Inflammatory Markers Associated with Male Genital Schistosomiasis and HIV Co-Infection.

By

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Declaration

I, Emilia Tariro Choto declare that the experimental work described in this thesis was carried out in the Biochemistry department, Veterinary Biochemistry Lab and at Parirenyatwa Medical Flow Cytometry at the University of Zimbabwe from January 2015 to November 2016. The work represents my original work and has not been reproduced from elsewhere or someone’s work. The use of other people’s work has been fully acknowledged in the text.

Signed…………………………………………………………………………………………………………………………

Date…………………………………………………………………………………………………………………………
Abstract

Schistosomiasis is a diseases caused by a water-borne parasitic trematode of different species of the genus *Schistosoma*. Male genital schistosomiasis is an infection caused by *Schistosoma haematobium* species which releases eggs into the reproductive tissues of the bladder. The released eggs cause granuloma lesions that induce an inflammatory response that include activation of CD4+ immune cells in the genitals leading to expression of receptors required by the HIV virus to attach and gain access into other cells. The prevalence of schistosomiasis and human immunodeficiency virus (HIV) are geographically concurrent and co-infection can have a higher unrecognized risk for progression and acquisition of the virus. In this study assessing CD4+ counts and the HIV viral load of infected individuals is important to know the impact that male genital schistosomiasis has on HIV progression. Assessing genetic variability of the pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α) and anti-inflammatory cytokine interleukin 10 (IL-10) in male individuals is a key factor in determining if there is an increased risk of HIV progression and transmission during co-infection. Samples (138) were collected from male of reproductive ages that are residing in Ngundu, Masvingo province. Individuals were initially screened for *S. haematobium* infection by urine filtration techniques and egg counts using a light microscope. Human immunodeficiency virus infection was determined by information from OI clinics and diagnosis was done voluntarily by serology using HIV rapid kits. The CD4+ counts in plasma and seminal viral loads were quantified using cell flow cytometry pre- and post-treatment with praziquantel. Cytokine levels in plasma were determined using ELISA methods. Extracted DNA from blood was used to detect single nucleotide polymorphisms of promoter sites -308 G/A of TNF-α and IL 10 G/A at position -1082 using Amplification Refractory Mutation System Polymerase Chain Reaction (ARMS-PCR). Plasma and seminal viral load decreased and CD4+ counts increased post treatment to prove the unknown impact of male genital schistosomiasis on HIV progression. The higher the infection intensity of male genital schistosomiasis, the higher the levels of TNF-α (P=0.51). Single nucleotide polymorphisms of the promoter site -308A/G TNF-α dominant A allele had the lowest frequency (11%) in the population but had the highest mean concentration levels (p=0.345). Interleukin 10 promoter position -1082 dominant G allele associated with the highest cytokine production had highest frequency but the cytokine levels were notably not different (p=0.44). Human immunodeficiency virus infected only individuals had lower TNF-α levels than MGS and HIV co-infected individuals as a result of MGS infection causing a continuous inflammatory environment therefore indirectly adding in HIV replication and progression. The levels of the TNF-α in circulation had an effect in the CD4+ counts showing that continuous inflammation as a result of male genital schistosomiasis infection has an effect on HIV infected individuals.

Extended inflammatory condition as a result of schistosomiasis infection indirectly activates latent HIV thereby increasing progression. The results showed that male genital schistosomiasis infection has an effect on the viral loads and CD4+ counts of HIV infected individuals. The study on host immuno-genetics of IL-10 and TNF-α for schistosomiasis and HIV co-infection did not have a significant effect on the cytokine levels. However an increase or decrease in particular cytokine levels as a result of genotypic trends may contribute to understanding the underlying mechanisms driving transmission and progression in areas prevalent with co-infection.
Dedication

I dedicate my work to my loving and beautiful mother, Ms. Margaret Chikoto and to my biggest motivator, my sweet son Junior Choto.
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<tr>
<td>α</td>
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<tr>
<td>γ</td>
<td>Gamma</td>
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<tr>
<td>°C</td>
<td>Degrees Celsius</td>
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<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
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<td>APC</td>
<td>Antigen presenting cells</td>
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<tr>
<td>ARMS</td>
<td>Amplification Refractory Mutation System</td>
</tr>
<tr>
<td>BP</td>
<td>Base Pairs</td>
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<td>CD4+</td>
<td>Cluster of differentiation 4</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme Linked immunosorbent assay</td>
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<tr>
<td>EDTA</td>
<td>Ethlenediaminetetraaceticacid</td>
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<td>Fig</td>
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<td>HIV</td>
<td>Human Immuno-deficiency virus</td>
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<tr>
<td>IFN-</td>
<td>Interferon</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>KDa</td>
<td>Kilo Dalton</td>
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<tr>
<td>mAb</td>
<td>Monoclonal antibodies</td>
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<tr>
<td>MGS</td>
<td>Male genital schistosomiasis</td>
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<tr>
<td>mL</td>
<td>Millilitre</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
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<tr>
<td>NTDs</td>
<td>Neglected tropical diseases</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OI</td>
<td>Opportunistic infections</td>
</tr>
<tr>
<td>OPD</td>
<td>Ortho-phenyldiamine dihydrochloride</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>S.h</td>
<td><em>Schistosoma haematobium</em></td>
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<tr>
<td>SNP</td>
<td>Single nucleotide polymorphisms</td>
</tr>
<tr>
<td>Th1/2</td>
<td>T helper Cell 1/2</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory thymus cell</td>
</tr>
<tr>
<td>μL</td>
<td>Microlitres</td>
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<tr>
<td>μg</td>
<td>Micrograms</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra Violet</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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CHAPTER 1

1.0 INTRODUCTION

Neglected tropical diseases have been defined as diseases that mainly affect the poor and marginalized populations living in low resources areas (WHO, 2016), normally with poor water and sanitation. Schistosomiasis is one of the devastating neglected tropical diseases that has been in the top ten diseases that cause admission in Zimbabwean hospitals (Midzi et al., 2014). Male genital schistosomiasis (MGS) has become a major health concern as a factor for human immunodeficiency virus (HIV) transmission among the male reproductive age group (Kjetland et al., 2014). In the past decade, the HIV endemic has emerged in full scale and has affected hundreds of millions of people worldwide and 25 million people are infected with the virus in sub-Saharan African Region (Erikstrup et al., 2008). The number of people who are co-infected with HIV and MGS is not known however in some areas of Zimbabwe with high prevalence of Schistosomiasis and HIV there is a high chance of co-infection incidences. Very little information on MGS regarding HIV transmission is known (Feldmeier et al., 1994) and it is important to acknowledge the association and the impact of MGS on HIV transmission. As a result of MGS infection, eggs penetrate vessel walls and move into the bladder and the genital tissues. In response to the embedded eggs, the body will orchestrate granuloma lesions formation in the genitals causing genital sores, which will result in abnormal blood vessels (Stecher et al., 2015, Pearce and MacDonald, 2002). This inflammatory response will result in ejaculatory pain, haematospermia, frequent urination, lumpy semen and leukocytospermia in men (Richens, 2004, Mbabazi et al., 2011, Stecher et al., 2015). The egg-induced inflammation will result in a great number of activated CD4+ immune cells in the genitals (Leutscher et al.,
2005). These CD4+ cells will express other receptors to bind and receive signals from different cytokine receptor that are also important for the HIV virus to attach and gain access into other cells (Stecher et al., 2015 and Kjetland et al., 2006) hence possibly an increment in the propagation rate of the virus. More HIV infected cells from the peripheral blood could be able to mix with the semen through eroded epithelium or broken blood vessels of the granuloma lesions (Kjetland et al., 2006) causing an increment of HIV replication. Therefore co-infection with MGS and HIV could cause an increase in inflammation possibly resulting in an increment of HIV progression than an individual with HIV infection only.

1.1.0 Cytokines in the Immunopathology of MGS

Cytokines are strongly associated with schistosomiasis infection, pathogenesis and the infection intensity. The Schistosoma adult worms/trematodes will reside in the pelvic region of an individual where it will reproduce and release some eggs/ova. Some of these eggs are passed out during urination and some eggs are embedded into the tissues surrounding the pelvic area such as the reproductive forming granuloma lesions. The granuloma lesions will contain different types of immune cells to fight off the eggs and they release cytokines which act as signaling molecules or immune messengers to recruit other immune cells to fight off the infection. However the cytokines also act as a suppressant signal to stop the production of other cytokines by different cells to control and balance inflammation. Cytokines are produced in response to cellular immunity cells known as Thymus helper 1 (Th1) and adaptive immunity mostly by thymus helper 2 (Th2) cells. A Th1 response is involved in acute infection and a Th2 response is mostly involved during chronic infection of schistosomiasis (Burke et al., 2009)
1.1.1 Single Nucleotide Polymorphisms (SNPs)

Single nucleotide polymorphisms are the most abundant and common form of genetic variation that occurs at approximately 1 in every 1000 bp (Mein et al., 2000, Anoosheh et al., 2011) and deoxyribonucleic acid (DNA) sequence differences are part of molecular genetics studies. Single nucleotide polymorphisms are variations that occur in the DNA sequence where one nucleotide sequence differs from the norm and they are markers of individuality but they do not have an effect on our health. However they can also be associated with how we respond to different drugs hence they are widely used in pharmacogenetics. These SNPs can fall in the coding or non-coding sequence of the DNA; sometimes they may not necessarily affect the protein coded. Cytokines are encoded by highly polymorphic genes and there is evidence of the cytokine produced by an individual as a genetically predetermined phenotype (Hajeer and Hutchinson, 2000). Single nucleotide polymorphisms in the promoter regions of some cytokines specify for low, medium and high cytokine production. In this study, the promoter sequence SNPs effects were determined if they have been known to have an effect on the production of IL-10 promoter region -1082A/G, of which the G allele have been associated with increased production of IL-10 and while -1082 A allele is associated with low levels (Afzal et al., 2011). Tumor necrosis factor-alpha promoter region -308 G/A, the A allele is known to be the dominant allele and it is associated with higher cytokine production than the G allele. These polymorphisms can help understand the severity of inflammation during schistosomiasis infection and also in other infections.

1.1.2 Antibodies in the Immunopathology of Male Genital Schistosomiasis

Antibodies are glycoproteins known as immunoglobulins that are produced by activated B-lymphocytes (B-cells) and they are part of the humoral acquired immunity. They are found in
bodily fluids such as blood and plasma also known as humors. Antibodies work in three ways in which they bind and neutralize the antigens, they also bind to the pathogen so as to opsonize the antigen hence inactivating the antigen and they activate the complement system so that it removes or destroys the pathogen. Different isotypes of antibodies work against different infections and hence they are considered to be specific. Antibodies present in the body directly reflect the infection that individual has possibly been exposed to. Immunoglobulins isotypes G and E have been shown to be elevated levels as a result of schistosomiasis and HIV infections (Tomaras and Haynes, 2009, Zhang and Mutapi, 2006).

1.1.3 Justification

Schistosomiasis infection results in inflammation that causes a continuous production of the inflammatory cytokines, possibly leading to an increment of viral loads and indirectly causing an impact on HIV transmission and diseases progression.

1.1.4 Hypothesis

Male genital schistosomiasis and HIV co-infection increases inflammation resulting in an unknown impact in increasing HIV progression and transmission.

1.1.5 Main Objective

To assess the markers of inflammation in S. haematobium and HIV co-infected individuals as an indirect risk factor for HIV transmission and progression.

1.1.6 Specific Objectives

1. To determine CD4+ cell counts, and HIV viral load (HIV-RNA) in semen and plasma of HIV and S. haematobium co-infected individuals.

2. To determine concentrations of TNF-α and IL-10 cytokines.
3. To assess genetic variability of the promoter positions of pro-inflammatory cytokine TNF-α position -308A/G and anti-inflammatory cytokine IL-10 position -1082G/A.

4. To determine the profiles of the HIV and S. haematobium specific antibodies. (IgE, IgG1, IgG4 and IgG) in the selected individuals.

1.2 LITERATURE REVIEW

1.2.1 Male Genital Schistosomiasis (MGS)

Schistosomiasis commonly known as bilharzia, is a disease caused by water-borne parasitic trematodes of six different species of the genus Schistosoma that are known to cause diseases in humans. These species are S. mansoni, S. japonicum, S. haematobium, S. intercalatum, S. makongi and S. guineensis (Colley et al., 2014). Schistosoma haematobium species infection is known as urogenital schistosomiasis (Female genital schistosomiasis and male genital schistosomiasis) because it causes damage to the urinary and genital tissues. Male genital schistosomiasis (MGS) has become a major health concern as a factor for human immunodeficiency virus (HIV) transmission among the male reproductive age group. The S. haematobium worms will reside in the venous plexus surrounding organs of the pelvis and lay eggs from about 300 to 3000 daily (Mbabazi et al., 2011). The eggs will penetrate the vessel walls and move into the bladder and result in some eggs being embedded into the tissue of the prostate glands, seminal vesicles and urinary bladder and this will induce a continuous inflammatory response. In response to the embedded eggs, the body will orchestrate granuloma lesions formation in the genitals causing genital sores (Stecher et al., 2015, Pearce and MacDonald, 2002) and this will result in clinical outcomes.
1.2.2 Epidemiology of Schistosomiasis

Schistosomiasis is a neglected tropical disease (NTDs) which have been defined as a disease that mainly affects the poor and marginalized populations living in low resources areas (WHO, 2016). Schistosoma parasite likelihood of infection has been linked to specific geographic locations which are normally areas with low socioeconomic conditions, poor water and sanitation where people are in the habit of urinating and defecation in the water and at the same time exposing themselves to this polluted water by bathing, swimming for recreation, washing utensils and clothes, walking bare-foot during irrigation, fishing and cattle herding. These are the areas where neglected tropical diseases are prevalent. Schistosomiasis has affected people in 76 countries and more than 200 million people are infected (Adenowoa et al., 2015) and it is prevalent in the tropical, and sub-tropical areas of Africa, Asia, South America and the Caribbean (El Saadany, 2008). Seven hundred and fifty million people are at risk of being affected with schistosomiasis (WHO, 2015a) because they reside in or close to areas that are schistosomiasis endemic. It is so unfortunate that 85% of the affected people reside in Africa (Kejtland et al., 2006). Schistosoma haematobium is endemic in 53 countries mostly in the Middle East and in sub-Saharan Africa (Chitsulo et al., 2000) and has affected 100 million people (Fenwick et al., 2003). Despite the fact that the disease is globally targeted to control its morbidity an estimated 20 thousand to 200 thousand people continue to die on a yearly basis (WHO, 2015b).

1.2.3 Life Cycle of Male Genital Schistosomiasis

There are two stages of Schistosoma haematobium life cycle:

1. The asexual stage is in the intermediate host (snails) specifically the Bulinus snail species.

2. The sexual stage in the definitive host (the humans).
1.2.3.1 Asexual Stage

The ova/eggs which are released by the *S. haematobium* adult paired worms residing in the pelvic area, are passed out in urine into the water where they will hatch into free living ciliated miracidia after a short period of incubation as. The miracidia will penetrate the bulinus or biomphalana snail species (Colley *et al*., 2014) where miracidia will lose its glycocalyx and develop into primary sporocysts. The primary sporocysts develop into secondary sporocysts and mature in the snail’s liver and become cercariae larva that has a tail, shown by figure 1 during the aquatic stages. This takes about 3 to 5 weeks then the cercariae will leave the snail in search of a human host. (Bamgbola, 2014).

1.2.3.2 Sexual Stage

Using its tail, the cercarie will attach to the epidermis of the human skin, penetrates the skin, gains entry inside the body and it loses its glycocalyx tail. The cercariae will then form a layer that protects and evades the human immune system as it becomes a schistosomula. The schistosomula will migrate through the venous circulation to the heart and the lungs and enter the portal hepatic circulation. The female and male worms eventually reach maturity and mating occurs where the female resides in the gneacophoric canal of the male (Colley *et al*., 2014). The adult worms will continue to copulate within the vessels and eventually migrate to the mesenteric venous plexus around the pelvic area such as the urinary bladder and ureters. The adult worms shown in figure 1 during intra-mammalian stages are known to be able to survive to up to 30 years inside the human body (El-Saadany, 2008, Bamgbola, 2014).
1.2.4 Clinical Manifestations of Male Genital Schistosomiasis

Due to the cercarial tail penetration into the human skin it causes cercarial dermatitis which is also known as the swimmers itch, itchy papules and local edema that appears within 12 hours (Akl, 2010). Acute schistosomiasis syndrome occurs between a week to 12 weeks post infection as a result of migration and maturation of the Schistosoma worm and egg deposits in the tissues. The eggs will penetrate the vessel walls and move towards the bladder, these eggs are found in the tissues of the pelvic organs such as the urinary bladder, lower ureters, prostate glands and seminal vesicles (Mbabazi et al., 2011). The cross reaction between the immune system and onset of the earliest egg production and the worm antigens will result in Katayama fever, snail fever and peripheral eosinophilia and the symptoms include chills, fever, headache, nausea, vomiting, diarrhea, dry cough, hepatosplenomegaly, lymphadenopathy. During the acute stage of
Schistosomiasis some eggs produced by the adult worms will be embedded inside the host tissues that will end up being incapacitated by the immune cells resulting in granuloma lesions mediated by T-cells. Chronic schistosomiasis is after 16 weeks post infection which will be an immune modulation of the granulomatory response to benefit the host elevated humoral response (Akl, 2010) therefore acute symptoms will become less intense. Chronic symptoms with time will include hematuria (blood in urine), ureteral dilation, hydrophrosis, bladder mucosa, bladder wall calcification which can result in frequent urination, urgency, dysuria, calcified semen (Bamgbola, 2014), orchitis, ejaculatory pain, haematospermia and leukocytospermia (Mbabazi et al., 2011). Other further complications will include urinary tract infections, sexual dysfunction, prostatitis, male infertility complications (oligospermia) and bladder cancer known as squamous cell carcinoma (SCC) which has a 30 fold higher risk of developing than an individual without male genital schistosomiasis (Bamgbola, 2014).

1.2.5 Immunopathology of Male Genital Schistosomiasis

*Schistosoma haematobium* initial infection causes acute infection of the host and initiate a cascade of immune reactions activating almost all parts of the immune defense system from innate to natural immunity. As the infection progresses to chronic infection the immune reaction will result in other immune responses such as the adaptive immune response.

1.2.5.1 Innate and Natural Immune Response to *Schistosoma haematobium*

*Schistosoma* cercariae which has penetrated an individual avoids destruction by the membrane attack complex by shedding its glycocalyx outer covering hence will be able to evade an attack from the immune system. The schistosomulae will adopt the host derived outer membrane to prevent elimination by the T helper 1 cells. As the schistosomulae mature into adult worms they offer the host immune system soluble worm antigen preparation (SWAP) but the immune
response to adult worms is not intense as opposed to soluble egg antigens (SEA) produced by the worm eggs. *Schistosoma haematobium* infection is not apparent until the egg deposit into the host tissue occurs however the immunity is unidentified (Odegaard and Hsiesh, 2014). During acute stages of schistosomiasis there are number of the eggs that are not excreted through urination but instead are trapped in the tissues of the pelvic areas and elicit the recruitment of innate immune cells such as the antigen presenting cells (APC) like the dendritic cells which are important for Th2 response and natural killer (NK) cells which result in primary and secondary immune response as shown in figure 2, basophils, mast cells which are the primary effector cell for IgE and macrophages which have a critical role of initiating and guarding the overall innate immune response resulting in maintaining and amplifying the inflammation response (Odegaard and Hsiesh 2014). Some of these cells will secrete tumor necrosis factor-alpha (TNF-α), interferon gamma (IFN-λ) and eotaxin that is believed to attract eosinophils and other type one (Th1) cytokines (Bamgbola 2013, Odegaard and Hsiesh 2014). Eventually the eggs will be surrounded by the eosinophils, a large amount of macrophages, giant cells and epithelioid macrophages forming granulomas (Odegaard and Hsiesh 2014). The immune system will attempt to eliminate the deposited eggs by the shift in immune response to pro-fibrotic T helper 2 cells (Bamgbola 2013).

**1.2.5.2 Adaptive Immune Response to Schistosoma haematobium**

As schistosomiasis becomes chronic, macrophages form the structural bulk of granulomas, T cells also infiltrate the egg exposed tissues to restrain the inflammation response (Odegaard and Heish, 2014). T helper cells play a critical role in both local and systemic response to infection. Th1 associated cytokines become less as Th2 cytokines elevate. Th2 associated cytokines such as IL-4, IL-5 which is essential for recruitment and maintaining eosinophils though IL-5 and IL-13
promotes fibrosis (Odegaard and Hseish, 2014), IL-8, IL-6, Th17 associated cytokines such as IL-17 and IL-23 are associated with increment in mortality and morbidity of the *S. haematobium* infection. Soluble egg antigens also recruit IL-10 which is produced by the Th2 cells T regulator cells (Treg) suppresses the T cells response therefore controlling the rate of inflammation. Some of the cytokines also recruit bone marrow cells (B cells) which are involved in infection clearance and resistance. The B cells will produce anti-schistosome immunoglobulins (Ig). Antibodies IgE, IgG1 and IgG3 in schistosome infection are associated with the reduction to susceptibility to reinfection whilst IgG4 has been associated with reinfection susceptibility (Colley and Secor, 2014).

**Figure 2:** Immune response from acute to chronic infection of *S. haematobium*.

**1.2.6 Treatment of Male Genital Schistosomiasis**

There are a number of drugs used to treat schistosomiasis which include niridazole, metrifonate, artemisinin derivatives and praziquantel. Praziquantel is considered the best treatment of choice (Ojurongbe *et al.*, 2014, Colley *et al.*, 2014) for its effectiveness, easy to administer, has low toxicity, low intense side effects, safe for pregnant women and it is also relatively inexpensive.
(da Silva, 2005, Doenhoff et al., 2008). The drug is administered at 40mg/kg in one oral dose and has been successfully used over 30 years (WHO 2011). The drug is currently used as a strategy against schistosomiasis morbidity control in school aged children and highly effective against the five schistosome species that infect humans (Ojurongbe et al., 2014). Praziquantel is believed to induce muscle contraction and tegumental disruption that leads to exposure of the surface antigens of Schistosoma trematode to the host immune system for total worm destruction (Tallima and Ridi, 2007).

1.2.7 Human Immunodeficiency Syndrome (HIV)

Human immunodeficiency virus is a virus that infects immune cells causing an immune deficiency of the infected individuals. The virus attacks and destroys the individuals immune system cells specifically the CD4+ cells. Without the CD4+ cells such as the macrophages and the T cells that are the central immune mediators, it makes it difficult for the immune system to fight off infections and this places the infected individual at risk for other life threatening infections and cancer (AIDS, 2012). An HIV infected individual can live with the virus without showing any symptoms for so many years until the viral load is very high and the CD4+ cell counts are low, leading to acquired immune deficiency syndrome (AIDS). AIDS is the advanced stage of the HIV infection status at which the individuals’ immune system cannot fight any infection leading to death. HIV is transmitted through HIV infected blood, semen, genital fluids and breast milk. In the past decade, the HIV endemic has emerged in full scale and has affected hundreds of millions of people worldwide and 25 million people are infected with the virus in sub-Saharan African Region (Erikstrup et al., 2008). It is believed that heterosexual transmission has been the cause for the HIV endemic. Even though HIV prevalence has declined in the sub-Saharan African countries (Gregson et al., 2007), Zimbabwe is the 5th highest HIV
prevalence in Africa (AvertInfoHIV, 2016, AIDS, 2015). It is believed that around 1, 4 million people are living with HIV in Zimbabwe, 15 % of the adults are infected with HIV and 51% of the adults are on antiretroviral treatment (ARV) (Avert Info HIV and AIDS, 2015).

Unfortunately and coincidentally high prevalence of Schistosomiasis and HIV are geographically concurrent (Kallestrup et al., 2006) in Zimbabwe.

1.2.8 Association of Male Genital Schistosomiasis to HIV

The number of people who are co-infected with HIV and MGS is unknown however in some areas of Zimbabwe with high prevalence Schistosomiasis and HIV there is a high chance of coinfection incidences. It is important to acknowledge the association and the impact of MGS on HIV transmission. The Schistosome egg induced inflammation which is orchestrated by Th cells will result in a great number of activated CD4+ immune cells not only in the plasma but also in the genital fluids (Leutscher et al., 2005). The schistosome egg and its excreta will attract the immune cells resulting in these immune cells and cytokines surrounding the schistosome egg resulting in the formation of granuloma. Among the immune cells, the CD4+ cells such as the T cells and the macrophages will express the chemokine receptors CCR5 and CXCR4 which are specific receptors that bind to certain cytokines but these chemokines are very necessary for the HIV virus to attach and gain access into other cells (Stecher et al., 2015 and Kjetland et al., 2006) leading to an increment in the propagation rate of the virus and destruction of the immune cells. More HIV infected cells from the peripheral blood could mix with the semen through eroded epithelium or broken blood vessels of the granuloma lesions (Kjetland et al., 2006) which will result in the HIV virus being in contact with immune cells for replication purposes. Therefore co-infected individuals with MGS and HIV could have an accelerated HIV
propagation rate in seminal fluids and plasma which will lead to an increase in viral load and rapid advancement to HIV AIDS than a HIV infection only.

1.2.9 Cytokines

Cytokines are short low molecular weight peptides of less than 30 kDa (Zhang et al., 2007, Khan, 2008). They serve as signaling agents between cells that modulate the immunity, inflammation and hematopoiesis. They are released by cells and have high affinity to receptors found on surfaces of the cells and cause specific effects on the interactions between cells. Cytokine is considered a generic name because different types of cells produce different molecules such as lymphokines produced by lymphocytes, monokines produced by monocytes, chemokines produced by chemotactic actins and interleukins produced by leucocytes however they are all considered cytokines. Cytokines also include interferons, colony stimulating factors and growth factors. They act in a cascading way and they are involved in the initiation of the production of other cytokines as well as inhibition of other cytokines resulting in an appropriate and necessary immune response to different infections. Different immune cell types can secrete the same cytokine and the same cytokine can act on different cell type a condition known as pleiotropy. The cytokines act in different ways which are autocrine, paracrine and endocrine. Autocrine manner is when they act on cells that act on them, paracrine is when they act on cells nearby and endocrine they act on distant cells. Cytokines are important in health and disease, host response to infection, immune responses, inflammation and trauma. They are grouped into pro-inflammatory cytokines and anti-inflammatory cytokines.

1.2.9.1 Pro-inflammatory Cytokines

These are cytokine involved in initiating and up regulating inflammatory reactions caused by injury and infections. Most of these cytokines are produced by activated macrophages and these
cytokines can recruit more HIV infected cells to semen upregulating viral replication and increases concentration of the HIV viral load in semen (Kjetland et al., 2012).

Table 1: Examples of pro-inflammatory cytokines and their mode of actions.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Source</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1</td>
<td>Macrophages, dendritic cells, endothelial cells</td>
<td>TH and B cells and various other tissues Activation</td>
</tr>
<tr>
<td>IL-2</td>
<td>TH1 cells and NK cells T cell</td>
<td>NK proliferation and induction of activity</td>
</tr>
<tr>
<td>IL-4</td>
<td>TH2 cells, mast cells, NK cells B cells, T cells, mast cells, macrophages</td>
<td>Proliferation, isotype switching, induction of MHC class II expression</td>
</tr>
<tr>
<td>IL-6</td>
<td>Neutrophils, Macrophages, TH2 cells Plasma cells, B cells</td>
<td>Differentiation and antibody secretion</td>
</tr>
<tr>
<td>IL-12</td>
<td>Macrophages, B cells TC, NK and LAK cells</td>
<td>Proliferation and differentiation in synergy with IL-2</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Th1, Macrophages Tumor cells, polymorphonuclear leukocytes, macrophages</td>
<td>Cytotoxicity, induction of cytokine secretion</td>
</tr>
</tbody>
</table>

1.2.9.1.1 Tumor Necrosis Factor- Alpha (TNF-α)

It is a pro-inflammatory cytokine that does not cause necrosis of tumors but instead can stimulate the augmentation of some tumors. Tumor necrosis factor-alpha gene is located on chromosome 6 at q21.1-21.2 (Al-Mohaya, 2015). It is secreted as a 25 kDa protein that is cleaved by the metalloprotease enzyme to a 17 kDa functional protein. It is mainly produced by macrophages and Th2 cells, it activates inflammation, cellular differentiation and it regulates signaling pathways resulting in apoptosis. Tumor necrosis factor-alpha is known to intensify the major histocompatibility class (MHC) 1 molecules against endogenous viruses and it induces acute phase responses by activating other molecules that can be detected in response to an infection such as C-reactive proteins produced by the hepatocytes. It is mainly known to be produced in
response to pathogen infections resulting in inflammation and it increases the translocation of immune cells to site of infection or damaged tissues by increasing expression of adhesion molecules (Elahi et al., 2009). It is also believed to activate HIV-1 in chronically latent HIV infected T-cell (Kumar et al., 2013) resulting in increased viral replication and depletion of CD4+ T-cells due to the replicating virus. Tumor necrosis factor-alpha has been associated indirectly to adding in viral replication by translocation of transcriptional activating factor called NF-KB23 which will bind to the long terminal repeat (LTR) of the HIV provirus hence transcribing the production of viral proteins such as tat and Nef necessary for the viral replication (Gounden et al., 2012 and Kumar et al., 2013). Tumor necrosis factor-alpha contributes to progression of schistosomiasis infection due to formation and maintenance of granulomas as well as adding in removing bacteria in the granuloma lesions which contain CD4+ T cells that are clonally expanded due to stimulation of high TNF-α concentrations therefore influencing HIV-1 replication.

1.2.9.1.2 TNF-α -308A/G (rs1800629) Polymorphism

Tumor necrosis factor-alpha -308A/G polymorphisms involves the substitution of the nucleotide Adenosine (A) wild type to Guanine (G) mutant in the promoter region located upstream of the transcribed gene at base number 308. Promoter polymorphisms control gene expression (Gounden et al., 2012). Tumor necrosis factor-alpha concentrations is controlled by the genotypic variations which may influence transcriptional regulation (Wang et al., 2008 and Gounden et al., 2012). Transcriptional activation is controlled by the proximal promoter region which result in differences in gene expression and protein secretion. Single nucleotide polymorphisms in the -308 promoter site G allele is associated with low TNF-α cytokine production and the A allele is associated with high cytokine production because it is believed
that the G to A nucleotide substitution changes the gene conformation in turn will have a higher binding affinity to transcriptional factors (Gounden *et al.*, 2012). Polymorphisms can affect the cytokine levels and how the disease progresses.

**1.2.9.2 Anti-Inflammatory Cytokines**

These are immuno-regulatory cytokines that regulate the pro-inflammatory cytokine response therefore down regulating and modulating the inflammatory response. These cytokines can also be multifunctional depending on the infection and the infection intensity.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Source</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL 10</td>
<td>TH2 cells Macrophages, APC , Treg</td>
<td>Anti-inflammatory cytokine inhibits other cytokine production</td>
</tr>
<tr>
<td>IL- 13</td>
<td>TH cells Macrophages, B cells</td>
<td>Regulation of inflammation, parasitic infections, Inhibition of inflammatory cytokines</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Treg,</td>
<td>Multifunctional: regulates cellular responses, cell differentiation, apoptosis, and response to angiogenesis, modulates inflammation pathways and differentiates cells.</td>
</tr>
</tbody>
</table>

**1.2.9.2.1 Interleukin 10 (IL-10)**

Interleukins are cytokines produced by leukocytes mostly CD4+ T lymphocytes as well as monocytes and macrophages and endothelial cells. Interleukin 10 is a 36 kDa glycoprotein with two 160 amino acids long chains and its gene is located on chromosome 1 on the long arm between 31 and 32 q31-32 and with 5 exons (Khan *et al.*, 2008). Interleukin 10 is secreted by Th2 cells, dendritic cells, B cells, Treg and Th17 cells. It is a multi-effect and anti-inflammatory cytokine and it regulates the immune system by inhibiting the secretion of other pro-inflammatory cytokines such as IL-2, IL-3, and TNF-α, IL-12 which are mostly produced by the
Th1 cells and antigen presenting cells such as macrophages and dendritic cells. It also inhibits cytotoxic T cells (CTL) production thus could inhibit the destruction of viral infected cells. IL-10 regulates the balance between the Th1 and Th2 immune response by promoting mast cells, B cells and Th2 responses and inhibiting Th1 immune response. The cytokine increases the intensity of receptors of other cytokines such as TNF-α and IL-1 and down regulates MHC class II molecules which are responsible for virus antigen presenting to CTL for destruction of the infected cells. During Schistosomiasis infection, IL-10 regulates the acute Th1 adaptive immunity resulting in a Th2 cell mediated immunity response as the schistosomiasis infection progresses to chronic state. (Spellberg and Edwards, 2001)

**1.2.9.2.2 IL-10 -1082G/A (rs18000896) Polymorphism**

Interleukin 10 -1082G/A polymorphisms involve the substitution of the nucleotide wild type Guanine (G) to mutant Adenosine (A) allele in the promoter region located upstream of the transcribed gene at base number 1082. Interleukin 10 promoter polymorphisms are associated with increased or decreased transcription rate resulting in affecting production rate of the cytokine. The G allele at -1082 is known to have high cytokine production and the A allele is associated with low cytokine concentrations (Turner *et al.*, 1997).

**1.2.10 Antibodies**

Antibodies are immunoglobulins (Ig) that are present in the body at certain level they are produced in response to certain antigen or epitopes of an infection. They are Y shaped molecules with variable regions that can fold symmetrical to form two identical heavy and light chains. The heavy and the light chains are bound by disulfide bonds and non-covalent bonds. The amino arm region of the Y shaped antibodies is known to be the variable region that consists of a heavy and light chain combination to form identical specific antigen binding sites (Janeway *et al.*, 2005).
There is a carboxyl end that is known as the constant or Fc region. Antibodies are considered to be a lifelong protective immunity and there are 5 major isotopes of antibodies which are immunoglobulin G (IgG), IgA, IgE, IgD and IgM. These isotopes differ in the heavy chain region which determines their structure and the function.

1.2.10.1 Antibody IgE

Antibodies isotope IgE is a major prominent antibody in schistosomiasis infection which is stimulated by a Th2 secreted cytokines such as IL-4 and IL-13 (Zhang and Mutapi, 2006). It has a molecular weight of 188 kDa consisting of two light chains and two heavy chains which have an additional carboxyl domain. The antibody IgE is mostly associated with allergic reactions and having a protective immunity. It is also considered to have a defense mechanisms against antigens such as parasites too large to be engulfed by phagocytes. The antibodies IgE is known to respond against schistosome trematodes and antigens and associated with resistance to re-infection (Negrao-Correa et al., 2014). Antibodies IgE serum levels are associated with early Th1 immunity and the higher the IgE levels indicate the greater the schistosomiasis infection intensity.

1.2.10.2 Antibody IgG

This antibody has a molecular weight of 150 kDa, the heavy chain is about 50 kDa and the light chain is about 25 kDa. It has a carboxyl terminal, it is made up of four polypeptide chains and has two antigen binding sites. It is the most abundant antibody, the only isotope antibody that can cross the placenta and enter extravascular spaces therefore protecting tissues and newborns (Janeway et al., 2005 pg. 617). It is present in the highest concentrations in the serum and it is responsible for removal of microorganisms and toxins. There are four sub-groups of the IgG which are IgG1, IgG2, IgG3 and IgG4 (Tomaras and Haynes, 2009). The sub groups differ in
their amino acid sequences and in the number and position of the disulphide bonds that hold the antibody together. IgG is known to initially work against HIV proteins called gag and the gp41 proteins during acute infection (Tomaras and Haynes, 2009). Both IgG1 and IgG4 have a molecular weight of 146 kDa. IgG1 is an antibody which has broad response towards HIV-1 infection both in acute and chronic infection and IgG4 has been shown to block IgE and therefore making an individual more prone to susceptibility to re-infection. IgG4 is predominately working towards antigens during the chronic stage of HIV-1 infection.

1.2.11 ELISA

Enzyme-linked immunosorbent assay (ELISA) is biochemical assay used to detect and quantify the presence of substances such as peptides, proteins, antibodies and hormones. It incorporates an enzyme conjugated with an antibody to react with a substrate to generate a colored product if the substance being tested is available hence the name enzyme linked. The intensity of the color gives an indication of the amount of antigen or antibody detected. The results are obtained using a spectrophotometer by determining optical densities (OD). There are different types of ELISA which include indirect/sandwich ELISA which is used to detect cytokine levels in which standards are incorporated for detection of cytokine concentrations and direct ELISA used to detect antibodies in which negative and positive controls are incorporated. From the negative control cutoff values are determined because they discriminate positively infected samples from negative samples.

1.2.12 ARMS-PCR

Allelic refractory mutation system polymerase chain reaction (ARMS-PCR) is a simple method that is used to detect single nucleotide polymorphisms (SNPs) in DNA. It is a simple, rapid and a relatively low cost PCR method. It does not require any after PCR work unlike restriction
fragment length polymorphisms (RFLP) PCR that involves post PCR work using enzymes to digest the PCR product amplified (amplicon). Amplification refractory mutation system-PCR incorporates two separate PCR reactions to amplify the two different alleles of SNP. In each PCR reaction, two sets of primers are used to amplify the targeted gene however one primer known as the generic reverse primer will be the same in both reaction tubes and the forward primers will be different in which one will be a primer to amplify the mutant allele and the other will be amplifying the wildtype allele and the procedure is illustrated in figure 3.
CHAPTER 2

2.0 MATERIALS AND METHODS

2.1 The Study Design, Area, Population and Recruitment Criteria

The study was conducted at the Biochemistry and Veterinary Science level 1 laboratories on samples that were collected from male reproductive aged (18 to 49) individuals residing in a schistosomiasis endemic area, Ngundu in Chivi district of Masvingo province in Zimbabwe. A total of 138 whole blood samples were collected and they were preserved in potassium EDTA and stored at -80 °C for later use. Urine was collected for parasitological assessment of *S. haematobium* infection. Venous blood was collected for HIV diagnosis, viral load, CD4+ counts and analysis of single nucleotide polymorphisms of infected individuals. Semen was collected for viral load detection and plasma used for cytokines levels determination.

2.2 Urine Collection and Schistosoma haematobium Detection

Male genital schistosomiasis is a proxy of *Schistosoma haematobium* infection which was the basis of initial diagnosis. Urine filtration technique is a process where by approximately 10 mL of urine collected during the day in a clean container was filtered using a syringe attached with a syringe filter holder (swinnex type, 13 mm diameter) containing filters (nytriel filters 13 mm diameter and 12 to 14 μm pore size diameter). The filter holder was carefully unscrewed, using forceps the filter paper was placed on the center of a clean microscope slide making sure the side with the eggs is facing upwards. A few drops of iodine solution were added to the filter paper to stain the eggs for easier visibility. The microscope slides were viewed under a light microscope and the shape of the eggs and the number of the eggs were counted. Patients identified positive
for *S. haematobium* infection were offered treatment with praziquantel given at 40 mg/kg as a single dose.

**2.3 Blood and Semen Collection and HIV Detection**

Whole blood (5 mL) was collected in potassium EDTA tubes according to WHO guidelines and used for CD4+ count, DNA extraction and HIV-RNA detection. Some of the male participants knew their HIV status through the opportunistic infections (OI) clinics of Zimbabwe. Individuals that had unknown HIV status were tested voluntarily using serological rapid HIV antibody determination kit (Bioline rapid tests). Participant’s semen was collected whereby they ejaculated in a clean container at site or in a condom at home and the sample was stored in cryovial at -80 °C.

**2.4 Plasma Harvesting**

Venous blood collected in EDTA tubes were centrifuged at 1800 RPM for 8 minutes to separate the plasma from the whole blood. The supernatant which will be the plasma was retained and stored in a cryovial for cytokine levels quantification.

**2.5 CD4+ Counts and Plasma and Seminal HIV-RNA Detection and Quantification**

Viral load (HIV-RNA) in plasma and semen detection and quantification was done at Parirenyatwa Flow Cytometry Medical Center in Harare using COBAS® Ampli-Prep and COBAS® Taq-Man HIV test machines (Roche Molecular diagnostics) which had a limit detection of <20 copies/mL and 10 000 000 copies/mL. CD4+ counts were also done at Parirenyatwa Flow Cytometry Medical Center in Harare by cell flow cytometry method using a D&B Fascalliber machine.
2.6 Enzyme Linked Immuno-sorbent Assays (ELISA)

ELISA is a direct binding assay for antibodies and can be used to detect certain secreted proteins such as cytokines. Quantification of cytokine levels was done by capture or sandwich ELISA assay. Detection of antibodies was done by direct ELISA.

2.6.1 Sandwich ELISA

Buffers and Reagents

Phosphate saline buffer (PBS, 1X) was made by mixing 8 g Sodium Chloride (137 mM), 0.2 g Potassium Chloride (2.7 mM), 2.9 g di-Sodium Hydrogen orthophosphate anhydrous (10 mM) and 0.2 g Potassium di-Hydrogen Phosphate (10 mM) to 1 litre of distilled water and the pH was adjusted to 7.4 with an automatic pH meter. Tris buffered saline (TBS, 1X) was made by mixing 6.05 g of Tris (50 mM) and 8.76 g of sodium chloride (150 mM) mixed to 1 litre of distilled water and the pH was adjusted to 9.6 by an automatic pH meter. Phosphate citrate buffer (1X) was made by mixing 7.19 g di-Sodium Hydrogen orthoPhosphate anhydrous and 5.19 g citric acid to 1 litre distilled water and the pH was adjusted to 5.0 by an automatic pH meter. The wash buffer was made by adding 1 litre TBS in a reagent bottles and mixing with 400 μL Tween-20 (0.04%). The blocking buffer was made by adding 0.5% bovine serum albumin (BSA) and 1 litre of TBS. The incubation buffer was made by mixing 0.5% BSA with 1 litre of TBS and 0.04% Tween-20. The substrate used was 0.4 mg/ml ortho-phenyldiamine dihydrochloride (OPD) which was added to phosphate citrate buffer and 30% hydrogen peroxide (H₂O₂) at concentration of 1 μg/L. Cytokine quantification kit for Human IL-10 and TNF-α ELISA development kit purchased from Mabtech Company from Sweden.

2.6.1.1 Interleukin 10 and TNF-α Quantification
A 96 well microtiter plate (Nunc MaxiSorb, Denmark) was coated with 100 μL in each well of specific cytokine monoclonal antibody (capture mAb) diluted 2 μg/mL in PBS and incubated overnight at 4 °C. After 24 hours the plate was washed twice with TBS (pH 7.4) and the wells were blocked by adding 200 μL of blocking buffer to block any nonspecific binding sites. The plate was left to incubate for 1 hour at room temperature on shaker at 200 rpm, then was washed 5X with TBS containing 0.05% Tween-20 (wash buffer) and gently tapped to remove excess blocking buffer. One hundred micro litres of the different samples and standards were added in the wells in distinctive known columns and rows. The plasma in the plate were left to incubate overnight at 4 °C to allow maximum binding of the cytokine to the capture antibody. The plasma was washed 5X with wash buffer to remove any unbound cytokines and tapped gently on a multi wipe to remove any excess wash buffer. Incubation buffer was mixed with biotinylated monoclonal antibody (detecting mAb) at 1 μg/mL and 100 μL of the mixture was added to each well to bind to the cytokine bound to the captures antibody and incubated for 1 hour. Streptavidin with horse-radish peroxide attached (conjugate mAb, enzyme-linked antibody) was mixed with incubation buffer at the ratio of 1:1000. One hundred microliters of the conjugate antibody used to quantitate the amount of bound detecting antibody was added to each well and left for incubation for 1 hour. After an hour the plate was washed 5X with the wash buffer and the and gently tapped to remove any excess wash buffer. Freshly prepared substrate was (100 μL) was added to each well and the plates were left to incubate in the dark for 30 minutes. If the enzyme linked antibody had bound to the antibody, the enzyme will catalase the substrate therefore a color change will occur. The absorbance of the samples were taken using a spectrophotometer at 450 nm using an ELISA plate analyzer (micro tire plate reader, Inqaba Biotec). (Protocol adapted from Mabtech Company manufactures instructions).
2.6.2 Direct ELISA

Reagents and Buffers

Antigens mixed in PBS were used for coating the plates and the following concentrations: HIV-1 MPER (gp41) at 5 mg/mL, HIV-1 gp120 at 5 mg/mL (AnaSpec Inc.) and Sh13 at 5 μg/ml (synthesized at Glasgow University). The antigen Sh13 is a recombinant protein antigen used to detect anti-Schistosomiasis antibodies in infected individuals. Phosphate saline buffer (PBS 1X) was made with 8 g Sodium Chloride, 0.2 g Potassium chloride, 2.9 g di-Sodium Hydrogen orthophosphate anhydrous and 0.2 g Potassium dihydrogen Phosphate were added to 1 litre of distilled water and the pH was adjusted to 7.4 by an automatic pH meter. The wash buffer was made by adding 0.01% Tween-20 to 1 litre of PBS and they were mixed until homogenization occurred. The blocking buffer was made by adding 5% of egg albumin in 1X PBS (25 g of egg albumin in 500 mL PBS). The plasma dilution buffer was made by mixing 5% BSA and 0.01% Tween-20 in PBS. The substrate was 0.4 mg/mL ortho-phenyldiamine dihydrochloride (OPD) which was added to phosphate citrate buffer and 30% hydrogen peroxide to a concentration of 1 μg/mL. Horseradish peroxidase labelled antibodies, IgE, IgG, IgG1 and IgG4 (HRP-Ab) purchased from Southern Biotech company.

2.6.2.1 Antibodies Detection

A 96 well microtiter plates Nunc Maxisorb (Nunc, Denmark) were coated with an antigen (HIV-1 gp120 /gp41 or Sh13 at 5 μg/mL in PBS) overnight at 4 °C. The plates were washed three times with PBS and 0.01 Tween-20 (wash buffer) and gently tapped on a multi-wipe. The plate was blocked with 5% egg albumin in PBS for 1 hour at room temperature on a shaker at 200 rpm. After an hour the plate was washed with 3X with wash buffer and 100 μL of the plasma diluted 1: 10 in incubation buffer was added in each distinctive well and left for incubation overnight for
After an hour the plate was washed 3X with wash buffer to remove any unbound antibodies. One hundred micro liters of peroxide conjugated monoclonal antibodies (IgE, IgG, IgG1, IgG4) diluted with the incubation buffer 1:100 was added to each well and incubated for 1 hour at room temperature. The plate was washed 6X with wash buffer and gently tapped to remove any excess wash buffer. Freshly prepared OPD substrate was then added to each well and left in the dark to incubate for 20 minutes. If the enzyme linked antibody had bound to the antibody bound to the antigen, the enzyme will catalise the substrate therefore a color change will occur. The absorbance of the samples were taken using a spectrophotometer at 450 nm using an ELISA plate analyzer (microplate reader, Inqaba Biotec). (Protocol adapted from southern biotech manufactures instructions).

2.7 DNA Extraction

DNA was isolated from stored whole human blood using a Qiagen DNA extraction flexigene kit as per manufacturer’s instructions as follows: Frozen blood stored in -80 °C was thawed at room temperature and kept on ice. From the thawed blood, 300 μL was pipetted in a 1.5 ml Eppendorf tube and was washed with added 750 μL FG1 buffer. The solutions were mixed by inverting the tube at least 5 times and this lysed the erythrocytes. The mixture was centrifuged at 10 000 rpm for 2 minutes, the supernatant was discarded and a white pellet was obtained. The tube was inverted on a clean absorbent multi-wipe for at least 2 mins and making sure the pellet remains in the tube. To the pellet, 150 μL of FG2 with protease buffer (denaturation buffer) was added and vortexed for 5 seconds at high speed immediately ensuring homogenization occurs. The mixture was then centrifuges at 10 000 rpm for 3-5 sec briefly to get all the solution at the bottom of the Eppendorf tube. The tube was placed in a preheated 65 °C water bath and the sample was incubated for 5 min, this ensures that all the protease enzyme
works at its optimum temperature for denaturation of proteins in the sample. The sample changes color from red to olive green. After 5 mins the sample was removed from the water bath and 150 μL of cold 100% isopropanol was added to the sample and mixed by inverting a couple of times to make sure that the DNA precipitates which can be seen as visible clumps or threads. The solution is then centrifuged for 3 minutes at 10 000 rpm. The supernatant was discarded and the pellet was retained. The Eppendorf was then briefly inverted on a clean absorbent multi-wipe, then 150 μL cold 70% ethanol was added and vortexed for 5 sec. The mixture was then centrifuged for 3 min at 10 000 rpm, the supernatant was discarded and the tube was inverted on a clean multi-wipe for at least 5 mins and left to air dry taking care that the pellet does not over dry. Finally 200 μL FG3 buffer was added to the pellet to suspend the DNA and left for incubation for 1 hour at 65 °C in a water bath. After 1 hour the DNA was analyzed by gel electrophoresis and stored at -20 °C.

2.7.1 Gel Electrophoresis
Multi-purpose agarose (CSL-AG500, from Clever Scientific Company) 0.8 % containing ethidium bromide to a final concentration of 0.5 μg/mL was used to visualize the DNA extracted. Agarose powder (0.8 g) was put in a 200 mL flask to which 100 mL 1X Tris borate EDTA (pH 8.0) and warmed in a microwave till it dissolved. The TBE-agarose mixture was left to cool down to about 60 degrees and 2 μL of ethidium bromide was added. The mixture was poured in a gel casting tray with a comb to create wells. The mixture was left to set at room temperature and slowly the combs were gently removed. The cast gel was placed into the electrophoresis tank and 1X TBE buffer was added to the tank to cover the gel. Extracted DNA (10 μL) was mixed with 2 μL loading dye (New England Biolabs) and each DNA sample was loaded in a different well. For each gel electrophoresed 5 μL of Lambda DNA HindIII digest ladder (New
England Biolabs) was placed in the first well to determine the size of the DNA extracted. Electrophoresis was done for 45 min at 120 volts. After 45 min the DNA was visualized on an ultra violet (UV) transilluminator.

### 2.8 Single Nucleotide Polymorphisms

Single nucleotide polymorphisms were studied on two different cytokine positions namely -1082G/A promoter region of IL-10 and -308A/G promoter region of TNF-α. Different sets of primers indicated in the table were used to amplify regions of the promoter gene sequences targeted by ARMS-PCR was performed in a thermocycler (Thermo Electron Corporation thermocycler). Human growth hormone (HGH) primers were also added to all the samples to eradicate the possibility of false positive results.

#### Table 3: Cytokine alleles, primer sequences and expected fragment size for ARMS-PCR.

<table>
<thead>
<tr>
<th>Cytokine Gene</th>
<th>SNP Position</th>
<th>Primer Sequences 5’ to 3’ end</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10</td>
<td>-1082 G/A</td>
<td>Common Reverse: CAGTGCCAAACTGAGAATTGG</td>
<td>258</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Forward G: CTACTAAGGCTTCTTTGGGAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Forward A: ACTACTAAGGCTTCTTTGGGAA</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>-308G/A</td>
<td>Common Reverse: TCTCGGTTTCTTTCTCCATCG</td>
<td>273</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Forward G: ATAGGTTTGTAGGGGATCG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Forward A: AATAGGTTTGTAGGGGATCG</td>
<td></td>
</tr>
<tr>
<td>HGH</td>
<td></td>
<td>Internal control primer 1: GCCTTCCCAACCATTCCCTTA</td>
<td>425</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Internal control primer 2: TCACGGATTCTGTGTGT TTC</td>
<td></td>
</tr>
</tbody>
</table>

(Mohindru et al., 2004 and Turner et al., 1997)

#### 2.8.1 ARMS-Polymerase Chain Reaction (PCR)

Polymerase chain reaction is a molecular technique used to amplify any targeted genome sequence by synthesizing a new strand of DNA complementary to the template strand. ARMS-
PCR is a PCR method based technique used to detect single nucleotide polymorphisms. Three sets of primers are required for the detection of SNPs, the generic primer, mutant primer and the wildtype primer. For each sample two tubes are used for which one tube will contain the generic and wild type primers and the other tube will contain the generic and the mutant primer for amplification. Hence there are three possible genotypes for the individual, mutant, wild type or having both.

**Reagents and buffers required for ARMS-PCR**

Taq master mix containing the following 20 mM Tris-HCL pH 25 °C, 1.8 mM Magnesium Chloride, 22 mM NH₄Cl, 22 mM Potassium Chloride, 0.2 mM Deoxynucleotide Triphosphates (dNTPs), 25 units/mL one Taq DNA polymerase, 5% glycerol, 0.06% IGEPAL CA-630 and 0.05% Tween-20, cytokine gene primers (10mM), internal control primers (human growth hormone, 10 mM), nuclease free distilled water, PCR tubes and 2 mL Eppendorf were all purchased from Inqaba biotechnical industries. DNA templates used were extracted from whole blood.

**2.8.1.1 Genotyping TNF-α promoter position -308A/G and IL-10 promoter position -1082G/A**

Two reaction master mixes were prepared for the, wild type allele primer and the mutant allele primers. Master reaction mix was prepared in an Eppendorf for 50 reactions for both the wild type and mutant allele and 20 μL of the mix was aliquoted in each PCR tube and 5 μL of the different DNA template was added. Each PCR had a final volume of 25 μL of the reaction mix. The reaction mix had the following components for one reaction mix: taq master mix 12.5 μL, internal control primer forward 0.5 μL, internal control primer reverse 0.5 μL, wild type/ mutant forward primer 1 μL, generic reverse primer 1 μL, distilled water 4.5 μL and the DNA template
5 μL. (Adapted from NewEngland Biolabs Taq 2X Master Mix protocol). The PCR tubes were then placed in a thermocycler (Thermo Electron Corporation) and the following conditions were set: For TNF-α (-308 A/G), pre-denaturation was set at 95 °C for 1 min for 1 cycle, denaturation was set at 95 °C for 15 seconds for 35 cycles, annealing was set at 56 °C for 45 seconds for 35 cycles, extension was set at 72 °C for 50 seconds for 35 cycles, final extension was set at 72 °C for 5 min for 1 cycle and the samples were held for 4 °C for 5 minutes. (Adapted from Mohindru et al., 2004, Chang et al., 2012 and New England BioLabs Taq 2X Master Mix Protocol). The following parameters were set for IL-10 (-1082 G/A), pre-denaturation was set at 95 °C for 1 min for 1 cycle, denaturation was set at 95 °C for 15 seconds for 30 cycles, annealing was set at 57 °C for 1 minute for 30 cycles, extension was set at 72 °C for 50 seconds for 30 cycles, final extension was set at 72 °C for 7 min for 1 cycle and the samples were held for 4 °C for 5 minutes. (Adapted from Turner et al., 1999, Little S. 1995 and New England BioLabs Taq 2X Master Mix Protocol.)
2.8.2 Detection of Amplicons (Gel electrophoresis)

The amplified DNA products were separated on 2% agarose gel stained with ethidium bromide. Multi-purpose agarose powder (2 g) was put in a 200 mL flask to which 100 mL 1X Tris borate EDTA (pH 8.0) and warmed in a microwave until it dissolved. The TBE-agarose mixture was left to cool down to about 60 °C and 2 μL ethidium bromide was added. The mixture was poured in a gel casting tray with a comb to create wells. The mixture was left to set at room temperature and slowly the combs were gently removed. The cast gel was placed into the electrophoresis tank and 1X TBE buffer was added to the tank to cover the gel. Amplified DNA (10 μL) was mixed with 2 μL loading dye and each DNA sample was loaded in a different well. For each gel electrophoresed 5 μL of 100 bp DNA ladder (New England Biolabs) was placed in the first well.
to determine the size of the DNA extracted. Electrophoresis was ran for one hour at 100 volts and then the amplicons were visualized on a UV transilluminator.

2.9 Statistical Analysis

Data was entered on Microsoft excel 2013 and two statistical programs were used for analysis of the data namely statistical package for social sciences (SPSS version 16.0, SPSS Inc., Chicago, IL, USA) and graph pad prism version 6.01. Pearson Chi-square test was performed to determine the significance of the genotype association to infection intensity, cytokine levels association to CD4+ counts and viral loads, CD4+ counts association to anti-HIV antibodies and the association of cytokine concentration to antibodies. One way analysis of variance (ANOVA) was performed to determine the significance of association of the infection intensity and cytokine levels. Kruskal-Wallis was performed to determine the significance of association of the genotype and the cytokine levels. The Mann-Whitney t-test was performed to determine the significance of anti-HIV antibodies differences, cytokine levels between infected and uninfected, viral load and CD4+ pre and post treatment differences, HIV+ and co-infected (HIV+& S.h+) cytokine levels.
CHAPTER 3

3.0 RESULTS

3.1 Study Population

Table 4: Total number of people recruited in the study and the numbers of *S. haematobium* infected and HIV positive status.

<table>
<thead>
<tr>
<th>Total population</th>
<th><em>S. haematobium</em> Infected</th>
<th><em>S. haematobium</em> and HIV Co-infected</th>
<th>HIV Infected only</th>
<th>No infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>138</td>
<td>61 (44%)</td>
<td>11 (8%)</td>
<td>4 (3%)</td>
<td>62 (45%)</td>
</tr>
</tbody>
</table>

Table 5: Guide to infection intensity quantitation according to WHO guidelines.

<table>
<thead>
<tr>
<th>Infection Intensity</th>
<th>Number of eggs</th>
</tr>
</thead>
<tbody>
<tr>
<td>No infection</td>
<td>0</td>
</tr>
<tr>
<td>Low/light</td>
<td>Less than 10</td>
</tr>
<tr>
<td>moderate</td>
<td>11 to 49</td>
</tr>
<tr>
<td>Heavy/severe</td>
<td>Greater than 50</td>
</tr>
</tbody>
</table>

Table 6: Infection intensity of *S. haematobium* infected individuals.

<table>
<thead>
<tr>
<th>Infection Intensity</th>
<th>Number of individuals (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Infection</td>
<td>66 (48)</td>
</tr>
<tr>
<td>Low</td>
<td>48 (35)</td>
</tr>
<tr>
<td>Moderate</td>
<td>17 (12)</td>
</tr>
<tr>
<td>Severe</td>
<td>7 (5)</td>
</tr>
</tbody>
</table>
Figure 4: MGS infection intensity percentage distribution, according to categories of low infection < 10 eggs/ml or urine; moderate infection 10<x<50 eggs/10 ml of urine and heavy infection >50 eggs/10 ml of urine.

Almost half of the participants did not have schistosomiasis infection and for the other half, a huge number of the participants had low infection intensity, less than 10 % individuals were heavily infected and around 12 % of the population were moderately infected with schistosomiasis.

3.2 DNA Extracted

DNA was successfully extracted and the expected band size was noted in figure 5, some DNA bands were faint meaning that DNA yield was low, some bands were seen as sheared meaning that DNA could been degrading or voltage was high for gel electrophoresis and some DNA bands had the perfect straight bands. However in all the samples, DNA extracted worked very well for PCR.
3.3 Cytokine Gene Polymorphisms (ARMS-PCR) Results

**IL-10 -1082G/A Polymorphisms**

![Figure 6: A representative photograph of IL-10 (-1082) polymorphisms on 2 % agarose gel stained with ethidium bromide, the 425 bp bands are amplified internal controls (HGH) and the 258 bp bands are the amplified IL-10. Lane M, marker DNA ladder, 100 bp; lane 1 to 19, samples 158, 267, 1717, 736, 1004, 911, 687, 888 and 1342 all genotype heterozygous AG.]
**Figure 7:** A representative photograph of IL-10 (-1082) polymorphisms on 2% agarose gel stained with ethidium bromide, the 425 bp bands are amplified internal controls (HGH) and the 258 bp are the amplified IL-10. Lane M, marker DNA ladder, 100 bp; lane 1 and 2, sample 794 genotype AG; lane 3 and 4, sample 310 genotype AG; lane 5 and 6, sample 807 genotype AA; lane 7 and 8, sample 238 genotype AG; lane 9 and 10, sample 158 genotype AG; lane 11 and 12, sample 849 genotype AG and lane 13 and 14, sample 799 genotype inconclusive; lane 15 and 16, sample 923 genotype not detected and lane 17 and 18, sample 546 genotype AG.

**TNF-α -308A/G Polymorphisms**

**Figure 8:** A representative photograph of TNF-α (-308) polymorphisms on 0.8% agarose gel stained with ethidium bromide, the top bands are amplified internal controls (HGH) and the bottom bands are the amplified TNF-α -308. Lane M, marker DNA ladder, 100 bp; lane 1 and 2, sample 158 genotype AG; lane 3 and 4, sample 309 genotype AG; lane 5 and 6, sample 1717 genotype AA; lane 7 and 8, sample 736 genotype AA; lane 9 and 10, sample 1004 genotype AG; lane 11 and 12, sample 687 genotype AA and lane 13 and 14, sample 888 genotype AG genotype AG.
Figure 9 and 10: Genotypes percentage distribution A: TNF-α (-308) and B: IL-10 (-1082).

Analysis of single nucleotide polymorphisms of the participants showed that the genotype GG had the highest frequency (70 %) of the population followed AG genotype with 19 % and lastly AA with 11 % for TNF-α promoter position -308. IL-10 promoter position -1082, genotype AG had the highest frequency with 88 % of the study population, genotype GG with 9 % and genotype AA having the lowest frequency of 3 % of the study population.

Table 7: Genotype and allele frequency distribution of TNF-α (-308) and IL-10 (-1082).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>TNF-α -308A/G frequency (%)</th>
<th>IL-10 -1082G/A frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>15 (10.9)</td>
<td>4 (2.9)</td>
</tr>
<tr>
<td>AG</td>
<td>27 (19.5)</td>
<td>121 (87.7)</td>
</tr>
<tr>
<td>GG</td>
<td>96 (69.6)</td>
<td>13 (9.4)</td>
</tr>
<tr>
<td>Total</td>
<td>138 (100)</td>
<td>138 (100)</td>
</tr>
</tbody>
</table>

Allele

- A: 20.65 %
- G: 79.35 %
The A allele for the promoter position for TNF-α -308 is the dominant allele and had the lowest frequency in the population and the G allele had the highest frequency. IL-10 promoter position -1082 G allele is the dominant allele however the distribution of the frequency was the almost the same with the A allele.

**Table 8:** Genotype and infection intensity frequency distribution association of TNF-α and IL-10. Pearson Chi-square: p value of 0.169 value 0.271 respectively.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Genotype</th>
<th>Infection Intensity</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>no infection</td>
<td>low</td>
</tr>
<tr>
<td>TNF-α</td>
<td>AA</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>50</td>
<td>30</td>
</tr>
<tr>
<td>IL-10</td>
<td>AA</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>57</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>9</td>
<td>3</td>
</tr>
</tbody>
</table>

The above table relates the genotype to the infection intensity of schistosomiasis infected participants even though there wasn’t a statistical significance. For both TNF-α and IL-10 SNPs for all genotypes, as the infection intensity increases the frequency of the population decreases except for the TNF-α AA genotype.

### 3.4 ELISA Results

**Antibody Assays**

Cutoff values were calculated using the following formulae:

\[
\text{Cutoff Value} = \text{mean value of negative control} + 3 \times \text{(standard deviation of the negative control)}.
\]
**Figure 11:** Schistosomiasis infection detection of IgE anti-Sh13 antigen in samples.

The samples with OD values on and below the cutoff value line are considered negative for schistosomiasis infection. Slightly below half of the samples were above the cutoff line meaning that these participants tested positive for schistosomiasis and these results were in sync with the parasitology data therefore I was working with the correct samples.

**Figure 12 and 13:** Anti-HIV antibody IgG1 using antigens A, gp41 and B, gp120 detected.

The known HIV positive samples and a few samples were also tested for HIV positive using ELISA by detecting IgG1 and using HIV gp41 and gp120 proteins antigens. The results had
corresponding information with the collected information of participants who tested positive however about 9 other samples also tested positive for HIV infection.

![Graph](image)

**Figure 14:** Different antibodies reaction towards anti-HIV gp41 and gp120 antigens. **Fig. 15:** Box plot comparison of IgG1 anti-HIV gp120 and gp41. Mann-Whitney test p value <0.0001.

Different antibodies work towards different antigens and detection of antibodies can confirm infection. Antibodies IgG react more towards HIV proteins than IgE. Antibodies IgG1 isotypes work more against HIV gp41 proteins better than HIV-gp120 proteins.

**Cytokine (Sandwich ELISA) Assay Results**

![Graph](image)

**Figure 16 and 17:** A. TNF-αand B. IL-10 standard curves used to determine cytokine concentrations of the samples.
R Square value of a plotted graph using graph pad prism is a value used to determine the accuracy of the concentration of the samples with 95% confidence interval. Using these graphs, concentrations of the cytokines could be determined from OD values.

**Figure 18:** Cytokine comparisons of infected (S. h, HIV+ and S. h and HIV+) and uninfected samples. Man-Whitney p value of 0.0053 for IL-10 and TNF-α p value of 0.8858.

The concentration levels of the TNF-α of all infected individuals with schistosomiasis and HIV were noticeably higher than individuals with no infection but the opposite is true for IL-10.

There is also a noticeably difference in the pro-inflammatory cytokine levels being higher than the anti-inflammatory cytokine levels.
Figure 19 and 20: Cytokine levels relation to infection intensity measured by the number of S. haematobium eggs in 10mls of urine. One way ANOVA, p value= 0.51 for TNF-α and 0.0168 for IL-10.

Correlating concentration of the cytokines to infection intensity of schistosomiasis, for TNF-α there was an increment of the concentration as the infection increases however there wasn’t a statistical significant association and for IL-10, individuals with no infection and moderate infection had the highest concentration of the cytokine and there was statistical significance.

Table 9: TNF-α (-308) and IL-10 (-1082) genotypic variations to cytokine concentrations. Kruskal-Wallis test, p value of 0.345 for TNF-α and 0.44 for IL-10.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>TNF-α -308A/G frequency (%)</th>
<th>TNF-α mean concentration (ng/ml)</th>
<th>IL-10 -1082G/A frequency (%)</th>
<th>IL-10 mean concentrations (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>15 (10.9)</td>
<td>2.005</td>
<td>13 (9.4)</td>
<td>0.3970</td>
</tr>
<tr>
<td>AG</td>
<td>27 (19.5)</td>
<td>2.121</td>
<td>121 (87.7)</td>
<td>0.4759</td>
</tr>
<tr>
<td>AA</td>
<td>96 (69.6)</td>
<td>3.114</td>
<td>4 (2.9)</td>
<td>0.483</td>
</tr>
<tr>
<td>Total</td>
<td>138 (100)</td>
<td>138 (100)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

There was higher cytokine levels associated with dominant A allele for TNF-α -308 position and there wasn’t a notable difference to the mean concentrations for IL-10 -1082 position to genotype.
**Figure 21 and 22:** TNF-α (-308) and IL-10 (-1082) genotypic variations to cytokine concentrations.

The genotype AG of -1082 IL-10 showed that there were a few individuals with higher concentrations for the cytokine and for TNF-α there was a higher concentration for the AA genotype.

**Figure 23 and 24:** Genotype, infection intensity and cytokine mean concentration.

The above graphs show the correlation of infection intensity to the genotype and the cytokine concentration moreover IL-10 -1082 AG genotype had the highest concentration value for the cytokine and for TNF-α the concentrations did not differ but for heavily infected individuals there was high concentration of the cytokine for the AA genotype.
3.5 Analysis of HIV viral load and CD4+ Cell Counts

**Figure 25**: HIV viral load differences pre- and post-treatment. Mann-Whitney, p value of 0.1415 for viral plasma and 0.8254 for viral seminal differences. **Fig 26**: CD4+ counts pre- and post-treatment. Mann-Whitney, p value of 0.5741.

There was a decline of viral loads in both plasma and seminal fluids (figure 25) and a slight increase of CD4+ counts post treatment with praziquantel (figure 26).

**Figure 27**: Box plot for HIV and HIV+SH infected CD4+ comparison. Mann-Whitney test, p value of 0.9931.

Participants that were infected with HIV only had a mean CD4+ count of around 400 cells/μL and participants that were co-infected with schistosomiasis and HIV had a lower mean of 300
cells/μL. This showed that schistosomiasis does have an impact on the CD4+ counts of HIV infected individuals.

![TNF-α Mean Level Comparisons][1]

**Figure 28 and 29:** HIV and HIV+ & S.h infected cytokine levels comparison. Mann-Whitney test, p value of 0.0571 for IL-10 and 0.108 for TNF-α.

Co-infected individuals had a higher mean concentration of the pro-inflammatory cytokine TNF-α than individuals that only were HIV infected and the vice versa is true for the anti-inflammatory cytokine IL-10.

![CD4+ and HIV Antibody Correlation][2]

**Figure 30:** CD4+ counts and HIV Correlation. Correlation analysis was performed using Pearson Chi-Square test showed a p value of 0.0971. **Figure 31:** TNF-α concentration relation to IgG anti-HIV gp14 levels. Correlation analysis was performed using Pearson Chi Square test showed a p value of 0.0010.

The above graphs are relating the antibodies detected in the body to CD4+ counts and TNF-α levels and there was no association of the antibody to the CD4+ counts and the TNF-α levels.
**Figure 32 and 33:** Cytokine concentrations correlation to CD4+ counts A, IL-10 and B, TNF-α. Correlation analysis was performed using Pearson Chi-square test showed a p value of 0.8104 for TNF-α and 0.8828 for IL-10.

Interleukin 10 and TNF-α could influence the HIV replication process and relating to them to the CD4+ counts was vital because as a result of continuous HIV replication, destruction of the immune cells occurs. Most HIV infected individuals had less than 400 cells/μL.

**Figure 34 and 35:** Correlation of viral load to cytokine concentrations A, TNF-α and B, IL-10. Correlation analysis was performed using Pearson Chi-square test showed a p-value of 0.6043 for TNF-α and 0.8829 for IL-10.

There wasn’t as association established between cytokine levels and the seminal viral load because weather the participants had high or low viral load they also had TNF-α and IL-10 different levels.
CHAPTER 4

4.0 DISCUSSION

4.1 Study Population

A lot of attention had been paid to study female genital schistosomiasis and it male genital schistosomiasis had been neglected so this study wanted to pay attention to MGS. Ngundu in Chivi district was chosen as a study area due to its high prevalence of S. haematobium and HIV infections in Zimbabwe hence the area was an ideal place to get high numbers of co-infected individuals. A lot of male individuals were initially screened for S. haematobium infection and then for HIV infection to fit into the study criteria for co-infected male reproductive age (18-49) individuals. However the co-infected male numbers were not satisfactory (table 4) and one reason could be that men are known to likely not visit clinics for health attention unlike females. HIV infected individuals were low in numbers since individuals were not forced to have HIV tests done because of ethical considerations.

4.2 Genotyping Polymorphisms of TNF-α (-308A/G) and IL-10 (-1082G/A)

It is important to access the relationship of circulating TNF-α levels and the genetic association of polymorphisms because this determines many diseases and clinical outcomes (Elahi et al., 2009). TNF-α -308 promoter positon genotype GG had the higher frequency in the population followed by AG and AA with the least frequency. The A wildtype allele had low number of frequencies but has been associated with high cytokine levels (figure 9). IL-10 -1082 promoter position genotype AG had the highest frequency in this study group followed by the GG and lastly AA (figure 10). Interleukin10 wildtype G allele had been associated with high cytokine production even though there wasn’t a difference in the distribution of the A and the G allele.
Single nucleotide polymorphisms are important in cytokine production because they determine the cytokine levels hence having the ability to control disease progression and clinical outcomes. However there wasn’t a statistical significance for correlation of cytokine levels to infection intensity but this information is important for future studies.

4.3 Antibodies Detection

Anti-Schistosomiasis Antibodies
Cut off values were determined to know exactly that the infected individuals had schistosomiasis and not any other infection since antibodies are specific. The individuals that had results above the cutoff value were consistent to the *S. haematobium* infection diagnosed by urine filtration technique. These samples were archived samples and antibody detection was a way of making sure we were working with the right samples and correct parasitology data (figure 11).

Anti-HIV Antibodies
Anti-HIV gp120 and gp41 antibodies were detected as a way of diagnosing HIV of the known HIV infected individuals and some random samples from the population were selected as controls. Of the 37 samples tested 9 samples were HIV negative due after cutoff values were determines and 28 samples were considered positive (figures 12 and 13). This showed that some participants were not truthful and some did not want their HIV status disclosed. As a researcher ethical consideration is very important in respecting participant’s wishes. However IgG antibodies work towards HIV and Anti-gp41 IgG1 antibodies had the highest OD values compared to the anti-gp120 IgG1 meaning that the antibodies detect and fight off gp41 antigen more than antigen gp120 even though gp 120 is the most outer layer of the HIV that is in first contact with the body cells.
4.4 Cytokine Levels

4.4.1 Infected and Uninfected Relation to Cytokine Level

Tumor necrosis factor-alpha cytokine mean levels were slightly higher in infected individuals than uninfected individuals as shown in figure 18 however there wasn’t a statistical significance of cytokine levels of infected and uninfected. High levels of TNF-α a pro-inflammatory cytokine of infected individuals were expected but in uninfected individuals the concentrations were also high one possible reason could be that anti-HIV antibodies were detected in individuals that were considered to be HIV negative but according to the results they were HIV positive therefore could be accounting for the high levels of TNF-α. The cytokine is also considered to have both beneficial and pathogenic effects (Burke et al., 2009) hence some levels of the cytokine is expected. A trend was noted in an increase in the cytokine levels as the infection intensity increases because as the disease progresses there is greater inflammation resulting in a higher cytokine secretion. TNF-α indicated that the higher the levels in circulation the more the disease progresses thus corresponding to the infection intensity. IL-10 levels differences in infected (HIV+, S. h and co-infected) and uninfected individuals had was statistically significant (figure 18). The levels of IL-10 were higher in uninfected individuals possibly due to the fact that IL-10 is considered to be multipotent cytokine and it is known to have non-immune effects like protecting neurons from toxin induced damage (Zhou et al., 2009), increasing the formation of new blood vessels a process known as angiogenesis (Dace et al., 2008) and it is involved in autophagy which is a self-degradative process to maintain homeostasis (Park et al., 2011) Infected individuals had low IL-10 levels probably because pro-inflammatory cytokines are suppressing the anti-inflammatory cytokine production so that the immune system can quickly and vigorously try to get rid of the infection. There wasn’t a trend in IL-10 levels in relation to
infection intensity of *S. haematobium* infection. High levels of IL-10 in individuals that were moderately infected was noted and this could be so due to the need to balance pro and anti-inflammatory cytokine environment since infection is moderate which is enough to suppress the anti-inflammatory cytokines.

4.5 Single Nucleotide Polymorphisms Association with Cytokine Levels

Host genetic factors are important to determine the differences in clinical outcomes of infectious diseases because of the host genetic influence to production of high or low cytokine levels which contribute to inflammation. TNF-α dominant A allele had the lowest frequency (11%) in the population studied but had the highest mean concentration levels and G allele had the highest frequency in the population but the low cytokine mean concentrations. The comparison between the SNPs, infection intensity and the cytokine levels showed that TNF-α levels were evenly distributed for the no-infection, low and moderately infected for the AG and GG genotypes (figure 24). However there was a notably increase in severely infected individuals for the AA genotype which has been associated with worse clinical symptoms and disease progression in diseases such as cerebral malaria. The higher the cytokine levels the greater the inflammation the more the HIV virus progresses. In this study there wasn’t a significant association of cytokine levels and genotypes but it was important to note the trend. Studies done by Govan *et al.* in 2006, showed there wasn’t an association between TNF-α gene polymorphisms as a risk to cervical cancer as well as another study done by Powell *et al.*, in 2001 showing there was no association between acute pancreatic but these studies were very important in studying host genetics. However in other studies there was an association of polymorphism of TNF-α -308 with increased risk to asthma (*Wittle et al.*, 2002) and diabetes type 2 (*Vendrell et al.*, 2003). TNF-α -308 polymorphisms have showed to have an effect on transcriptional activity increasing cytokine
production however there are different studies with different outcomes that did not have absolute conclusive results (Anoosheh et al., 2011).

IL-10 -1082 positon dominant G allele associated with the highest cytokine production had highest frequency but the cytokine levels were not notably different. There wasn’t a trend in the distribution of IL-10 between infection intensity status and a study done in Pakistan showed that there was not an association of SNP IL-10 in Hepatitis C virus infected patients (Afzal et al., 2011). A combination of several SNPs of IL-10 showed they influence the levels secreted shown by a study done by (AL-Mahaya et al., 2015). Association of single nucleotide polymorphisms to different diseases is incomplete and has contradicting results. This study could also help shed some light on the impact of SNPs polymorphisms on parasitic infections. Moreover a combination of SNPs would have a strong association in schistosomiasis infection progression just like the study done in Indonesia by Lamis et al., in 2002 which showed that in parasitic infection, a combination of different distinctive alleles could have an influence in cytokine secretion. Several factors such as sample size, selection of the study and the age of participants could possibly be a reason for differences in SNPs results.

4.6 HIV and Co-Infected (HIV and MGS)

It was very important to relate MGS and HIV co-infection having an impact in HIV progression by investigating the cytokine levels that causes inflammation leading to HIV replication. The balance between the pro-inflammatory and anti-inflammatory cytokine is critical because it could influence HIV replication and the disease outcome. There was a huge noticeable reduction of plasma viral load pre and post treatment of schistosomiasis infection as shown in figure 25. The reduction in seminal viral load was noted but didn’t drastically change because da Silva et al. in 2005 showed that even after praziquantel treatment individuals still had granulomas with
degenerating viable eggs at 24 months post-treatment (da Silva et al., 2005). Kjetland et al. study showed that there wasn’t a change in the sandy patches caused by embedded eggs over a year in females with FGS (Kjetland et al., 2012) and this could be the same case with this study population. This could mean that could have been therapeutic urogenital failure to get rid of the embedded eggs resulting in continuous inflammation recruiting the CD4+ cells that are infected by the HIV, hence continuous HIV replication occurring though at a lower rate. The CD4+ counts increased post treatment increasing the immune strength and this did show that schistosomiasis infection contributes to the HIV replication by destroying the immune cells thus adding to HIV progression. Therefore MGS and HIV co-infection can aid in HIV progression leading to rapid advancement to AIDS status.

4.6.1 CD4+ count comparisons

The CD4+ receptor is found on immune cells such as the T-cells, macrophages, dendritic cell and microglia cells which are important in fighting any infection an individual encounters. These cells are a way of monitoring a fully functional immune system. The CD4+ counts are immune cell counts with the CD4+ receptors that the HIV virus infects and destroys. CD4+ counts are monitored closely in individuals that HIV infected as a way of monitoring HIV progression to AIDS status. According to WHO CD4+ count below 400 cell/μL is considered to be immune compromised and can be infected with opportunistic infections that could eventually kill the individual. These individuals are started on anti-retroviral therapy (ART) to stop replication of HIV that destroys CD4+ cells so as to have a CD4+ count above 400 cell/μL. Figure 27 shows that HIV infected only individuals had a higher mean CD4+ count which was above 400 cell/μL as opposed to CD4+ counts of individuals that are co-infected had a lower mean value which was below 400 cell/μL and this shows that MGS has an impact in HIV infected individuals that
causes destruction of CD4+ immune cells resulting in a lower mean CD4+ count hence low immune cells to fight off infection. Therefore MGS infection has an impact on HIV infected individuals.

### 4.6.2 Cytokine levels comparisons

*Schistosoma haematobium* infection activates the CD4+ cells that are linked to host genotypes of TNF-α high cytokine production that confers the individual with an inflammatory environment. Extended inflammatory condition indirectly activates latent HIV thereby increasing replication most likely transmission as well. Human immunodeficiency virus infected individuals had lower TNF-α levels than co-infected individuals, this is so because MGS infection causes a continuous inflammatory environment due to the pro-inflammatory cytokine production. Tumor necrosis factor-alpha will indirectly aid in HIV progression by activating latent HIV and adding in HIV proteins production. Human immunodeficiency virus infected individuals had higher IL-10 indicating the ability of IL-10 to control inflammation caused by just HIV only hence balancing the pro and anti-inflammatory cytokines and most importantly being able to manage disease progression. Interleukin10 levels were high but they do not progress HIV infection but instead it prevents the development of severe pathology (Burke *et al.*, 2009). The reason may be that the high pro-inflammatory response of TNF-α may be inhibited by high IL-10 production, which might be involved in natural defense resulting in Th1/Th-2 balance being beneficial to these contact (Joshi *et al.*, 2015). In co-infected individuals IL-10 levels were low possibly because there could be an antagonistic relationship of IL-10 and TNF-α or other pro-inflammatory cytokines during co-infection and hence constant inflammation to fight off infection could result in lower anti-inflammatory cytokines (figure 28 and 29). Another study done by Erikstrup *et al.* in 2008 showed that production of IL-10 was impaired among schistosomiasis and HIV-infected
patients compared with those infected only with schistosomiasis. The balance between the pro and anti-inflammatory cytokines might adjust the benefits of the antiretroviral therapy influencing the outcome of the disease.

4.7 Cytokine levels association to anti-HIV Antibodies, CD4+ counts and HIV viral load

Statistical association between the anti-HIV antibodies and CD4+ counts could not be established however it was noted that the higher the anti-HIV IgG antibodies the lower the HIV replication resulting in a high CD4+ count. Tumor necrosis factor-alpha concentrations relating to IgG in circulation had a statistical significant (P = 0.0010) and it showed the antibodies in circulation and mostly subtype IgG1 (figure 31). This study showed that the higher the TNF-α more the HIV replicates the higher the antibody IgG levels to fight off infection. However TNF-α and IL-10 levels in relation to anti-gp41 IgG1, IgG and anti-gp120 IgG4 (figure 14) also did not have a statistical significance association. Individuals with any high IL-10 concentrations had low viral load indicating that these individuals had the ability to control inflammation resulting in low viral load with the exception of one individual who had a notably high viral load (figure 35). A trend could not be established for TNF-α levels in relation to seminal viral load. The viral loads of individuals in semen and plasma were in sync, the higher the plasma viral load the higher the seminal viral load. Seminal viral load and cytokine present in the semen are a definite determination of MGS diagnosis because S. haematobium eggs are embedded in the reproductive system of the male individual such as the prostate glands and seminal vesicle causing granulomas.
4.7 CONCLUSION AND RECOMMENDATIONS

4.7.1 Conclusion

This study revealed that MGS has an impact on HIV progression as a trend even though statistical significance could not be established. The CD4+ counts increased and viral load decreased after MGS treatment showing that MGS infection has an impact on HIV infection. The levels of TNF-α increased as the levels of MGS increased as well showing that as the infection becomes chronic the greater the inflammation resulting in an indirect HIV progression. The study on host genetics of Schistosomiasis and HIV co-infection revealed single nucleotide polymorphism genotypic trends that may contribute to understanding the underlying mechanisms driving transmission and progression in areas prevalent with co-infection. Tumor necrosis factor-alpha -308 A allele indicated the production of high to moderate cytokine levels contributing to HIV replication resulting in a high viral load. Interleukin 10 -1082 G allele showed high or low producers indicating the ability to control an inflammatory environment thereby reducing the progression of HIV in infected individuals. Understanding the relationship between the balance of IL-10 and TNF-α levels is important in managing any disease progression. Male genital schistosomiasis and host-immunogenetics could be fueling the transmission and progression of HIV in regions endemic with co-infections and it is important to acknowledge the impact of Co-infection. The results indirectly point to the need to incorporate urogenital schistosomiasis control among the reproductive age groups as a strategy to control HIV transmission.

4.7.2 Study limitations

Out of a large number of participants recruited only a few individuals had HIV probably because male individuals are not likely to visit a health center than women therefore could account the lack of huge numbers of individuals that are MGS and HIV co-infected. It is important to
encourage male individuals to pay very close attention to their health. Ethical consideration has to be properly observed so individuals cannot be forced to have HIV tests or any other test without their consent.

**4.7.3 Recommendations**

There is still a need to further investigate MGS and HIV co-infection therefore a country wide study of MGS is necessary to have a clear representation of the impact of MGS on HIV in Zimbabwe. Monitoring cytokine levels as a way of monitoring clinical outcomes is necessary so as to help manage HIV progression. There is also a need to detect all the possible cytokines in circulation due to MGS and HIV co-infection and possibly could be used as diagnostic tool for quick detection of MGS and a need to have better diagnostic tools for MGS. There is a huge need to study therapeutic measures of tissue embedded schistosome eggs because age, infection intensity, the location of the granuloma lesions and the seriousness of the granuloma are some of the parameters to be considered to have a successful therapeutic measure. Most importantly there is a need to inform the need to intensify Schistosoma parasite control that could have an indirect impact on HIV transmission in adults. If there is an improved and better Zimbabwe to stop the life cycle of schistosomiasis by eradicating snail species, having better sanitations and incorporating boreholes in endemic areas as a way of stopping the point of schistosomiasis infection. If Japan, Tunisia and Morocco were able to eliminate completely schistosomiasis by incorporating molluscides to kill/control snails (Adenowo et al., 2015) so can Zimbabwe. There is a need to continue schistosomiasis studies to aid in valuable information for the development of a vaccine and a need to pay attention all the other neglected tropical diseases that devastate the endemic areas for they possibly could have an unknown huge impact.
CHAPTER 5

5.0 REFERENCES AND APPENDIX

5.1 References


5.2 Appendices

Appendix A

Agarose gel picture for DNA extracted for some of the samples.

Appendix B

Agarose gel pictures for some of the samples genotyped for IL-10 (-1082) polymorphism, the product size is 258 bp.
Appendix C

Agarose gel pictures for some samples genotyped for TNF-α polymorphism, the product size is 273 bp.
Appendix D

ELISA data for some of the samples for cytokine quantification O.D values read at 450 nm.
Appendix E

ELISA data for some of the samples for antibody detection O.D values read at 450nm.