CYTOKINES LEVELS AND THEIR RELATIONSHIP WITH CLINICAL CORRELATES IN HUMAN IMMUNODEFICIENCY VIRUS/HEPATITIS C CO-INFECTED DRUG USERS FROM MOMBASA COUNTY, KENYA

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NOVEMBER, 2015
DECLARATION

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

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DEDICATION

This thesis is dedicated to my sponsor, Peter Brice, whose sacrifice and financial support has seen me through my Masters program; and to my beloved mother, Jane W. Karanja, and my siblings for their support and encouragement during my entire study period.
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ABBREVIATIONS AND ACRONYMS

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<tr>
<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
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<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
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<tr>
<td>ART</td>
<td>Anti-Retroviral Therapy</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
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<tr>
<td>BMI</td>
<td>Body Mass Index</td>
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<td>CD4</td>
<td>Cluster of Differentiation 4</td>
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<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
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<td>ESLD</td>
<td>End Stage Liver Disease</td>
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<tr>
<td>HAART</td>
<td>Highly active Anti-Retroviral Therapy</td>
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<td>HC</td>
<td>Healthy Control</td>
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<td>HCC</td>
<td>Hepatocellular Carcinoma</td>
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<td>HCV</td>
<td>Hepatitis C Virus</td>
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<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HS</td>
<td>Hepatic Steatosis</td>
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<tr>
<td>IDUS</td>
<td>Injection Drug Users</td>
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<tr>
<td>IFN-γ</td>
<td>Interferon Gamma</td>
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<tr>
<td>IL-10</td>
<td>Interleukin-10</td>
</tr>
<tr>
<td>IVDU</td>
<td>Intravenous Drug Use</td>
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<tr>
<td>KAIS</td>
<td>Kenya AIDS Indicator Survey</td>
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<tr>
<td>MARPS</td>
<td>Most At Risk Populations</td>
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<td>MOH</td>
<td>Ministry of Health</td>
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NACC  National AIDS Control Council
NASCOP  National AIDS and STI Control Programme
PCR  Polymerase chain reaction
PI  Protease Inhibitor
UNAIDS  United Nations Programme on HIV and AIDS
UNEP  United Nations Environmental Programme
UNGASS  United Nations General Assembly Special Session
WHO  World Health Organization
ABSTRACT

Human immunodeficiency virus-1 (HIV-1) and hepatitis C virus (HCV) co-infection are two rapidly growing epidemics and health concerns strongly driven by injection drug use (IDU) in Kenya and the entire world. Inflammatory cytokines are important mediators of the host response to HIV-1 and HCV infections including injection drugs. However, the molecular interaction between HIV-1 and HCV co-infection and correlation with injection drug use is largely undefined. Therefore, this cross-sectional study determined plasma levels of interferon (IFN)-γ, interleukin (IL)-10, adiponectin and their association with CD4+ T cell count, HIV-1 viral load and basal metabolic index (BMI) in HIV-1 and HCV mono-infected antiretroviral (ART)-naive (n=18) and -exposed (n=38); and co-infected ART-naive (n=5) and -exposed (n=14); and uninfected (n=24) IDUs and healthy controls (n=27). One hundred and thirty five injection drug users and 27 healthy controls were recruited from Bomu Medical Center, Mombasa, Kenya through outreach and respondent driven sampling methods. Social demographic information including drug use histories were collected from the study subjects upon enrolment into the study through structured interview schedules with open- and closed-ended questions. Plasma cytokines levels were determined using Enzyme linked Immunosorbent assay (ELISA) technique, CD4 T cell count were enumerated using FacsCalibur™ flow cytometry, HIV-1 viral load through polymerase chain reaction (PCR), and HIV-1 and HCV infections determined by rapid sero-diagnosis. Interferon (IFN)-γ levels differed across-group (P<0.0001) and were significantly elevated in HIV-1 mono-infected ART-exposed and uninfected individuals relative to healthy controls (p<0.0001). Interleukin (IL)-10 levels also varied across-group (P<0.0001) and were higher in HIV-1 and HCV co-infected ART-exposed, HCV mono-infected and uninfected IDUs compared to healthy controls (p<0.0001). Adiponectin levels varied across-group (p<0.0001) and were significantly higher in HCV mono-infected compared to HIV-1 mono-infected ART-exposed or -naive (p<0.0001). Interferon-γ (IFN-γ)/IL-10 ratio differed across-group (P<0.0001) and was increased in HIV-1 and HCV co-infected ART-exposed, HCV mono-infected, HIV-1 mono-infected ART-exposed, and uninfected groups relative to healthy controls (P<0.0001). Interferon-γ (IFN-γ) was correlated with BMI (r=0.628; P=0.029) in HIV-1 and HCV co-infected ART-exposed, viral load (r= -0.998; P=0.004) in HIV-1 and HCV co-infected ART-naive, CD4+ T cell count (r=0.393; P=0.018) in HCV mono-infected IDUs. Interferon-γ (IFN-γ) was also correlated with IL-10 in HIV/HCV co-infected ART-exposed (r=0.711, p=0.010), HIV mono-infected ART-naive (r=0.616, p=0.011), healthy controls (r=0.877, p< 0.0001), adiponectin (r= -0.422, p=0.013) in ART-exposed HIV-1 mono-infected IDUs. Taken together, these results suggest profound dysregulation in IFN-γ and IL-10 production that is associated with clinical outcomes in both HIV-1 and HCV mono- and co-infected and uninfected IDUs. The implications of these findings include their utility in initiation and monitoring of treatment in IDUs.
CHAPTER ONE: INTRODUCTION

1.1 Background information
Human immunodeficiency virus (HIV)-1 infection is one of the leading causes of morbidity and mortality by infectious disease origin in the world. In Kenya, an estimated 1.6 million people are infected with HIV (UNAIDS, 2012). In addition, HIV infections caused about 62,000 deaths in 2011 (UNAIDS, 2012). The burden of HIV-1 infections is frequently higher among key populations, including sex workers, injection drug users (IDUs), men who have sex with men (MSM), truck drivers and cross-border mobile populations (UNGASS, 2008). Injection drug use has been increasing in Kenya mainly in the larger towns and cities such as Mombasa and Nairobi (UNODC, 2013). The prevalence of HIV-1 among IDUs in Kenya ranges from 43-49%, with new infections occurring at the rate of 17% (NASCOP, 2008). Thus, IDUs appear to experience high burden of HIV-1 infections, largely due to increased exposure through heterosexual and parenteral routes, lack of adherence to treatment and increased antiretroviral resistance (Mizukoshi et al., 2008). In addition, IDUs are at a higher risk of acquiring multiple infections with blood-borne pathogens.

Hepatitis C virus (HCV) infections frequently occur among injection drug users, and in Kenya, HCV prevalence rates range from 82-93% among injection drug users (Larsen et al., 2008). The rates of HCV infections are even higher in IDUs infected with HIV-1. The prevalence of HCV in HIV-1 infected IDUs in Kenya is 42.29-90% (Aceijas et al., 2007). While the interaction between HCV and HIV-1 is complex and poorly delineated, it appears that HCV promotes rapid CD4 count decline and disease progression during HIV-1 co-infection (Thomas, 2002). Moreover, HCV co-infection with HIV-1 also accelerates HCV disease progression and risk of antiretroviral drug associated hepatotoxicity (Greub, 2000). Taken together, these studies suggest
that interactions between HCV and HIV-1 drive progression of both diseases leading to increased morbidity and mortality.

A network of cytokines regulates the growth and function of the cells of the immune system. T cells possess a dominant role in this network since they are the main source of many cytokines. The patterns of cytokines produced by T-helper (Th) cells have led to the differentiation of T-helper cells into two different subsets, Th1 and Th2. Th1 cells produce cytokines such as interleukin-2 (IL-2), tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) that promote cell-mediated immunity, while Th2 cells produce cytokines such as IL-4, IL-5, IL-6, IL-10 and IL-13 that promote antibody-mediated immunity (Mosmann et al., 2003). A hypothesis has been introduced suggesting that a shift in the balance of production of Th1 versus Th2 cytokines is a major contributor to HIV-1 disease progression. The hypothesis suggests that early in HIV-1 infection, a vigorous cell-mediated immune response facilitated by Th1 cells effectively controls the amount of HIV-1 in the body (Turk et al., 2012). However, with time, the predominant cytokine response shifts to a Th2 type, leading to a loss of effective cell-mediated immunity against HIV-1, permitting increased levels of viral replication, extensive damage to the immune system, and progression to AIDS (Clerici and Shearer, 1994). However, other investigators have either found no change in the cytokine levels or in some cases the opposite result (Graziosi et al., 2006).

Interferon-gamma (IFN-γ) is the hallmark cytokine of T helper 1 cells. Natural killer cells and CD8 cytotoxic T cells also produce IFN-γ which has antiviral, immunoregulatory, and anti-tumor
properties (Schroder et al., 2004). It is an important activator of macrophages. Its importance in the immune system stems from its ability to inhibit viral replication directly.

Interleukin-10 (IL-10), also known as human cytokine synthesis inhibitory factor (CSIF), is an anti-inflammatory cytokine. The IL-10 protein is a homodimer; each of its subunits is 178-amino acids long. Interleukin-10 is classified as a class-2 cytokine, a set of cytokines including IL-19, IL-20, IL-22, IL-24 and IL-26 (Said et al., 2010). In humans, IL-10 is encoded by IL-10 gene, which is located on chromosome 1 and comprises of 5 exons and is primarily produced by monocytes and, to a lesser extent, lymphocytes, namely T helper 2, mastocytes, CD4 regulatory cells, and by a certain subset of activated T cells and B cells. Interleukin-10 (IL-10) is a cytokine with pleiotropic effects in immunoregulation and inflammation. It down-regulates the expression of Th1 cytokines, MHC class II antigens, and co-stimulatory molecules on macrophages. It also enhances B cell survival, proliferation, and antibody production (Eskdale et al., 1997).

Adiponectin is a protein hormone that modulates a number of metabolic processes, including glucose regulation and fatty acid oxidation (Diez et al., 2003). Adiponectin is exclusively secreted from adipose tissue and also from the placenta in pregnancy (Chen et al., 2006) into the bloodstream and is very abundant in plasma relative to many hormones. Levels of the hormone are inversely correlated with body fat percentage in adults (Chen et al., 2006), while the association in infants and young children is less clear. Transgenic mice with increased adiponectin show impaired adipocyte differentiation and increased energy expenditure associated with protein uncoupling (Bauche et al., 2007). The hormone plays a role in the suppression of the metabolic derangements that may result in type 2 diabetes, obesity, and atherosclerosis (Diez
Adiponectin is secreted into the bloodstream where it accounts for approximately 0.01% of all plasma protein and exerts some of its weight reduction effects via the brain. This is similar to the action of leptin (Hainer et al., 2005), but the two hormones perform complementary actions, and can have synergistic effects.

The objective of the study was to determine cytokines levels and their interactions with baseline predictors of disease progression and treatment outcome among HIV-1/HCV co-infected Kenyan injection drug users.

1.2 Problem statement
Human immunodeficiency virus type 1 (HIV-1) and HCV are the agents behind two viral epidemics causing huge morbidity and mortality worldwide especially among IDUs (Falconer et al., 2009). Previous research findings have reported on the prevalence of HIV-1/HCV co-infections globally, but few have gone further to assess the immunological profiles among injection drug users. Cytokines, especially IFN-γ, IL-10 and adiponectin are immunological factors that play critical roles in immune responses during HIV-1/HCV co-infection and mono-infections. Hence, there is need for further evaluation of levels of these cytokines among Kenyan IDUs with the ultimate goal of trying to understand the interaction and alterations occasioned by drugs if any. Interferon-gamma though used as an adjunctive treatment for HCV infection, the determination of its utility in HIV-1/HCV co-infected IDUs calls for clinical studies in humans. This can only be assessed by defining the levels of IFN-γ in HIV-1/HCV co-infected IDUs. Currently, there is minimal or no data on IFN-γ, IL-10 and adiponectin levels in HIV-1/HCV co-infected IDUs. This study sought to quantify the levels of IFN-γ, IL-10 and
adiponectin among Kenyan IDUs in order to give a greater insight into care and treatment of HIV-1/HCV co-infected IDUs.

1.3 Study justification
Human immunodeficiency virus (HIV)-1 is a common viral infection among the injection drug users. Co-infection with hepatitis C virus (HCV) is also common because HIV-1 and HCV share similar routes of transmission. Injection drug users have been shown to be at a higher risk of acquiring HIV-1, HCV and other blood borne pathogens owing to their risky sexual behavior as well as sharing of needles and hence the need to evaluate and characterize the immunological correlates that underlie disease progression among HIV-1/HCV co-infected IDUs. On the other hand, HCV complicates the management of HIV-1 and is one of the leading causes of hospital admission and death among HIV-1 patients (Eva et al., 2011). Mombasa is the portal entry for illicit drugs and happens to harbor the highest number of injection drug users in Kenya, making it a suitable site to carry out the study. Likewise, Bomu medical centre hosts a major rehabilitation centre that draws injection drug users from different parts of Mombasa making it a good representative of the County. Interferon-gamma has been used as an adjunctive treatment for HCV infection while IL-10 and adiponectin antagonize IFN-γ during host immune responses. The determination of IFN-γ levels in HIV-1/HCV co-infected IDUs, therefore, will help define its utility and how it works in concert with IL-10 and adiponectin as its inhibitors.
1.4 Research questions

i. What are the levels of IFN-\(\gamma\), IL-10 and adiponectin in HIV-1 and HCV co-infected injection drug users from Mombasa County?

ii. What is the relationship between circulating IFN-\(\gamma\), IL-10, adiponectin and CD4+ T cell counts, the body mass index, and HIV-1 viral load in HIV-1 and HCV co-infected injection drug users from Mombasa County?

iii. What are the effects of antiretroviral treatment on circulating IFN-\(\gamma\), IL-10 and adiponectin levels in HIV-1 and HCV co-infected injection drug users from Mombasa County?

1.5 Null Hypotheses

i. Levels of IFN-\(\gamma\), IL-10 or adiponectin in HIV-1 and HCV co-infected injection drug users are not similar

ii. Levels of IFN-\(\gamma\), IL-10 or adiponectin have no association with CD4+ T cell counts, HIV-1 viral load and body mass index in HIV-1 and HCV co-infected injection drug users

iii. Antiretroviral treatment has no effect on IFN-\(\gamma\), IL-10 or adiponectin levels in HIV-1 and HCV co-infected injection drug users
1.6 Objectives

1.6.1 General objective
To determine plasma IFN-γ, IL-10, adiponectin levels and their relationship with CD4+ T cell counts, HIV-1 viral load and basal metabolic index in HIV-1 and HCV co-infected injection drug users from Mombasa County.

1.6.2 Specific objectives
i. To determine plasma IFN-γ, IL-10 and adiponectin levels in HIV-1 and HCV co-infected injection drug users from Mombasa County

ii. To establish the relationship between CD4+ T cell counts, HIV-1 viral loads and body mass index in HIV-1 and HCV co-infected injection drug users from Mombasa County

iii. To determine the effect of ART on plasma IFN-γ, IL-10 and adiponectin levels in HIV-1 and HCV co-infected injection drug users from Mombasa County

1.7 Expected significance of the study
Results obtained in this study will provide clear guidelines on how exactly treatment and care should be administered to injection drug users. Findings will inform whether interferon-based treatment of HCV infection among IDUs will be customized. Data will also be useful in determining whether routine HCV diagnosis together with HIV-1 testing should be recommended in IDUs, thus enabling proper management of these infections. This information can be used in formulating policies on management of the viral diseases affecting Kenyan IDUs.
CHAPTER TWO: LITERATURE REVIEW

2.1 Hepatitis C virus (HCV) and HIV-1 burden

Hepatitis C virus infection is a major health concern globally. It is estimated that 150-200 million people, or ~3% of the world’s population is living with chronic hepatitis C (WHO, 2011). Rates of HCV are high (>3.5% population infected) in Central and East Asia, North Africa and the Middle East and they are low (<1.5%) in Asia Pacific, Tropical Latin America and North America (Mohd and Flaxman, 2013). As of 2011, approximately 34 million people had HIV worldwide (UNAIDS, 2011). This means that about 5% of the adult population is infected. In Kenya, HIV-1 prevalence among adults aged 15 to 64 years decreased nationally from 7.2% to 5.6% between 2007 and 2012. Human Immunodeficiency Virus (HIV)-1 prevalence among adults varied by regions, with the highest prevalence in Nyanza and lowest prevalence in the Eastern North region. While most regions showed a decreased prevalence from 2007 to 2012, substantial drops up to 4.3%, 4.9% and 3.1% were identified in the Coast, Nairobi and Rift Valley regions respectively (KAIS, 2012).

2.2 Immunopathogenesis of human immunodeficiency virus (HIV)-1

The human immunodeficiency virus (HIV)-1 is a lentivirus (a subgroup of retrovirus) that causes the acquired immunodeficiency syndrome (AIDS) (Weiss et al., 1993; Douek et al., 2009), a condition in humans in which progressive failure of the immune system allows life-threatening opportunistic infections and cancers to thrive. After the virus enters the body, there is a period of rapid viral replication, leading to an abundance of virus in the peripheral blood. During primary infection, the level of HIV-1 may reach several million virus particles per milliliter of blood (Piatak et al., 1993). This response is accompanied by a marked drop in the numbers of
circulating CD4$^+$ T cells. This acute viremia is virtually associated with all people with the activation of CD8 T cells, which kill HIV-1-infected cells, and subsequently with antibody production, or seroconversion. The CD8$^+$ T cell response is thought to be important in controlling virus levels, which peak and then decline, as the CD4$^+$ T cell counts rebound (Benito et al., 2004).

Normal CD8$^+$ T cell response has been linked to slower HIV-1 disease progression and a better prognosis, though it does not eliminate the virus (Graziosi et al., 2006). Ultimately, HIV-1 causes AIDS by depleting CD4$^+$ T helper lymphocytes. This weakens the immune system and allows opportunistic infections. T lymphocytes are essential to the immune response and without which the body cannot fight infections or kill cancerous cells. The mechanism of CD4$^+$ T cell depletion differs in the acute and chronic phases (Hel et al., 2006). During the acute phase, HIV-induced cell lysis and killing of infected cells by cytotoxic T cells accounts for CD4$^+$ T cell depletion, although apoptosis may also be a factor. During the chronic phase, the consequences of generalized immune activation coupled with the gradual loss of the ability of the immune system to generate new T cells appear to account for the slow decline in CD4$^+$ T cell numbers. Although the symptoms of immune deficiency, characteristic of AIDS do not appear for years after a person is infected, the bulk of CD4$^+$ T cell loss occurs during the first weeks of infection, especially in the intestinal mucosa, which harbors the majority of the lymphocytes found in the body (Mehandru et al., 2004). The reason for the preferential loss of mucosal CD4$^+$ T cells is that a majority of mucosal CD4$^+$ T cells express the CCR5 co-receptor, whereas only a small fraction of CD4$^+$ T cells in the bloodstream do so (Douek et al., 2009). Human immunodeficiency virus seeks out and destroys CCR5 expressing CD4$^+$ cells during acute
infection. A vigorous immune response eventually controls the infection and initiates the clinically latent phase. However, CD4$^+$ T cells in mucosal tissues remain depleted throughout the infection, although enough remain to initially ward off life-threatening infections.

Continuous HIV-1 replication results in a state of generalized immune activation persisting throughout the chronic phase (Appay et al., 2008). Immune activation, which is reflected by the increased activation state of immune cells and release of pro-inflammatory cytokines, results from the activity of several HIV-1 gene products and the immune response to ongoing HIV-1 replication. Another cause is the breakdown of the immune surveillance system of the mucosal barrier caused by the depletion of mucosal CD4$^+$ T cells during the acute phase of disease (Rodriguez et al., 2006). This results in the systemic exposure of the immune system to microbial components of the gut’s normal flora, which in a healthy person is kept in check by the mucosal immune system. The activation and proliferation of T cells that results from immune activation provides fresh targets for HIV-1 infection. However, direct killing by HIV-1 alone cannot account for the observed depletion of CD4$^+$ T cells since only 0.01–0.10% of CD4$^+$ T cells in the blood are infected (Fauci, 2007). A major cause of CD4$^+$ T cell loss appears to result from their heightened susceptibility to apoptosis when the immune system remains activated. Although new T cells are continuously produced by the thymus to replace the ones lost, the regenerative capacity of the thymus is slowly destroyed by direct infection of its thymocytes by HIV-1 (Ye et al., 2004). Eventually, the minimal number of CD4$^+$ T cells necessary to maintain a sufficient immune response is lost, leading to AIDS (Rodriguez et al., 2006).
2.2.1 Diagnosis of HIV-1 infection

Tests used for the diagnosis of HIV-1 infection in a particular person require a high degree of both sensitivity and specificity. If antibodies are detected by an initial test based on the enzyme linked Immunosorbent assay (ELISA) method, then a second test using the Western blot procedure determines the size of the antigens in the test kit binding to the antibodies. The combination of these two methods is highly accurate (UNAIDS and WHO, 2006).

2.2.1.1 Antibody tests

Human immunodeficiency virus (HIV)-1 antibody tests are specifically designed for routine diagnostic testing of adults; these tests are inexpensive and extremely accurate. Antibody tests may give false negative (no antibodies were detected despite the presence of HIV-1) results during the window period, an interval of three weeks to six months between the time of HIV-1 infection and the production of measurable antibodies to HIV-1 seroconversions (Hare et al., 2004). Most people develop detectable antibodies approximately 30 days after infection, although some seroconvert later. The vast majority of people (97%) have detectable antibodies by three months after HIV-1 infection; a six-month window is extremely rare with modern antibody testing (CDC, 2007). During the window period, an infected person can transmit HIV-1 to others although their HIV-1 infection may not be detectable with an antibody test. Anti-retroviral therapy during the window period can delay the formation of antibodies and extend the window period beyond 12 months (Hare et al., 2004). This was not the case with patients that underwent treatment with post-exposure prophylaxis (PEP) (Hare et al., 2004). Those patients must take ELISA tests at various intervals after the usual 28 day course of treatment, sometimes extending outside of the conservative window period of 6 months. Antibody tests may also yield
false negative results in patients with X-linked agammaglobulinemia; other diagnostic tests should be used in such patients. Three instances of delayed HIV seroconversion occurring in health-care workers have been reported (Ridzon et al., 1997); in these instances, the health-care workers tested negative for HIV-1 antibodies greater than 6 months post exposure but were seropositive within 12 months after the exposure (Gerberding, 1997). DNA sequencing confirmed the source of infection in one instance. Two of the delayed seroconversions were associated with simultaneous exposure to hepatitis C virus (HCV). In one case, co-infection was associated with a rapidly fatal HCV disease course; however, it is not known whether HCV directly influences the risk for or course of HIV-1 infection or is a marker for other exposure-related factors.

The enzyme-linked Immunosorbent assay (ELISA), or enzyme immunoassay (EIA) was the first screening test commonly employed for HIV-1. It has a high sensitivity. In an ELISA test, a person's serum is diluted 400-fold and applied to a plate to which HIV-1 antigens have been attached. If antibodies to HIV-1 are present in the serum, they may bind to these HIV-1 antigens (Bartlett, 2006). The plate is then washed to remove all other components of the serum. A specially prepared "secondary antibody" - an antibody that binds to human antibodies is then applied to the plate, followed by another wash. This secondary antibody is chemically linked in advance to an enzyme. Thus, the plate will contain enzyme in proportion to the amount of secondary antibody bound to the plate. A substrate for the enzyme is applied, and catalysis by the enzyme leads to a change in color or fluorescence (Bartlett, 2006). ELISA results are reported as a number; the most controversial aspect of this test is determining the "cut-off" point between a positive and a negative result.
2.2.1.2 Western blot

Like the ELISA procedure, the western blot is an antibody detection test. However, unlike the ELISA method, the viral proteins are separated first and immobilized. In subsequent steps, the binding of serum antibodies to specific HIV-1 proteins is visualized (Bartlett, 2006). Specifically, cells that may be HIV-1-infected are opened and the proteins within are placed into a slab of gel, to which an electrical current is applied. Different proteins will move with different velocities in this field, depending on their size, while their electrical charge is leveled by a surfactant called sodium lauryl sulfate (Bartlett et al., 2006). Some commercially prepared Western blot test kits contain the HIV-1 proteins already on a cellulose acetate strip. Once the proteins are well-separated, they are transferred to a membrane and the procedure continues similar to an ELISA: the person's diluted serum is applied to the membrane and antibodies in the serum may attach to some of the HIV-1 proteins. Antibodies that do not attach are washed away, and enzyme-linked antibodies with the capability to attach to the person's antibodies determine to which HIV-1 proteins the person has antibodies.

There are no universal criteria for interpreting the western blot test; the number of viral bands that must be present may vary. If no viral bands are detected, the result is negative. If at least one viral band for each of the GAG, POL, and ENV gene-product groups is present, the result is positive (Soriano et al., 1997). The three-gene-product approach to western blot interpretation has not been adopted for public health or clinical practice. Tests in which less than the required numbers of viral bands are detected are reported as indeterminate: a person who has an indeterminate result should be retested, as later tests may be more conclusive. Almost all HIV-1-infected persons with indeterminate western blot results will develop a positive result when
tested after one month; persistently indeterminate results over a period of six months suggest the results are not due to HIV-1 infection. In a generally healthy low-risk population, indeterminate results on western blot occur on the order of 1 in 5,000 patients (Bartlett, 2006).

### 2.2.2 Treatment for HIV infection

There are currently five different classes of HIV-1 drugs namely: entry inhibitors, fusion inhibitors, reverse transcriptase inhibitors, integrase inhibitors, and protease inhibitors. Each class of drug attacks the virus at different points in its life cycle. Taking more than one drug also offers protection against HIV-1 drug resistance (FDA, 2009). When HIV-1 reproduces, it can make copies of itself that are imperfect and these mutations may not respond to the drugs taken to control HIV-1. If the 3-drug regimen is followed, HIV-1 is less likely to make new copies that don’t respond to HIV-1 drugs. Nucleoside/Nucleotide Reverse Transcriptase Inhibitors (NRTIs) sometimes called "nukes", work to block a very important step in HIV’s reproduction process by acting as faulty building blocks in production of viral DNA. This blocks HIV’s ability to use a special type of enzyme (reverse transcriptase) to correctly build new genetic material (DNA) that the virus needs to make copies of itself (FDA, 2009).

Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs) also called "non-nukes", block the enzyme reverse transcriptase, and prevent HIV-1 from making copies of its own DNA. Unlike the nukes, which work on the genetic material, non-nukes act directly on the enzyme itself to prevent it from functioning correctly. Protease Inhibitors (PIs) or stoppers block protease enzyme and prevent the long strands of HIV-1 genetic material from being cut up into shorter functional pieces (FDA, 2009).
2.3 Immunopathogenesis of HCV infection

The hepatitis C virus (HCV) is a small, enveloped, single-stranded, positive-sense RNA virus (Rosen, 2011). It is a member of the *Hepacivirus* genus in the family *Flaviviridae* (Stuart *et al*., 2009). There are seven major genotypes of HCV, which are known as genotypes one to seven (Lau *et al*., 2011). The primary route of transmission in the developed world is intravenous drug use (IDU), while in the developing world the main methods are blood transfusions and unsafe medical procedures (Maheshwari *et al*., 2010). The cause of transmission remains unknown in 20% of cases (Ponde, 2011); however, many of these are believed to be accounted for by IDU.

Most cases of chronic HCV are asymptomatic without episode of clinically apparent jaundice (Calleri, 2007). In 15% to 45% of exposed individuals, acute HCV disease completely resolves, with clearance of HCV RNA from serum within 4 months (Brown *et al*., 2001). Hepatitis C viral RNA becomes detectable in serum 7 to 21 days after exposure, while HCV antibodies are present from 20 to 150 days. Clinical syndrome may occur consisting of nausea, fatigue, myalgias, fevers, upper abdominal discomfort, and jaundice which occurs within 2 to 12 weeks after exposure, and may not be related to viral titers (Orland, 2001). HCV-specific CD4+ and CD8+ T cells appear within 2 to 3 months following acute infection. A strong T cell response, with increased production of IFN-γ and IL-2 is characteristic of HCV-mono-infected subjects who clear viremia.

Approximately 55% to 85% of exposed individuals fail to spontaneously clear the virus and are at risk of liver damage. Progression of HCV-induced liver disease has been associated with many systemic diseases and their complications, including autoimmune hepatitis and
glomerulonephritis. The pathogenetic mechanisms that result in chronic hepatitis are unknown; Tsai et al. (1997) proposed that abnormalities in early events, involving innate immunity, may lead to the impaired cellular immune responses. Thus, loss of sustained CD4+ reaction (Gerlach et al., 1999) and lower IFN-α response to HCV proteins (Danta, 2006) lead to chronicity. In HIV-1/HCV co-infection, there is increased apoptosis of CD4+ and CD8+ naive and memory cells and reduced activation of CD8+ memory cells, suggesting a permissive effect of HIV-1, regardless of viral load or CD4 counts, establishing chronic HCV infection (Yonkers, 2006).

In chronic HCV infection, fibrosis results from the activation of hepatic stellate cells by cytokines and other signaling molecules induced by the inflammatory process. Hepatic steatosis is a concurrent factor in the progression to advanced fibrosis and is associated with HCV genotype 3, and in other patients it is associated with metabolic factors such as higher body mass index, type 2 diabetes, and hyperlipidemia (Castera, 2006). Hepatitis C virus (HCV), with or without HIV-1 increases the risk of insulin resistance and diabetes (Duong, 2001; Mehta, 2003). Insulin resistance is a common denominator for steatosis, fibrosis, and elevated circulating tumor necrosis factor (TNF), which adversely affect sustained virologic response (SVR) rates of genotype 1 HCV-infected patients (Romero-Gomez et al., 2005). In contrast to mono-infected patients, insulin resistance and diabetes are not contributing factors to fibrosis progression in HIV-1/HCV co-infected patients (Merchante, 2006; Monto, 2006), although a recent report has shown hyperglycemic patients to be more likely to have advanced fibrosis (Barreiro et al., 2006).

At least 20% of those with chronic hepatitis progress to cirrhosis after 20 years, with risk of liver failure and hepatocellular carcinoma (HCC). The disease rapidly progresses to cirrhosis in those
with other risk factors, such as alcohol consumption, aging, and HBV and/or HIV-1 co-infection (Barreiro et al., 2006). Older patients without access to medical care may have advanced liver disease, cirrhosis, and/or HCC (Thimme et al., 2009). Currently, HCV-induced cirrhosis is the most common indication for liver transplantation in the United States and Europe (Barreiro et al., 2006).

2.3.1 Diagnosis of HCV infection

Anti-HCV antibody is usually detectable within 3 weeks of exposure, whereas HCV-RNA is detectable in blood 1 to 3 weeks after exposure (Orland et al., 2001). Patients with positive serology for antibodies against HCV should have a qualitative RNA level measured to confirm viremia. Elevated alanine aminotransferase (ALT), history of intravenous drug use and CD4 levels below 200 cells per mm$^3$ are predictors of seronegative HCV (Chamie, 2006) and may indicate the need for further screening for HCV RNA.

Two polymerase chain reaction (PCR)-based tests for qualitative detection of HCV RNA are currently approved by the FDA: Amplicor Hepatitis C Virus Test, version 2.0, and Cobas Amplicor Hepatitis C Virus Test, version 2.0 (Roche Molecular Systems, Branchburg, NJ), which have lower limits of detection of approximately 50 IU per ml. Serum aminotransferases (AST and ALT) remain abnormal after 12 months in 60% to 85% of patients with post transfusion or sporadic hepatitis. These enzymes decline from the peak values encountered in the acute phase of the disease, but typically remain abnormal by 2- to 8-fold. Serum ALT concentrations may fluctuate during the course of the disease, but they can also be intermittently or consistently normal. As chronic disease progresses, laboratory values continue to become
more abnormal. Serum aspartate aminotransferase (AST) greater than ALT, hypoalbuminemia, thrombocytopenia, and prolonged prothrombin time all suggest cirrhosis. A liver biopsy is helpful in grading the degree of inflammation and staging the degree of fibrosis. Biopsy has prognostic value, since all patients with initial periportal fibrosis are likely to develop cirrhosis after 2 decades of untreated infection (Yano, 1996). In patients with less-severe histologic disease who may never develop cirrhosis, careful clinical monitoring is an alternative to antiviral therapy. In addition, liver biopsy may be repeated in 5 years to assess progression rate (Strader, 2004) in mono-infected patients.

2.3.2 Treatment for HCV infection

In general, treatment is recommended for those with proven HCV infection liver abnormalities; as of 2010, treatments consist of a combination of pegylated (coupled to polyethylene glycol) interferon alpha and the antiviral drug ribavirin (guanosine nucleoside analogue) for a period of 24 or 48 weeks. Treatment is administered through subcutaneous injection, and the dosage is 1.5 grams per kilogram per week depending on weight and HCV genotype (Wilkins et al., 2010). When combined with ribavirin, pegylated interferon-alpha-2a may be superior to pegylated interferon- alpha-2b, though the evidence is not strong (Awad et al., 2010). Combining either boceprevir or telaprevir with ribavirin and peginterferon alpha improves antiviral response for hepatitis C genotype 1 (Foote et al., 2011). Adverse effects with treatment are common, with half of people getting flu like symptoms and a third experiencing emotional problems (Wilkins et al., 2010). Treatment during the first six months is more effective than once hepatitis C has become chronic (Ozaras et al., 2009). If someone develops a new infection and it has not cleared after eight to twelve weeks, 24 weeks of pegylated interferon is recommended. In people with
thallasemia, ribavirin appears to be useful but increases the need for transfusions (Alavian et al., 2010).

2.4 Injection drug use and HIV-1/HCV co-infection in Kenya

There are few immunological data regarding hepatitis C virus (HCV) and HIV-1 co-infection among IDUs (Modi, 2007). In 2008, Kenyan Demographic and Health Survey reported that 0.7% of Kenyan population was co-infected with HIV-1/HCV. Notwithstanding the assessment of the UN International Drug Control Program (Mwenesi, 1996), the international organizations have been slow to recognize either the spread of heroin use in Kenya or the existence of injection drug use. Injection drug users (IDUs) are a high risk or core group for HIV-1 infection. Many IDUs share needles and syringes as well as having unprotected sex, and have been identified as a ‘bridging population’, speeding the spread of HIV-1 to the general population (Dehne et al., 2001). Heroin injection appears to be occurring in most large towns of Kenya (ODEk-Ogunde et al., 2003), and is regarded as a street drug at the Kenyan Coast, especially Mombasa and Malindi since the 1980s (Beckerleg, 2000). Much has been done on the prevalence of HIV-1/HCV co-infection but the immunological outcomes in injection drug users (IDUs) remains scanty.

2.5 Immunopathogenesis of HIV-1/HCV co-infection

2.5.1 Epidemiology

The reported prevalence of HIV-1/HCV co-infection varies significantly among studies. Although HIV-1 and HCV are both transmitted through parenteral, sexual, and vertical exposure, they differ in the transmission efficiencies of these routes (Tedaldi et al., 2003). Thus, the risk
factors of the population under study directly influence the prevalence in that particular population. Parenteral exposure modes such as intravenous drug use (IVDU) or multiple transfusions have consistently been found to be the most important risk factors for co-infection (Perez et al., 2003). In HIV-1 positive patients with a history of IVDU, the rate of HCV infection is reported to be 82 to 93% (Larsen et al., 2008). On the other hand, sexual transmission of HCV is relatively inefficient, and the rate of co-infection among HIV-1-infected patients with a sexual risk factor is less than 10% (Larsen et al., 2008). Men who have sex with men do not have an overall - increased risk for co-infection (Bollepalli et al., 2007), although epidemics of acute HCV have been described for HIV-1-infected men who have sex with men with high risk behaviors (Danta and Brown, 2007). The overall burden of co-infection is estimated at 4 to 5 million people worldwide (Alter, 2006). HIV-1-infected patients exposed to HCV are less likely to clear the acute infections (Thomas et al., 2000). This scenario seems to be especially relevant to transmission via IVDU (Shores and Soriano, 2008). On the other hand, co-infected individuals are more likely to transmit HCV.

The rate of vertical transmission of HCV is increased about threefold for co-infected mothers (Polis et al., 2007) compared to that for HCV mono-infected ones; this effect may be limited to women with low HCV RNA levels (>10⁶ IU/ml) (Marine-Barjoan et al., 2007). Percutaneous exposure of health care workers to blood from co-infected patients has been shown to increase the risk of acquiring HCV (De Carli et al., 2003). Although co-infected individuals have been shown to have a higher prevalence of HCV RNA in cervicovaginal secretion (Nowicki et al., 2005) and semen (Briot et al., 2005), sexual transmission of HCV is rare even to partners of co-infected patients (Marincovich and Rodriguez, 2003). Co-infection with HIV-1 and HCV is a
global problem that is more prevalent in injection drug users because they have a higher risk for acquiring both viruses (Nazira et al., 2007). Although little is known about the mechanisms by which HIV-1 and HCV directly interact at cellular and molecular levels, recent studies using direct virus–virus interactions in vitro provide additional insight into the events underlying the accelerated liver disease progression observed in HIV-1/HCV co-infection (Jang et al., 2011).

2.5.2 HCV-associated liver disease progression in setting of HIV-1 co-infections

Despite reductions of morbidity and mortality in HIV-1-infected individuals on HAART, liver-related deaths now represents a leading cause of death in this population, primarily due to HCV co-infection. HCV-associated liver diseases including fibrosis, cirrhosis and end stage liver disease (ESLD) are accelerated in HIV-1-infected individuals. Prospective studies show increased risk of hepatitis and liver related deaths despite HAART among co-infected IDUs suggesting that HIV-1 accelerates liver disease during HAART (Geskus et al., 2008). Hepatitis steatosis (HS) occurs as a complication in 40%-75% of HCV mono-infected and HIV-1 co-infected patients. Moreover, HS is associated with increased body mass index (BMI) and fibrosis (Smith and Sterling, 2008).

Mechanisms to explain accelerated liver disease in co-infected patients are not well understood but may include direct viral effects and immunological alterations such as immune activation, apoptosis and diminished HCV-specific T-cell responses (Rotman et al., 2009). Immune activation by HIV-1 induces cytokine changes (e.g., IL-4, IL-5 and IL-13, TGF-β) that increase liver inflammation and fibrosis (Roe et al., 2008). Co-infection increases apoptosis of hepatocytes through a Fas/FasL pathway that could account for accelerated liver disease (Roe et
al., 2008). Accumulation of cytotoxic CD8 T cells in the liver that increases inflammatory mediators in co-infected compared to HCV–mono infected patients may also lead to increased tissue damage in co-infected patients (Kuntzen et al., 2008). New evidence shows HIV-specific CD8 T cells accumulate in the liver in co-infection and produce TNF-α which is associated with liver fibrosis (Vali et al., 2008).

2.6 Markers associated with progression of HIV-1 infection

Initial infection with HIV-1 is followed by an asymptomatic period of variable duration characterized by low or absent virus replication, stable or slowly decreasing numbers of CD4+ T-helper cells, and qualitative defects in T-cell function (Marc et al., 2012). The pathogenesis of HIV-1 infection involves dynamic interactions between the virus and the host immune system which result in immune activation throughout the course of infection. The degree of activation of the immune system can be monitored by measuring the serum levels of a variety of molecules such as β2-microglobulin and neopterin as well as other serum and cellular markers that correlate with clinical progression of HIV-1 disease (Fahey et al., 1990). Because of the varied timing of the development of clinical AIDS following seroconversion for any particular individual, the use of nonclinical disease markers has become critically important to patient management (Dar and Singh, 1999).

Data on the high viral turnover in HIV-1 infection at all stages of the disease and the value of viral load measurements in antiretroviral drug trials have led to the use of markers that directly measure viral load or other viral characteristics in clinical practice (Saag et al., 1996). Measurement of levels of HIV-1 RNA over time has been of great value in delineating the
relationship between levels of virus and rates of disease progression, the rate of viral turnover, the relationship between immune system activation and viral replication, and the time to development of antiretroviral drug resistance (Dar et al., 1999). Viral load determination is instrumental in several aspects of clinical management of HIV-1 infection. First, in early infection at the set point, it serves to assess the likely course the infection will take. Based on the risk of progression, which depends on the viral load, appropriate treatment decisions can be made. The short term efficacy of a specific antiretroviral treatment can then be assessed by measuring the reduction in virus concentrations achieved within the first 2 to 4 weeks after treatment initiation. Long-term efficacy is achieved when virus levels continuously decrease below the level of detection and remain undetectable by the most sensitive assays (Saag et al., 1996).

Non-HIV-1 specific cellular markers have also been used for staging, monitoring progression of HIV infection and assessing response to therapy but the most commonly used cellular marker is the CD4 lymphocyte count (Fahey et al., 1990). Though CD4 cell count is widely used by clinicians, it is a vague predictor of progression. A single abnormal result is not usually a sufficient reason to introduce or change treatment as there are many physiological variables which may affect the count, including the time of day the sample is collected, concurrent infections, and recent exercise (Melone et al., 1990). Higher HIV-1 RNA levels correlate with lower baseline CD4+ T-cell counts, a more rapid decline in CD4+ T-cell counts, and more rapid disease progression. Patients with more than 100,000 copies/ml of plasma HIV-1 RNA within 6 months of seroconversion have been shown to be 10-fold more likely to progress to AIDS over 5 years than those with fewer copies of plasma HIV-1 RNA. Maintenance of less than 10,000
copies/ml of plasma HIV-1 RNA in early HIV-1 infection is associated with a decreased risk of progression to AIDS. However, in patients with more advanced disease, a low RNA count does not protect from progression; up to 30% of patients with less than 10,000 copies/ml have been found to progress to AIDS (Saag et al., 1996; O’Brien et al., 1997).

2.7 Virological, immunological and clinical outcomes of HIV-1/HCV co-infections

It is unclear whether HIV-1/HCV co-infection is associated with impaired CD4 cell recovery following HAART initiation. Individual studies reported conflicting reports, while a Meta-analysis (Miller et al., 2005) found only a modest effect: the increase in CD4 cells was 33 cells/mm$^3$ less in co-infected patients. Some studies suggested a unique genotype effect: genotype 3 seemed to be associated with HIV-1 progression based on comparison of progressors and non progressors (Morsica et al., 2007) and with slower recovery of CD4 cells (Antonucci et al., 2005). Potential explanations on the mechanisms of increased HCV replication and accelerated fibrosis leading to the liver mortality in HIV-1/HCV co-infected patients have been proposed including the generalized immune suppression resulting from the loss of CD4 T cells, an intra-hepatic interaction between the viruses or their gene products and an indirect effect on the liver, secondary to HIV-1 infection of other organs (Lin et al., 2013).

2.7.1 Alteration of cytokine network in HIV-1/HCV co-infection

It is thought that immune dysfunction is due not only to a loss of CD4 T-cells, but also to an imbalance of many cytokines that augment or suppress immunologic functions (Eva et al., 2011). The T helper cells comprise several subjects with distinct functions, which are affected
differentially by HIV-1 infection (Clerici and Shearer, 1994). Among the cytokines, IL-12 is one of the most critical because it enhances natural killer (NK) and T-helper functions and induces production of other cytokines, particularly IFN-γ and IL-2 and the generation of cytotoxic lymphocytes (Rahman et al., 2011). It has been reported that IL-12 production is increased in HIV-1-infected patients (Rahman et al., 2011).

Type 1 helper cells produce IL-2, IFN-γ and Lymphotoxin and are important for an effective antiviral defense. Type 2 helper cells secrete IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 and can act as counterparts, down regulating antiviral activity by inhibiting T helper 1 cytokines formation (Douglas et al., 2003). Interferon-α, IL-2, IL-3, IL-6, IL-8, IL-12, IL-15 and RANTES cytokines are expressed at higher levels in the co-infected individuals (Chehimi et al., 2011). In case of HIV-1 mono-infected individuals, the levels of inflammatory cytokines, IFN-γ and TNF-α are increased (Rahman et al., 2011). These data suggest that a defect in cytokine activation may occur in HIV-1/HCV co-infected person that limits efficient clearance of HCV from the liver (Blackard et al., 2006).

### 2.8 IFN-γ, IL-10 and adiponectin levels in HIV-1/HCV co-infection

In co-infected patients, IFN-γ mRNA levels increase linearly with increasing peripheral CD4+ cell counts by around 1.23 times for every 100 CD4+ cells/mm³. No other cytokines are significantly associated with CD4+ cell counts (Chung et al., 2006). No association has been noted between intra-hepatic IFN-γ levels and other variables including age, race, HCV genotype, BMI and HCV risk factors (Chung et al., 2006; Myers et al., 2008). Interleukin (IL)-10 levels in HCV patients are higher relative to healthy controls (Mohamed et al., 2013) while the levels are
lower in both HCV and HIV-1 mono-infections compared to HIV-1/HCV co-infected patients (Gonzalez et al., 2008; Chung et al., 2008). Adiponectin levels are lower in HCV and HIV-1 mono-infections relative to healthy controls (Norton et al., 2013) while in HIV-1/HCV patients they are even more reduced.

### 2.8.1 Impact of ART on IFN-γ, IL-10 and adiponectin levels in HIV-1/HCV Co-infection

Standard treatment consists of at least three drugs often called ‘highly active antiretroviral therapy’ or HAART that suppress HIV-1 replication. Results from previous studies indicate that livers from naïve HIV-1/HCV co-infected patients show a significant increase in IFN-γ levels when compared with livers from co-infected patients treated with ART or naive HCV mono-infected patients (Thimme et al., 2001; Shoukry et al., 2004). Interleukin-10 levels in HIV-1/HCV co-infected ART-naive and -exposed and, HCV naive patients have been found to be comparable (Sitia et al., 2006). Levels of IL-10 in HIV-1 mono-infection are not altered by ART treatment (Orsilles et al., 2006).
CHAPTER THREE: MATERIALS AND METHODS

3.1 Study area

The study subjects were recruited at Bomu Medical centre in Mombasa County. Mombasa County is one of the 47 counties of Kenya. It is the smallest County in Kenya covering an area of 229.7 km². The County is situated in the South Eastern part of the former Coast province (UNEP, 2013). The port in Mombasa is an ideal drug trafficking center both because of its geographical location and probably due to corruption. Mombasa County happens to harbor the highest number of IDUs in Kenya (UNODC, 2013). Mombasa has the fifth highest HIV-1/AIDS burden among all the counties in Kenya (NACC, 2012). It is estimated that 58,100 residents of Mombasa are living with HIV-1, 7,400 of them being children below 14 years. Injection drug users contribute 6% of new infections (NACC, 2012).

3.2 Study design

The study utilized a cross-sectional experimental design to investigate the immunological correlates of HIV-1 co-infection in injection drug users. The study participants were grouped into seven study groups (Table 3.1).
Table 3.1: Experimental groups

<table>
<thead>
<tr>
<th></th>
<th>HIV-/HCV-</th>
<th>HIV+/ART-</th>
<th>HIV+/ART+</th>
<th>HCV+</th>
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<td>n=36</td>
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</tbody>
</table>


3.3 Study population and sample size

3.3.1 Study population

Study population comprised of HIV-1/HCV co-infected injection drug users in Mombasa County while the target population consisted of individuals suffering from the dual viral infection; individuals who were injecting drugs but were free from the viruses as well as healthy controls who were recruited into the study.

3.3.2 Sample size determination

Sample size was calculated based on the methods for the independent t-tests (Dallal, 2012). The formula below was used to calculate the sample size. Type I error (α=1.96), type II error and power (1-beta) are applicable in sample size calculation that give 80% power at the 0.05 level of significance (two tailed). Previous studies in healthy individuals showed average IFN-γ levels of 10.02±0.62 pg/ml while those with infection had an average of 10.62±1.09 pg/ml (Prohic et al., 2010); the values were used to calculate sample size.
Sample size \( n \) = \[16S^2 / d^2 \] + 1

Where, \( S \) = standard deviation = 0.62
\( d \) = mean difference = 10.62 - 10.02 = 0.60

\[ n = 16(0.62)^2 / 0.6^2 \] + 1 = 17.08 + 1 = 18

Therefore, a minimum of 18 samples were used for each of the study groups. This made a total of 162 study subjects.

### 3.3.3 Sampling technique

Study subjects were recruited from Bomu Medical Center through snow ball sampling technique, whereby existing study subjects were used to recruit more subjects into the sample. Bomu Medical Center is a major rehabilitation center in Mombasa with approximately 800 patients every month. Sampling criteria: needle scars and injecting illicit drugs in previous months, 18 years and above, lived in Mombasa and written informed consent.

### 3.3.4 Blood Sample collection

Venous blood samples were collected from each study participant. Five (5) millilitres of each sample were dispensed into ethylenediaminetetra acetic acid (EDTA)-containing tubes (vacutainer; BD Sciences, USA) and 5 mL into plain tubes (Greiner, Bio-One, Austria). Some of the EDTA anti-coagulated blood was assayed for HIV-1 antibody by Determine HIV-1/2 Antibody test (ABBOT, Tokyo, Japan) and for CD4 T lymphocyte numbers. From the remaining EDTA anti-coagulated blood, approximately 2 mL of plasma was obtained by centrifugation at 5,000 r.p.m. Aliquots of cell-free plasma were collected from each sample and stored at -70°C until assayed for HIV-1 viral load quantity. Aliquots of plasma were collected from each sample
and stored at -80°C until assayed for IFN-γ, IL-10 and adiponectin levels. Frozen samples were thawed only once.

3.4 Data collection techniques

3.4.1 Clinical data

Upon enrolment into the study, information on demographic characteristics (age and gender) was recorded using structured forms while height and weight were measured using a wall mounted stadiometer and balanced beam scale respectively. The study subjects were requested to complete an informed consent form, their baseline characteristics were obtained and they were identified using codes.

3.4.2 Basal metabolic index

Body mass index (BMI) of the study subjects was calculated using height and weight according to (Freedman et al., 2013). BMI was calculated as follows:

$$\text{BMI} = \left[ \frac{\text{Weight (kg)}}{\text{Height (m)}^2} \right]$$

3.4.3 CD4+ T cell measurements

CD4 lymphocytes were enumerated using the four-color BD FACScalibur according to the manufacturer’s protocols (BD Biosciences, USA) (WHO, 2007). In the test procedure, 20 μL of Mutitest Reagent was pipetted into TruCOUNT tubes (BD Biosciences, USA) labeled according
to the samples. Fifty microlitres (50 μL) 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, 450 μL of FACS Lysing Solution (BD Biosciences, San Jose, CA, USA) was added to each tube and the mixture was 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 appropriate software on the FACStation computer, the samples were run to identify lymphocytes and lymphocyte subsets. The CD4 lymphocyte count of each sample was then recorded from the results obtained.

3.4.4 HIV-1 viral load determination

The COBAS AMPLICOR HIV-1 MONITOR test version 1.5 (Roche Molecular Systems, Branchburg, USA) was used to quantify plasma viral load in the cell-free plasma. Briefly, the quantitation of HIV-1 viral RNA was performed using Quantitation Standard RNA (Puren et al., 2010). The HIV-1 Quantitation Standard was incorporated into each individual specimen at a known copy number and was carried through the specimen preparation, reverse transcription, PCR amplification, hybridization and detection steps along with the HIV-1 target and was amplified together with the HIV-1 target. The COBAS AMPLICOR Analyzer then calculated the HIV-1 RNA levels in the test samples by comparing the HIV-1 signal to the Quantitation Standard signal for each sample.
3.4.5 Enzyme Linked Immunosorbent assays (ELISAs) for cytokines

The circulating IFN-γ level was determined in plasma samples using a quantitative sandwich ELISA according to the manufacturer’s protocols (R&D Systems, USA) (Leng et al., 2008). Human IFN-γ ELISA kit (R&D Systems, USA) was used to measure IFN-γ level in the plasma sample from HIV-1/HCV co-infected adults and healthy controls. ELISA microtitre plate wells were coated with 100 μl/well of anti-human IFN-γ capture antibody (R&D Systems, USA) diluted in coating buffer, PBS (phosphate buffered saline). The plates were sealed and incubated overnight at 4°C. After overnight incubation, excess coating buffer was discarded and the wells blocked with 100 μl/well blocking buffer [1% BSA (bovine serum albumin) in PBS] followed by incubation at 37°C for 1 h. After washing the plates 3 times in washing buffer (0.05% tween-20 in PBS), 100 μl of plasma samples and IFN-γ standards diluted in buffer + 0.5% BSA were added to appropriate wells followed by incubation of plates at 37°C for 2 h.

Serial dilutions of the top standards were done to make a standard curve. Plates were washed as before and blotted on absorbent paper to remove any residual buffer. Biotinylated antibody (100 μl) was added to each well followed by incubation at 37°C for 1 h, and washed as before. Horse-Radish Peroxidase (100 μl) conjugated to streptavidin was added to each well and the plates incubated at 37°C for 1 h, and washed 3 times. Tetrathenylbenzidine (TMB) substrate (100 μl) wasadded to each well followed by incubation at room temperature for 15 minutes. Stop solution (1 N H2SO4), 50 μl was added to each well to stop the reaction. The absorbance (optical densities, OD) for each well was read at 450 nm using an ELISA reader. From the results, a standard curve was run on each assay plate to extrapolate results for samples.
The circulating IL-10 levels were determined using a quantitative sandwich ELISA according to the manufacturer’s instructions (R&D Systems, USA). ELISA plate wells were coated with 100 μl/well of anti-human IL-10 capture antibody in coating buffer and incubated overnight at 4°C. After incubation, excess coating buffer was discarded and wells blocked with 100 μl/well of blocking buffer (1% BSA in PBS) followed by incubation at 37°C for 1 h. Plates were washed 3 times in washing buffer (0.05% tween-20 in PBS), 100 μl of plasma samples and IL-10 standards diluted in buffer were added to appropriate wells, and incubation at 37°C for 2 h followed. Plates were washed as before and detector biotinylated antibody (100 μl) added to each well followed by incubation at 37°C for 1 h, then washed as before. Streptavidin-HRP (Horse-Radish Peroxidase) conjugate (100 μl) was added to each well followed by incubation of plates at 37°C for 1 h, and washing as before. Substrate solution, 100 μl was added to each well and plates incubated at room temperature for 15 minutes for colour development. Stop solution (50 μl) was added to each well and plates were read at 450 nm.

Circulating adiponectin levels were determined using a sandwich ELISA. Plates were coated with 100 μl/well of anti-human adiponectin capture antibody in coating buffer followed by incubation overnight at 4°C. After incubation, excess coating buffer was discarded and wells were blocked with 100 μl/well blocking buffer (1% BSA in PBS) followed by incubation at 37°C for 1 h. Plates were washed 4 times in washing buffer (0.05% tween-20 in PBS) and plasma samples and adiponectin standards (100 μl) added to appropriate wells followed by incubation for 2 h. Plates were washed as before, biotinylated antibody (100 μl) was added to each well followed by incubation at 37°C for 1 h, and 4 further washes. Horse-Radish Peroxidase (100 μl) conjugated to streptavidin was added to each well and plates incubated at 37°C for 1 h, and
washed as before. Substrate solution (100 µl) was added to each well followed by incubation at room temperature for 15 minutes. Reaction was stopped by addition of 1 N H₂SO₄ (50 µl) to each well. Plates were read at 450 nm.

3.5 Ethical considerations

Ethical approval for this study was obtained from Kenyatta University Ethical Review Committee (PKU 019/116 of 2012). Written informed consent was obtained from each participant before enrollment.

3.6 Data processing and analysis

Statistical analyses were performed using SPSS software (19.0 version). Inter-group comparison of continuous data such as IFN-γ, IL-10, adiponectin levels, and body mass index was determined using the non-parametric ANOVA (i.e. Kruskal Wallis) tests followed by post-hoc Bonferroni for multiple comparisons. Spearman`s rank correlation tests was used to examine the relationship between cytokine levels and clinical outcomes (i.e., CD4+ T cell counts, viral loads, body mass index). All tests were two-tailed and a P value ≤ 0.05 was considered statistically significant.
CHAPTER FOUR: RESULTS

4.1 Demographic and clinical characteristics of the study participants

Mean age of the study participants was highest in HCV mono-infected (35.90±8.6 years) IDUs while HIV-1/HCV co-infected ART-exposed (33.65±6.2 years) and –naive (33.34±4.6 years) recorded intermediate mean ages. Healthy controls (27.37±5.9 years) recorded lowest mean age. Mean ages of the study participants differed significantly across-groups ($P < 0.0001$) with HIV-1/HCV co-infected, HCV mono-infected or HIV-1 mono-infected injection drug users having significantly higher ages when each of the groups was compared to healthy controls ($P = 0.005$).

Among the study participants, mean height was highest in HCV mono-infected (1.72±0.1 m) and uninfected IDUs (1.71±0.1 m). Other study groups indicated average mean heights with HIV-1/HCV co-infected ART-naive, HIV-1 mono-infected ART-exposed and healthy controls recording 1.64±0.1 m, 1.66±0.1 m and 1.64±0.1 m respectively.

Mean body heights of the study subjects were significantly different across-groups ($P = 0.001$) whereby HCV mono-infected or uninfected IDUs showed significantly higher ($P = 0.004$) heights when each of the groups was compared to healthy controls. Mean body weight of the study subjects was highest in healthy controls (62.07±11.3 kg). Intermediate weights were observed in HIV-1/HCV co-infected ART-exposed (55.64±7.0 kg), HCV mono-infected (54.06±6.2 kg) and uninfected IDUs (54.67±5.8 kg) while HIV-1/HCV co-infected ART-naive (50.00±10.3 kg) and HIV-1 mono-infected ART-exposed (50.00±5.3 kg) had lowest mean body weights. The mean body weights of study participants differed significantly across-groups ($P < 0.0001$) with healthy controls having significantly higher ($P = 0.003$) weights when compared to HCV mono-infected or HIV-1 mono-infected ART-exposed IDUs whereas significantly higher
(P = 0.004) body weights were noted in uninfected or HCV mono-infected IDUs when each of the groups was compared to HIV-1 mono-infected ART-exposed IDUs.

Healthy controls exhibited highest mean BMI (22.87±4.6 kg/m²) while other experimental groups recorded lower values: HIV-1/HCV co-infected, HCV mono-infected and HIV-1 mono-infected had mean values of 18.94±1.8 kg/m², 18.26±2.1 kg/m², 18.22±1.5 kg/m² respectively. Body mass index of study subjects differed significantly across-groups (P < 0.0001) with healthy controls indicating significantly higher (P < 0.05) BMI when compared to HIV-1/HCV co-infected, HCV mono-infected, HIV-1 mono-infected or uninfected IDUs. The CD4+ T cell counts were highest among uninfected IDUs (935±402 counts/µl). Other experimental groups recorded lower mean values: HIV-1/HCV co-infected ART-exposed and co-infected ART-naive indicated mean values of 512±145 counts/µl and 668±464 counts/µl respectively while HCV mono-infected recorded a mean value of 915±348 counts/µl. Least CD4+ T cell counts were recorded amongst HIV-1 mono-infected ART-exposed (453±309 counts/µl) and mono-infected ART-naive IDUs (484±319 counts/µl). Healthy controls recorded a mean value of 751±297 counts/µl. Among the HIV-1 disease markers, CD4+ T cell counts were significantly different across-groups (P < 0.0001), such that HCV mono-infected, uninfected IDUs or healthy controls presented with significantly higher (P = 0.007) CD4+ T cell counts compared to HIV-1 mono-infected ART-exposed IDUs. Mean values of HIV-1 RNA loads were comparable across-groups (P = 0.063).
The demographic and clinical characteristics of the study participants including age, body heights and weights, BMI, CD4+ T cell counts and HIV-1 RNA load are summarized in table 4.1.
### Table 4.1: Baseline demographic and clinical characteristics of the study participants

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Study groups</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yrs.</td>
<td>27.37±5.9</td>
<td>30.40±6.1</td>
</tr>
<tr>
<td>Male/female, %</td>
<td>33.3/66.7</td>
<td>66.7/33.3</td>
</tr>
<tr>
<td>Height, m</td>
<td>1.64±0.1</td>
<td>1.71±0.1</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>62.07±11.3</td>
<td>54.67±5.8</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>22.87±4.6</td>
<td>18.73±2.2</td>
</tr>
<tr>
<td>CD4+ T cells/µl</td>
<td>751±297</td>
<td>935±402</td>
</tr>
<tr>
<td>HIV-1 RNA, copies/ml</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Data are presented as means± SD (standard deviation) or as number (n) and proportions (%) of subjects. ART, anti-retroviral therapy. HIV-1, human immunodeficiency virus-1. BMI, body mass index. HCV, hepatitis C virus. HC, healthy controls. Across-group analysis of continuous variables (age, height, weight, BMI, CD4+ T cell counts, and HIV-1 viral load) was performed using Kruskal Wallis tests followed by post-hoc Bonferroni corrections for multiple comparisons. Significant p-values are indicated using superscripts a, b, c, d and e. Differences in proportions of gender across the groups were compared using the chi-square test.
4.2 Circulating IFN-γ levels of study participants

Mean level of IFN-γ was highest in HIV-1 mono-infected ART-exposed (54.26±18.6 pg/ml). Other study groups indicated lower IFN-γ levels: HIV-1/HCV co-infected ART-exposed had a mean value of 36.79±1.1 pg/ml while uninfected IDUs had a mean value of 38.12±1.3 pg/ml. Least amounts of IFN-γ levels were recorded in HIV-1/HCV co-infected ART-naive (36.10±1.1 pg/ml) and HCV mono-infected (36.84±2.8 pg/ml). Healthy controls had a mean IFN-γ level of 40.62±13.3 pg/ml. Statistical analysis through analysis of variance (ANOVA) showed that circulating IFN-γ levels were significantly different across the study groups ($P < 0.0001$). Post-hoc analysis indicated significantly higher ($P = 0.004$) IFN-γ levels in uninfected IDUs when compared to HIV-1/HCV co-infected ART-naive or co-infected ART-exposed. Hepatitis C (HCV) mono-infected IDUs had significantly higher IFN-γ levels compared to healthy controls ($P < 0.0001$) while the IFN-γ levels were significantly lower in HCV mono-infected when compared to uninfected IDUs ($P < 0.0001$; Figure 4.2).
Figure 4.2: Plasma IFN-γ levels in HIV-1 and hepatitis C mono- and co-infected injection drug users. Data is presented as box plots with the line through the box showing the median, cross sign designate the mean, the box indicates the interquartile range, whiskers denote the 10th and 90th percentile, and closed circles denote outliers. HIV-1[+]/HCV [+]/ART [+] (n=14) and ART [-] (n=5); HCV [+](n=36); HIV-1[+]/ART [+] (n=38) and ART [-] (n=18); HIV-1[-]/HCV [-] (n=24); HC, healthy controls (n=27).
4.3 Circulating IL-10 levels of the study participants

Mean value of circulating IL-10 levels was highest in uninfected IDUs (149.76±20.9 pg/ml). Other experimental groups recorded lower IL-10 levels: HIV-1/HCV co-infected ART-naive showed a mean value of 99.60±21.6 pg/ml while the co-infected ART-exposed had a mean value of 127.30±28.8 pg/ml. Other study groups recorded least amounts of IL-10 levels of 73.68±13.4 pg/ml and 71.40±11.4 pg/ml in HIV-1 mono-infected ART-naive and mono-infected ART-exposed respectively. Healthy controls exhibited a mean value of 72.72±26.9 pg/ml. Analysis of variance (ANOVA) indicated that circulating IL-10 levels differed significantly across the study groups (P = 0.0001). Further post-hoc analysis showed that HIV-1/HCV co-infected ART-exposed, HCV mono-infected or uninfected IDUs presented with significantly higher (P < 0.0001) circulating IL-10 levels when each of the groups was compared to healthy controls. It was noted that IL-10 levels in uninfected IDUs were significantly higher than in HIV-1 mono-infected ART-exposed or HIV-1 mono-infected ART-naive IDUs (P < 0.0001; Figure 4.3).
Figure 4.3: Plasma IL-10 levels in HIV-1 and hepatitis C mono- and co-infected injection drug users. Data is presented as box plots with the line through the box showing the median, cross sign designate the mean, the box indicates the interquartile range, whiskers denote the 10th and 90th percentile, and closed circles denote outliers. HIV-1[+] / HCV [+]/ ART [+] (n=14) and ART [-] (n=5); HCV [+](n=36); HIV-1 [+] / ART [+] (n=38) and ART [-] (n=18); HIV-1[-]/HCV [-] (n=24); HC, healthy controls (n=27).
4.4 Interferon-gamma (IFN-γ)/IL-10 ratios of the study participants

Mean level of IFN-γ/IL-10 ratio was highest in HIV-1 mono-infected ART-exposed (0.8±0.30). Other experimental groups had lower IFN-γ/IL-10 ratios: HIV-1/HCV co-infected ART-exposed recorded a mean value of 0.3±0.08 while co-infected ART-naive group had a cytokine ratio of 0.4±0.09. Hepatitis C (HCV) mono-infected and uninfected IDUs recorded cytokine ratios of 0.3±0.04 and 0.3±0.03 respectively. Healthy control group had a cytokine ratio of 0.6±0.16. Analysis of variance (ANOVA) showed that IFN-γ/IL-10 ratios differed significantly across the study groups ($P < 0.0001$). Following post-hoc analysis, significantly higher ($P = 0.005$) IFN-γ/IL-10 ratio was noted in healthy controls when compared to HIV-1/HCV co-infected, HCV mono-infected or uninfected IDUs. However, HIV-1 mono-infected ART-exposed presented a significantly higher ($P = 0.005$) cytokine ratio compared to healthy controls (Figure 4.4).
Figure 4.4: Plasma IFN-γ/IL-10 ratio in HIV-1 and hepatitis C mono- and co-infected injection drug users. Data is presented as box plots with the line through the box showing the median, cross sign designate the mean, the box indicates the interquartile range, whiskers denote the 10th and 90th percentile, and closed circles denote outliers. HIV-1[+]/HCV [+]/ART [+] (n=14) and ART [-] (n=5); HCV [+] (n=36); HIV-1 [+]/ART [+] (n=38) and ART [-] (n=18); HIV-1[-]/HCV [-] (n=24); HC, healthy controls (n=27).
4.5 Circulating Adiponectin levels of the study participants

Mean value of adiponectin was highest in HCV mono-infected (24.08±3.6 ng/ml) while HIV-1/HCV co-infected ART-exposed recorded mean value of 21.06±7.1 ng/ml. Human immunodeficiency virus-1 (HIV-1) mono-infected ART-exposed and uninfected IDUs recorded mean values of 17.41±7.4 ng/ml and 22.67±3.6 ng/ml respectively. Lower amounts of adiponectin levels were noted in HIV-1/HCV co-infected ART-naive (11.68±8.7 ng/ml) and HIV-1 mono-infected ART-naive (16.55±8.2 ng/ml). Healthy control group recorded a mean value of 19.93±6.4 ng/ml. Statistical analysis through ANOVA indicated that circulating adiponectin levels differed significantly across the study groups ($P < 0.0001$) with HCV mono-infected indicating significantly higher ($P = 0.001$) adiponectin levels compared to HIV-1 mono-infected ART-exposed or mono-infected ART-naive IDUs. Other study groups had similar adiponectin levels (Figure 4.5).
Figure 4.5: Plasma adiponectin levels in HIV-1 and hepatitis C mono- and co-infected injection drug users. Data are presented as box plots with the line through the box showing the median, cross sign designate the mean, the box indicates the interquartile range, whiskers denote the 10th and 90th percentile, and closed circles denote outliers. Acrp30, adiponectin. HIV-1[+]/HCV [+]/ART [+] (n=14) and ART [-] (n=5); HCV [+] (n=36); HIV-1 [+] /ART [+] (n=38) and ART [-] (n=18); HIV-1[-]/HCV [-] (n=24); HC, healthy controls (n=27).
4.6 Associations between cytokines, CD4+ T cell counts, HIV-1 viral load and body mass index

Correlation analysis revealed that plasma IFN-\(\gamma\) levels were significantly associated inversely with CD4+ T cell count in the HCV mono-infected group (\(\rho\), rho = -0.393; \(P = 0.018\)), HIV-1 viral load in HIV-1/HCV co-infected ART-naive group (\(\rho = -1.000; P = 0.004\)), and BMI in the HIV-1/HCV co-infected ART-exposed group (\(\rho = -0.628; P = 0.029\)). However, IFN-\(\gamma\) levels were not significantly associated with CD4+ T cell counts, HIV-1 viral load and BMI in HIV-1 mono-infected ART-exposed or -naive groups (\(P > 0.05\) for all comparisons).

Circulating IL-10 levels indicated a significant inverse correlation with BMI in healthy controls (\(\rho = -0.407; P = 0.035\)). However, IL-10 was not significantly correlated with either HIV-1 viral load or CD4 T cell counts in HIV-1 and HCV mono- and co-infected or uninfected IDUs (\(P > 0.05\) for all comparisons). Adiponectin levels were positively correlated with BMI in HIV-1 mono-infected ART-naive IDUs (\(\rho = 0.598; P = 0.014\)).

Plasma IFN-\(\gamma\) levels and IL-10 levels showed significant positive correlations in HIV-1/HCV co-infected ART-exposed (\(\rho = 0.711; P = 0.010\)), HIV-1 mono-infected ART-naive (\(\rho = 0.616; P = 0.011\)) IDUs and healthy controls (\(\rho = 0.877; P < 0.0001\)). In addition, a significant inverse correlation was exhibited between circulating IFN-\(\gamma\) and adiponectin levels in HIV-1 mono-infected ART-exposed IDUs (\(\rho = -0.422; P = 0.013\)).
CHAPTER FIVE: DISCUSSION

Results in the present study showing that injection drug users were older relative to healthy controls, suggest an older age for initiating substance injection. These findings are consistent with previous studies in Mombasa County showing that drug and substance users start the practice at an older age of 36-40 years (Kahuthia-Gathu et al., 2013). The lower BMI and body weights observed among the HIV-1 and HCV mono- and co-infected ART-naive and -exposed, and uninfected injection drug users are consistent with previous studies indicating low body weights and BMI among injection drug users (Birk et al., 2006). The weight loss may be linked to inadequate food intake leading to malnutrition and reduced adipose tissue promoting loss of body fat mass probably as a result of utilization of glycogen as a form of energy source. Wasting in HIV-1 patients is also attributable to the high levels of pro-inflammatory cytokines that promote wasting and metabolic shifting to lipid and sugar production with loss of proteins (Giralt et al., 2011).

The present study found comparable HIV-1 viremia in HIV-1 mono-infected and HIV-1/HCV co-infected individuals. Contrary to this, previous studies conducted among Chinese subjects showed higher HIV-1 viremia in HIV-1 mono-infected individuals than in HIV-1 and HCV co-infected patients (Morsica et al., 2007). The scenario may be attributed to differences in sample characteristics with subjects in the present study having “better” immunity, as indicated by the higher CD4+ T cell counts of the current subjects under investigation compared to Chinese subjects in the previous studies who had < 350 counts/µl. Poor ART adherence in IDUs may also account for poor CD4+ T cell count recovery which functions to suppress HIV-1 viral load.
Since HIV-specific CD4 T lymphocytes mediate control of HIV-1 infection, an initiating early treatment in the acute phase preserves HIV-specific CD4 T cells that protect amounts of IFN-γ (Altfeld et al., 2001).

Profound depressions of CD4+ T cell counts in the HIV-1 mono-infected injection drug users versus HIV-seronegative injection drug users and healthy controls suggests poor immune reconstitution and reductions in the circulating lymphocytes probably resulting from poor adherence to ART and/or illicit drugs. These findings are further supported by reduced CD4+ T cell numbers and T cell function observed in HIV-1 mono-infected compared to uninfected injection drug users (Mizukoshi et al., 2008). Therefore, the fact that HIV-1 infected injection drug users present with higher depressions in the CD4+ T cell counts than uninfected injection drug users may be an indication that reduced CD4+ T cell counts is a function of both illicit substances and HIV-1 infection. Moreover, the fact that ART treatment did not promote CD4+ T cell recovery among HIV-1 infected injection drug users in the present study indicates an impaired reconstitution of T cell compartment due to poor adherence to the ART treatment and increased drug resistance among injection drug users; previous studies found similar results (Mizukoshi et al., 2008).

Results in the present study showing higher IFN-γ levels in HIV-1 mono-infected ART-exposed individuals compared to healthy controls mirror findings of previous studies illustrating higher IFN-γ levels in HIV-1 mono-infected patients compared to HIV-1/HCV co-infected patients or healthy controls (Silvia et al., 2006). These results underscore the role of IFN-γ in inhibiting HIV-1 replication during the early stages of the host immune response to HIV-1 infection (Norris
et al., 2006). However, elevated IFN-\(\gamma\) levels in the uninfected injection drug users suggests that IFN-\(\gamma\) is a novel predictor of the pro-inflammatory response to illicit/narcotic drugs such as heroin that are commonly used among injection drug users from Mombasa, Kenya. Research findings have shown that illicit drugs like opiates which include opium, heroin and morphine suppress host immunity while cannabinoids have ability to shift developing immune response from Th1 towards Th2 immunity causing a decrease in levels of key cytokines like IFN-\(\gamma\) and IL-12 (Roy et al., 2001).

Higher levels of IL-10 observed in HIV-1/HCV co-infected and HCV mono-infected versus HIV-1 mono-infected IDUs or healthy controls are consistent with previous studies showing higher plasma IL-10 levels in HIV-1/HCV co-infected individuals compared to HIV-1 mono-infected non-injection drug users or healthy controls (Hodowanec et al., 2013; Mohamed et al., 2013). High IL-10 levels in HIV-1/HCV co-infected and HCV mono-infected injection drug users underscores the anti-inflammatory role of IL-10 against heightened inflammation possibly resulting from type-1 cytokines such as IFN-\(\gamma\) among virally-infected and uninfected injection drug users. In line with previous studies, HCV induces selective IL-10 production leading to chronic infection and hepatic pathology (Barrett et al., 2008). Taken together, it appears that heightened immune activation during injection drug use promotes increased IL-10 production but the deranged immune system in the infected individuals does not exhibit similar response to injection drug use.

Lower IFN-\(\gamma\)/IL-10 ratio exhibited in all IDUs and healthy controls as compared to HIV-1 mono-infected IDUs in the present study concurs with other studies (Kang et al., 2012) which
indicated that IFN-γ/IL-10 ratio remained higher in HIV-1 mono-infected patients than all other groups, suggesting that the ratio may be a better marker of disease progression than individual cytokines. In summary, these findings suggest that co-infection with HCV can affect Th1/Th2 balance by decreasing expression of Th1-type cytokines especially IFN-γ levels (Loffreda et al., 2003). Other studies have found out that the expression of Th1 and Th2 cytokines levels in HIV-1/HCV co-infection and their dynamic changes during ART is a rarely understood mechanism (Kang et al., 2012).

Depressed adiponectin levels observed in HIV-1 infected IDUs as compared to all other study groups conforms to an earlier report in normal population, which noted the same depression (Norton et al., 2013). This could probably be explained by the fact that HIV-1 infection interferes with adipose tissue homeostasis independent of ART-induced lipodystrophy (Grunfeld et al., 1992), which in turn impairs the secretion of adiponectin from adipocytes. Co-infection with HCV did not affect the levels of adiponectin since they were comparable to healthy controls unlike in previous reports which indicate a drop in the level of adiponectin in co-infected group compared to healthy controls. It may be valid to hypothesize that inducer cell activation by IFN-γ or drug injection may be involved in activation of adipocytes to produce adiponectin maintaining it at the same level as in the healthy controls.

Observation in the present study that CD4+ T cell counts inversely correlate with IFN-γ in HCV mono-infected injection drug users may draw some analogy from other studies that have reported diminishing frequency of HCV-specific IFN-γ-secreting CD4+ T cells in HIV-1/HCV co-infected patients though it is not a very clear relationship (Harcourt et al., 2006). Loss of other T
helper functions rather than IFN-\(\gamma\)-secretion may be more critical in explaining the decline in CD4+ T cell counts, which is a marker of degree of immunological impairment. Abnormally high T cell activation may have induced T cell anergy leading to a decline in CD4+ T cell counts.

Inverse association observed between IFN-\(\gamma\) levels versus HIV-1 viral load in HIV-1/HCV co-infected ART-naive injection drug users in the current study contradicts other reports which found a positive correlation between HIV-1 viral load and most Th1 cytokines especially IFN-\(\gamma\) (Kang et al., 2012). In addition, findings by Sheila et al. (2011) also found a weak positive association of HIV-1 viral load and IFN-\(\gamma\) levels. This implies that HIV-1 viral replication is high in the absence of anti-retroviral treatment and as such, viral load compromises the proliferation of the T lymphocytes that secrete IFN-\(\gamma\) for antiviral activity; this coupled with anti-inflammatory effects of other cytokines may down regulate expression of IFN-\(\gamma\) levels among injection drug users. Taken together, these findings indicate that HIV-1 is an essential influencing factor on the reactivity of Th1 cytokine cells as well as a key surrogate marker of disease progression.

Previous studies by Chung et al. (2002) indicate that no association has been noted between intra-hepatic IFN-\(\gamma\) levels and other variables including age, race and BMI. However, the present study contrasts these findings in that BMI showed a negative correlation with IFN-\(\gamma\) levels in HIV-1/HCV co-infected ART-exposed injection drug users. Possible proposition is that, IFN-\(\gamma\) levels may have been down-regulated by ART treatment as viral replication goes down, being part of a normal immune restoration and reconstitution elicited by treatment intervention. BMI
on the other hand may have increased due to impaired adipocytes differentiation impairing secretion of adiponectin which in turn controls fat and glucose metabolism in HIV-1 infected patients. Lipodystrophy is a common metabolic abnormality observed in HIV-1 infected patients, while an association between lipodystrophy and low circulating levels of adiponectin has been described (Mynarcik et al., 2002). In the light of these events, BMI levels spirals out of control.

Observation in the current study showing a negative association between BMI and IL-10 in healthy controls agrees with a recent study that weight loss leads to increased IL-10 production (Jung et al., 2008). In these metabolically healthy individuals, this could be an effective immunoregulation to counter any form of inflammation-induced weight loss through increased production of IL-10 but the mechanisms involved warrant further investigations.

Positive correlation noted between adiponectin levels and BMI in HIV-1 mono-infected ART-naive injection drug users in the current study suggests possible abnormalities in fat metabolism and distribution during HIV-1 infection. A lipodystrophic syndrome and metabolic abnormalities have been observed in HIV-infected patients and lipodystrophy is associated with decreased levels of adiponectin (Grinspoon et al., 2005). In addition, HIV-1 infected patients especially injection drug users have low BMI owing to malnutrition caused by poor dietary intake among other causes like adverse drug effects.

In the present study, an intriguing observation was made to the effect that IFN-γ positively correlates with IL-10 in HIV-1/HCV co-infected ART-exposed or HIV-1 mono-infected ART-naive injection drug users despite the fact the two cytokines are antagonists in terms of immune
responses (Chehimi et al., 2011). Most viral infections induce humoral and cellular immune responses that act in concert to limit viral spread, clear infection and provide protective immunity against re-infection with the same virus. However, a number of viruses have evolved varied and sophisticated mechanisms like manipulation of cytokine responses to establish persistent infection, even in immunocompetent hosts (Alcami et al., 2000). The virus may have invoked some of these mechanisms to elevate the levels of IL-10 amid high levels of IFN-γ in HIV-1 infections, or there may have been T cells proliferation that induced production of the two cytokines by the same cell during the acute phase of the infection. Drug injection has also been shown to cause elevation of both IFN-γ and IL-10 levels in the present study independent of viral infections. This phenomenon may imply that there is more rapid disease progression among injection drug users.

Inverse correlation exhibited between IFN-γ and adiponectin levels in HIV-1 mono-infected ART-exposed injection drug users supports previous studies indicating that adiponectin protects cells from IFN-γ-induced and spontaneous apoptosis dependent upon adenosine mono-phosphate kinase (AMPK) phosphorylation at position 172 of threonine (Thr^{172}). This provides a potential pathway through which AMPK may regulate cell survival under energy stress conditions like autoimmune inflammation (Stergios et al., 2010). These findings point to metabolic disturbances and depressed immune reconstitution during HIV-1 infection (Grinspoon et al., 2005) as a result of reduced adiponectin levels. Furthermore, adiponectin may have been exerting its anti-inflammatory role against inflammation by IFN-γ, even though this may not be very clear since adiponectin is said to play both pro- and anti-inflammatory roles in various diseases (Tilg et al., 2006).
Findings of similar IFN-γ levels in both mono- and co-infected individuals whether ART-exposed or -naive in the present study differ from previous studies indicating associations between ART and reduced IFN-γ levels in HIV-1/HCV co-infected patients (Shoukry et al., 2004). When a subject is infected with HIV-1, IFN-γ is secreted to combat the invading HIV-1 with its pro-inflammatory activities contributing to proliferation and activation of naive CD4+ T cells (Pflanz et al., 2002). In this anti-HIV feedback arc set, IFN-γ acts as the mediator; IFN-γ elevates when HIV-1 replicates, and decreases upon inhibition of HIV-1 replication. However, in the HCV-co-infected group, the anti-HIV feedback arc set may be interrupted, resulting in uncontrolled or less effective suppression of HIV-1 replication (Stebbing et al., 2005). Drug injection also seems to interfere with immune reconstitution during ART treatment (Mizukoshi et al., 2008) and the effect is more profound in the co-infected group as indicated by the lower HIV-1 viral load and higher IFN-γ levels in the HIV-1 mono-infected ART-exposed individuals. This may explain why the circulating levels of IFN-γ did not decrease as expected in the co-infected group on ART which inhibits viral replication in HIV-1 infection.

Results presented here showing similar levels of IL-10 in HIV-1 mono-infected and HIV-1/HCV co-infected IDUs regardless of ART status are consistent with previous studies conducted in non-injection drug users indicating similar plasma IL-10 levels in HIV-1 and HCV mono- and co-infected non-injection drug using patients (Orsilles et al., 2006; Sitia et al., 2006). Observation of similar adiponectin levels in HIV-1/HCV co-infected ART-exposed and -naive injection drug users in the present study contradicts previous findings where adiponectin levels were reduced in patients who were ART-exposed relative to healthy controls (Das et al., 2006).
T cell responses play a critical role in antiviral defense, a function also performed by antiviral cytokines, and cytotoxic T lymphocytes (CTL) act to destroy infected cells during HIV-1 and HCV infections (Eckels et al., 1999). In the light of this, the quantity and quality of T cells are vital in disease pathology (Gigi et al., 2008). Cytokines secreted by CD4+ T lymphocytes play a crucial role in viral infection by promoting elimination of intracellular pathogen and host antiviral activities including CTL generation and natural killer cell activation, or initiating humoral immune responses that inhibit development of Th1 responses (Nelson et al., 1999). The data from the current study coupled with earlier findings by Kang et al. (2012) hypothesize that the possible reason for increasing cytokine expression was HIV-1 itself as indicated by low cytokine levels in HIV-1 uninfected individuals. It was the main stimulating factor on T cells that caused high cytokine expressions, although further research would be required to determine the molecular mechanism of this effect. Drug injection has been shown to cause heightened immune activation independent of viral infections, which results in elevated levels of cytokines as demonstrated by uninfected IDUs in the present study.
CHAPTER SIX: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The study established that:

i. IFN-γ levels were significantly higher in uninfected IDUs relative to healthy controls, HIV-1/HCV co-infected ART-exposed and -naive or HCV mono-infected IDUs. Similarly, IL-10 levels were significantly higher in HIV-1/HCV co-infected, HCV mono-infected and uninfected IDUs compared to healthy controls or HIV-1 mono-infected IDUs while adiponectin levels were comparable in healthy controls, HIV-1/HCV co-infected and uninfected IDUs.

ii. The CD4+ T cell counts were inversely associated with circulating IFN-γ in HCV mono-infected IDUs, indicating rapid HCV disease progression among infected injection drug users.

iii. ART treatment in injection drug users was not associated with the circulating levels of IFN-γ, IL-10 or adiponectin.

6.2 Recommendations

6.2.1 Recommendations from the study

i. Based on the high levels of IFN-γ among IDUs, interferon treatment for HCV infection should be scaled down and customized among injection drug users.

ii. Following rapid HCV disease progression among IDUs, routine HCV diagnosis together with HIV-1 testing should be carried out by healthcare providers. Treatment should be easily accessible to IDUs, and adherence to treatment monitored closely to reduce chances of drug resistance.
iii. Cytokines may not be the best measures for evaluating host immune responses during treatment in IDUs. Therefore, other clinical parameters such as CD4+ T cell counts and clinical chemistry profiles should be considered in monitoring treatment course among IDUs.

6.2.2 Recommendations for further research

Prospective studies are warranted in order to better understand the impact of the complex interactions of HCV, HIV-1, ART and substance use on cytokine profiles among injection drug users. In addition, analyses of an array of cytokines including HIV and HCV disease markers in a longitudinal approach will provide additional insights into the immunology of HCV and HIV disease progression among injection drug users.
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

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P. O. Box 43844,
Nairobi, 00100
Tel: 8710301/12

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

Date: 12th July 2012

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 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 then you can decide if you want to join this study or not. The Investigators in this study are Kenyatta University and Technical University of Mombasa. A study team will be working closely with the investigators and the study will run for 3 years.

The purpose of this study is to determine the factors associated with HIV and pulmonary TB co-infections among intravenous drug users. If you choose to participate in this study, the team will require 3ml of blood (HIV voluntary testing and Complete Blood Count) and three early morning sputum (for TB testing) from you. No drug or chemical will be introduced into your body.

You can decide whether to take part in this study or not. You are free 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 any cost implications to you. 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 drug users.

The risks in this study include possible discomfort due to questions on health and personal behaviour/history. In addition, discomfort may be experienced while a blood sample is being obtained. Every effort will be made to keep 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 think that you were harmed because of this study, contact the Principle 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, risks 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 study procedures as directed. I have been told that I can withdraw from the study at any time.

Participant's Name.................................. Signature..................................Date..............................

Principal Investigator............................. Signature..........................Date........................... Or supervisor

Note: Below are some of the key contacts

Principle investigator – Dr Tom Were 0720326127; Co-Investigator – Valentine Budambula 07222822448; KU-ERC kuerc.chairman@ku.ac.ke

Kiswahili Version

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

Kwako mhusika:


Nia hasa ya utafiti huu nikutathimini au kuamua sababu zinazohusisha na uambukizo pamoja wa virusi vya HIV na kifua kikuu kati ya watumiaji wa mihadarati kwa kujidunga. Ukichagua kushiriki kwenye utafiti huu, hii timu ya watafiti itahitaji kiasi cha mililita 3 za damu kutoka kwako (kwa ajili ya upimaji wa hiyari wa virusi vya HIV na hesabu ya kiwango cha damu) na pia watahitaji makohozo ya asubuhi

(kwa ajili ya upimaji wa maambukizi ya kifua kikuu). Hakuna dawa ama kemikali zozote zitakazoekwa kwa mwili wako.


Kwa kuweka sahihi jina langu nathibitisha yafuatayo:

1) nimesoma (ama nimesomewa) karatasi hii ya kutoa idhini ya kukubali, na maswali yako yote yamejibiwa na nimeridhika; 2) nia, mitindo, hatari pamoja na faida zinazoambatana na utafiti huu zimeelezwa kwangu; 3) nakubali na kufuata mitindo ya utafiti kutumia na kugawa habari za kiafya ama aina yoyote ya habari zitakazo kifedha kwa kushiriki katika utafiti huu; 4) nimekubali kwa hili kushiriki wakati wowote. Nakubali kufuata mitindo ya utafiti huu; 5) nimeelezwa kwamba unaweza kukoma kushiriki kwenye utafiti huu.

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, 07222822448; KU-ERC kupitia barua pepe kuerc.chairman@ku.ac.ke