative to healthy controls. This observation contrasts with previous clinical findings reporting lower IFN-γ levels among the non-drug using ART-naive HIV-1 and TB co-infected individuals compared to uninfected individuals (Hertoghe et al., 2000). The higher levels of IFN-γ in the HIV/TB co-infected ART-naive IDUs relative to healthy individuals in the present study may be attributable to injection and non-injection substance use. In support of this interpretation, previous studies have shown that regular khat and alcohol consumption increases IFN-γ production (Murdock et al., 2011; Gonzalez-Reimers et al., 2012), which is suggestive of the role of substances in inducing inflammation. This could result in immunological impairment as a result of T cell activation, lymphocyte apoptosis and depletion plus generalised hyperresponsiveness in the T-cell compartment among co-infected individuals (Katsikis et al., 1995; Gehri et al., 1996; Hirsch et al., 1999a). HIV replicates within activated CD4+ T cells and macrophages, hence this enhances expression and synthesis of IFN-γ cytokine. Additionally, chronic HIV infection renders the cell-mediated immunity ineffective against MTB with overall effects that are reflected by altered cytokine expression levels (Clerici et al., 1993; Graziosi et al., 1996).

Likewise, the HIV-1/TB co-infected ART-experienced IDUs in the current study expressed high IFN-γ levels compared to healthy controls despite being on ART.
The initiation of ART has been described in many studies to lower the levels of plasma IFN-γ to quantities that are comparable to that of healthy controls (Kang et al., 2012; Malherbe et al., 2014). However, in the current investigation, plasma IFN-γ levels remained markedly high despite antiretroviral use. This observation may be linked to the effect of injection drug use that summatively inhibits HIV-1 viral load suppression. For example, impaired virological response to HAART has been associated with frequent drug injection (Lucas et al., 2002; Palepu et al., 2003). Alternatively, the development of immune reconstitution inflammatory syndrome (IRIS) on initiation of ART, which has been suggested to be triggered by opportunistic pathogens such as MTB (Lai et al., 2013), may also partly explain the observed findings.

Nonetheless, underlying HIV-1 associated opportunistic infections such as Pneumocystis carinii, but which in this case was not determined may also have possibly undermined the efficacy of antiretroviral agents, thereby interfering with immune cytokine regulation. Lastly, the compositions of different ART regimen have been demonstrated to elicit different therapeutic outcomes among HIV-1 infected individuals (Gulick et al., 2004). This may have also predisposed observed IFN-γ levels in the current study. Hence, the integral role in immune regulation coupled with pro-inflammatory antiviral responses makes IFN-γ an attractive biomarker in evaluating immune competence and antiviral response in HIV-1 and possibly TB infected individuals.
In the current study, plasma levels of IL-10 were two-fold elevated among the HIV-1 negative and TB uninfected IDUs relative to healthy controls. This finding corroborates previous studies that reported elevated expression of IL-10 among heroin addicts compared to healthy controls (Zaki et al., 2005; Azarang et al., 2007). Likewise, high IL-10 levels have also been reported among individuals using anti-depressant drugs such as clomipramine and excessive alcohol consumption (Maes et al., 1999; Gonzalez-Reimers et al., 2012). Moreover, cannabinoids from bhang have also been described to stimulate an increase in systemic IL-10 production (Weiss et al., 2006). Hence, summatively, illicit drug and substance use possess immunosuppressive effects (Miyagi et al., 2000; Friedman et al., 2003; Edelman et al., 2014).

The 2-fold increase in IL-10 levels among the HIV-1 negative and TB uninfected IDUs against healthy controls portrays profound inflammatory response possibly due to systemic effects of illicit drugs. Systemic inflammation may have led to the activation of macrophages with subsequent release of anti-inflammatory cytokines such as IL-10 (Moor et al., 2001). In addition, elevated IL-10 levels has been shown to reflect high magnitude of immune inflammation possibly through illicit drug mediated necrosis, cell apoptosis and other immune mechanisms (Singhal et al., 1998; Murdock et al., 2011). It is also possible that illicit substance use focuses the CD4 T cell responses to a Th-2-type anti-inflammatory response. This
hypothesis can be explained by previous murine studies showing that chronic morphine exposure promotes CD4+ Th-2 cells differentiation (Abbas et al., 1996; Roy et al., 2005). It is well known that a shift in the balance from a Th-1 to Th-2 response is characterised by the expression of anti-inflammatory cytokines such as IL-10 which cause immune down-regulatory effects (Klein et al., 1997). Therefore, the observed raised IL-10 levels among the HIV-1 negative and TB uninfected IDUs is attributable to illicit drug use and thus may be suggestive of the utility of IL-10 as a novel predictor of inflammatory events as a result of illicit drugs use.

The plasma IL-10 levels were also elevated among the HIV-1 negative and TB uninfected IDUs as compared to the TB mono-infected, HIV-1 mono-infected ART-naive and HIV-1/TB co-infected ART-naive and -experienced IDUs. This observation fails to concur with past findings that reported high IL-10 production among HIV-1 and TB co-infected individuals (Geldmacher et al., 2010). Various clinical case studies have described the role of IL-10 in providing an effective auto-regulatory mechanism that protects the host from excessive inflammation and tissue damage that is in part initiated by the Th-1 driven pro-inflammatory immune responses during infections such as TB and HIV-1 (Sunder et al., 2012).

Interferon-γ primarily regulates host immune responses against viral and intracellular bacterial pathogens (Roff et al., 2013). Hence, excessive immune
activation experienced in HIV and TB infections is likely to cause a shift in T-helper cell subsets balance to favour an enhanced Type-1 pro-inflammatory cytokine response that in part antagonizes Th-2 anti-inflammatory responses thereby resulting in lowered IL-10 levels. This effect may account for the decreased IL-10 levels observed among the TB and HIV-1 infected IDUs against the HIV-1 negative and TB uninfected IDUs. Furthermore, it has been previously reported that IFN-γ suppresses Toll-like receptor mediated induction of IL-10 expression (Hu et al., 2006). Hence, this observation reinforces the hypothesis that IFN-γ by itself does indeed suppress IL-10 production. All the same, substance abuse seems to exert robust and reciprocal effects on the immune system cytokines, which are powerful modulators of immunity. Hence, this may immensely contribute towards IL-10 cytokine response pattern exhibited by the HIV-1 negative and TB uninfected IDUs.

The IFN-γ to IL-10 ratio were markedly reduced in the HIV-1 negative and TB uninfected compared to TB mono-infected, HIV-1 mono-infected ART-naive and HIV-1/TB co-infected ART-naive and -experienced IDUs. These observations reflect upon the lower IFN-γ and higher IL-10 levels expressed among the HIV-1 negative TB uninfected IDUs. These results are in agreement with previous studies showing depressed IFN-γ to IL-10 production ratio that was linked to the anti-inflammatory effects of anti-depressant drugs (Kubera et al., 2001). The mechanisms governing the lower IFN-γ to IL-10 ratio may be linked to illicit drug
injection and polysubstance abuse which may act synergistically and alter cytokine expression skewing it towards an anti-inflammatory Th-2 cytokine milieu independent of viral and mycobacterial infections (Roy et al., 2011).

Furthermore, the functionality of different immune cells such as macrophages and T-cells is altered among HIV-1 and TB co-infected individuals (Patel et al., 2007; Geldmacher et al., 2008; Kumawat et al., 2010). Both HIV-1 and TB have additive effects; each infection provides specific immune changes, which may contribute to the mutually unfavourable effects of co-infection. This may partly explain the altered balance between Th-1 and Th-2 cytokine expression observed among the HIV-1 and TB-infected compared to HIV-1 negative and TB uninfected IDUs. Nonetheless, the observed IFN-γ to IL-10 ratio in HIV-1/TB co-infected and HIV-1 mono-infected ART-experienced IDUs highlights the important role of IL-10 in exerting immunosuppressive effects against heightened immune inflammation rendered by IFN-γ during viral and/or mycobacterial infections.

The HIV-1/TB co-infected ART-naive presented with higher IFN-γ to IL-10 ratio relative to HIV-1 mono-infected ART-naive and TB mono-infected IDUs. This finding may be supported by previous studies that have identified HIV to be the main stimulatory factor of T cells in co-infection (Hoshino et al., 2002; Kang et al., 2012). HIV replication is increased at sites of MTB infection (Lawn et al.,
Consequently, co-infection with HIV and TB up-regulates production of pro-inflammatory cytokines specifically IFN-γ to a higher magnitude compared to individual infection, which in turn results in a raised IFN-γ to IL-10 ratio among the HIV-1/TB co-infected ART-naive IDUs against the HIV-1 and TB mono-infected IDUs. Besides, individuals with HIV-1 and TB co-infection are reported to also exhibit polyfunctional T lymphocyte immune responses (Sutherland et al., 2010), which may also partly explain the elevated IFN-γ to IL-10 ratio in co-infection as opposed to mono-infection. However, the hypothesis that HIV-1 itself is the main stimulatory factor on T cells that trigger high cytokine expression warrants further research so as to determine the molecular mechanisms leading to this effect.

Elevated IFN-γ to IL-10 ratio was also observed among the HIV-1/TB co-infected ART-naive IDUs compared to healthy controls. The alteration in IFN-γ to IL-10 ratio in the HIV-1/TB co-infected ART-naive IDUs relative to healthy controls may reflect an accelerated disease progression that is in part driven by infections, immunological reactions and polysubstance abuse. Interferon-gamma with its pro-inflammatory and antiviral properties acts as an immune modulator (Schroder et al., 2004); hence it becomes elevated when HIV replicates, and subsequently decreases when HIV-1 replication is inhibited (Watanabe et al., 2010). Further co-infection with TB up-regulates IFN-γ production by the lung resident CD4+ T cells (Diedrich and Flynn, 2011). In contrast, IL-10 an anti-inflammatory cytokine...
exerts an immunoregulatory role that is largely associated with suppression of host antiviral immune responses, partly by down-regulating expression of Th-1 cytokines (Klein, 1997). However, pro-inflammatory cytokine responses predominate early in HIV/TB co-infection as opposed to anti-inflammatory responses which increase with advanced clinical disease (Srikanth et al., 2000). These immune mechanisms may help explain the higher IFN-γ to IL-10 ratio elicited by the HIV-1/TB co-infected ART-naive IDU compared to healthy controls.

Overall, the IFN-γ to IL-10 ratio may have an ancillary role as a biological marker suggestive of a dysregulation of the immunological properties involved in the regulatory mechanisms of IDUs in response to HIV and TB co-infection. Hence, the IFN-γ to IL-10 ratio presents as a good biomarker for predicting the extent of disease severity and progression as compared to individual cytokines since it possesses a much higher predictive power as opposed to individual cytokines.

In the current study, levels of Acrp30, an anti-inflammatory cytokine were reduced among the TB mono-infected IDUs relative to healthy controls. This premise agrees with previous studies that demonstrated reduced Acrp30 levels among individuals with active pulmonary TB relative to healthy controls (Xu et al., 2007). Adiponectin is a hormone secreted almost exclusively by the adipocytes (Ouchi et al., 2011); hence its plasma concentrations are directly
correlated with adipose fat stores. The significantly low Acrp30 levels in the TB mono-infected IDUs may possibly have been triggered as a result of the increased loss of adipose tissue (cachexia) among TB mono-infected IDUs, since TB is usually characterised by body wasting (Eddleston, et al., 2008; Fauci et al., 2009).

Additionally, it is possible that use of hard drugs and other non-injection substances such as alcohol and khat may accelerate depletion of Acrp30 levels through dysregulation of appetite-regulatory hormones (Chang et al., 2013), with consequent appetite suppression leading to weight loss.

Furthermore, in the present investigation, Acrp30 levels were significantly decreased in the HIV-1/TB co-infected ART-experienced IDUs compared to healthy controls. These results corroborate previous observations that demonstrated reduced serum Acrp30 levels among heroin addicts (Housova et al., 2005). The lower levels of Acrp30 observed among the HIV-1/TB co-infected ART-experienced IDUs may have been influenced by the high magnitude of adipose tissue depletion resulting from lypodystrophy and impaired fat redistribution effects (Carr, 2003; de Luis et al., 2012), possibly due to the negative adverse drug reactions of antiretroviral agents (de Waal, et al., 2013). Additionally, antiretroviral medication can negatively interact with recreational drugs when used concomitantly (Tseng and Antoniou, 2002), which may further dampen normal Acrp30 cytokine expression pattern leading to poor clinical
outcomes. Hence, the interplay of signals regulated by Acrp30 may determine its net effect as a modulator of immune anti-inflammatory responses.

On a similar note, the HIV-1 mono-infected ART-experienced, HIV-1/TB co-infected ART-naive and -experienced IDUs presented with higher IFN-γ to Acrp30 ratio relative to healthy controls, and the HIV-1 negative and TB uninfected IDUs, respectively. Being a pro-inflammatory cytokine, IFN-γ is pivotal in the regulation of host immune responses against viral and intracellular bacterial pathogens (Roff et al., 2013). Hence, due to HIV-1 and TB infections, the HIV-1 and TB co-infected ART-naive and -experienced IDUs elicited higher IFN-γ levels compared to controls. Contrastingly, the HIV-1 and TB co-infected IDUs expressed lower Acrp30 levels relative to healthy controls possibly due to the significant loss of adipose tissue that is associated with decreased levels of Acrp30 (Luo et al., 2009).

Consequently, this phenomenon of enhanced Th-1 responses in HIV-1 and TB co-infection coupled with depressed Acrp30 responses, results in higher IFN-γ to Acrp30 ratio among the HIV-1 mono-infected ART-experienced, HIV-1/TB co-infected ART-naive and -experienced IDUs relative to controls. The fact that IFN-γ is also produced by NK cells during active infection (Spellberg and Edwards, 2001), and Acrp30 enhances NK cells activity (O’Shea et al., 2010), may also cause increased IFN-γ production. Therefore, these actions may contribute to the
observed lower IFN-γ to Acrp30 ratio in the current study. Ultimately, the IFN-γ to Acrp30 ratio may be proposed as an index for assessing the balance between the two markers (IFN-γ and Acrp30) bearing apparently opposite functions in inflammation.

Significant correlations were observed between the cytokine markers (IFN-γ, IL-10, Acrp30) and the disease progression outcomes including (CD4+ T cells counts, HIV-1 viral load and BMI) within the current study. The BMI correlated positively with Acrp30 levels and inversely with IFN-γ to Acrp-30 ratio, respectively in the HIV-1/TB co-infected ART-naive IDUs. The body mass index is an indicator of body fat percentage (Wellens et al., 1996); whereas Acrp30 is a novel protein hormone exclusively secreted from adipose tissue and is associated with adiposity (Scherer et al., 1995; Ouchi et al., 2011; Adamszczak and Wiecek, 2013). In the advent of HIV and TB co-infection, loss of body fat mass is accelerated which in turn results in depleted Acrp30 levels that positively correlates with low body weight and overall reduced BMI.

The premise is in agreement with previous studies showing associations between decreased Acrp30 levels and peripheral fat loss among HIV infected Chinese patients (Luo et al., 2009). Furthermore, decreased Acrp30 levels have been detected in experimental animal models manifesting lipoatrophy (Sutinen et al., 2003). Therefore, subcutaneous fat loss may actually be responsible for decreased
Acrp30 levels in the HIV-1/TB co-infected ART-naive group of injection drug users. Altogether, this study suggests that low fat store and underlying inflammation may regulate metabolic markers such as Acrp30 in TB and HIV co-infection.

The inverse correlation of BMI with the IFN-γ to Acrp30 ratio observed among the HIV-1/TB co-infected ART-naive IDUs in the current study may be an indication of failure of host immune regulation in the setting of HIV-1/TB co-infection cascade. The current study identified a positive correlation between BMI and circulating plasma Acrp30, an anti-inflammatory cytokine. Hence, this implies that lowered BMI levels mirrors reduced plasma Acrp30 levels in HIV-TB co-infected IDUs. When the IFN-γ to Acrp30 ratio is elevated a pro-inflammatory (IFN-γ), over an anti-inflammatory (Acrp30) response predominates, thus Acrp30 expression is shadowed, which ultimately results in an inverse correlation between IFN-γ to Acrp30 ratio and BMI.

Similarly, inverse correlation between Acrp30 levels and the CD4+ T cell counts was found in the TB mono-infected IDUs. The observation in the current study fails to conform to previous findings that reported Acrp30 to elicit a pro-inflammatory response in human macrophages while promoting a Th-1 differentiation of isolated CD4+ T cells (Cheng et al., 2012). These discordant results may be owed to injection drugs and polysubstance abuse among study
subjects, which interferes with kinetics of type-1 and type-2 cytokine specific expression. For instance, the circulating concentrations of Acrp30 are markedly altered in individuals with chronic heroin addiction (Housova et al., 2005; Shahouzehi et al., 2013). Nonetheless, injecting drug use has previously been described to cause a more rapid decline in CD4+ T cell counts (Meijerink et al., 2014). Hence, there is substantial evidence that injection drug use by itself is associated with marked metabolic and immunologic derangements in TB mono-infected IDUs.

Taken together, the observed levels of IFN-γ and IL-10 seem to be entirely opposing factors that govern the nature and shift of immune responses. The pro-inflammatory (IFN-γ) cytokines down regulates anti-inflammatory (IL-10) cytokine responses and vice-versa, which has overall aim of regulating balance between protection and immunopathology.

It is noteworthy that this cross-sectional study is part of a larger investigation that provides initial baseline information with regards to cytokine levels observed in concurrent HIV-1 and TB co-infection among Kenyan IDU population. However, a prospective (longitudinal) study encompassing a larger sample size would have been more ideal in enhancing understanding of biological interactions observed during ongoing drug injection, antiretroviral treatment and co-infection state. Additionally, the relatively small number of cytokines measured in the current
study precludes a definitive conclusion. On the other hand, individual history of substance abuse and injection drug use was self-reported by the IDUs and therefore subject to recall and reporting bias.

5.2 Conclusions

In summary, findings of the present study revealed marked dysregulation in the production of inflammatory profiles. The plasma IFN-γ levels were markedly elevated among HIV-1 and TB mono- and co-infected IDUs relative to healthy controls. The observation suggests that both HIV-1 and TB infections suppress and/or inhibit normal host immune processes by inducing increased IFN-γ production. The IL-10 levels were predominant among the HIV-1 negative and TB uninfected IDUs compared to HIV-1/TB infected IDUs and healthy controls, indicating that injection drugs cause substantives damage to tissues leading to systemic inflammation and subsequent immune activation. On the other hand, there were lower circulating Acrp30 levels in TB mono-infected IDUs relative to healthy controls, depicting the detrimental effect of TB infection on Acrp30 production.

In the present study, antiretroviral (HAART) use significantly influenced plasma cytokine levels among the HIV-1 positive IDUs. For instance, HIV-1/TB co-infected ART-experienced IDUs presented with relatively lower Acrp30 levels relative to healthy controls, which may be attributable to lypodystrophy and other
associated side effects of antiretroviral drugs. On the other hand, studies demonstrate ART to lower plasma IFN-\(\gamma\) levels to those comparable to healthy controls. However, the HIV-1 infected IDUs on ART and also concomitantly consuming injection drugs showed elevated levels of IFN-\(\gamma\) despite therapeutic intervention. This observation may indicate that injection drugs possibly interfere with immune reconstitution during ART, through impairment of lymphocyte reactivity among HIV positive drug users.

Correlation analysis indicated that BMI measurements correlated positively with Acrp30 levels and inversely with IFN-\(\gamma\) to Acrp30 ratio among the HIV-1/TB co-infected ART-naive IDUs. This implies that loss of adipose tissue during HIV-1 and TB co-infection directly affects Acrp30 secretion, an adipocyte derived hormone. The significant inverse correlation of BMI with the IFN-\(\gamma\) to Acrp30 ratio is as a consequence of immune regulatory mechanisms that are pro-inflammatory versus the anti-inflammatory immune responses that function to maintain immune integrity. On the other hand, the inverse correlation between Acrp30 and CD4+ T cell counts in TB mono-infected IDUs may be due to injection drugs and polysubstance abuse among study subjects, which interferes with kinetics of type-1 and type-2 cytokine specific expression.
5.3 Recommendations

1) Since plasma IFN-γ, IL-10 and Acrp30 demonstrated marked dysregulation during HIV-1, TB and substance use, more extensive evaluation should be performed in order to validate the adoption of these cytokine levels as disease surrogate markers in the assessment of inflammatory profiles during HIV-1 and TB co-infection in IDUs with a goal of providing better disease progression patterns and response to therapeutic intervention.

2) The Ministry of Health and Medical Services, clinicians and other relevant stakeholders involved in providing healthcare and support to IDUs should consider incorporating plasma Acrp30 besides BMI in the assessment of nutritional profiles of TB mono-infection among injection drug users.

5.4 Suggestions for further research

1) Future investigations should identify sources and define the mechanisms of elevated circulating IFN-γ levels among HIV-1 and TB co-infected IDUs.

2) Define mechanisms through which ART restores IFN-γ production among HIV-1 infected ART-experienced IDUs for improving therapeutic options in this population.
3) Perform a longitudinal study comparing different HAART regimens among IDUs in order to identify the contributions of each drug upon the cytokine profiles.
REFERENCES


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APPENDICES

Appendix I: Map of the study area in Mombasa County
Appendix II: Ethical approval

KENYATTA UNIVERSITY
ETHICS REVIEW COMMITTEE

Fax: 8711242/8711575
Email: kuerc.chairman@ku.ac.ke
Website: www.ku.ac.ke

P. O. Box 43844,
Nairobi, 00100
Tel: 8710901/12

Our Ref: KU/R/COMM/51/32-4

Date: June 6th, 2012

Valentine Budambula
School of Public Health,
Kenyatta University
P.O. Box 43844, Nairobi.

Dear Ms. Valentine


1. IDENTIFICATION OF PROTOCOL

The application before the committee is with a research topic ‘HIV/Pulmonary TB co-infection amongst intravenous drug users in Mombasa, Kenya’, Version 4. Dated 19th May, 2012.

2. APPLICANT

Valentine Budambula
School of Public Health,
Kenyatta University
P.O. Box 43844, Nairobi.

3. SITE

Mombasa County, Kenya.

4. DECISION REACHED.

The committee has considered the research protocol in accordance with the Kenyatta University Research Policy (section 7.2.1.3) and the Kenyatta University Ethics Review Committee Guidelines, and is of the view that against the following elements of review,

i. Scientific design and conduct of study,
ii. Recruitment of research participant,
iii. Care and protection of research participants,
iv. Protection of research participant’s confidentiality,
v. Informed consent process,
vi. Community considerations.

AND APPROVED that the research may proceed for a period of ONE year from 6th June, 2012.
Appendix III: Research permit

MINISTRY OF PUBLIC HEALTH & SANITATION

Office of
The Provincial Director
of Public Health &
Sanitation Services /Coast
Province
P.O. Box 90233-80100
Mombasa

Date: 12th July 2012

Ref: ADM.3/5/37/121

District Medical Officers of Health
>
Mombasa
>
Kilindini

RE: REQUEST TO CARRY OUT DATA COLLECTION ON HIV/PULMONARY
TB CO-INFECTION AMONGST INTRAVENOUS DRUG USERS IN
MOMBASA

The bearer of this letter, Valentine Budambula is a PhD student at the
Department of Community Health, Kenyatta University (KU), Nairobi and also a
lecturer at Department of Environment and Health, Mombasa Polytechnic
University College.

She wishes to carry out data collection in health institutions and rehabilitations
centres within Mombasa County for a period of one (1) year with effect from 6th
July 2012.

Kindly accord her the necessary support.

Dr. Anisa Omar, OGW
M, B, Ch, B M, Med (paed) H. Dip.HSM (Israel)
Provincial Director of Public Health and Sanitation,
COAST PROVINCE
Appendix IV: Consent to participate in the research

English Version

Title: HIV/Pulmonary TB co-infection amongst Intravenous Drug Users in Mombasa, Kenya.

Dear participant:
You are invited to take part in this research study because you have a history of intravenous drug use. This form tells you why this research study is being done. Please read through then you can decide if you are interested in participating in the study or not. The investigators in this study are from Kenyatta University and Technical University of Mombasa. A study team will be working closely with the investigators and the study will run for a period of two years.

The purpose of the study is to determine the factors associated with HIV and pulmonary Tuberculosis co-infections among intravenous drug users. If you choose to participate in this study, the research team will require 5ml of whole blood (HIV voluntary testing and Complete Blood Count) and three early morning sputum (TB testing) from you. No drug or chemical will be introduced into your body.

You can decide whether to take part in the study or not. You have the right to say Yes or No. If you say No, your regular medical care will not change. Even if you join this study, you do not have to stay in it, you may stop at any time. It is important to note that there is no financial benefit for participating in this study. At the same time there will be no cost implications to you the study participant. Participation in this study is important as the findings of the study have the potential of being used to lobby for funding for antiretroviral drugs (ARVs) and primary healthcare for the intravenous drug users.

The risks of this study include possible discomfort due to questions on health and personal history/behaviour. In addition, discomfort may be experienced while a blood sample is being obtained. Every effort will be made to your study records confidential but we cannot guarantee it. No funds have been set aside to pay any costs if you are harmed because of this study. If you feel that you were harmed because of this study, contact the Principal or Co-investigator.

By signing my name below, I confirm the following:
I have read or been read to) this entire consent document. All of my questions have been answered to my satisfaction. The study’s purpose, procedures, risk and possible benefits have been explained to me. I agree to let the study team use and share the health information and other information gathered for this study. I voluntarily agree to participate in this research study. I agree to follow the research study procedures as directed. I have been informed that I can withdraw from the study at any time.

Participant’s Name ..................................Signature..............................
Date................................

Principal investigator/Supervisor................................Signature..........................Date..........

Note: Below are some of the key contacts

Principal investigator- Dr Tom Were 0720326127; Co-investigator- Valentine Budambula 0722822448; KU-ERC kuerc.chairman@ku.ac.ke
Kiswahili Version

Mada ya Utafiti: Uambukizo pamoja wa virusi vya HIV na Kifua kikuu kati ya watumiaji mihadarati kwa kujidunga, Mombasa Kenya.

Kwako mhusika:

Nia hasa ya utafiti huu ni kutathmini au kuamua sababu zinazohusishwa na uambukizo pamoja wa virusi vya HIV na kifua kikuu kati ya watumiaji mihadarati kwa kujidunga sindano. Ukichagua kushiriki kwenye utafiti huu, timu ya utafiti itahitaji mililita tano (5) ya damu kutoka kwako (kwa ajili ya kwenye kiwango cha damu) na pia watahitaji kohozu cha asubuhi (kwa ajili ya kwenye kifua kikuu). Hakuna dawa ama kemikali zozote zitazotaka ndani ya mwili wako.

Unaweza kutoa uamuzi wa kushiriki kwenye utafiti huu au la, pia uko na uhuru wa kusema ndio au la. Ukisema la, matibabu yako yake yana maambo zaidi zaidi ya kujidunga kwa sababu ni kuamua sababu zinazohusishwa na uambukizo pamoja wa virusi vya HIV na kifua kikuu. Utafiti huu ni ufikiajili muhimu kwa sababu katika historia ya kujidunga kwa sababu ni kuamua sababu zinazohusishwa na uambukizo pamoja wa virusi vya HIV na kifua kikuu.

Hatari zinazoambatana na kushiriki katika utafiti huu ni kama usumbufu kutokana na maswali ya kiafya ya kifua kikuu. Kadhalika utahisi usumbu kwa kutolewa damu. Juhudi zote zitafanywa kwa ajili ya kuamua sababu zinazohusishwa na uambukizo pamoja wa virusi vya HIV na kifua kikuu.
Kwa kuweka sahihi jina langu nathibitisha yafuatayo:
1) Nimesoma (ama nimesomewa) idhini hii ya kukubali, na maswali yangu yote yamejibiwa na nimeridhika; 2) Nia, mitindo, hatari pamoja na faida zinazoambatana na na utafiti huu zimeeleza kwangu; 3) Nakubali kwa kuruhusu timu ya utafiti huu kutumia na kugawa za kiafya ama aina yoyote ya habari zitakazo kusanywa kutokana na utafiti huu; 4) Nimekubali kwa hiyari yangu kushiriki kwenye utafiti huu. Nakubali kufuata mitindo ya utafiti huu; 5) Nimeelezwa ya kwamba ninaweza kukoma kushiriki wowote.

Jina la mshiriki ....................................Sahihi ..................................Tarehe ...........................................

Mtafiti mkuu/Msaidizi .................................Sahihi ..................................Tarehe ...........................................

Zaidi; wasiliana na wafuatao
Mtafiti mkuu- Dr Tom Were 0720326127; Mtafiti msaidizi- Valentine Budambula nambari ya rununu, 0722822448; KU-ERC kupitia barua pepe kuerc.chairman@ku.ac.ke
Appendix V: Study questionnaire

**Questionnaire for HIV/TB co-infection in IDUs**

Study participant code __________                        Interview
date______/______/______

1. Age _______ (Years)                             Birth date______/______/______

2. Gender: □ Male  □ Female

3. Do you inject drugs?                            □ Yes   □ No

4. If yes, which drug(s) do you routinely inject? _________

5. How long have you injected drugs? _________ (Months/Years)

6. Do you share needles and syringes while injecting drugs?  □ Yes   □ No

7. Do you practice blood flashing?                 □ Yes   □ No

8. Do you know your HIV status?                    □ Yes   □ No

9. Are you on antiretroviral treatment?           □ Yes   □ No

10. If yes, how long have you been on antiretroviral treatment? (Months/Years)

11. Have you been diagnosed with Tuberculosis before? □ Yes   □ No

12. Do you suffer from both HIV and TB infections? □ Yes   □ No