PATHOGENESIS OF SCHISTOSOMIASIS AND HIV CO-INFECTION: POLYMORPHISMS IN IL-23 RECEPTOR IN SCHISTOSOMIASIS PATIENTS UNDERGOING HIGHLY ACTIVE ANTIRETROVIRAL THERAPY (HAART)

BY

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ABSTRACT

HIV and schistosomiasis co-infection is common around Lake Victoria region, raising the possibility of schistosomiasis-related immune reconstitution inflammatory syndrome (IRIS) in patients undergoing HIV medication. Some patients develop schistosome-related IRIS while others do not, and the associated immunogenetic markers are not known. This longitudinal study aimed at identifying a possible role of Interleukin-23 receptor (IL-23R) gene polymorphism in the pathogenesis of schistosomiasis-associated IRIS. In addition, the study established the relationship between plasma viral load and CD4+ cell counts. Ninety adults, occupationally exposed to water-infested with the infective stage of *Schistosoma mansoni,* working in L. Victoria in Uyoma Rarieda and undergoing Highly Active Antiretroviral Therapy (HAART), were sampled at baseline and followed up for six months to establish if they developed schistosome-related IRIS. Twenty six (26) persons who developed schistosomiasis-associated IRIS and forty five (45) who did not were included in data analysis, while those with incomplete follow-up data (n=19) were excluded. Genotyping for variants (rs188444, rs7539625, rs7530511, rs11465754 and rs6682925) within the IL-23R was carried out on whole blood samples drawn from participants while Kato-Katz was used to measure *S.mansoni* infestation from stool. Determinations of plasma viral load and CD4+ cell counts were done both at baseline (n=71) and post-treatment (n=71) to access response to HAART. The results showed an increase in CD4+ cell counts coupled with a decrease in HIV viral load in patients undergoing HAART. However, across group comparison using Kruskal-Wallis test revealed comparable levels in HIV viral load and CD4+ counts between the variants ($P>0.05$). A logistic regression analyses, while controlling for age and sex, revealed that homozygous G/G of IL-23R (rs1884444), relative to G/T genotype, was significantly associated with susceptibility to IRIS (OR, 0.25, 95% CI, 0.07-0.96, P=0.043). Pearson's Chi-Square analyses did not yield any significant association between IRIS cases and non-IRIS controls ($P>0.05$). This study showed that majority of schistosomiasis/ HIV co-infected patients responded well to HAART but their recovery was often complicated by the onset of IRIS, creating a need for health care providers to plan for its management. Homozygous G/G allele of IL-23R (rs1884444) could be one of the possible predictors of schistosomiasis-associated IRIS. Further testing will be needed to determine the feasibility of using this gene variant by health care providers in screening patients for advance preparation in the management of the syndrome.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

About 800 million individuals are at risk of schistosomiasis, and over 200 million are infected in many parts of South America, the Middle East and Southeast Asia. However, the infections are particularly pronounced in sub-Sahara Africa (Burke *et al.*, 2009). On the other hand, 33.2 million people are infected with HIV, with 7.2% Kenyans living with the virus (UNAIDS, 2009). A study carried out on car-washers along the shores of Lake Victoria revealed that about 100% of the study population was infected with *Schistosoma mansoni* and that 30% of this population had HIV co-infection (Secor, 2006).

The current treatment regimen for HIV/AIDS is known as Highly Active Antiretroviral Therapy (HAART). However, a debilitating inflammatory syndrome has been linked to HAART which causes a rapid immune reconstitution that may result in acute inflammatory responses to chronic infections such as schistosomiasis (Stoll and Schmidt, 2004). This may result in *S. mansoni*-associated immune reconstitution inflammatory syndrome (IRIS), characterized by eosinophilia and eosinophilic enteritis (Fernando and Miller, 2002).

Chronic infection by schistosomes tilts the Th1-like/Th2-like balance towards a Th2-like immune response (Mwinzi *et al.*, 2001) . This shift is characterized by increased production of interleukin-13 (IL-13), a cytokine whose production is associated with increased hepatic fibrosis (MacDonald *et al.*, 2002). The Th2 response is triggered by schistosome eggs or soluble egg antigens (Pearce *et al.*, 1991). IL-23 contributes to immunopathology in schistosomiasis both by boosting predominantly lesional Th1 cell development and

restraining regulatory networks involving IL-10, which results in pro-inflammatory cytokines and chemokines release, neutrophils recruitment and phagocytes-associated nitrogen oxide (NO) release (Rutitzky *et al.*, 2008).

Interleukin-23 receptor (IL-23R) is a type 1 cytokine receptor, which interacts with IL-23 in a lock-and-key fashion. Polymorphism in IL-23R gene has been linked to the development of acute inflammatory responses associated with IRIS (Duerr *et al.*, 2006). For example, variations at the IL23R gene at position 381 (Arg381Glu) reduces the risk of ankylosing spondilitis, Crohn's disease and psoriasis (Ban *et al.*, 2004). However, the association between IL-23R gene polymorphism and IRIS in HIV/*S. mansoni* co-infected patients undergoing HAART has not been investigated. The study was aimed at identifying the prevalence of variant forms of IL-23R gene in *S. mansoni*-infected patients undergoing HAART and to associate IL-23R gene polymorphism with acute inflammatory responses in *S. mansoni* infected patients undergoing HAART. In addition, the study also determined association between IL23R gene polymorphism with CD4+ and HIV viral loads levels in *S. mansoni*-infected patients undergoing HAART.

1.2 Statement of the Problem

Many persons in schistosomiasis-endemic regions who contract HIV already have an underlying schistosome infection, which is characterized by biased Th2 response and down-regulated Th1 and cytotoxic-T-lymphocytes (CTL) activity, raising the possibility of accelerated progression of HIV infection. In addition, successful schistosome de-worming has not been associated with decreased plasma viral load (Lawn *et al.*, 2000). Since both *S. mansoni* and HIV infections, independently, have been associated with acute inflammatory responses, and because HAART regimen may exacerbate such responses (DeSimone *et al.*, 2000), it was critical to elucidate these inflammatory responses in HIV *and S. mansoni*/ co-

infected patients undergoing HAART treatment. It is also known that variations within key inflammatory molecules, like IL-23R, can functionally increase or decrease their levels of expression (Price *et al.*, 2002), thereby affecting disease outcome. As such, variations within the IL-23R gene were investigated in patients co-infected with *S. mansoni* and HIV and undergoing HAART. In addition, comparisons in the frequency of these variants were carried out between persons who developed IRIS and those who did not.

1.3.1 General Objective

To determine the association between IL-23R gene polymorphism, CD4+ cell counts and HIV RNA levels and IRIS in HIV and *S. mansoni* co-infected patients undergoing HAART.

1.3.2 Specific Objectives

1. To determine the prevalence of the IL-23R gene variants in *S. mansoni*-infected patients undergoing HAART.
2. To establish the association between IL-23R gene variants and susceptibility to acute inflammatory responses in *S. mansoni* infected patients undergoing HAART.
3. To establish the association between changes in CD4+ cell counts and HIV RNA levels with IL-23R gene variants in *S. mansoni*-infected patients undergoing HAART.

1.3.3 Research Questions

1. What is the prevalence of the IL-23R gene variants in *S. mansoni*-infected patients undergoing HAART?
2. What are the associations between IL-23R gene variants and susceptibility to acute inflammatory responses in *S. mansoni* infected patients undergoing HAART?
3. What are the associations between changes in CD4+ cell counts and HIV RNA levels with IL-23R gene variants in *S. mansoni*-infected patients undergoing HAART?

1.3.4 Justification

HIV and schistosomiasis are common in Kenya, particularly around Lake Victoria, where the intermediate host of *S.mansoni*, *Biomphalaria,* is also common (Secor, 2006). HAART is the current treatment regimen used to suppress HIV viral replication and progression of HIV disease. This treatment regimen has proven to reduce the amount of active virus and in some cases, lower the viral load to undetectable levels by current testing techniques (Tattevin *et al.*, 2008). About 10-30% of those beginning HAART will experience immune reconstitution inflammatory syndrome (IRIS) during the first three months of treatment (Boulware *et al.*, 2008). This may lead to stoppage of medication which may give the virus time to mutate and become resistant to available drugs. The anti-retroviral (ARV) drugs help the patients' immune system to recover, causing it to respond excessively to pre-existing and dormant infections such as schistosomiasis. Since IL-23R gene is a major susceptibility gene for several autoimmune and inflammatory diseases (Huber *et al.*, 2008), variations within the IL-23R gene are likely to affect inflammatory responses to schistosomiasis. Therefore, this study investigated whether genetic variations at the IL-23R would have any association with schistosomiasis-IRIS.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 HIV treatment and IRIS

HIV is an uncommon retrovirus and drugs developed to disrupt the action of HIV are known as antiretroviral (ARV). The hallmark of HIV disease is the continuous depletion of CD4+ T cells leading to progressive immunodeficiency, opportunistic diseases, and death (Saag *et al.*, 1996). The AIDS virus mutates rapidly, which makes it extremely skillful at developing resistance to drugs. To minimize this risk, people with HIV are generally treated with a combination of ARVs, a regimen known as highly active antiretroviral therapy (HAART) (UNAIDS, 2009). The HAART combines three or more different drugs, usually two nucleoside reverse transcriptase inhibitors and a protease inhibitor (UNAIDS, 2009). The regimen reduces the viral load, in some cases to undetectable levels by most current blood testing techniques. The use of HAART results in suppression of HIV replication and an increase in CD4+ cell count, effects that correlate with improved T-cell responses to antigens and mitogens (Hirsch *et al.*, 2004).

HAART has resulted in a marked decrease in morbidity and improved survival in HIV-infected patients. However, patients and their providers are increasingly confronted with many potential complications of HAART. One such complication is immune reconstitution inflammatory syndrome (IRIS) (Kaufmann *et al.*, 1999). The prevalence of IRIS is known to vary depending on the causative agent- tuberculosis (45%), Kaposi sarcoma (7%) and *Cryptococcus* (8% to 31%) (Bower *et al.*, 2005; Lawn *et al.*, 2005; Lortholary *et al.*, 2005; Shelburne *et al.*, 2006). However, no study has been carried out to determine the prevalence of schistosomiasis-associated IRIS.

IRIS occurs as a direct consequence of a rapid and dysregulated restoration of antigen-specific immune responses during the first three months of initiation of HAART (Dhasmana *et al.*, 2008). This period also marks the first phase of immune reconstitution which is characterized by rapid increases in CD4+ cells, redeployment of activated CD4+CD45RO+ memory cells previously sequestered in lymphoid tissue and reduction in apoptotic cell death (Bucy *et al.*, 1999; Lederman, 2001). IRIS may manifest with diverse range of clinical presentations and is associated with a number of antigenic targets (Dhasmana *et al.*, 2008). These include viable replicating infective antigen (unmasking IRIS), dead and dying infective antigen (paradoxical IRIS), host antigen (autoimmune disease), tumour antigen and other inflammatory conditions (Dhasmana *et al.*, 2008). The pathogenesis of IRIS is poorly understood, however polymorphism in human leukocyte antigen (HLA) and cytokine-related genes have been associated with this clinical condition (Price *et al.*, 2001; Price *et al.*, 2002). As such, this study focused on the pathogenesis of schistosomiasis-associated IRIS particularly on the role of IL-23R polymorphism on the disease outcome.

2. 2 HIV and *S.mansoni* co-infection and their association with IRIS development

The progression of HIV infection does not seem to be affected by schistosomiasis (Brown *et al.*, 2004). In addition, the presence of HIV co-infection has no influence on the degree of severity of schistosomiasis (Brown *et al.*, 2004). However, HIV infection leads to lower eggs excretion, with the efficiency of egg excretion having a positive correlation with CD4+ cell levels in HIV-1 positive patients (Karanja *et al.*, 1998). In a few cases of co-infected patients who were immigrants from Africa to United Kingdom, initiation of HAART led to IRIS characterized with new clinical presentation or deterioration of schistosomiasis (Lawn and Wilkinson, 2006). One case involved a male who had a viral load of 49,700 copies/mL and CD4+ cell count of 170 × 10^6 cells/L at the time of initiation of HAART. His CD4+ cells increased to 223 ×10 cells/L while his viral load decreased to 1330 copies/mL in the

preceding four weeks but he had to stop HAART five times because he developed fever, vomiting, diarrhea and abdominal pains (de Silva *et al.*, 2006). After elimination of other possible causes of these symptoms, it was concluded that the patient developed *S.mansoni*-associated IRIS (de Silva *et al.*, 2006). The above studies were case studies from which it was difficult to make generalized conclusions on the symptoms of schistosomiasis- associated IRIS. As such, the current study targeted a well phenotypically defined population to attempt to identify symptoms of schistosomiasis-related IRIS.

2.3 IL-23 receptor polymorphisms

Interleukin 23 receptor (IL-23R) is a type 1 trans-membrane protein found on activated T cells, natural killer (NK) cells, monocytes and dendritic cells (Parham *et al.*, 2002). The human IL-23R gene is located on the short arm of chromosome 1 at position 31.3 (base pairs 67404756 to 67498249). The IL-23R is a heterodimer, comprising the IL-12Rβ1 and a novel subunit named IL-23R (Parham *et al.*, 2002). The IL-12Rβ1 subunit of IL-23R is similar to that of IL-12 receptor (IL-12R). However, the IL-23R lacks IL-12Rβ2 subunit found in IL-12R **(Figure 1)** (Langrish *et al.*, 2004). IL-23R has an extracellular domain which contains a signal sequence, an N-terminal immunoglobulin like domain, and two cytokine receptor domains containing a WQPWS motif. The intracellular domain of IL-23R has seven tyrosine residues that are phosphorylated; three are *src* homology, two domain-binding sites and two signal transducers and activators of transcription (STAT) binding sites (Lankford and Frucht, 2003). Binding of IL-23 to its receptor leads to activation of Janus kinases (Jaks) which phosphorylates IL-23R at certain locations, thus forming docking sites for the STATs. Subsequently, the Jaks phosphorylate the STATs, allowing them to translocate to the nucleus where they activate the transcription of pro-inflammatory genes such as IL-17 and interferon-γ (IFN-γ) genes (Lankford and Frucht, 2003). Signaling of IL-23 through STAT-4

contributes to autoimmune responses thus, it is possible that up-regulation of IL-23R, due to variations in the gene, could increase STAT-4 signaling, thereby conferring the risk for auto-immunity (McKenzie *et al.*, 2006).

IL-23 is a hetero-dimeric cytokine that comprises a p19 subunit which associates with the IL-12p40 subunit produced by macrophages and dendritic cells (Langrish *et al.*, 2004). IL-23 is responsible for the differentiation and expansion of $Th_{17}/Th_{IL\text{-}17}$ cells from naive $CD4^+$ T cells (Iwakura *et al.*, 2008). Studies have shown that IL-23 deficient mice were resistant to central nervous system (CNS) autoimmune inflammation because they lack the ability to develop IL-17 producing $Th_{IL\text{-}17}$ cells (Langrish *et al.*, 2004). Studies also show that IL-23/IL-17 pathway is strongly regulated by the IL-12/IFN-γ axis of the immune regulatory functions. Failure to suppress the expansion of the activated CD4+ T cells population in IFN-γ-deficient mice leads to collagen-induced arthritis exacerbation (Iwakura *et al.*, 2008). Different variants of IL-23R may predispose to different autoimmune diseases. For example, variants within the IL-23R on exon three (rs1884444; T>G), is associated with decreased susceptibility to gastric cancer (Chen *et al.*, 2010) whereas IL-23R haplotype rs7530511 (L310P) showed a significant association with psoriasis in both cases and controls (Nair *et al.*, 2008) and IL-23R (rs2201841; located in intron 7) have been shown to confer risk to both Crohn's disease and rheumatoid arthritis (Huber *et al.*, 2008). However, no studies have been carried out to determine the association between IL-23R polymorphisms and IRIS associated with schistosomiasis. As such, this study investigated the association between these polymorphism and schistosomiasis-associated IRIS.

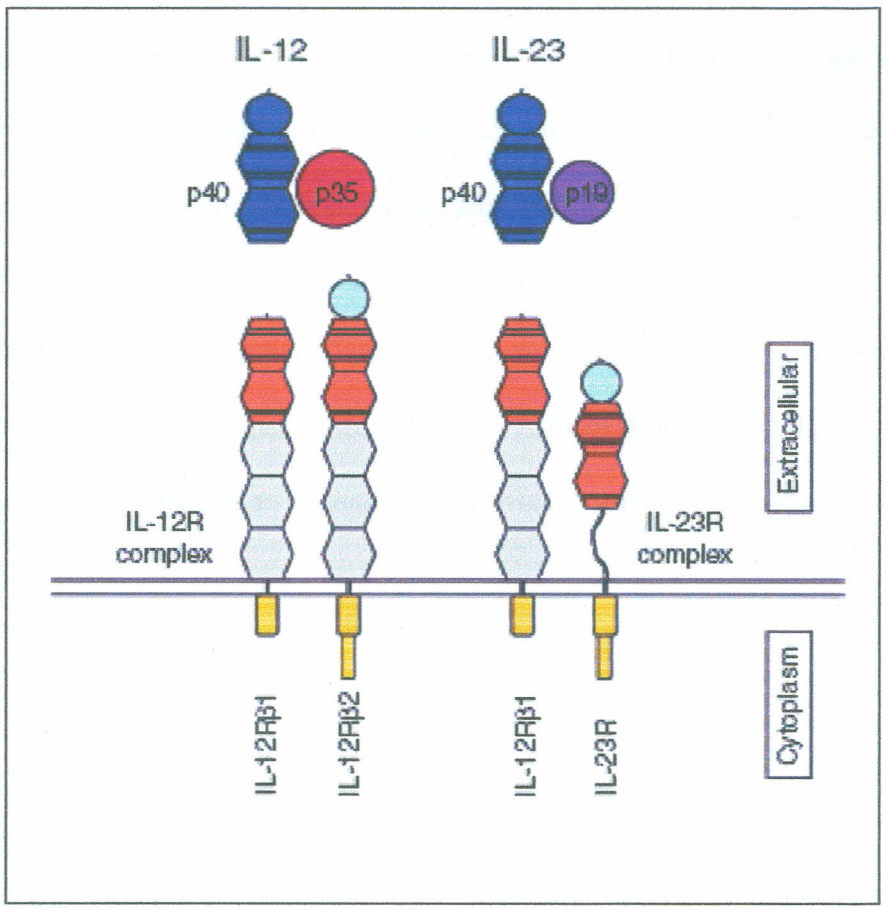

Figure 1: Comparison of IL-23 and IL-23R with IL-12 and IL-12R (adopted from (Langrish *et al.*, 2004))

2.4. Selection of SNPs for genotyping

A total of five polymorphic sites located in IL-23R, rs1884444, rs7530511, rs11465754, rs6682925 and rs7539625, were selected considering their location and allelic frequencies based on public data bases (dbSNP; http.ncbi.nlm.nih.gov/SNP/, HAP-MAP;http:// www.hapmap.org/index.html.en). IRIS has been associated with distinct human leukocyte antigen (HLA) profiles and regulatory cytokine genes. For instance, cytomegalovirus (CMV)

retinitis IRIS has been associated with HLA-B44 while patients with mycobacterial IRIS rarely carry TNF-α-308*2 and IL-6-174*G (Kestens *et al.*, 2008). Although IL-23 is a known cellular marker of inflammation (Nixon and Landay, 2010), no previous study has ever been done to determine if this cytokine or its receptor (IL-23R) has any association with IRIS. This necessitated the need to investigate the association between IL-23R and schistosomiasis-associated IRIS. The particular SNPs selected for the current study were those that had at least >5% mutant allele frequency in reference African Yoruba population since these have been previously established to be undergoing disease selective pressure (Ouma *et al.*, 2008; Ouma *et al.*, 2010).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1. Study site and population

The study was carried out on fishing community in Uyoma, Rarieda District, along the shores of Lake Victoria (**Appendix III**). These groups of people are occupationally exposed to water infested with the infective stage of *S. mansoni* parasite. The prevalence of schistosomiasis in this fishing community is nearly 100% with about a third of them being co-infected with HIV-1 (Karanja *et al.*, 1998; 1997; Mwinzi *et al.*, 2001; Watanabe *et al.*, 2007)

3.2. Study design

The study was a 6-month longitudinal study in which 90 HIV and *S.mansoni* co-infected persons were enrolled, started on HAART and paraziquantel (PZQ), and followed for the development of schistosomiasis-related IRIS. Seventy one (71) people qualified for data analysis while nineteen (19) were lost due to incomplete follow up. Twenty six (36.62%) patients developed IRIS, a number higher than the postulated eleven patients (15%). Forty five (63.38%) patients did not develop IRIS. The study was designed to identify the candidate IRIS susceptibility variants within IL-23R gene in patients (who develop IRIS) verses controls (who did not develop IRIS).

3.3. Inclusion criteria

1. Adults over 18 years of age.
2. Schistosome infection with or without HIV co-infection.
3. Permanent residents of the area.
4. Must have undergone HIV voluntary counseling and testing in a recognized government institution.
5. Must be on HAART naive at the beginning of the study.

6. A written informed consent obtained from each participant.

3.4. Exclusion criteria

1. Children (less than 18 of age).
2. People not infected with schistosomes.
3. Temporary residents of the area.
4. People not willing to undergo HIV voluntary testing and counseling in a recognized government institution.
5. People already on HAART.
6. People not willing to give a written consent.

3.5. VCT, HIV treatment and care

Participants were required to go for voluntary HIV counseling and testing (VCT) in a government recognized institution as a mandatory requirement before being enrolled into the study. The participants were also required to accept to be initiated on HAART for HIV if found to be HIV positive. They were provided with VCT client's card that were signed and dated by the VCT counselor as a proof of having been counseled. Those who accepted to be enrolled into the study were tested to determine their HIV viral load and CD4+ cell counts.

3.6. Ethical considerations

Voluntary recruitment and consenting process of participants by KEMRI employees was carried out in a language of the participant's choice (Kiswahili or English or Dholuo). Prospective participants were allowed to read the consent document by themselves (for those who were able to read) while the document was read to those unable to read. Written informed consent was obtained prior to enrolment. Confidential information gathered from

participants was used only for the purpose of the study and were kept in a coded language to ensure that the confidentiality is maintained. The study was part of larger study which was reviewed by the Scientific Steering Committee (SSC) at the departmental level at the Center for Global Health Research (CGHR) of KEMRI before it was forwarded for approval to the Institutional Scientific Steering Committee (SSC) of KEMRI, the National/KEMRI Ethical Clearance Committee (ERC) (Appendix III).

3.7. Sample collection and handling

Urine and three stool samples (for Kato-Katz testing and microscopy) were collected from participants to determine *S. mansoni,* and soil-transmitted helminthes as well as to evaluate cure following treatment. Venous blood was drawn from participants using sterile techniques by qualified KEMRI personnel to minimize risk of infection. Participants found to be positive for schistosomiasis were treated with PZQ while those positive for other helminthes were treated with albendazole.

A total volume of 10 mL of blood samples were collected from participants by venipuncture into EDTA tubes at baseline, one month and three months post-enrollment and after treatment commencement. The blood was transported in ice packs to Kisian-KEMRI schistosomiasis laboratory. One part of the collected blood (5 mL) was subjected to Ficoll-Hypaque density centrifugation to isolate peripheral blood mononuclear cells (PBMC) and plasma. The plasma was used to confirm HIV status and to determine viral load. The other part of whole blood was used to determine CD4+ counts and for genotyping assays. Patients were followed for three months post-enrolment to monitor development of IRIS. After IRIS phenotypes were identified, genotyping for IL-23R gene was carried out on whole blood samples.

In the study IRIS was defined as: A HIV/schistosomiasis patient who develops within six months of starting a successful HAART regimen, an increase of at least one of the following schistosomiasis-related symptoms namely: hepato-splenomegaly, ultra-sound finding or clinical signs of hypertension, ultra-sound finding or clinical signs of increased granuloma formation/liver fibrosis, hematuria and at least one laboratory finding: an increase of liver test abnormalities or egg production in stool.

3.7.1. Genotyping IL-23R single nucleotide polymorphism (SNPs)

Genomic DNA was isolated from 2 mL of peripheral whole blood using Qi-Amp Midi kit (Qiagen, Hilden, Germany) by centrifugation. About 20µl QIAGEN Protease was pipetted into a 1.5 ml microcentrifuge tube and 200 µl samples added to it. This was followed by addition of 200 µl Buffer AL to the sample and vortexing for 15 sec. The sample was incubated at 56°C and spun down. Thereafter, 200 µl ethanol (96-100%) was added to it before it was quickly vortexed and spun again. The lysate was placed in a QIAamp column carefully without wetting the rim and centrifuged for 1 min at full speed before addition of 500µl of AW1 buffer with a further one minute centrifugation at full speed. The column was placed in new collection tube and 500 µl AW2 added to it. A further centrifugation at full speed for 3 min was done and the resultant sample placed in a clean 1.5 ml microcentrifuge tube. About 200µl buffer AE was added to it before it was incubated at room temperature for 1 min and the DNA eluted.

The IL-23R polymorphisms rs7530511, rs1884444, rs11465754, rs6682925 and rs7539625 were determined using TaqMan 5' Allelic Discrimination–Assay-By-Design (Applied Biosystems, Foster City, CA). Allelic Discrimination (AD) is a multiplexed (probe pair per reaction), end data point assay that detects variants of a single nucleic acid. For each AD assay, a unique pair of fluorescent dye detector is used (one dye a perfect match to the wild

type and the other a perfect match to the mutant) AD measures the change in fluorescence of dye associated with the probes. The assays were performed in a total volume of 5µl with the following amplification protocol: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60 °C for 1 min. Allele-calling was carried out using allelic-specific fluorescence on the ABI prism 7900H sequence detection system. The automated sequence detection software (SDS) was then used for allelic discrimination (Applied Biosystems, Inc.).

3.7.2. Blood cell counts

Absolute CD4+cells count was determined by BD TruCount (BD Bioscience) to monitor the response of study population to HAART. In this process absolute number of leucocytes is determined my mixing appropriate monoclonal antibody reagent with whole blood in a TruCount tube. Lyophilized pellets in the tube dissolves, releasing a known number of fluorescent beads. Analysis of the positive cells is done by comparing cellular events to bead events.

Fifty micro liters of whole blood was pipette in to each tube and incubated in the dark for 15 minutes and vortexed. To each tube, fifty micro liters appropriate corresponding antibody was added. The sample was then vortexed and incubated in the dark for 15 minutes before addition of four hundred fifty micro liters of FACS lysing solution. The mixture was then incubated for 10 minutes at room temperature before addition of fifty micro liters of TruCount control beads and a final incubation for 15 minutes in the dark. Thereafter, the sample was acquired and analyzed in a FACS machine.

3.7.3. Determination of viral load

HIV-1 RNA load was determined using Amplicor HIV-1 Monitor Test (version 1.5) (Roche, Basel, Switzerland) from plasma obtained by centrifugation of whole blood and stored at -80°C. The Amplicor HIV-1 monitor test was designed to quantitate viral load by utilizing a

second target sequence (QS) added to the amplification mixture at a known concentration. The optical density in each well of the micro-well plate (MWP) is proportional to the amount of HIV- 1 or QS amplicon in the well, and the total optical density. The amount of HIV-1 RNA in each specimen is calculated from the ratio of the total optical density for the HIV-I specific well to the total optical density for the QS-specific well and the input number of QS RNA molecules. HIV-1 positive plasma was lysed under highly denaturing conditions to inactivate RNase and ensure intact isolation of viral RNA. Viral RNA was precipitated by addition of 70% ethanol. A known number of quantitation standard RNA molecules were then introduced into each specimen with the lysis reagent. Reverse transcription and PCR of the isolated viral RNA was done in a single tube containing the enzyme *Thermus thermophilus DNA* polymerase, Mn^{2+} and excess deoxynucleoside trisphosphates (dNTPs). Following PCR amplification, the HIV-1 amplicon and the HIV-1 quantitation standard amplicon was denatured by addition of denaturation solution (EDTA solution containing I.6% sodium hydroxide and amaranth dye). Denatured amplicon was placed in separate wells of microwell plate (MWP) coated with HIV-1 specific and HIV-1 quantitation standard specific oligonucleotide probes. Thereafter, the MWP was washed with wash solution, composed of sodium phosphate containing EDTA, before addition of avidin-horseradish peroxidase to each well. A second wash was done to remove unbound conjugate, followed by addition of substrate solution to the wells to form a colored complex. The reactions in the well were stopped by addition of 4.9% sulfuric acid and optical density (OD) was measured using an automated microwell plate reader. Quantitation of HIV-1 viral RNA was done according to the manufacturer's protocol (Roche, Basel, Switzerland) using the formula:-

Total HIV-1 OD × input HIV-1 QS copies/PCR × sample volume Factor = HIV-1 RNA copies/mL × Total QS OD.

Where QS copies/PCR = the number of copies of Quantitation Standard in each reaction.

Sample Volume Factor = factor to convert copies/PCR to copies/mL = 40

3.7.4. Determination of schistosome infection in Stool and Urine

Forty seven milligrams of stool was sieved and stained with 10% malachite green soaked in cellophane for twenty four hours. The slide with the stool was in mounted on a microscope for identification of schistosome eggs under magnification of X10. To identify schistosome eggs in urine, 10 milliliters of urine was filtered and the residue placed on microscope slide before staining with iodine. The stained sample was viewed under magnification of X10 to identify *S. heamatobium* eggs.

3.7.5. Sample size calculation

The appropriate sample size (n) was determined based on the prevalence of schistosomiasis-associated IRIS (0.015) (UNAIDS, 2009: Secor, 2006), desired confidence level (1.96) and acceptable margin of error (0.05).The sample size was calculated using a web based formula below.

$n= t^2 \times p\ (1-p)/m^2$

$n=1.96^2 \times 0.015\ (1-0.015)/0.05^2$

$n=23$

Where;

- n is the sample size
- p is the prevalence of Schistosomiasis associated IRIS
- m is the acceptable margin of error
- t is the desired confidence level

3.8. Statistical Analysis

Data was initially entered in Microsoft Excel, coded and later analyzed in SPSS (version 17.0 Chicago, IL). Proportions and allele frequencies between IRIS patients and controls were assessed by Pearson χ^2 test while Krukal-Wallis test was used to analyze differences across groups. Multivariate logistic regression analyses were used to determine the association between each SNP with susceptibility to IRIS by calculating odds ratios (OR), 95% confidence interval (CI) and corresponding *P*-values, while controlling for the confounding effects of age and sex. These confounders were controlled for since both of them are known to influence levels cytokines expressions (Pietschmann *et al.*, 2003). All analyses were two-tailed and *P*-values of <0.05 were considered statistically significant.

CHAPTER FOUR

4.0 RESULTS

4.1. Demographic characteristics of the study population

Table 1 shows the demographic characteristics of the study population. A total of seventy one (71) participants were enrolled in the study (thirty five males and thirty six females). The distribution of the males vs. females were comparable in both IRIS and non-IRIS groups (P=0.154). Twenty six participants (36.62%) developed schistosomiasis-associated IRIS (fourteen males and twelve females) while 63.38% did not. The mean age, baseline CD4+ cell counts and baseline HIV plasma viral loads were comparable between the IRIS and non-IRIS patients (P=0.867, P=0.101 and P=0.116, respectively).

Table 1. Demographic characteristics of the study participants

	IRIS	NON IRIS	P
Age in Years at time Of enrolment	35.5(28-45)	34(29-41)	0.867[a]
Male (n) (%)	14(53.8)	21(46.7)	0.154[b]
Base-line CD4 count	171.5(112-251)	216(144-317)	0.101[a]
Base-line Viral Load (copies/ml)	93200(9825-213000)	234000(11990-531000)	0.116[a]

Data are median and interquartile range unless stated otherwise

[a]Statistical significance determined by Mann-Whitney U test

[b]Statistical significance determined by Pearsons Chi-square analyses

4.2. Prevalence of IL-23R genotypes in *S. mansoni*-infected patients undergoing HAART

In order to determine the prevalence of IL-23R genotypes in *S. mansoni*-infected patients undergoing HAART, a Pearson's chi-square analysis was carried out in a total population of seventy one (71) individuals. The targeted IL-23R genotypes targeted in the current analyses were rs7530511, rs1884444, rs11465754, rs6682925, and rs7539625 (Table 2). Results revealed a comparable distribution of variants within the rs7530511 (P=0.518), rs1884444 (P=0.091), rs11465754 (P=0.426), rs6682925 (P=0.393), and (rs6682925 (P=0.088) between the IRIS and non-IRIS patients (Table 2).

Table 2. The prevalence of IL-23R genotypes in patients undergoing HAART (IRIS) and non-IRIS.

Genotypes	IRIS	Non-IRIS	*P*-value
rs7530511			
CC	17 (70.83)	33 (76.74)	0.518[a]
CT	5 (20.83)	9 (20.93)	
TT	2 (8.33)	1 (2.33)	
rs1884444			
GG	4 (18.18)	16 (39.02)	0.091[a]
GT	16 (72.73)	18 (43.90)	
TT	2 (9.09)	7 (17.07)	
rs11465754			
AA	7 (26.92)	16 (38.10)	0.426[a]
AG	16 (61.54)	19 (45.24)	
GG	3 (11.54)	7 (16.67)	
rs6682925			
CC	10 (38.46)	19 (48.72)	0.393[a]
CG	13 (50.00)	13 (33.33)	
GG	3 (11.54)	7 (17.95)	
rs7539625			
GG	5 (20.83)	16 (36.36)	0.088[a]
GA	17 (70.83)	19 (43.18)	
AA	2 (8.33)	9 (20.45)	

[a]Data are proportions (%) as determined by χ^2. The targeted variants were those that had a prevalence of >10% in reference to African Yoruba population (dbSNP; http.ncbi.nlm.nih.gov/SNP/, HAP-MAP;http:// www.hapmap.org/index.html.en).

There were no significant differences in proportions of the variants in IRIS versus non-IRIS patients.

4.3. Association between IL-23R Variants and susceptibility to IRIS

In order to determine the association between the IL-23R variants and susceptibility to IRIS, a multivariate logistic regression analyses was carried out while controlling for the confounding effects of age and sex. Results revealed that relative to the GT (most prevalent in the population), carriers of GG genotype at the rs1884444 gene had a reduced risk in the development of IRIS (OR, 0.25, 95% CI, 0.07-0.96, P=0.043). However, none of the other variants altered susceptibility to IRIS (Table 3).

Table 3: The association between IL-23R variants and susceptibility to IRIS

	IRIS		
Genotypes	OR	95%CI	*P*
rs7530511			
CC	1.00	(reference)	
CT	1.05	0.30– 3.68	0.308
TT	3.64	0.30 – 43.62	0.386
rs1884444			
GT	1.00	(reference)	
TT	0.24	0.04– 1.51	0.129
GG	0.25	0.07 – 0.96	**0.043**
rs11465754			
AG	1.00	(reference)	
AA	0.40	0.12– 1.33	0.136
GG	0.37	0.07 – 1.84	0.222
rs6682925			
CC	1.00	(reference)	
CG	1.95	0.64– 5.94	0.240
GG	0.77	0.16 – 3.81	0.748
rs7539625			
GA	1.00	(reference)	
GG	0.34	0.10– 1.14	0.080
AA	0.25	0.05 – 1.40	0.116

Data are presented as Odds Ratio (OR) and 95% Confidence Interval (95% CI). Data analyzed through multivariate logistic regression analyses controlling for the confounding effects of age and sex. *P*-values in bold are significant at $P \leq 0.05.4.4$. The association between rs7530511, rs1884444, rs11465754, rs6682925, rs7539625 and changes in CD4+ cell counts and HIV Loads in blood

In order to determine the association between variants of rs7530511, rs1884444, rs11465754, rs6682925, rs7539625 and changes in CD4+ cell counts and HIV viral loads in patients, Kruskal-Wallis test was carried across the different variants per polymorphism. Results revealed comparable levels in CD4+ cell counts and HIV viral loads for variants in rs7530511 (P=0.500, P=0.405), rs1884444 (P=0.414, P=0.648), rs11465754 (P=0.782, P=0.975), rs6682925 (P=0.700, P=0.818), rs7539625 (P=0.715, P=0.863), respectively (Figures 2: a-e).

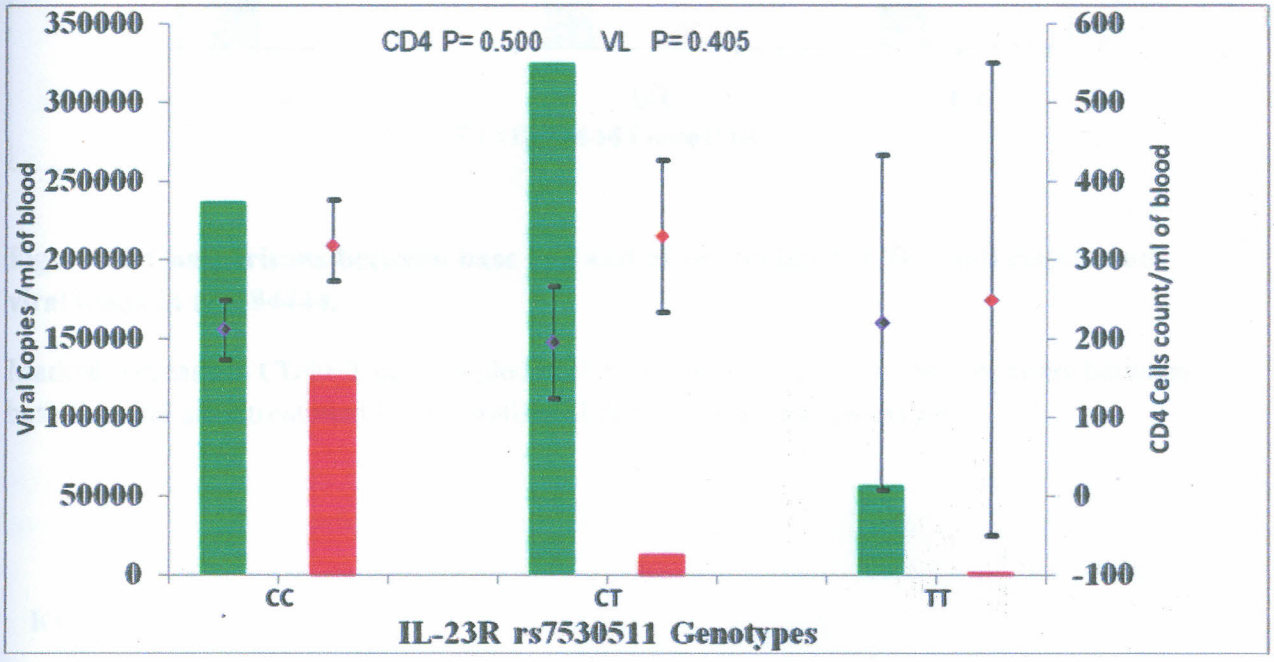

Figure 2a: Comparisons between base line and after treatment CD4+ cell counts and viral loads in rs7530511.

Marked increase in CD4+ T cell coupled with marked decrease HIV copy numbers between base line and after treatment but no statistical difference across genotypes.

KEY

Base line viral load

After 3 viral load

Baseline CD4+ count

After 3 months CD4+ count

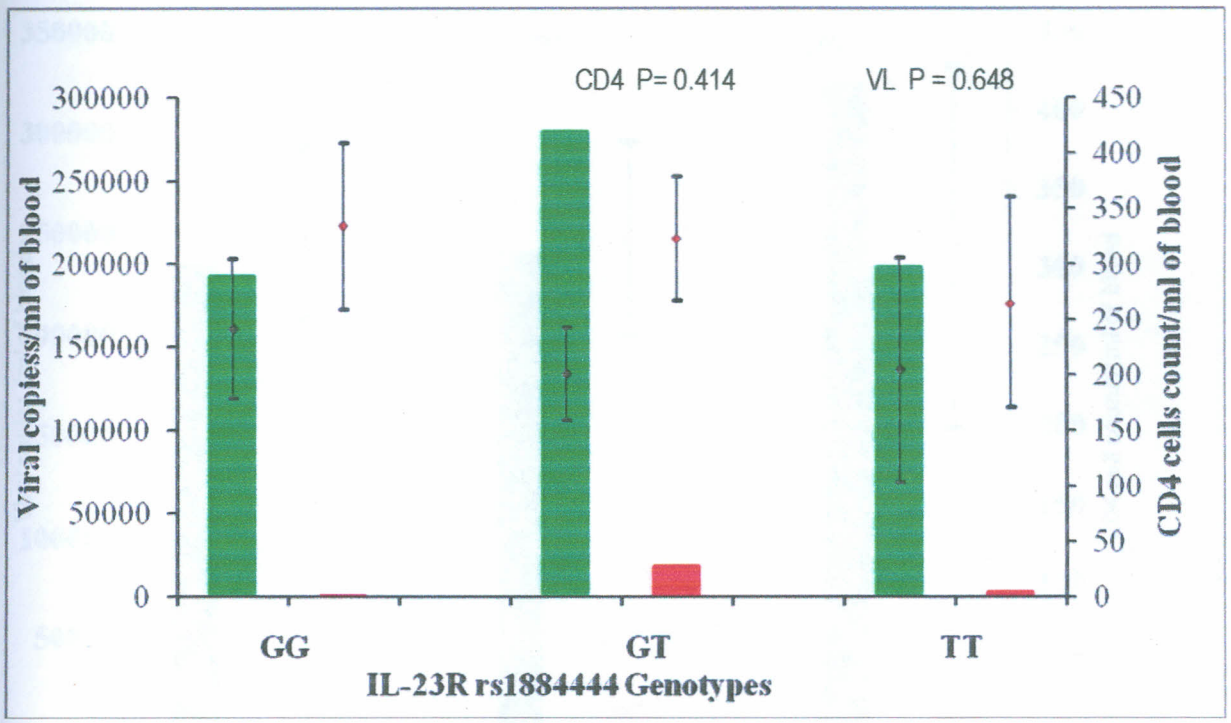

Figure 2b Comparisons between base line and after treatment CD4+ cell counts and viral loads in rs1884444.

Marked increase in CD4+ T cell coupled with marked decrease HIV copy numbers between base line and after treatment but no statistical difference across genotypes.

KEY

Base line viral load

After 3 viral load

Baseline CD4+ count

After 3 months CD4+ count

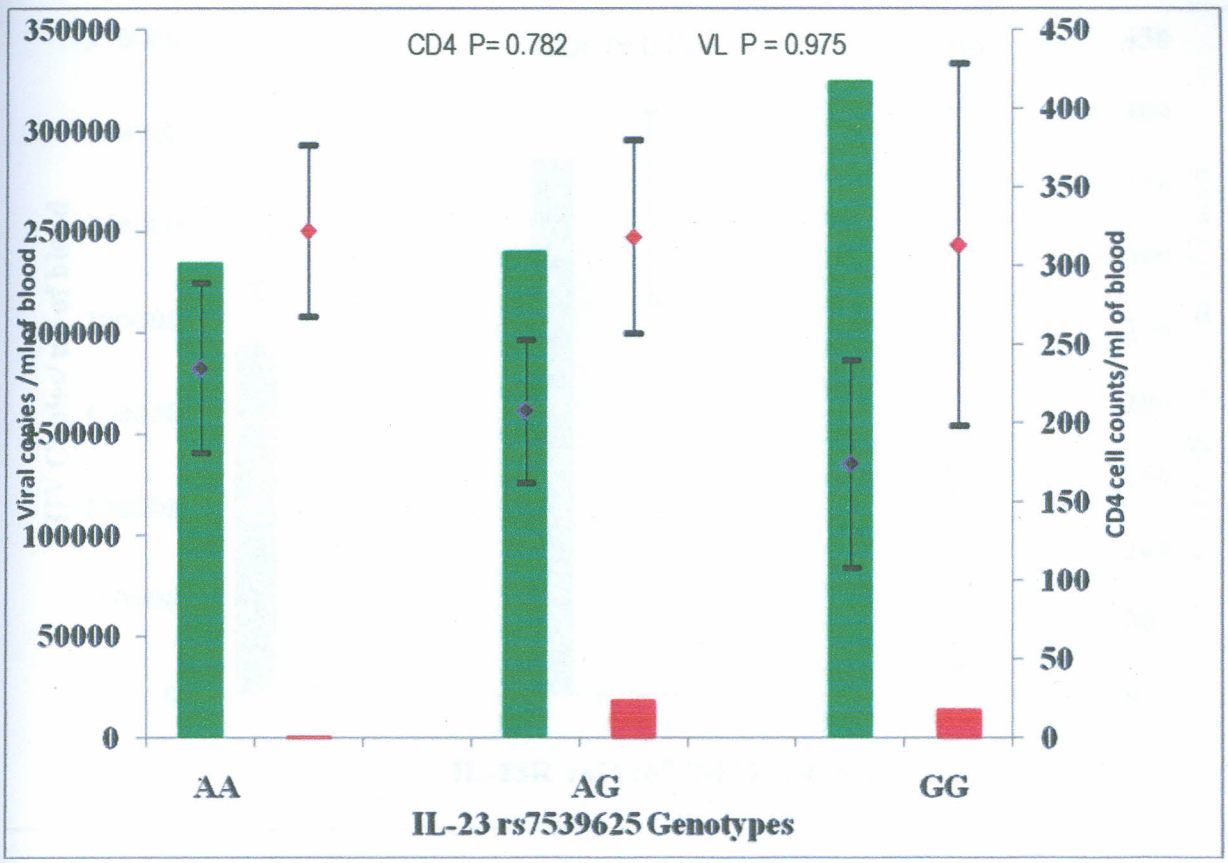

Figure 2c. Comparisons between base line and after treatment CD4+ cell counts and viral loads in rs7539625

Marked increase in CD4+ T cell coupled with marked decrease HIV copy numbers between base line and after treatment but no statistical difference across genotypes.

KEY

Base line viral load

After 3 viral load

Baseline CD4+ count

After 3 months CD4+ count

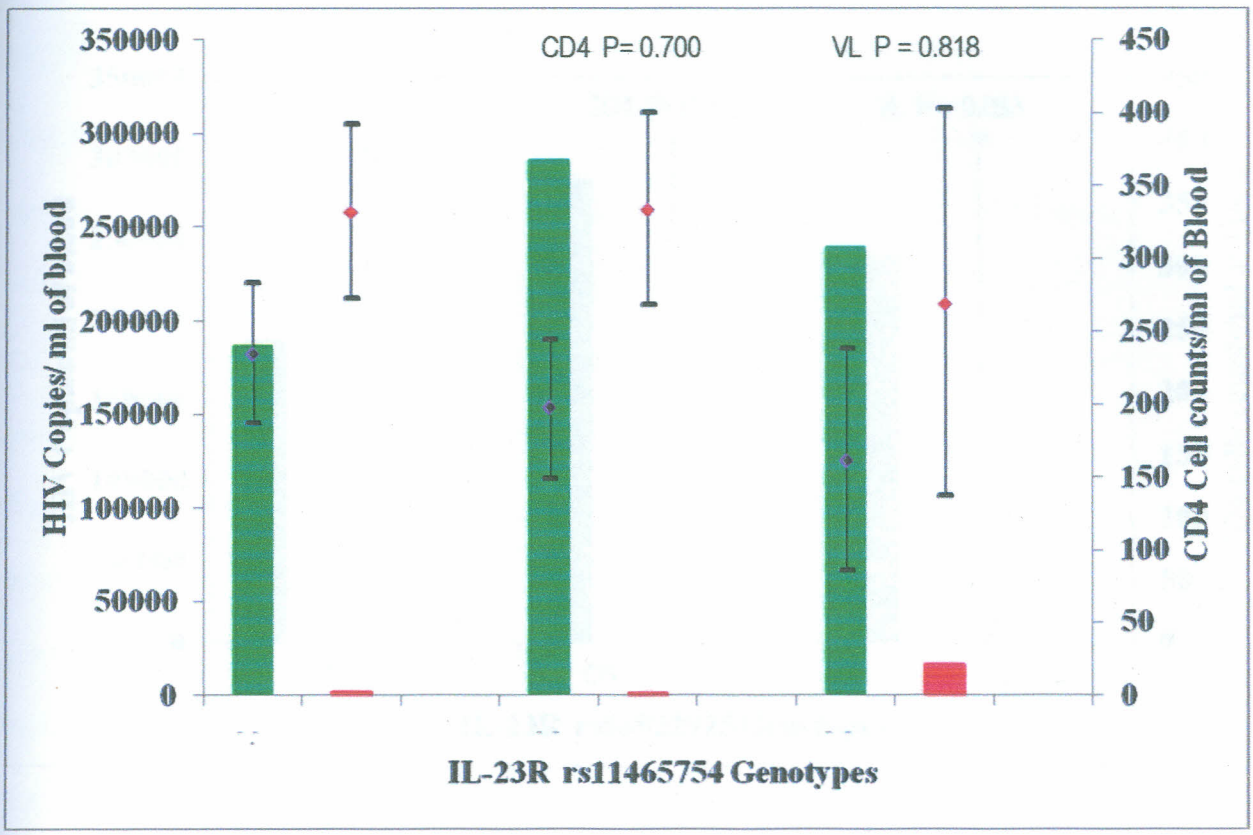

Figure 2d. Comparisons between base line and after treatment CD4+ cell counts and viral loads in rs11465754.

Marked increase in CD4+ T cell coupled with marked decrease HIV copy numbers between base line and after treatment but no statistical difference across genotypes.

KEY

Base line viral load

After 3 viral load

Baseline CD4 + count

After 3 months CD4+ count

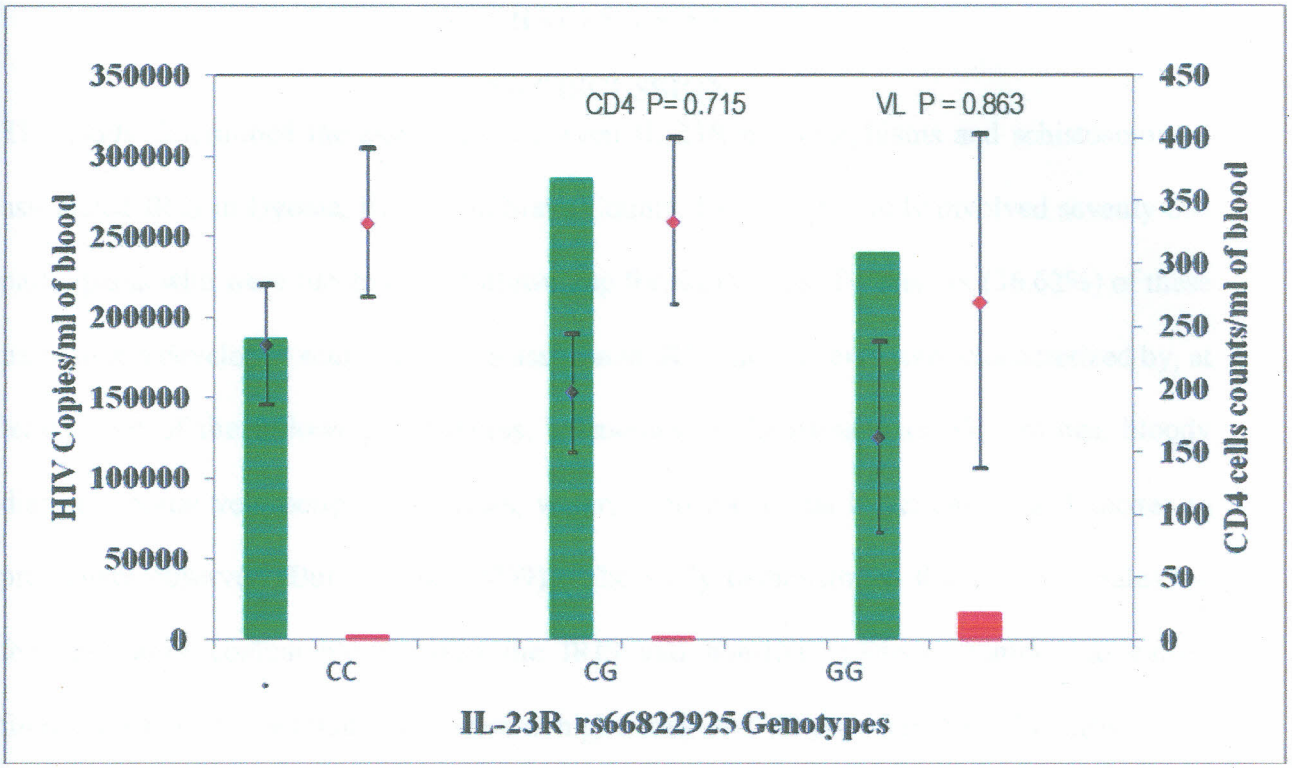

Figure 2e Comparisons between base line and after treatment CD4+ cell counts and viral loads in rs66822925.

Marked increase in CD4+ T cell coupled with marked decrease HIV copy numbers between base line and after treatment but no statistical difference across genotypes.

KEY

Base line viral load

After 3 viral load

Baseline CD4+ count

After 3 months CD4+ count

CHAPTER FIVE

5.0 DISCUSSION

This study determined the association between IL-23R polymorphisms and schistosomiasis associated-IRIS in Uyoma, Rarieda in Siaya County, Kenya. The study involved seventy one participants who were successfully followed up for six months. Twenty six (36.62%) of these participants developed schistosomiasis-associated IRIS. IRIS events were characterized by, at least, three of the following symptoms; splenomegaly, hepatomegaly, skin rashes, bloody diarrhea, portal vein periportal fibrosis, watery diarrhea, portal hypertension and ascites as previously observed (Burke *et al.*, 2009). The study demonstrated that gender (males vs. females) were comparable between the IRIS and non-IRIS patients contrary to earlier findings which showed that males were at higher risk of IRIS than females (Shelburne *et al.*, 2005). Further analyses demonstrated that age, CD4+ cell counts and the HIV viral loads were also comparable between the IRIS and non-IRIS groups. A logistic regression analysis however, showed that relative to the GT, carriers of GG genotype at the rs1884444 polymorphism had a reduced risk in the development of IRIS.

5.1. The prevalence of the IL-23R gene variants in *S. mansoni*-infected patients undergoing HAART

This study revealed that there was no significant difference in distribution of variants of IL-23R between IRIS and non-IRIS controls. An earlier study revealed that the T allele in the rs11465804 of the IL-23R was more prevalent in patients with inflammatory bowel disease in a Canadian population (Murdoch *et al.*, 2012). In addition, another study involving a Thai population demonstrated that rs7539625 was associated with risk to psoriasis (Stuart *et al.*, 2010). However, the current study demonstrated comparable distributions of the IL-23R variants in the IRIS and non-IRIS groups. This observation could be due to low sample sizes that were logistically available in the current settings.

5.2. Association between IL-23R Variants and susceptibility to IRIS

The current study demonstrated a significant association between the rs188444 and susceptibility to IRIS. For example, the homozygous GG individuals relative to the wild type (GT) were at a significant reduced risk to schistosomiasis-associated IRIS. Consistent with the current observation, a previous study indicated that this gene variant is associated with decreased risk of gastric cancer in a Chinese population (Chen *et al.*, 2010). According to web-based SNP analysis tool Pupa-suite 2, T to G base of rs1884444 may disrupt an exonic splicing enhancer, resulting in exon skipping, malformation or transcript alternative splicing (Chen *et al.*, 2010). This discovery is consistent with the theory that IL-23R interacts with its cytokine IL-23 which is a critical signal necessary to support the pro-inflammatory Th_{17} subset involved in elevated schistosomiasis pathology (Rutitzky and Stadecker, 2006). The IL-23/IL-23R pathway may modulate STAT-3 transcription activity which is an essential regulator of both immune-mediated inflammation and inflammatory-associated apoptosis resistance (Gaj *et al.*, 2008). .

5.3. The association between rs7530511, rs1884444, rs11465754, rs6682925, rs7539625 and changes in CD4+ cell counts and HIV loads in patients with IRIS vs. non-IRIS

The current study demonstrated comparable levels in CD4+ cell counts and HIV loads across all the IL-23R variants in IRIS and non-IRIS patients. The initiation of participants on HAART led to remarkable reduction in HIV replication and recovery in CD4+ cell numbers in all the carriers of the different gene variants. Despite the immune recovery, twenty six individuals experienced clinical deterioration characterized by symptoms usually associated with AIDS and schistosomiasis. The possible explanation for this observation is that these groups of people developed schistosomiasis-associated IRIS. On comparison, none of variants had a significantly high increase in CD4+ counts or decrease in HIV viral load. It has been previously established that host genetics impact on infectious disease by influencing host immune response through cytokine genes thereby influencing response to HAART

(Langford *et al.*, 2007). The current study was carried out in a 6-month follow-up period. It would be important to extend such studies over longer periods to assess whether the CD4+ cell counts and HIV viral loads would change as one progress to IRIS. In addition, it would be important to have an all inclusive panel of immune mediators in the analyses to further identify additional immune mediators that may alter changes in CD4+ counts and viral loads in patients presenting with IRIS.

CHAPTER SIX

6.0. CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions

1. There is no difference in distribution of the IL-23R variants between IRIS and non-IRIS controls.
2. The IL-23R genotype (rs1884444) is associated with susceptibility to schistosomiasis-related IRIS.
3. The changes in plasma CD4+ and HIV viral loads are not significantly associated with IL-23R gene variants.

6.2. Recommendations

1. Health care providers should place less focus on the prevalence of IL-23R gene variants while trying to minimize the effect of schistosomiasis-associated IRIS.
2. Patients should be screened for the presence of the homozygous G/G allele of IL-23R rs1884444 before the onset of HAART.
3. It is important to investigate other predictors of schistosomiasis-associated IRIS in order to improve its management.

6.3. Recommendations for future studies.

Future studies should:

1. Investigate the role IL-23 rs1884444 in the pathogenesis of schistosomiasis-associated IRIS in a population with varied geographical origin.
2. Investigate if IL-23R rs1884444 has a role on the levels of expression of IL-17, a critical cytokine in the pathogenesis of schistosomiasis.

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