Role of local or systemic *Schistosoma* infections in driving inflammation and HIV risk in women enrolled in the CAPRISA 004 cohort

Sahil Tulsi

Student No: 200309440

B.Sc., B.Med.Sc. (Hons)

Supervisor: Dr Derseree Archary

Co-Supervisors: William Horsnell and Jo-Ann Passmore

Submitted in fulfilment of the requirements for the degree of M.Med.Science in the Discipline of Medical Microbiology, Faculty of Health Sciences

University of KwaZulu-Natal

2016

TABLE OF CONTENTS

Page

Chapter:

DECLARATION

This study represents the original work by the author and has not been submitted in any form to another University. The use of work by others has been duly acknowledged in the text.

The research described in this study was carried out in the Discipline of Medical Microbiology, Faculty of Health Sciences, University of KwaZulu-Natal, under the supervision of Dr Derseree Archary, Prof Jo-Ann Passmore & Prof William Horsnell.

S. Tulsi

ACKNOWLDEGEMENTS

I would like to express sincere gratitude and appreciation to Dr. Derseree Archary for her guidance, valuable criticism and encouragement throughout the study.

I am sincerely grateful to Prof Jo-Ann Passmore, Prof William Horsnell & Dr. Lenine Liebenberg for their guidance and support during this research endeavour; and for their contribution towards this thesis.

I am grateful to the National Research Foundation (Free-standing Scarce Skills, Grant Reference: 97979) for awarding me their scholarship, the College of Health Sciences for funding this study & Dr Ravesh Singh for his laboratory training during this study

Finally, I would like to thank my family for their encouragement, support and understanding throughout this study.

LIST OF ABBREVIATIONS

°C	Degrees Celsius
µg	Microgram
Ml	Microliter
Ab	Antibody
ACD	Anticoagulant dextrose
Ag	Antigen
AIDS	Acquired Immune Deficiency Syndrome
ARV	Antiretroviral
Basic FGF	Basic fibroblast growth factor
B-NGF	Beta nerve growth factor
Bp	base pair
CAPRISA	Centre for the AIDS Programme of Research in South Africa
CD4, CD8	Cluster of Differentiation
CD4 T_H lymphocyte	CD4 T-helper lymphocyte
CI	Confidence Intervals
CTL	Cytotoxic T Lymphocytes
CVL	Cervico-vaginal lavage
CSF	Colony stimulating factor
CTACK	Cell attracting chemokine
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
FGS	Female genital schistosomiasis
FUS	Female urogenital schistosomiasis

H	Hour
HGF	Hepatocyte growth factor
HIV	Human Immunodeficiency Virus
IL	Interleukin
IFN	Interferon
ITS	Internal Transcription Spacer
IQR	Interquartile range
LIF	Leukaemia inhibitory factor
MCP	Monocyte chemo attractant protein
MCSF	Macrophage colony-stimulating factor
Mg	Milligram
$MgCl_2$	Magnesium Chloride
min/s	minute/s
MIF	Migration inhibitory factor
Ml	Millilitre
mM	Millimolar
NaCl	Sodium Chloride
Ng	Nanogram
PDGF	Platelet-derived growth factor
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
Pg	Pictogram
RANTES	Regulated on activation, normal T expressed & secreted
RNA	Ribonucleic Acid

rRNA	Ribosomal RNA
SCF	Stem cell factor
SDF	Stromal derived cell factor
SNP	Single Nucleotide Polymorphism
Taq	*Thermus aquaticus*
TNFα	Tumour Necrosis Factor alpha
TNFR1/2	TNF receptor 1 / 2
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
U	Unit
UV	Ultraviolet
V	Volts
VEGF	Vascular endothelial growth factor

List of figures

Legend **Page**

Chapter 2

Chapter 3

Chapter 4

List of tables

Legend **Page**

Chapter 3:

Chapter 4:

Chapter 8:

Chapter 1: Abstract

Female genitourinary schistosomiasis (FGS) has been associated with increased HIV susceptibility, presumably through lesions secondary to parasitic eggs *in situ* in the female genital tract. We determined the prevalence of FGS infection by real-time PCR (indicative of local involvement of parasitic eggs in the genital mucosae) and sero-prevalence of schistosomiasis (indicating prior exposure to parasitic infection) in HIV-uninfected KZN women (n=383) who had participated in the CAPRISA004 trial. The hypothesis for this study was that FGS, and genital tract inflammation are risk factors for HIV-acquisition. DNA PCR was used to confirm the presence of FGS, ELISAs were used for detection of *Schistosoma* spp IgG and multiplex technology was used to detect genital tract cytokines in the cervicovaginal lavages (CVLs). The median age of the women in this study was 23 years (range 20-26 years). Of the 383 HIV negative women, 52/383 (13.8%) became HIV-infected by study exit with an HIV incidence rate of 9.1 per 100 women-years (95% CI: 6.8 – 11.9). Nine of 383 (2.3%) women had a positive DNA PCR for *Schistosoma* spp indicative of prevalent genital schistosomiasis. Of these 9 women, 4/9 (44%) acquired HIV infection by study exit with a 4.0 times increased risk for HIV-infection (OR of 4.05- 95% CI 1.8-8.9, p=0.01) than PCR-negative women. *Schistosoma haematobium*, the endemic species in KZN has high sequence homology with *S. mansoni* antigen which was used to detect IgG in the plasma samples. Of the 383 plasma samples from study entry, 21/383 (5.5%) and 19/383 (4.96%) of study participants had detectable levels of IgG to *S. mansoni* at study exit. . Only, MCP-3, a chemokine was significantly higher in FGS+ compared to FGS- healthy HIV negative women. Genital tract pro-inflammatory cytokines at study exit were significantly higher in FGS-HIV+ women for IL-1β, MIF, IL-1A & IL-6 compared to FGS-HIV- women (p<0.0001, p=0.0014, p=0.0088, p=0.0069 respectively). Anti-inflammatory cytokine data in FGS-HIV+ women showed higher median levels of IL-1RA & IL-2RA compared to FGS – HIV- women (p<0.0001 & p=0.0012 respectively). Mixed responses of both pro and anti-inflammatory cytokines in the presence or absence of FGS may be an indication that HIV infection is driving these signatures and causing dysregulation. The presence of parasite DNA in the genital tract was significantly associated with increased risk for HIV acquistion. Taken together, these results highlight the importance of understanding the complex interplay of parasitic infections, and host immunity as potential risk factors for HIV acquistion in regions with high HIV and parasite burden.

Hypothesis

Female genitourinary schistosoma infection increases risk to HIV-1 acquisition, possibly by causing local lesions in mucosae, thereby increasing inflammatory responses in the female genital tract. The role of genital tract and systemic inflammation during *Schistosoma* spp infections in determing HIV risk in women is not completely understood. Therefore, this study sought to investigate the prevalence and incidence of *Schistosoma* spp infections by identifying the presence of schistosomal DNA and *Schistosoma* spp antibodies in women who were part of the placebo arm of the CAPRISA 004 trial. Specifically, the hypothesis was that women with detectable DNA specific to this parasitic infection in the genital mucosae had elevated genital cytokine responses compared to women who did not show evidence of infection, placing these women at higher risk for HIV acquisition.

Chapter 2: Literature review

2.1.1: Worldwide prevalence of Schistosomiasis

Schistosomiasis or bilharziasis is a waterborne parasitic infection caused by three main species of worms: *Schistosoma mansoni*, *Schistosoma haematobium* and *Schistosoma japonicum*. *Schistosoma* spp infections are a major global health problem; tropical and sub-tropical countries (Saathoff et al., 2004), at least 200 million individuals may be infected worldwide with ~90% of these cases (~180 million) occurring in sub-Saharan Africa (SSA) (WHO, 2012, WHO, 2015). In SSA, the two main species responsible for infections are *S. mansoni* and *S. haematobium*, accounting for 280 000 infections that occur every year [reviewed by (Lustigman et al., 2012)]. *Schistosoma mansoni* and *S. haematobium* in SSA are the species that cause both hepatic-intestinal and urinary schistosomiasis [Figure 2.1].

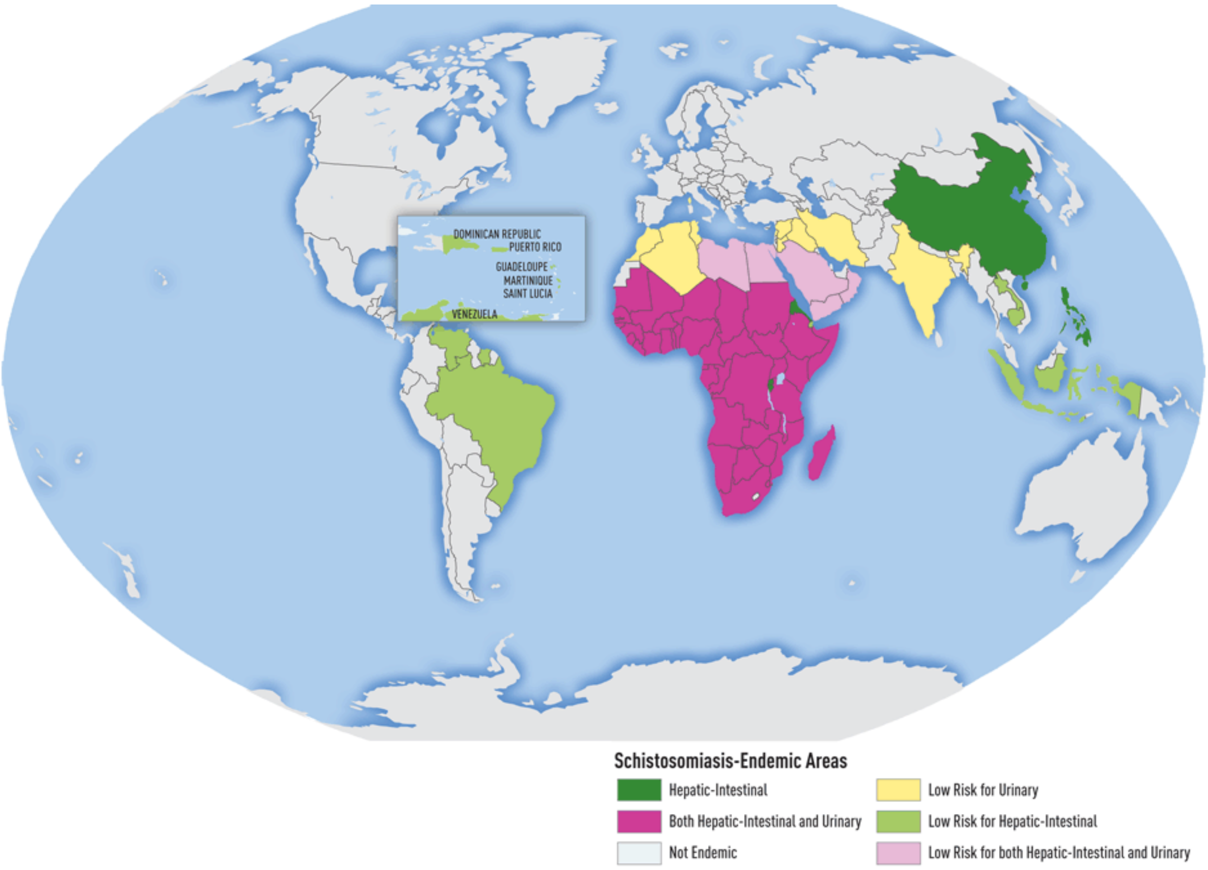


Figure 2.1: Geographical distribution of schistosomiasis. Sub-Saharan Africa has the majority of both hepatic-intestinal and urinary parasitic infections caused by *Schistoma* spp. (Center for Disease Control and Prevention, 2016). Illustration adapted in November 2016,

from http://wwwnc.cdc.gov/travel/yellowbook/2016/infectious-diseases-related-to-travel/schistosomiasis.

Schistosome infections have been the cause of many deaths worldwide, with SSA bearing the major burden of infection with attributable deaths (Figure 2.2).

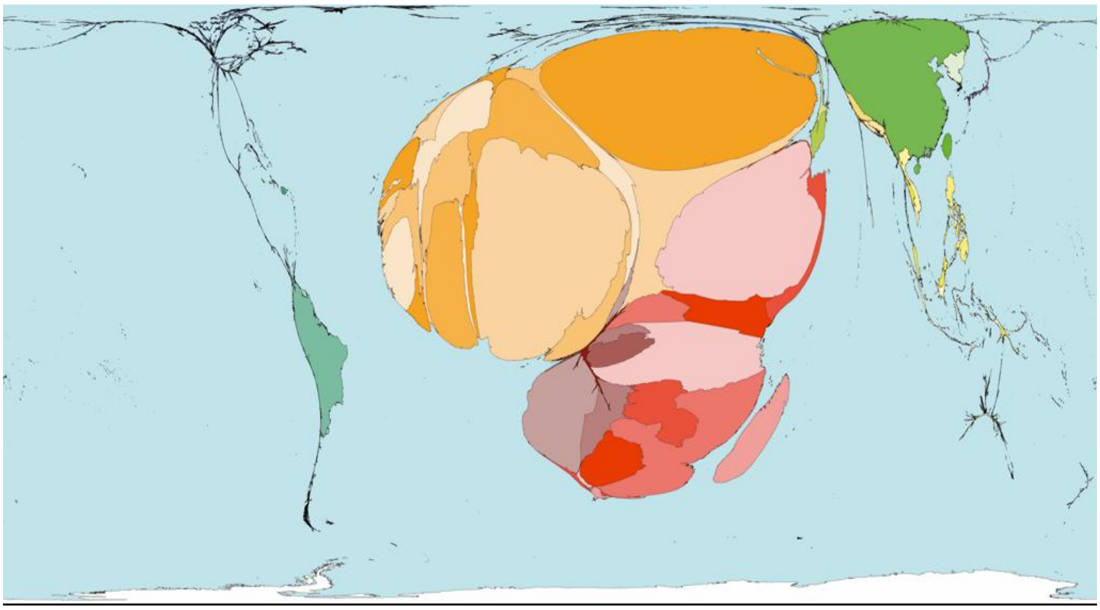


Figure 2.2: Burden of disease and deaths caused by schistosomiasis. Disease burden in Africa is highlighted by its disproportionate size relative to other continents. High prevalence of schistosoma infections (indicated in red) exist in SSA, with medium prevalence (indicated in orange and yellow) occurring in North African countries and low prevalence (indicated in green) in South America & Eastern countries . Figure shows deaths that occurred due to schistosomiasis in 2002 (0.08%), with an average of 7 deaths per million people (figure adapted in January 2015 from www.worldmapper.org).

Schistosomiasis is recognized as a disease of poverty, being highly preventable and curable (Olds and Dasarathy, 2001). Poor sanitation remains a key driver of this disease, particularly where people come into contact with human faeces or urine-contaminated water supplies (Center for Disease Control and Prevention, 2012). It has been proposed that women are at a higher risk of contracting this parasitic disease than men because they typically carry out domestic chores, including washing clothes, which may increase their exposure (Mutengo et al., 2014).

2.1.2 Prevalence of schistosomiasis in South Africa

Surveillance for schistosomiasis in South Africa is not routine, seroprevalence has been reported to be high, ranging from 71 to 100% in Limpopo and KwaZulu-Natal (KZN) provinces (Moodley et al., 2003). The prevalence of urinary schistosomiasis in KZN was 7.2% in 2004 [reviewed in (Johnson and Appleton, 2005)], however no more recent studies have been conducted on the parasitic burden in this province. Given the high sero-prevelence rates, schistosoma remains a major but neglected public health concern in South Africa, particularly in KZN (Kjetland et al., 2012).

2.1.3 Converging epidemics of HIV and helminth infections

It has been suggested that helminth infections impact negatively on the HIV epidemic (Mkhize-Kwitshana et al., 2011). In 2013, South Africa had ~6.4 million (range 5.8-6.1 million) individuals infected with HIV, with KZN province having a substantial portion of these individuals [~1.2 (1.1-1.3) million individuals] (WHO, 2013). Furthermore, women are at over two-fold higher risk to acquire HIV than males in South Africa (CDC, 2014), (Abdool Karim et al., 2010a). The majority of HIV infections that occur in South Africa are sexually transmitted (Abdool Karim et al., 2010b). Like schistosomiasis, KZN also has the highest prevalence of HIV (16.9%) than other provinces in South Africa (Shisana and Rehle, 2014).

Since cervicovaginal inflammation has been shown to significantly increase women’s risk for acquiring HIV infection (Masson et al., 2015) by ensuring higher numbers of HIV target cells being recruited to the genital tract (Anahtar et al., 2015), convergence of the HIV and *Schistosoma* spp epidemics in KZN may predispose women to HIV infection and is therefore important to investigate. Further recent evidence shows that systemic immune activation induced by helminth infections through broad T cell activation can lead to significantly increased susceptibility to HIV (Kroidl et al., 2016) and HPV infection (Gravitt et al., 2016).

2.2 *Schistosoma* life cycle

Schistosomes belong to an ancient lineage of the animal kingdom, known as Platyhelminthes. They occur in both parasitic and free-living forms (reviewed (Rollinson and Johnston, 1996). Snails are the intermediate hosts for schistosomes and are an essential component of the parasitic life cycle. Three major species that are responsible for causing *Schistosoma* infection are *S. haematobium* (usually but not confined to causing urinary schistosomiasis), *S. mansoni* and *S. japonicum* (usually but not confined to causing intestinal schistosomiasis). Even though the transmission routes are similar for the various species, they can be distinguished from each other based on egg morphology and also on the main anatomical sites they locate to and cause pathology (Pitchford, 1965). *S. haematobium* has a terminal spike on its ova whereas *S. mansoni* has a lateral spike. *S. japonicum* has a round or pear-shaped ova that are pale yellow as shown in Figure 2.3.

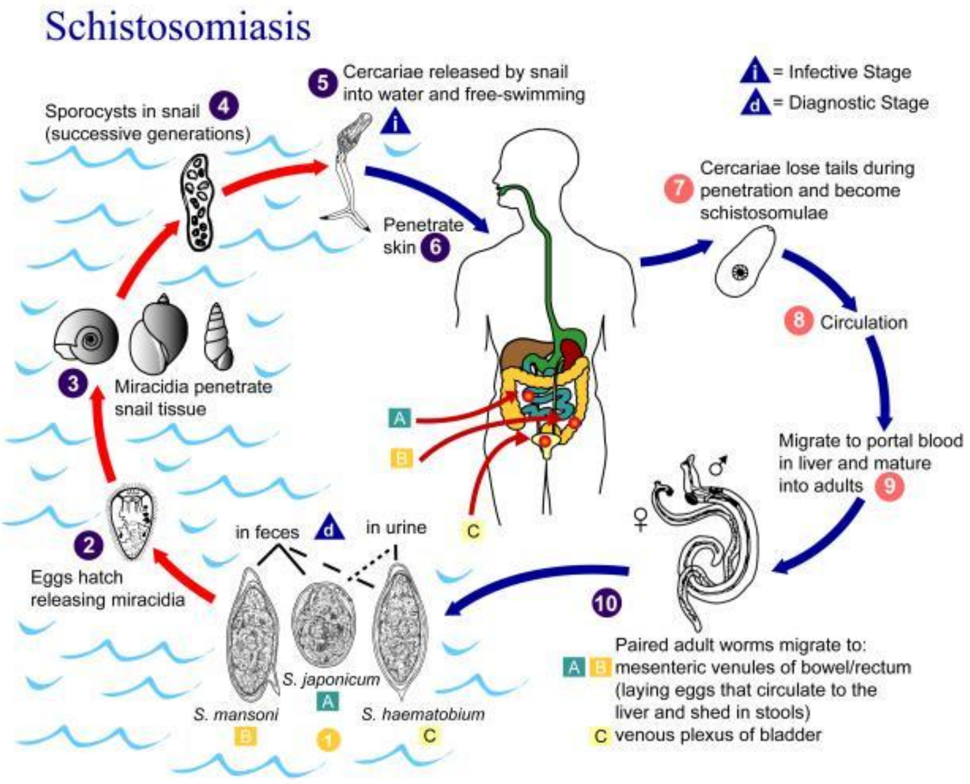


Figure 2.3: Parasitic life cycle of schistosoma species. Schistosoma cercariae penetrate skin of the host and cercariae lose their tails, forming schistosomules. These circulate in the host bloodstream to the liver where they mature into adult worms. Paired adult worms then move

to mesenteric vessels of the bowel or rectum, or to the venus plexus of the bladder and egg laying occurs through this passage. Eggs are shed either through the urine or through the stool of the human host. The eggs hatch in the water to release the miracidia, which then penetrate snails as their intermediate hosts, these miracidia then develop into sporocysts. Through several cycles, these mature into cercaria in order to complete the life cycle of the worm (Center for Disease Control and Prevention, 2012). Illustration adapted in January 2015, from http:// http://www.cdc.gov/parasites/schistosomiasis/biology.html

Schistosoma infection occurs when free-swimming larvae, called cercariae, penetrate the skin of individuals who come into contact with contaminated water (reviewed by (Rollinson and Johnston, 1996). The local site at which the cercariae penetrates the human host skin can lead to a condition called "swimmer's itch" (reviewed by (Rollinson and Johnston, 1996) which lasts for one week (Verbrugge et al., 2004). The cercariae otherwise pass unnoticed into the body and migrate via blood and lymph systems through the lungs to vessels of the liver (Harvie et al., 2007). In the liver of their host, worms pair-up, mature and move to preferred egg laying sites to complete their life cycle.

Sites of infection and manifestation of pathology are specific to the particular schistosoma species causing the infection. *S. mansoni* is known to cause intestinal schistosomiaisis (reviewed by (Rollinson and Johnston, 1996), whilst *S. haemotobium* causes mainly urinary or genital schistosomiasis (Kleppa et al., 2014). Often the schistosomules (immature form of schistosome after entry of the hosts blood vessels) migrate via the bloodstream and ultimately manifest in the bladder (Kjetland et al., 2012). Eggs are deposited into the tissue and may be passed through the stool or urine to carry on their life cycle depending on the site of infection (Figure 2.3).

2.2.1 Pulmonary schistosomiasis

Schistosomes may also migrate to and cause pathology in the lungs of human hosts. Pulmonary schistosomiasis is usually associated with miliary mottling or diffuse nodular infiltrates, detectable by X-ray (Schaberg et al., 1991). Infection usually develops in people residing or travelling in endemic areas (Niemann et al., 2010). Pulmonary schistosomiasis occurs 3–8 weeks after infection and symptoms such as fever, headache malaise, myalgia, cough, hepatomegaly and peripheral eosinophilia are experienced (Cooke et al., 1999).

Granuloma formation in the lungs and fibrosis around the schistosome eggs retained in the pulmonary vasculature may cause pulmonary hypertension leading to cor pulmonale, defined as abnormal enlargement of the right hand side of the heart caused by lung disease (Niemann et al., 2010).

2.2.2 Female genital schistosomiasis

Female genital schistosomiasis is defined by the presence of sandy patches visible in the lower female genital tract (Figure 2.4), or by microscopic confirmation of *S. haematobium* eggs in genital tissue *in situ*. In the lower reproductive tract, genital schistosomiasis may involve the vulva, vagina, ecto- and endocervix, and bladder, leading to pathogenesis and disease.

One mechanism proposed by which genital schistosomiasis symptoms could occur in the vagina is through transmission via the urinary tract in the infected individual. During urination, the ova are excreted out of the female genital tract (Kjetland et al., 2012). As the ova are excreted through the urine (depending on the load of ova in the urine), these ova can become trapped in tissues and be subsequently recognized and surrounded by immune cells that may have migrated to the site of ova in the tissues of the lower female genital tract. This results in an inflammatory reaction, which can lead to fibrotic scarring (Willey et al., 2008). An alternative mechanism that has been proposed may be spill-over of eggs into the genital tract and cervix through blood vessel anastomoses (where two blood vessels reconnect, that previously branched out) between the pelvic organs (Figure 2.4). These lesions or sandy patches (Figure 2.4B) and abnormal blood vessels in the genital tissue are visible when colposcopy is performed (Kjetland et al., 2012).

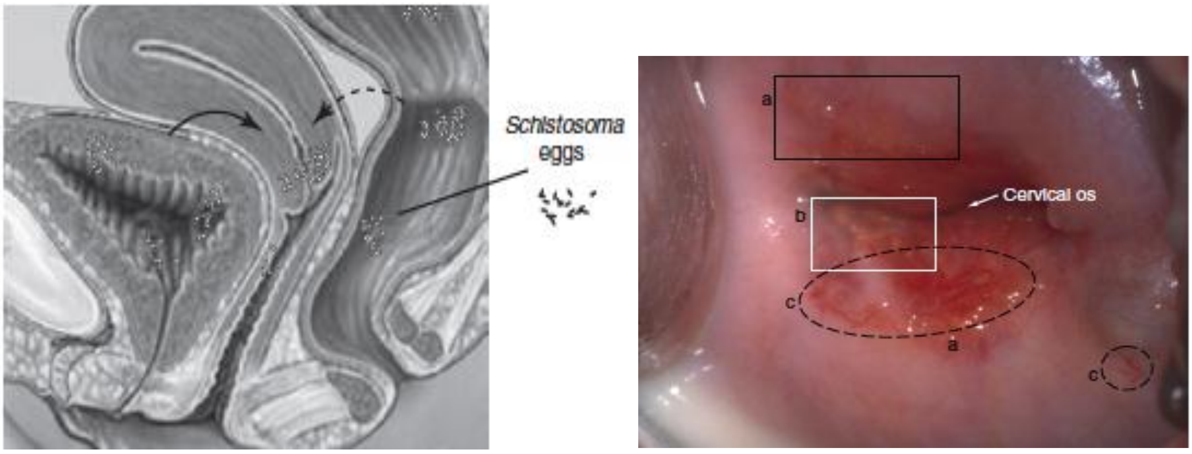


Figure 2.4: Urinary, genital and intestinal tracts of a female with schistosomiasis. (A) Diagram showing eggs of the helminth transmitted through the blood vessels anastomoses in the pelvic organs to the genital tract. This process results because of the proximity to the pelvic organs in the female [reviewed in (Kjetland et al., 2012)]. (B) Colposcopy image of the female genital tract from a Schistosoma-infected women, showing (a) homogenous yellow sandy patches (sandy areas with no distinct grains), (b) grainy sandy patches (forming oblong yellow to white grains, approximately 0.05 0.2 mm in size); (c) abnormal blood vessels [also referred to as 'neo-vascularization', are pathological convoluted (corkscrew)]; and/or branched and uneven-calibered blood vessels that are visible (under 15X magnification) on the mucosal surface. They are deeply or superficially situated in the mucosa. Diagnosis for female genital shistosomaisis is often quite difficult to make in the absence of colposcopy (Norseth et al., 2014).

Symptoms associated with female genital schistosomiasis, particularly cervical schistosomiasis, are non-specific and may be unremarkable. They may be associated with post-coital bleeding, lower abdominal pain or, in more severe cases, menorrhagia, which involves additional abnormal bleeding during menstruation (reviewed by (Rollinson and Johnston, 1996). Therefore, genital pathology caused by *S. haematobium* may go unnoticed and remain under-reported and under-treated. Colposcopy is the current gold standard for confirming the presence of female genital schistosomiasis (Kjetland et al., 2012). During colposcopy, cervical schistosomiasis can appear as cauliflower-like growths which are ulcerative, homogenous yellow areas, known as "sandy patches" (Mosunjac et al., 2003).

2.2.3 Gastrointestinal schistosomiasis

Some schistosome eggs pass through the wall of the intestine and is the most common manifestation and is usually caused due to *S. mansoni* [reviewed in (Rollinson and Johnston, 1996)]. *Schistosoma mansoni* lives in the bloodstream in the portal circulatory system (Pearce and MacDonald, 2002). In some rare cases, *S. haematobium* was the causative agent (Ata et al., 1970). Paired adult worms migrate to the mesenteric venules of the bowel/rectum and during this process eggs are laid which then circulate to the liver and are shed in stool. In the large intestine, the schistosoma ova are mainly distributed in the loose sub-mucosa, and fewer in the sub-serosa (which is the layer of tissue between the muscularis and serosa) where there is infrequent formation of multiple granulomas (Strickland, 1994). In the gastrointestinal mucosa, sandy patches usually occur if the sub-mucosa thickens due to fibrous tissue containing large numbers of calcified eggs. The mucosa then takes on a yellow dirty appearance (Strickland, 1994). Long term involvement of the gut can lead to bloody diarrhoea and abdominal pains (Nour, 2010). Eggs present in the intestinal veins migrate via portal blood flow to the liver causing inflammation which results in hepatic enlargement and periportal fibrosis (Harvie et al., 2007).

2.2.4 Helminth induced immune responses in humans

Schistosoma infections involve diverse organs and systems in the human host and can therefore elicit diverse immune responses. Cytokines are soluble proteins that exert both broad and targeted immune responses (Berger, 2000). T helper type 1 (Th1) cytokines are known to enhance pro-inflammatory cellular immune responses that account for killing intracellular parasites and eliciting autoimmune responses (Berger, 2000). T helper type 2 (Th2) cytokines control helminth infections, as well as act in allergic responses and enhance antibody responses, through the upregulation of IgE and eosinophilic responses typically found in atopy (Berger, 2000).

Helminth infections in humans are generally associated with Th2 immune responses (T-cells that produce 1L-4 and IL-13) (Adachi et al., 2014). In the early phase of infection, Th1 immune responses are present while Th2 immune responses dominate after oviposition (deposition of eggs by *Schistosoma* spp) (Figure 2.5) (Adachi et al., 2014). Infection with parasites (such as the schistosoma species) usually result in the production of interleukin (IL)-4, IL-5, IL-9 and IL-13, typically associated with Th2-type responses (Adachi et al., 2014). These Th2-associated cytokines mediate immune responses by inducing differentiation of T cells, production of IgE and IgG by activated B cells, and recruitment and activation of innate effector cells (Maizels et al., 1993).

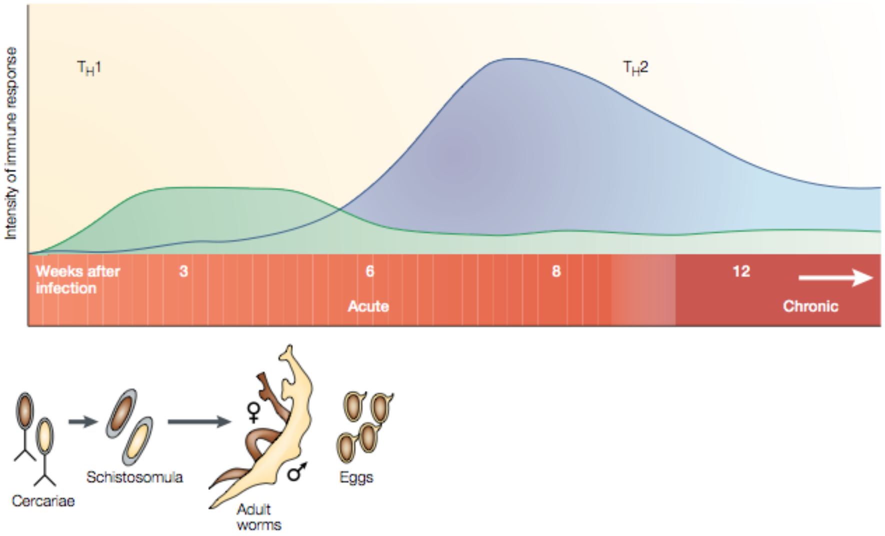


Figure 2.5: Th1- and Th2-immune response phases during *Schistosoma* infection. Th1 immune responses are initiated during the initial acute phase of infection, and wane after 5-6 weeks. Th2-immune responses emerge at 6 weeks post infection, where they dominate and reach a steady state into the chronic stage of infection [adapted from (Pearce and MacDonald, 2002)].

2.2.5 Helminth immune modulation increases susceptibility to other diseases

Helminth infections are thought to impair host immune responses to many diseases, because they skew host immunity to Th2 rather than Th1-type responses typically associated with protection from infectious diseases (Chen et al., 2012, Horsnell and Brombacher, 2008, Horsnell and Brombacher, 2010). Parasitic infection play a role in altering susceptibility to autoimmune disorders and allergies, (Santiago Hda et al., 2015) through increased IgE and eosinophilic responses (Huang et al., 2015). This suggests that parasitic infection impairs host immune function through ongoing antigenic stimulation in various biological compartments (Bashir et al., 2002). Infection by helminths are known to increase the susceptibility for HIV acquisition (Kroidl et al., 2016) and HPV infection (Gravitt et al., 2016)due to the systemic immune activation caused by these parasites.

2.2.6 Schistosomiasis as a risk factor for HIV acquisition

Understanding mucosal risk factors in the female genital tract is critical for understanding HIV susceptibility. Female urogenital schistosomiasis (FUS) could pose as a risk factor for HIV acquisition (Downs et al., 2011). A few epidemiological studies have highlighted increased risk for HIV acquistion in areas highly endemic for schistoma parasites (Downs et al., 2011, Kjetland et al., 2006, Ndhlovu et al., 2007). Furthermore, female genital schistosomaisis along with sexually transmitted infections (STIs) are associated with an increased susceptibility to HIV-1 acquisition, presumably because they create lesions in the genital mucosa allowing entry of the virus (Kleppa et al., 2014, Mbabazi et al., 2011).

Immunological studies have provided mechanistic insight into the relationship between schistosomiasis and risk for HIV acquisition. In addition to causing lesions in the genital mucosa, schistosomiasis also appears to increase HIV susceptibility through inducing chronic immune modulation (Secor et al., 2003). The various mechanisms put forward show that there is a bias towards the Th2 immune response and at the same time there is a downregulation of the Th1 response which may then increase the susceptibility to HIV infection (Pearce et al., 1991). This is further supported by evidence that CD4+ T-cells with a Th2 phenotype rather than a Th1 phenotype were more likely to be HIV-infected (Maggi et al., 1994), and in the presence of a background of a *S. mansoni* –HIV co-infection, these Th2 CD4+ T-cells are preferentially depleted [reviewed in (Mbabazi et al., 2011, Mwinzi et al., 2001)]. In addition, *S. mansoni* –HIV co-infection also depletes HIV specific CD8 T-cell responses further compromising immunity (McElroy et al., 2005). However, despite the decline of CD4+ and CD8+ T-cells with *S. mansoni*-HIV co-infection, upregulated surface expression of CCR5 and CXCR4 (major co-receptors that facilitate the entry of HIV into CD4+ T-cells) on target cells increase HIV infection of cells and replication (Secor et al., 2003). However praziquantel treatment in *Schistosoma*-HIV co-infected individuals have been shown downregulate CCR5 and CXCR4 expression (Secor et al., 2003), suggesting that such treatment may indeed impact on the spread of HIV in high prevalence areas where HIV and *Schistosoma spp* prevail.

HIV-1 and STIs remain a public health challenge and the interplay between these and the presence of female genital schistosomiasis remains largely undefined. The CAPRISA 002 study focused on women that were in the acute phase of HIV infection and aimed to characterize the viral set point, clinical progression of subtype C infection and identify host genetic, viral and immune factors that may predict disease progression. In the CAPRISA002 Acute Infection cohort, Mlisana et al., (2012) demonstrated that both symptomatic and asymptomatic STIs resulted in increased concentrations of several soluble cytokines at cervicovaginal lavages (CVLs). These inflammatory cytokines were independent predictors of HIV acquisition in prospective follow-up, after accounting for multiple comparisons, including: IL-6 (HR 1.25, 95% CI 1.05-1.50), IL-1β (HR 1.28, 95% CI 1.07-1.54), IL-8 (HR 1.39, 95% CI 1.04-1.87) and sCD40L (HR 1.45, 95% CI 1.02-2.07; per 1 log10 pg/ml increase in cytokine concentration.

Pre-infection, Masson et al. (2015) further showed that in women with genital inflammation there was a >3.0 fold increased risk for HIV acquisition compared to women with no genital tract inflammation. Inflammation was defined as having 5 of 9 key pro-inflammatory cytokines (MIP-1α, MIP-1β, IP-10, IL-8, MCP-1, IL-1α, IL-1β, IL-6, and TNF-α) elevated in the genital tract. Genital tract inflammation, defined as being in the highest quartile for any combination of 5 of 9 inflammatory cytokines (including IL-1α, IL-1β, TNFα, IL-6, IL-8, IP-10, MCP-1, MIP-1α, and MIP-1β) was associated with a 2.9-fold increase in risk of HIV acquisition overall.

Kedzierska and Crowe (2001) reported that Th1 cytokines (IL-2 and IFNγ) are generally suppressed during HIV infection, whilst Th2 cytokines (IL-4 and IL-10) are elevated. IL-10 treatment inhibited HIV replication in monocytes (Masood et al., 1994). The interplay between the Th1 and Th2 responses in the presence of genital inflammation due to STIs and parasitic infection remains poorly understood.

2.2.7 Anatomy and physiology of the female genital tract

The female reproductive tract has distinct anatomical compartments composed of the upper tract including the endocervix and uterus, and the lower tract including the ectocervix and vagina. The upper genital tract is a predominantly sterile environment, whilst the lower genital tract comprises a complex biome of commensal flora, including lactobacillus species, and sometimes pathogens (Huszar, 1991). *Lactobacillus* species promote vaginal health by producing lactic acid that lowers pH that is inhospitable to many non-commensal bacteria. Predominance of Lactobacilli is negatively correlated with bacterial vaginosis (BV), a dysbiotic outgrowth of anaerobic bacteria (Lamont et al., 2011). Lactobacilli in the genital tract also produce hydrogen peroxide (H_2O_2), antibiotic toxic hydroxyl radicals, bacteriocins and probiotics (Lamont et al., 2011) which have antimicrobial activity.

The female vaginal tract has two types of epithelia: type I and type II. The type I epithelium of the endocervix is comprised of the simple columnar epithelium while the type II epithelium of the vagina and ectocervix mucosa comprises non-keratinized stratified squamous epithelium. This forms a natural barrier against pathogens (Rose et al., 2012). The region at which the two types of epithelia transition is known as the transformation zone, and is thought to be particularly susceptible to infections including human papillomavirus (HPV) and HIV. The sub-mucosa lies beneath the epithelium, which is the site of innate and adaptive immune cells.

2.2.8 Innate immune responses in the female genital tract

At mucosal barriers to the outside world, one of the first barriers against infection is mucus, which acts as a defense barrier for most invading pathogens. The lower reproductive tract in women is lined with mucus which covers the luminal surface (Iwasaki, 2010). In an aqueous matrix, the thick consistency of the mucus is due to its constituents such as glycoproteins and gel-forming mucins (Pudney et al., 2005). Goblet cells are responsible for producing and secreting mucus in type I epithelia, whilst local mucus-secreting epithelial cells produce mucus in type II epithelia (Pudney et al., 2005). In the lower reproductive tract, goblet cells are found in the endocervical canal. Vaginal mucus also contains other immune defense molecules such as antibodies, antimicrobial peptides and enzymes.

In the lower reproductive tract, a variety of immune defense molecules [such as complement proteins, antimicrobial peptides which includes mannose binding lectins (MBLs) and secretory leukocyte protease inhibitors (SLPI)] are responsible for protecting the vagina against invading pathogens [reviewed in (Iwasaki, 2010)]. MBLs bind to carbohydrates on various, parasites, pathogens & bacteria. Complement proteins have been detected in cervicovaginal secretions, providing first-line defence against infectious agents colonizing the lower region of the reproductive system (Bulla et al., 2010). These molecules prevent interaction between the pathogens and vaginal epithelium by directly combating and killing foreign pathogens. These antimicrobial compounds are constantly produced by vaginal mucosal epithelial cells, and neutrophils present in the sub-mucosa and cervix [reviewed in (Iwasaki, 2010)]. Vaginal fluid also contains defensins and lysozyme that contribute to antimicrobial defense (Valore et al., 2002).

The presence of commensal flora at the vaginal mucosa has recently been shown to be central in maintaining genital health in women [reviewed (Spurbeck and Arvidson, 2011)]. Commensal bacteria typically found in the lower reproductive tract include *Lactobacillus, Corynebacterium* and *Peptostreptococcus* spp., with *Lactobacillus* species being the most prevalent [reviewed in (Spurbeck and Arvidson, 2011)].

Innate immune cells that provide protection against pathogens in the vaginal mucosa include macrophages, dendritic cells (DC), langerhans cells and γδ T cells [reviewed in (Milligan, 1999)]. Additional innate immune cells are recruited to the site of pathogen invasion, which include natural killer cells (NK cells), neutrophils, monocytes and plasmacytoid DCs (pDCs) (Pudney et al., 2005). Dendritic cells patrol and protect the vaginal environment through phagocytosis [reviewed in (Savina and Amigorena, 2007)]. Upon DC activation (Figure 2.6) by an invading pathogen, they migrate to the draining lymph node where they prime naïve T and B cells, initiating an adaptive immune response [reviewed in (Iwasaki and Medzhitov, 2004)].

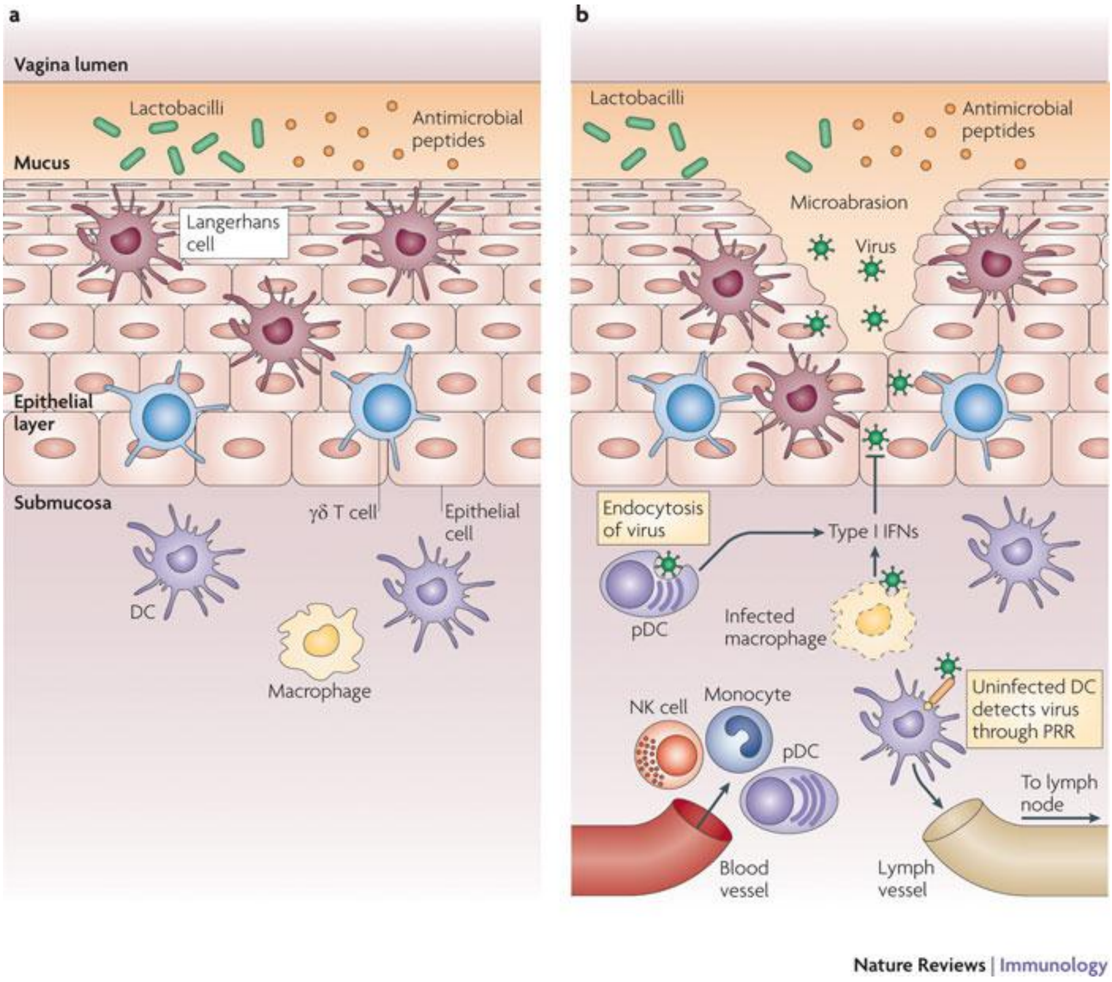


Figure 2.6: Innate immune responses at vaginal mucosa. a) Innate immune cells such as DCs and macrophages patrol the vaginal lumen during a relaxed state whilst the epithelial layer of the vaginal lumen is still intact (no invading pathogen). b) Microabrasions of the epithelial layer occurs upon invasion of antimicrobial peptides, viruses and pathogens. Antigen presentations by DCs that detect pathogens occur and migration to the lymph node takes place. Infected macrophages release type I IFNs, whilst NK cells and monocytes are also released from the blood stream [taken from (Iwasaki, 2010)].

2.2.9 Adaptive immune responses in vaginal mucosa to pathogens

Upon detection of an invading pathogen, antigen presenting cells such as DCs take up foreign antigen at the mucosal surface, migrate to the lymph nodes and present to naïve CD4$^+$ and CD8$^+$T cells (Lee et al., 2009). Activated CD8+ T cells, which are also known to be cytotoxic, migrate to the site of infection and kill virus-infected cells expressing cognate antigen (Altfeld et al., 2002). CD4+ Th1 effector cells have the ability to protect against viruses by migrating to the mucosa and secreting interferon gamma (IFN-γ) (Iijima et al., 2008). Following infection, Th2 cells induce class switching (biological process that changes B cell's immunoglobulin production from one type to another) in B cells, leading to antibody production (Toellner et al., 1998). Antibody-producing B cells constitute an important arm of the adaptive immune response in the genital mucosa. Sexual transmission of pathogenic viruses, parasites and bacteria are the most common causes of infection in the genital tract, with HIV being the most prevalent sexually-transmitted viral infection among women in SSA [reviewed by (Quinn and Overbaugh, 2005)].

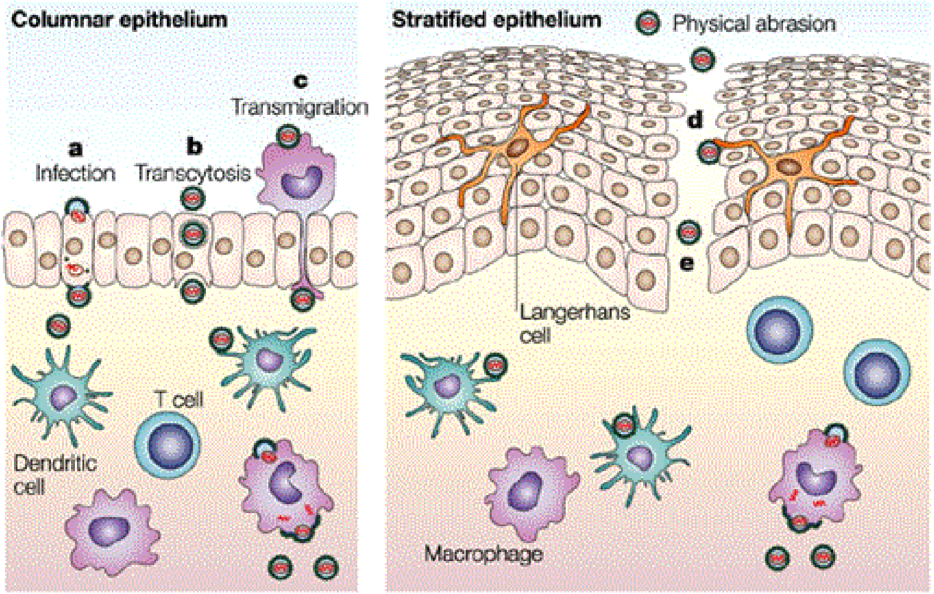
Although less studied and less common, parasitic infection of the female genital tract, causing genital inflammation, could lead to an increased susceptibility to HIV-1 infection (Feldmeier et al., 1998).

2.2.10 HIV transmission across the vaginal mucosal barrier

The vaginal epithelium is a multi-layered thick, mucus-coated barrier that is almost impenetrable to invading viruses, bacteria and parasites. Lesions caused by female genital schistomomiasis provides opportunity for HIV to enter the mucosal barrier through these microabrasions and gain access to target cells in the submucosa, including CD4+ T cells, DCs, langerhans cells and macrophages [reviewed in (Magaisa et al., 2015)]. About 80% of new HIV infections are caused by a single HIV variant following vaginal transmission, usually referred to as a “bottleneck” (Keele et al., 2008).

However, in the presence of vaginal microabrasions, multiple viral strains may have a chance to permeate and enter the genital mucosae (Iwasaki, 2010).

Several mechanisms have been proposed for how HIV is able to cross the mucosal barrier and gain access to CD4+ target cells, including transcytosis of the epithelial layer, or entry through micro-lesions or microabrasions in the epithelial barrier [Figure 2.7; reviewed by (Shattock and Moore, 2003)]. Infection of Langerhans cells within the epithelium which can transmit virus to CD4+ target cells in the submucosa is another hypothesized method of transmission (Miller and Shattock, 2003). The transformation zone between the multi-layered squamous epithelia of the vagina and single-layered columnar epithelium in the endocervix was previously thought to be the most susceptible point for HIV infection because of the presence of high numbers of potential target cells like the CD4+ cells in this region (Carias et al., 2013). More recently, Stieh et al. (2014) showed that HIV also caused infection of the upper reproductive tract.



Nature Reviews | Microbiology

Figure 2.7: Mechanisms by which HIV crosses the vaginal mucosal epithelium [taken from (Shattock and Moore, 2003)]. Left figure panel represents HIV crossing the mucosal barrier in columnar epithelium (a) infection by HIV with viral RNA integrating into the DNA of host cells, (b) transcytosis of HIV virion through mucosal barrier & (c) transmigration of HIV virion that's attached to a macrophage. The right panel of the figure represents the HIV mechanism of entry in

stratified epithelium, (d) HIV virions attach to Langerhans cells between the mucosal barriers due to (e) physical abrasions that allow HIV entry to occur with ease. As pathogens invade the mucosal barrier, dendritic cells come into contact with the viral particles for antigen presentation [reviewed in (Neutra and Kozlowski, 2006)].

Recruitment of HIV target cells in the female genital tract under cytokine gradients can strongly influence susceptibility to HIV acquisition. Other co-factors including STIs and parasitic infections have been associated with increased risk for HIV acquisition (Feldmeier et al., 1998). Parasitic infections may cause increased immune activation, particularly in co-endemic regions where STIs such as HIV and parasites co-exist (Bentwich et al., 1995). Helminths that induce systemic immune responses could also alter the local mucosal immunity of the genital tract (Witkin et al., 2000).

Infection by *Schistosoma* spp remains a global challenge. Sub-Saharan Africa has the largest burden for both the *Schistosoma* infections and HIV. In particular, in South Africa, Kwa-Zulu Natal has the highest prevalence of HIV infections countrywide and is therefore epicenter for HIV. At the same time, KZN is also endemic for *Schistosoma* spp. and has the highest prevalence of this disease countrywide too. This study that was undertaken in two sites KwaZulu-Natal therefore focuses on the interplay between these two diseases and explores the immunological risk factors for HIV-acquisition in the presence of *Schistosoma* infection in the female genital tract.

The aims of this dissertation were to:

- Determine the prevalence and incidence of HIV in women with/without *Schistosoma* spp infections, by indentifying presence of schistosome DNA and *Schistosoma* spp antibodies in women who were part of the placebo arm of the CAPRISA 004 trial.

- Determine the differences in cytokine levels that play a role in genital inflammation in women with/without FGS and with/without HIV. This could help determine whether genital schistosomiasis influenced inflammation was associated with increased risk of HIV infection.

Specifically, the hypothesis was that women with detectable DNA specific for parasitic infection in the genital mucosae had elevated genital cytokine responses compared to women who did not show evidence of infection placing these women at higher risk for HIV acquisition.

Chapter 3: Methodology

3.1 Study design and population

This study was a retrospective study of 383 women from the placebo arm of the CAPRISA 004 1% tenofovir micobicide gel trial (Abdool Karim et al., 2010a). Participants for the study were from both a rural community (Vulindlela, Kwa-Zulu Natal) and an urban community (eThekwini Clinic, Durban). All women consented to the study. This sub-study included specimens from two time-points for each women: study entry time-point (3 months post-enrollment) and study exit, with an average period of 3 years between the study entry and the study exit time-points. Cervicovaginal lavage (CVL) supernatants, CVL pellets and anti-coagulant dextrose plasma (ACD) samples were selected from both time-points for this sub-study.

3.2 Ethical considerations

The CAPRISA 004 trial, which assessed the safety and effectiveness of the vaginal microbicide 1% Tenofovir gel (Abdool Karim et al., 2010a) was approved by the Biomedical Research Ethics Committee (BREC 111/06). This sub-study underwent expedited ethics approval (BREC 333/13). A copy of the original ethics approval letter and expedited BREC approval letter for the sub-study are attached as Annexure 1 (Appendix).

3.3 CVL collection and processing

CVLs were obtained and processed as previously reported (Bebell et al., 2008, Mlisana et al., 2012). A volume of 5ml of sterile PBS was used to irrigate and repeatedly bathe the cervix. The pooled cervicovaginal fluids that were aspirated from the vaginal vault using a sterile plastic bulb pipette were dispensed into sterile conical tubes and transported on ice to the laboratory for further processing. CVLs were centrifuged, and the supernatant was stored at −80°C. CVL samples were not collected from menstruating participants, for whom sampling was postponed to the following week.

3.4 Plasma preparation

Whole blood collected into ACD tubes were spun down at 800 x g for 30min for the processing and storage of the ACD plasma samples. After centrifugation, the ACD plasma was pipetted out and stored at -80°C until further use.

3.5 HIV screening and testing

At each study visit of the CAPRISA 004 trial, two HIV rapid tests [Determine HIV 1/2 (Abbott Laboratories, Illinois, USA) and Uni-Gold Recombigen® HIV test (Trinity Biotech, Wicklow, Ireland)] were performed for each participant to confirm their serostatus. Study participants with concordantly positive, discordant or indeterminate results were assessed for possible seroconversion by means of two separate RNA PCRs (Roche Cobas Amplicor HIV-1 Monitor v1.5, Roche Diagnostics, Branchburg, New Jersey, USA), approximately one week apart. When HIV seroconversion was confirmed, use of the microbicide or placebo product was discontinued and women were enrolled in local AIDS treatment services, including the CAPRISA AIDS Treatment Program which provided free antiretroviral therapy for HIV infected participants.

3.6 DNA isolation: Template for downstream Real-time PCR

DNA for real-time PCR was isolated from all CVL pellet samples using the MagNA pure isolation kits (Roche, Germany). Volumes of 50µl of reconstituted cervical pellets per sample were aliquoted into 32-well cartridges (Roche, Germany). According to the manufacturer's instructions, the samples were lysed with lysis buffer, followed by a series of wash and precipitation steps with ethanol-based wash buffers using an automated extraction method involving the use of the MagNA pure 32 (Roche, Germany) for the extraction and the isolation of the DNA. The DNA was then eluted in 100µl of elution buffer that was supplied in the kit and was stored at -80^{o}C until utilized. The concentration of DNA was quantified using the NanoDrop 2000 UV-Vis spectrophotometer (Thermo Fischer Scientific, USA) and only the DNA samples with a OD_{260}/OD_{280} ratio of 1.8 were used. The DNA samples were then standardised to 1µg of DNA for each sample and for downstream use in real-time PCR amplification.

3.7 Preparation of *S. mansoni* DNA for PCR positive control

In order to verify the presence of the parasite in the clinical isolates of the DNA samples from the participants in this sub-study we amplified a region of Schistosome genome common to both *S. mansoni* and *S. haematobium*. This allowed the use of cercarial *S. mansoni* DNA as a postive control. Total DNA was extracted manually from the cercariae of *S. mansoni,* (obtained from the Division of Immunology, IDM, University of Cape Town) according to the manufacturer's instruction using QIAamp DNA mini kits (Qiagen, USA). This cercarial DNA was used as a positive control for the real-time PCR assay when screening for the presence of both *S. haematobium* and *S. mansoni* DNA.

3.8 Primer Design, cycling conditions and Real-time amplification of target schistosomal DNA

Primers are short nucleic acid sequences that serve as a target specific starting point for PCR or DNA replication processes. Specific primers Ssp48F and Ssp124R (Kjetland et al., 2009) were designed and synthesized to detect a target region common to both *Schistosoma* species (Roche, Germany) and were validated in the Hasso-Plattner Laboratory at the HIV Pathogenesis Programme laboratory, University of KwaZulu-Natal. Oligonucleotides for Ssp48F &Ssp124R shown below in table 3.1 were used to amplify the 77-base pair (bp) fragment of the internal transcription spacer (ITS), homologous to both *S. haematobium* and *S. mansoni* species from DNA extracted from the CVL pellets. The ITS is the DNA spaced between small sub-unit ribosomal RNA (rRNA) and large sub-unit rRNA.

Table 3.1: Primer sequences

Specific primers	Sequence 5'- 3'
Ssp48F	5′-GGT CTA GAT GAC TTG ATY GAG ATG CT-3′
Ssp124R	5′-TCC CGA GCG YGT ATA ATG TCA TTA-3′

3.9 Real-time polymerase chain reaction for amplification of schistosomal DNA

Real-time PCR was performed using the Light Cycler LC 480 (Roche, Germany) to detect *Schistosoma spp.* DNA in the CVL pellets from genital samples obtained through cervico-vaginal lavage. Samples that tested positive for schistosome DNA in the pellet of the CVLs were defined as women who were FGS PCR positive for the purposes of this study. Furthermore in this study, women that were found to be schistosome DNA PCR positive in their CVL do not necessarily have FGS and not all women that have FGS would have tested positive by PCR for schistosome DNA in the CVL due to the insensitivity of the genital tract PCR (Kjetland et al., 2009, Randrianasolo et al., 2015). Cycling conditions used were as follows: 95°C for 5 minutes, 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds with a total of 55 cycles.

Each PCR reaction contained 10µl according to the Master mix reagents and volumes are shown below in table 3.2. Each of the DNA samples were run in in duplicate on a 96-well real-time PCR plate purchased from Roche. Each 96-well plate for PCR was run with a positive and a negative control. SYBR green was used as an intercalating fluorescent dye to detect the presence of double stranded DNA amplicons which would represent a positive result. In addition, the melting temperature of 81.2°C, or within range of 1°C when compared to the melting temperature of the positive control (81.2°C) were also used to confirm a positive PCR result. Furthermore, samples deemed as positive were also validated on a 1% agarose gel to confirm molecular weights of PCR products with the use of a molecular base pair ladder (GeneRuler, ThermoFisher Scientific, USA).

Table 3.2: Real-time PCR reaction volumes (10µl)

Master mix reagents	Master Mix for 1X	Master Mix for 100X
H_2O	2.5µl	250µl
Ssp48F (2.5pmol/µl)	0.25µl	25µl
Ssp124R (2.5pmol/µl)	0.25µl	25µl
SYBR green	5µl	500µl
DNA template	2µl	

Real-time PCR in general has specific advantages over conventional PCR. Conventional PCR is only measured at the end-point of the assay (plateau), whilst real-time PCR provides data in the exponential growth phase of a reaction. Monitoring of melt curves also provides a definitive tool for the identification of PCR positive samples when looking at fluorescence history and melting temperatures. In real-time PCR, an increase in reporter fluorescence signal (Figure 3.1) is directly proportional to the amount of double-stranded DNA (amplicons) present.

Real-time PCR is therefore quantitative while conventional PCR is largely qualitative. The cleaved probe provides a permanent record amplification of the amplicons. For real-time PCR, there is an increase in dynamic range of detection and does not require post PCR processing.

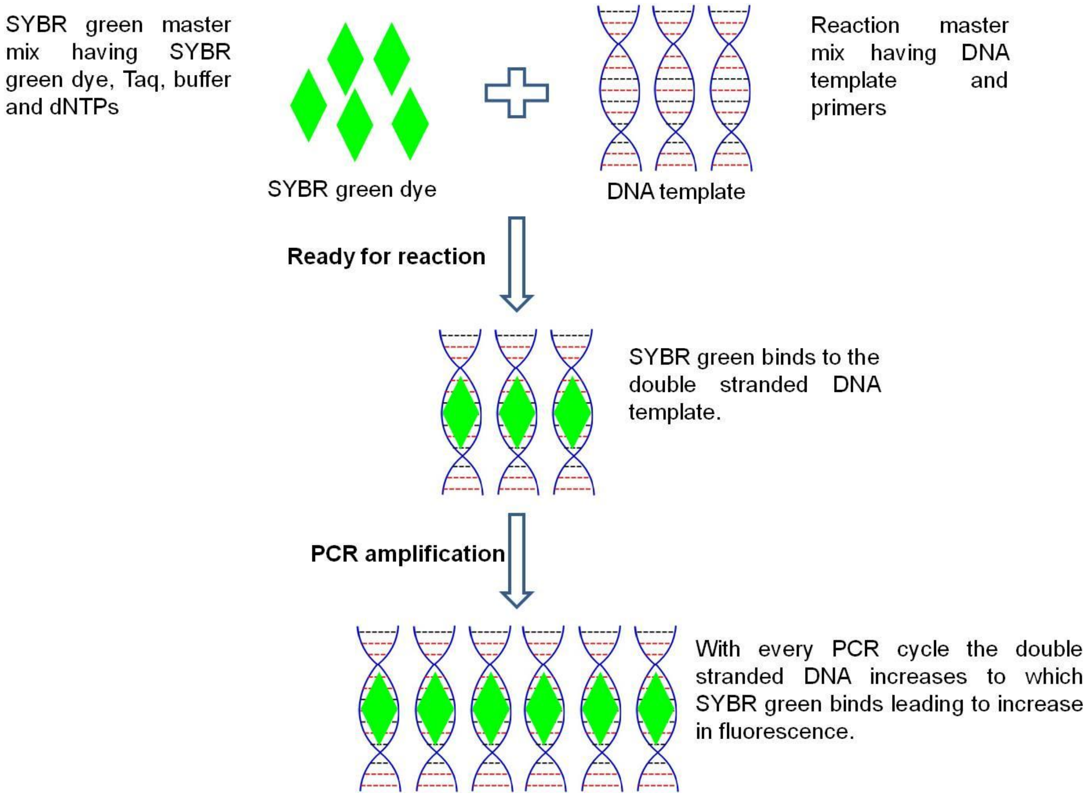


Figure 3.1: RT-PCR principle using universal intercalating dye SYBR green (SG). As the amplification of DNA increases with every cycle, more SG is bound to the double stranded DNA, corresponding to a proportional increase in fluorescence [adapted from (Ali et al., 2011)].

Positive FGS PCR samples were identified by Ct (cycle threshold) values between cycles 15-30 (Figure 4.1). Cycle threshold values are the fractional cycle of a PCR at which the normalized signal of the reporter dye intercepts the threshold. No Ct values were observed for Negative FGS PCR samples as the amplification curve (representing the reporter dye of the target) of the negative samples did not intercept the threshold.

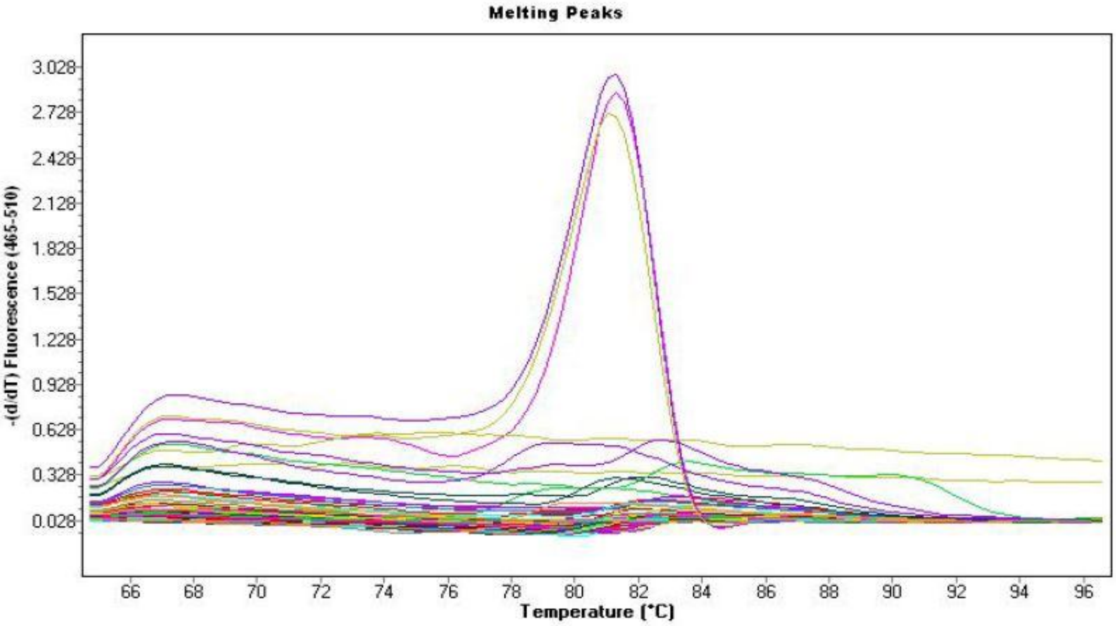


Figure 3.2: Melt peaks of real-time PCR assay showing melting curves temperatures of amplicons from nine participants positive for FGS at 81.5°C, thus validating specific PCR product.

Distinct peaks on melt peak plots are representative of the specific targeted PCR product. Samples positive for FGS PCR that share the same melting temperature on melt peaks, are a clear indication of a specific PCR product. Melting temperature is the temperature at which the double stranded DNA product separates into two single strands. Polymerase chain reaction products of equal size share common melting temperatures. In this study, amplicons with a melting temperature of 81.5°C (Figure 3.2) were identified as FGS positive PCR products and confirmatory tests of the RT PCR products through agarose gel electrophoresis were performed in order to further validate that the amplicons were the same molecular mass as that of the positive control. Figure 3.3 represents the 77 base pair FGS PCR amplicons that were amplified from the RT PCR reactions of the genital CVL specimens.

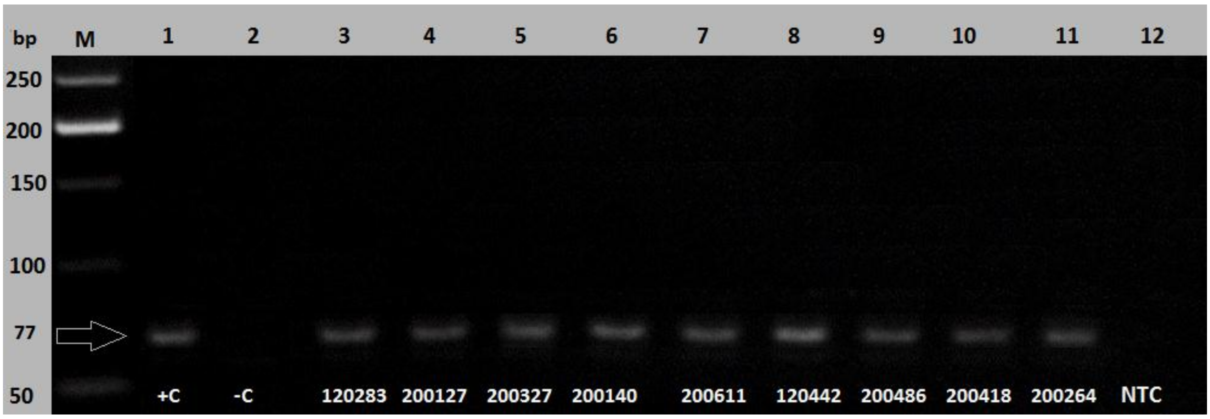


Figure 3.3: FGS PCR amplicons (77 bp) performed using a 1% agarose gel as a confirmation to the analysis of melt peaks from the real-time PCR data. The positive control together with the molecular weight ladder confirmed the positive results obtained. NTC = None-template control, +C = Positive control, -C = Negative control.

3.10 Cycle sequencing of PCR products and sequencing clean-up reaction prior to capillary electrophoresis

In order to validate FGS PCR amplicons, products were also amplified conventionally [Table 8.1 & 8.2, (Annexure 2, Appendix)] using Platinum *Taq* DNA polymerase (Invitrogen, USA) and subjected to DNA sequencing. DNA obtained from the genital CVL samples with the same molecular mass and sharing the same melt peaks as the positive control were then subjected to DNA sequencing with BigDye v3.1 terminator kits (Applied Biosystems, USA) on a 3130XL Genetic Analyzer (Applied Biosystems, USA). Sequencing clean-up reactions were performed using the 3M sodium acetate/100% ethanol precipitation with a dilution of 1:10. Sequencing was performed on the 3130 XL genetic analyzer (Applied Biosystems, USA). Sequence reaction volumes are shown below in table 3.3.

Table 3.3: DNA sequencing reaction volumes

Sequencing reagents	Master Mix for 1X	Master Mix for 12X
H_2O	3.6µl	43.2µl
BigDye	0.4µl	4.8µl
5X sequencing buffer	2µl	24µl
Primer (1.6pmol/µl)	2µl	24µl
Amplified DNA from CVL pellets	2µl	

3.11 Measurement of *Schistosoma* antibodies by ELISA

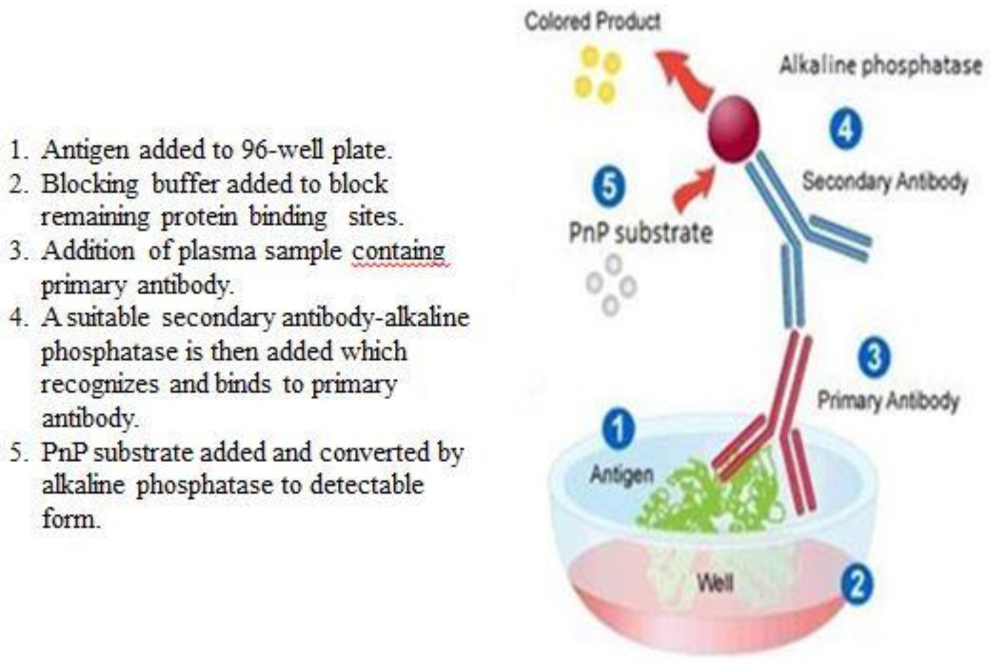
In order to detect the presence of *Schistosoma haematobium/mansoni* IgG antibodies (as a surrogate for exposure to the parasites), qualitative enzyme-linked immunosorbent assays (ELISA) were performed on all ACD plasma samples at both study time points. Due to the cross-reactivity of the IgG between the species, *S. mansoni* antigen used to coat the plates was supplied by the Division of Immunology at the University of Cape Town in order to detect antibodies to both *S. haematobium* or *S. mansoni* species. Plasma samples that were obtained from the Division of Immunology at the University of Cape Town, were from individuals confirmed to be sero-positive for schistosome infections. These samples were used as a positive control for each ELISA, on every plate run in order to validate the assay for detecting human IgG responses to *S. mansoni* antigen. Plasma samples that had no history of exposure to helminth infections as well as standard PBS were used as negative controls for each ELISA plate.

To pre-coat the ELISA plates (day 1), 96 well Nunc-Maxisorp plates (Nunc Maxisorp™; ThermoFisher Scientific, Roskilde, Denmark) were coated with 50µl of 5µg/ml *S. mansoni* antigen and incubated for 3 hours at 37°C in a 5% CO_2 incubator (ThermoFisher Scientific, USA). The antigen used to coat the plates was diluted 1:100 in carbonate buffer (the methods for the various wash and blocking buffers for this procedure is attached as Annexure 2). Plates were then washed three times with each well containing 200µl of the 1X wash buffer to wash off any excess or unbound antigen. Blocking buffer was then prepared as a 2% milk powder in PBS and all wells were then blocked with 200 µl of blocking buffer overnight at 4°C and plates were wrapped in cling wrap to prevent evaporation.

On day 2, ELISA plates were washed three times with 200µl wash buffer 24 hours later. Dilution buffer (made up using 1% bovine serum albumin PBS solution) was used to dilute ACD plasma samples. Dilutions of the plasma samples (80µl/reaction) were done on V-bottom 96 well plates, first a 1:50 dilution of ACD plasma sample into dilution buffer, followed by 1:5 dilution for a further seven serial dilutions in dilution buffer. The pre-diluted samples (50µl) were then transferred onto the ELISA plates and incubated at 4^oC overnight for further processing.

On day 3, plates were washed three times each with 200µl/well of wash buffer 24 hours later. Secondary antibody, mouse anti-human IgG (Fc)-AP (Clone JDC-10) (Biocom, Biotech, Berlin, Germany) was prepared as a 1:1000 dilution in dilution buffer and 50µl was added to each reaction well. Secondary antibodies were alkaline phosphatase conjugated, 4-Nitrophenyl phosphate disodium salt hexahydrate (PnP) was used as a substrate to develop the colour reaction. PnP (Sigma Aldrich, USA) was diluted in substrate buffer and 50µl of substrate buffer containing PnP was added to each reaction well (Figure 3.2). Plates were incubated at room temperature for 15 minutes to develop, and thereafter reactions were stopped with 25µl 1M NaOH once developed.

Plates were then read on the VersaMax ELISA microplate reader plate reader (Molecular devices, USA) with the use of Software Max Pro with using the following dual wavelength laser modules (LM). LM1 was set at 405nm and LM2= 492nm as the (reference wavelength). Optical density values > 1.0 in this study were regarded as positive and detectable IgG levels for *S. mansoni* (Vendrame et al., 2001) and was used to confirm that participants had exposure to this parasite and had experienced schistosoma infections. Participants with *S. mansoni* IgG O.D values <1.0 were recorded as undetectable in this study.



1. Antigen added to 96-well plate.
2. Blocking buffer added to block remaining protein binding sites.
3. Addition of plasma sample containg primary antibody.
4. A suitable secondary antibody-alkaline phosphatase is then added which recognizes and binds to primary antibody.
5. PnP substrate added and converted by alkaline phosphatase to detectable form.

Figure 3.4: Principle of indirect antibody ELISA used in this study. Alkaline phosphatase conjugated secondary antibody was used, that binds to primary antibody, causing substrate PnP to produce a yellow colour (Figure taken from http://www.leinco.com/indirect_elisa, March 2015,).

3.12 Measurement of CVL cytokine concentrations by Luminex Multiplex assay

In order to quantify for multiple cytokines in a limited sample volume, a multiplex assay was used for the quantitative determination the concentrations of 48 cytokines, chemokines, and growth factors in a relatively small volume of CVL sample. The advantage of using this multiplex assay is that a number of analytes can be quantified under the same laboratory conditions, decreasing the impact of inter-assay variability. In this approach, magnetic microscopic beads were coated with antibodies specific to cytokines that were bound using a chemical process of covalent binding (Figure 3.3). CVL was then added to the antibody-coated bead and antibody complex where the respective soluble cytokines present in the CVL would then bind. Fluorescent-labeled reporters are then able to bind to the captured cytokines. Target cytokines were identified and quantified by lasers that detected different wavelengths of the fluorescent labeled beads. Prior to the cytokine multiplex assays, each

CVL sample (300µl) was filtered using 0.5ml Corning Costar Spin-X columns, (Scientific Group, South Africa), to remove cervical mucus and particulate antigen that could block the fluidics of the Luminex plate reader from the soluble protein fraction in which cytokines were to be measured.

For this study, the concentrations of the 48 cytokines were determined using two separate Bioplex cytokine kits (27 plex and 21 plex kits, Bio-Rad, USA). The 27 plex kit was used to screen for interleukin (IL)-1β, IL-1ra, IL-2 , IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, basic fibroblast growth factor (FGF), eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ, inducible protein (IP-10), monocyte chemo attractant protein-1 (MCP-1/MCAF), macrophage inflammatory protein (MIP)-1α, MIP-1β, platelet-derived growth factor (PDGF)-BB, regulated on activation, normal T cell excreted and secreted (RANTES), tumor necrosis factor (TNF-α) and vascular endothelial growth factor (VEGF). The 21-plex cytokine kit was used to screen for IL-1α, IL-2Rα, IL-3, IL-12 (p40), IL-16, IL-18, cutaneous T cell attracting chemokine (CTACK), growth related oncogene (GRO)-α, human growth factor (HGF), intracellular adhesion molecule (ICAM)-1, IFN-α2, leukemia inhibitory factor (LIF), MCP-3, macrophage colony-stimulating factor (M-CSF), macrophage migration inhibitory factor (MIF), monocyte induced by interferon gamma (MIG), beta nerve growth factor (β-NGF), stem cell factor (SCF), stem cell growth factor (SCGF)-β, stromal derived cell factor (SDF)-1α, TNF-β, TNF related apoptosis inducing ligand (TRAIL) and vascular cell adhesion molecule (VCAM)-1. For standards and reagent preparation, samples and standards were thawed to room temperature. The reconstituted lyophilized standard was prepared by adding 500µl of standard diluent to form the master standard stock solution. Stock solution was then vortexed and incubated on ice for 30 min for use in the serial four-fold dilution to generate a standard curve. Samples were prepared during incubation of the standard. Nine 1.5 ml eppendorf tubes were labelled (S1-S8) with its respective concentrations. Volumes of standard diluent were pipetted into each tube as per manufactures protocol. A volume of of 128µl of was then added to S1, which contained 72µl of standard diluent. Fifty microliters (50µl) of the diluted standard from S1 was then transferred into tube S2, and thereafter the process was repeated for a serial four-fold dilution in tubes S3 to S8 to generate a broad range standard curve. To prepare the beads for the multiplex assay, beads were protected from light at all time and adjusted to room temperature prior to useage.

Beads were vortexed for 30 seconds before dilution and prepared by adding 5,175µl of assay buffer to a 15mL tube. A volume of 575µl of 10X beads was transferred into tube containing the 5,175µl (5,175 ml) of assay buffer which was provided in the kit. The diluted beads were vortexed for 30 sec and a volume of 50µL was added into each well on the 96 well plate. The wells containing the magnetic beads were then washed twice using the Bio-Plex Pro™ Wash Station (Bio-Rad, USA). The standard dilutions, samples and controls were vortexed gently for 1-3 sec and 50µL were added to the appropriate wells. The plate was then sealed and incubated on a shaker at room temperature for 30min at 300 rpm. During the last 10min of the incubation time, 1X detection antibody was prepared by adding 300µL of 10X detection antibody to 2,700µL of detection antibody diluent in a 15mL tube. After incubation, the sealing tape was removed and the plate was washed three times in order to proceed with the addition of the detection antibody to all wells of the 96 well plate. The diluted detection antibody was vortexed and 25µL was added into each well. The plate was covered and incubated on a shaker at 300 rpm for 30min at room temperature. With 10min remaining of this incubation time, 1X Streptavidin-Phycoerythrin (PE) was prepared by adding 60µL of the concentrated 100X Streptavidin-PE to 5.940µl assay buffer in a 15mL tube. After incubation of the detection antibody, the sealing tape was removed and the plate was washed three times. The diluted Streptavidin-PE was vortexed and 50µl were added into each well. The plate was sealed and incubated on a shaker for 10min at room temperature. After incubation the plate was washed three times and 125µl of assay buffer was added to each well. The plate then sealed and incubated on shaker at room temperature at 300rpm for 30 sec. The plate cover was removed and the plate read using the Bio-Plex 200 Multiplex system (Bio-Rad Laboratories Inc, USA). Plates were run on low PMT setttings (photomultiplier).

Data was collected using a Bio-Plex manager software version 6 (Bio-Rad Laboratories Inc, USA) and a 5 PL regression formula was used to calculate cytokine concentrations from the standard curves. Raw data files were saved as extension RBX files, and thereafter using the documents export function on the software, the files were exported to Microsoft excel format (.xml) files containing all the information in the RBX file.

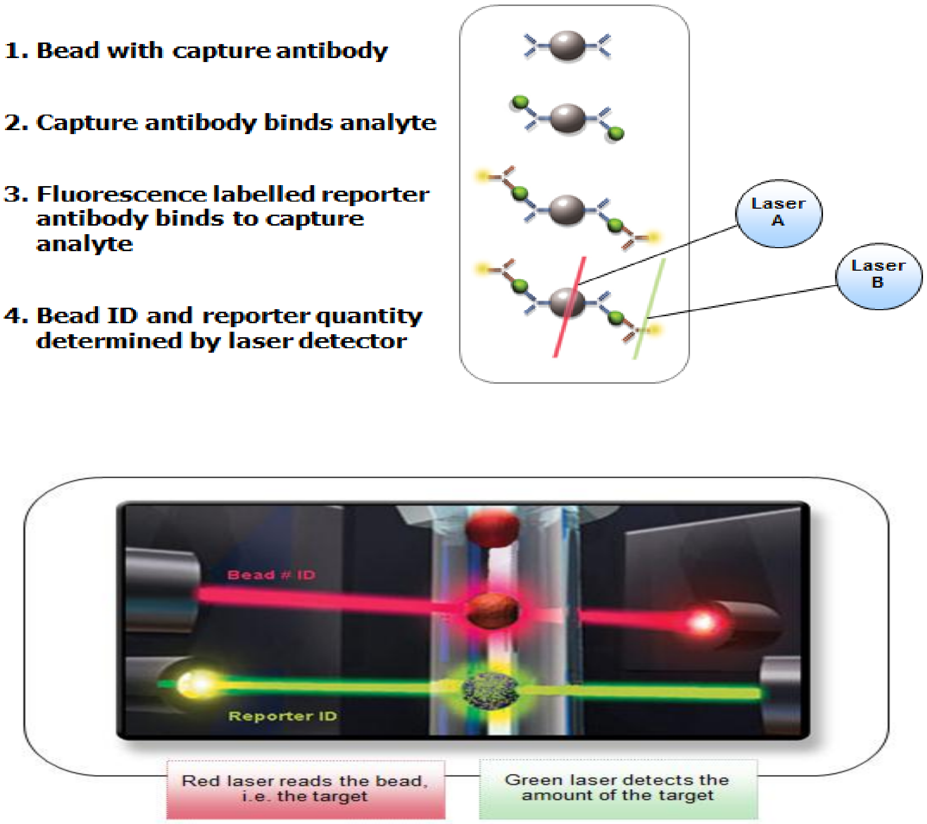


Figure 3.5: Illustration of the process of magnetic beads bound with with antibodies, followed by soluble cytokine binding from sample with and bound fluorescent labelling reporters. As magnetic beads pass through the flowcell, the red laser identifies the captured cytokines bound to the bead whilst the green laser quantifies the cytokines present in the sample [adapted from (Nolan and Sklar, 2002)].

3.13 Statistical analysis

Generation of dot plots, non-parametric statistical analysis were performed using Graphpad Prism V.5. Differences between groups were evaluated using a Student's t-test or ANOVA, as indicated. Mann- Whitney U and Kruskal Wallis tests were used for unmatched and matched comparisons, respectively were used. Bonferroni correction to adjust for multiple comparisons was also applied to prevent a Type 1 error and circumvents over estimating statistical significance. P-values less < 0.05 were considered significant.

Chapter 4: Results

4.1. Description of the cohort

To evaluate exposure to Schistosoma in the rural and urban parts of Kwa-Zulu Natal, this retrospective study included a total of 383 female participants from the placebo arm of the CAPRISA 004 1% tenofovir microbicide gel trial (Abdool Karim et al., 2010a). Table 4.1 summarizes the clinical characteristics for this cohort. The median follow-up time for the 383 women from study entry to study exit was 19.1 months (IQR:13.3 to 22.8 months).

The inclusion criteria for women in the CAPRISA 004 study were as follows: Women aged from 18 to 40 years, who were HIV negative, who were sexually active (at least twice within 30 days prior to screening), having engaged in unprotected sexual intercourse and who were not pregnant were eligible for study enrolment. The median age of the women was 22 years (IQR 20-26 years). Of the 383 women in this study, 274/383 were from rural Vulindlela (71.5%) and 109/383 were from urban eThekwini in the city centre of Durban (28.3%). The majority of the cohort (354/383; 92.4%) had a stable partner and all were sexually active and on some form of hormonal contraception with 75.9% on Depo Provera and 17.6% on Nuristerate. As a marker of socioeconomic status, less than half the women (45.4%; 174/383) had access to potable water.

Most women reported being unemployed (91%). Only 6% of participants owned a house. Prevalence of self-reported genitourinary symptoms was low, with 6% reporting vaginal itching, 0.01% reporting vaginal burning, and 0.03% had genitourinary ulcers. Exposure to helminth infection within the placebo arm of the CAPRISA 004 cohort had not previously been investigated. During the trial, 52/383 of the women in this sub-study became HIV-infected (13.6%), yielding an HIV incidence rate of 9.1 per 100 women-years (95% CI: 6.8 – 11.9).

Table 4.1: Demographic data of study participants enrolled in the placebo arm of the CAPRISA 004 1% tenofovir microbicide gel trial.

Variables	N=383
Age (Median, IQR)	22 (20-26)
Clinical site (n, %)	
Vulindlela	274 (71.54%)
eThekwini	109 (28.26%)
HIV status (n, %)	
HIV negative	331 (86.42%)
Sero-converters	52 (13.58%)
Stable partners (n, %)	
No stable partners(s)	29 (7.57%)
Stable partner(s)	354 (92.43%)
Access to potable water	
	174/383 (45.43%)
Other Variables	N=108 (Data available)
Employment status	
Unemployed	98 (91%)
Self-employed	10 (9%)
Participants owing a house	
Not owning a house	101 (94%)
Owning a house	7 (6%)
Symptoms/ Clinical data	
Vaginal Itching	7 (6%)
Vaginal Burning	1 (0.01%)
Genitourinary ulcers	3 (0.03%)
Use of Depo Provera	82 (75.9%)
Use of injectable Nuristerate	19 (17.6%)

4.2 Prevalence of female genital schistosomiasis in CVL

Of the 383 HIV negative women screened, 52 (13.6%) became HIV-infected later in the study (Table 4.2). Nine of 383 (2.35%) women had detectable FGS DNA in their CVLs. Six of these women were from the Vulindlela site, while 3 women were from the eThekwini site. Of these 9 women, 3/9 (33%) women had prevalent FGS DNA at study entry (at study entry all women were HIV-uninfected). At study exit, 6/9 (67%) of these women were FGS PCR+ve. Of these 9 women, 5/9 (56%) acquired HIV infection by study exit with 4/9 women (44%) remaining HIV-uninfected despite the presence of FGS DNA. These data indicated that women positive for FGS, were 4.0 times more likely to become HIV-infected than FGS-negative women (OR 4.05- 95% CI 1.8-8.9, p=0.01). Forty-eight of the 52 women that sero-converted in this study, tested PCR negative for schistosome DNA from the pellets of their lavage samples.

None of the women were FGS PCR+ve at both study entry and study exit. No information of praziquantal treatment was recorded for all women including those who tested positive for schistosome DNA in the pellet of their lavage samples in this study.

Table 4.2: Prevalence of female genital schistosomiasis DNA

Variables	**N=383**
HIV sero-converters	52/383 (13.6%)
Total FGS PCR +ve	9/383 (2.35%)
Vulindlela site	6/383 (1.57%)
eThekwini site	3/383 (0.78%)
FGS PCR +ve at study entry	3/9 (33%)
FGS PCR +ve at study exit	6/9 (67%)
FGS PCR +ve at study entry & exit	0/9 (0%)
FGS PCR+ve that acquired HIV at study exit	4/9 (44%)
FGS PCR+ve, HIV negative at study exit	5/9 (56%)

4.3 DNA sequencing of amplicons and alignment to *S. haematobium* sequence

Sequences of all FGS PCR positive samples showed similar homology to the *S. haematobium* control sequence, with no differences in the nucleotide sequences in any of the participant's sequences for the 77 bases [Figure 4.3].

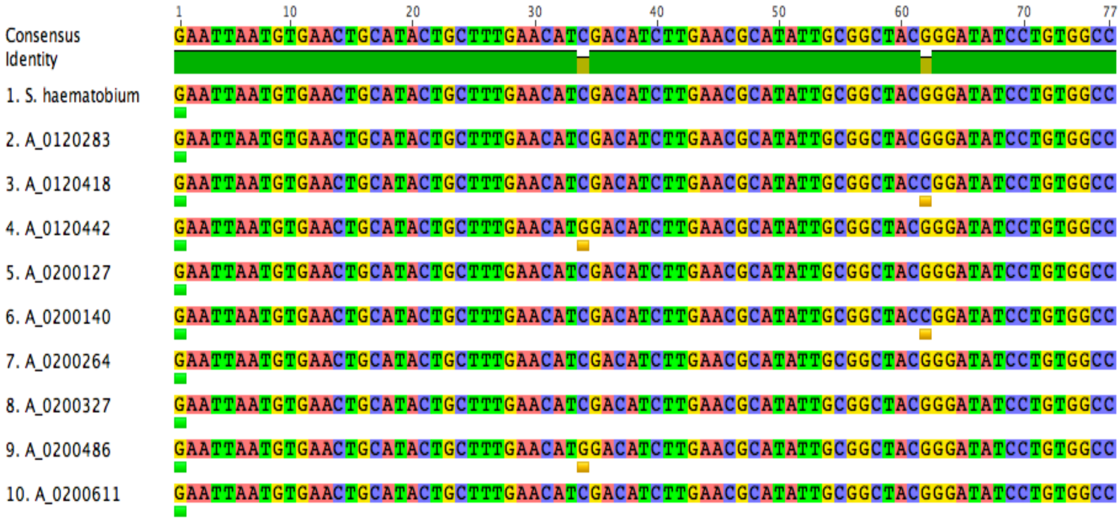


Figure 4.1: Multiple alignments of FGS PCR products, validating that FGS PCR positive sequences showed similar nuclear base composition when compared to *S. haematobium* control sequence. The 77-bp internal transcription spacer is homologous to both *S. mansoni* & *S. haematobium* species.

4.4 Seroprevalence of schistosomiasis

Levels of *Schistosoma spp* specific IgG from n= 383 female participants were measured at both study entry and study exit time points (Table 4.3).

Table 4.3: Optical densities based on raw data for *Schistosoma* spp IgG in IgG-HIV-, IgG+HIV-, IgG+HIV- & IgG+HIV+ women at entry & exit of study before controlling for an OD >1.0.

Study entry (month 3)	***S. mansoni* IgG-HIV- (N=362)**	***S. mansoni* IgG + HIV- (N=21)**
Median OD values	0.363	0.742
Range OD values	0.065-0.782	0.066-1.426
Study exit (month 52)	***S. mansoni* IgG-HIV- (N=323)**	***S. mansoni* IgG+HIV- (N=15)**
Median OD values	0.289	0.783
Range OD values	0.0061-0.491	0.068-1.498

Study exit (month 52)	***S. mansoni* IgG-HIV+ (N=41)**	***S. mansoni* IgG+HIV+ (N=4)**
Median OD values	0.312	0.738
Range OD values	0.052-0.562	0.059-1.369

Of the 383 women at study entry, 21 (5.5%) had detectable *S. mansoni* IgG in their plasma samples [Figure 4.2 (A)]. At study exit, 19/383 women (5.0%) had detectable IgG [Figure 4.2 (B)]. Only 17 women had IgG at both time points. Of the 9 FGS PCR+ women (Table 4.2 above), 7 were also positive for *S. mansoni* IgG indicating a concordance between the systemic compartment and the genital tract. From the 21/383 women that tested IgG sero-positive for *S. mansoni* at study entry, only 4 of these women had HIV sero-converted by study exit. No significant differences in the optical densities for the IgG titres were found between women who became HIV-infected compared to those who remained uninfected (p=0.47) [Figure 4.2 (C)].

Table 4.4: *S. mansoni*-specific IgG sero-prevalence of women in the placebo arm of the CAPRISA 004 cohort.

Participants *S. mansoni* IgG +ve at study entry	21/383 (5.5%)
Participants remained *S. mansoni* IgG+ve at study exit	2/21 (9.5%)
Participants *S. mansoni* IgG +ve at study exit	19/383 (5.0%)
Participants *S. mansoni* IgG -ve at study entry	17/19 (89.5%)

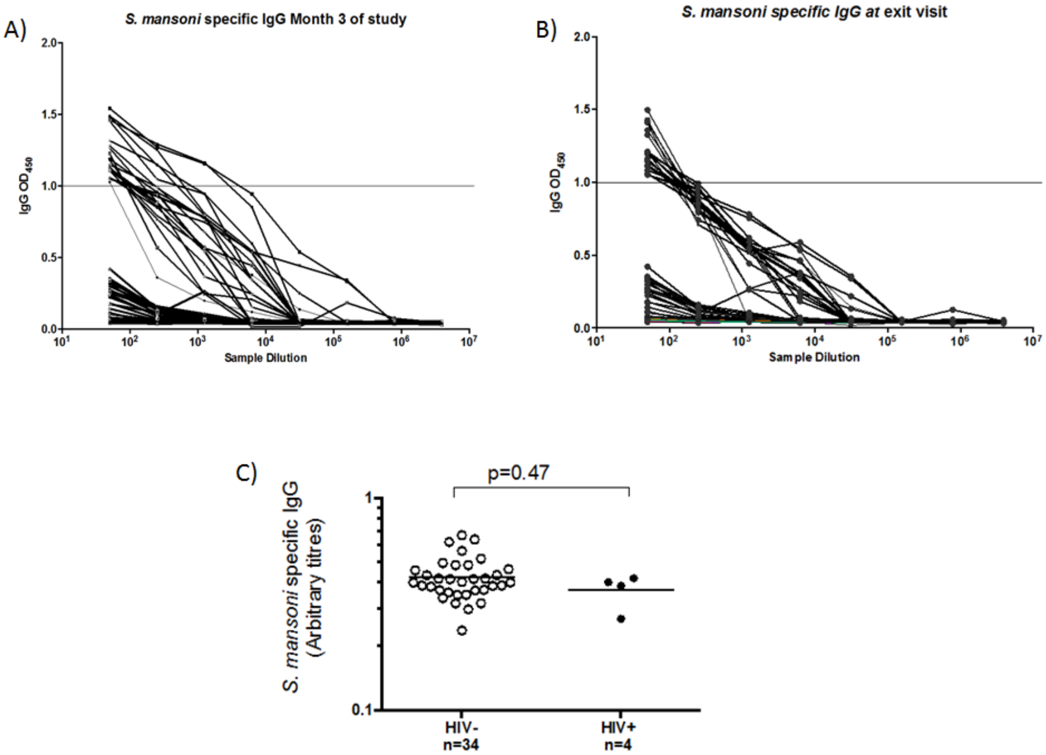


Figure 4.2: (A) *S. mansoni* specific IgG levels in women at month 3 of study (study entry). Detectable Ab levels were found for 21/383 (5.5%) of study participants. Horizontal line represents cut-off Ab titres. (B) Total *S. mansoni*-specific IgG antibody titres were measured in study participants at study exit. 19/383 (5.0%) of women had detectable plasma Ab levels. (C) Schistosomiasis IgG titres from HIV uninfected *S. mansoni* positive women (n=34) compared to *S. mansoni* positive women who later became HIV-infected (n=4). *S. mansoni* arbitrary IgG titres from study participants that seroconverted showed that HIV had no significant effect on IgG levels of study participants that were exposed to *S. mansoni*.

4.5 Genital cytokine concentrations associated with FGS or HIV infection at study entry (month 3)

To investigate genital tract inflammation, screening of 48 pro- and anti-inflammatory cytokines and chemokines of CVLs from women in the CAPRISA 004 cohort was conducted at study entry (month 3-Table 4.5) and at study exit (month 52 on average-Table 4.6). In women who were FGS PCR+ significantly higher levels of MCP-3 was observed compared to the FGS- women, and in contrast, FGS- women showed significantly higher basic FGF than in FGS+ women [figure 4.3].

Study Entry: Cytokine levels of women were screened at month 3 of the study in order to investigate their role in genital inflammation. At month 3, all women participating in the CAPRISA 004 trial were all HIV negative (Table 4.5).

Table 4.5: Cytokine concentrations amongst FGS-HIV- & FGS+HIV- women at study entry

Cytokine	FGS-HIV- Median (IQR) (N=380) [pg/ml]	FGS+HIV- Median (IQR) (N=3) [pg/ml]	P value	FDR adjusted p-value
BASIC_FGF	25.1 (18.1-31.8)	12.0 (7.2-15.3)	**0.025**	0.5837
EOTAXIN	7.0 (0.9-12.9)	1.7 (0.1-4.4)	0.165	0.5837
G-CSF	209.3 (72.1-885.2)	36.9 (5.3-782.0)	0.235	0.5837
GM_CSF	94.3 (65.5-122.4)	51.2 (38.5-119.9)	0.183	0.5837
IFN_G	40.9 (21.7-70.8)	47.2 (22.4-68.2)	0.875	0.9547
IL_10	23.5 (7.3-38.3)	6.6 (0.6-11.9)	0.064	0.5837
IL_12P70	48.0 (23.4-95.5)	11.4 (0.9-59.0)	0.105	0.5837
IL_13	3.0 (1.6-4.7)	1.8 (0.4-3.0)	0.207	0.5837
IL_15	2.7 (0.0-8.9)	2.6 (0.0-8.9)	0.645	0.8465
IL_17A	17.9 (10.6-28.9)	10.7 (5.3-22.0)	0.265	0.5837
IL_1B	38.9 (11.3-154.1)	40.9 (3.5-118.4)	0.688	0.8465
IL_1RA	9158.2 (4989.4-13321.1)	21962.6 (1957.7-29027.6)	0.376	0.6224
IL_2	3.2 (1.8-5.3)	3.2 (0.0-7.0)	0.844	0.9425
IL_4	0.8 (0.5-1.3)	0.7 (0.3-0.8)	0.306	0.5870
IL_5	0.4 (0.1-1.6)	0.4 (0.2-0.7)	0.981	0.9806
IL_6	7.6 (3.0-20.7)	2.2 (0.5-10.9)	0.170	0.5837
IL_7	2.8 (1.4-5.1)	1.0 (0.3-2.8)	0.107	0.5837
IL_8	392.6 (138.7-1092.8)	129.2 (0.9-1228.3)	0.360	0.6224
IL_9	5.7 (3.3-11.0)	2.5 (0.1-5.7)	0.105	0.5837
IP_10	169.8 (35.7-688.1)	34.0 (7.3-833.1)	0.349	0.6224
MCP_1	17.6 (9.0-45.3)	10.6 (2.0-51.6)	0.409	0.6298
MIP_1A	2.1 (1.0-2.9)	0.6 (0.6-0.7)	0.052	0.5837
MIP_1B	13.2 (5.6-31.5)	12.9 (2.3-55.0)	0.840	0.9425
PDGF_BB	10.2 (5.7-18.5)	6.3 (0.9-12.0)	0.197	0.5837
RANTES	9.6 (3.8-18.1)	7.0 (0.8-10.2)	0.300	0.5870
TNF_A	10.2 (5.4-20.9)	4.7 (4.1-6.0)	0.092	0.5837
VEGF	375.4 (175.7-761.9)	140.5 (22.7-721.4)	0.255	0.5837
CTACK	19.6 (11.4-30.9)	31.1 (15.7-34.3)	0.394	0.6298
GRO_A	394.0 (54.8-1509.1)	235.3 (32.1-3527.7)	0.901	0.9615

Table 4.5 continued

HGF	224.1 (75.3-637.0)	105.1 (28.7-632.5)	0.536	0.7563
IFN_A2	24.4 (17.5-35.2)	28.2 (21.6-43.9)	0.467	0.6786
IL_12P40	345.1 (253.2-454.6)	335.8 (182.1-681.0)	0.969	0.9806
IL_16	36.3 (18.2-79.6)	58.0 (54.1-88.3)	0.268	0.5837
IL_18	156.4 (51.2-487.7)	260.1 (9.6-997.7)	0.964	0.9806
IL_1A	158.1 (69.7-413.5)	99.8 (44.9-224.7)	0.420	0.6298
IL_2RA	27.4 (16.9-44.7)	42.2 (29.9-44.3)	0.296	0.5870
IL_3	134.1 (85.6-210.8)	147.1 (78.1-147.2)	0.712	0.8549
LIF	19.1 (13.3-33.1)	14.7 (12.7-18.8)	0.370	0.6224
M_CSF	84.4 (45.1-174.1)	90.8 (39.5-91.7)	0.653	0.8465
MCP_3	13.1 (6.6-26.0)	46.7 (19.6-87.8)	**0.038**	0.5837
MIF	5025.1 (963.5-10371.2)	3587.3 (169.3-10549.9)	0.679	0.8465
MIG	597.0 (203.5-1732.6)	192.5 (128.1-616.6)	0.268	0.5837
SCF	9.0 (3.8-19.2)	6.5 (0.0-7.3)	0.204	0.5837
SCGF_B	146.0 (1.6-547.2)	176.2 (1.6-1180.5)	0.805	0.9425
SDF_1A	107.3 (71.4-142.9)	139.5 (108.6-198.5)	0.198	0.5837
TNF_B	4.9 (3.0-7.6)	3.0 (2.7-3.2)	0.148	0.5837
TRAIL	25.5 (8.7-69.6)	33.5 (0.0-35.0)	0.627	0.8465
B_NGF	1.1 (0.5-3.4)	0.6 (0.3-1.0)	0.226	0.5837

Kruskal Wallis tests was performed for FGS-HIV- & FGS+HIV- study groups at study entry. Samples were not adjusted for STIs. Significant p values and those withstanding false discovery rate adjustment are indicated in bold text.

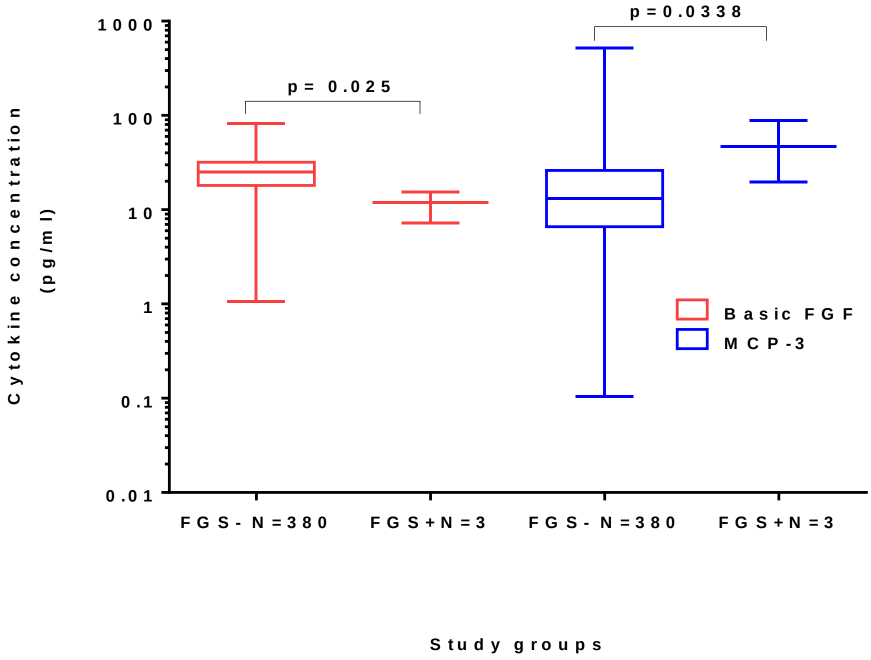


Figure 4.3: Comparison of CVL Basic-FGF and MCP-3 concentrations in FGS- (n=380) and FGS+ women (n=3) at study entry. Mann Whitney test was used to test for differences between groups.

Th1 (IFN-γ, IP-10, IL-8 & TNF-α) and Th2 cytokines (IL-4, IL-5, IL-9 & IL-10) that are usually associated with helminth infection were also investigated at study entry in the presence or the absence of FGS as shown in table 4.5, figure 8.1 & 8.2 (Appendix). Women who were FGS- had a higher median of IP-10 (median 169.8 pg/ml, IQR 35.7-688.1 pg/ml) [figure 8.1] compared to the FGS+ women (median 34.0 pg/ml, IQR 7.3-833.1 pg/ml), with no significant difference noted. Median cytokine levels of IL-9 & IL-10 (median 5.7 pg/ml, IQR 3.3-11.0 pg/ml & median 23.5 pg/ml, IQR 7.3-38.3 pg/ml) [figure 8.2] were higher in FGS- women compared to FGS+ women (median 2.5 pg/ml, IQR 0.1-5.7 pg/ml & median 6.6 pg/ml, IQR 0.6-11.9 pg/ml), however no significant differences were observed.

4.6 Genital cytokine concentrations associated with FGS or HIV infection at study exit (month 52)

To investigate genital tract inflammation, screening of 48 pro- and anti-inflammatory cytokines and chemokines of CVLs from women in the CAPRISA 004 cohort was conducted at study exit (month 52 on average-Table 4.6).

Study Exit: Cytokine & chemokine concentrations of these women were also investigated at month 52 of the study in order to investigate their role in inflammation in the presence/absence of FGS and or with/without HIV as 52/383 (13.58%) women had sero-converted by study exit.

Table 4.6: Comparison of cytokine concentrations at study exit amongst FGS-HIV-, FGS+HIV-, FGS-HIV+ & FGS+HIV+ study women

Cytokine	FGS-HIV- Median (IQR) (N=332) [pg/ml]	FGS-HIV+ Median (IQR) (N=45) [pg/ml]	FGS+HIV- Median (IQR) (N=3) [pg/ml]	FGS+HIV+ Median (IQR) (N=3) [pg/ml]	p-value	False Discovery Rate p-value
BASIC_FGF	25.2 (15.7-33.8)	27.3 (15.7-34.2)	16.8 (8.3-27.9)	22.1 (18.2-34.3)	0.712	0.777
EOTAXIN	6.6 (0.9-12.7)	6.0 (0.1-14.6)	0.1 (0.1-10.1)	3.4 (0.1-14.3)	0.456	0.5842
G-CSF	126.2 (38.8-560.3)	525.5 (117.2-1813.2)	150.3 (8.0-1629.2)	1230.2 (45.9-1928.1)	***0.004**	****0.0151**
GM_CSF	84.9 (55.2-109.5)	89.1 (57.4-110.6)	71.6 (46.8-113.6)	101.1 (83.0-119.2)	0.812	0.8664
IFN_G	36.9 (19.8-62.2)	36.0 (20.9-69.3)	26.3 (14.5-96.3)	17.4 (16.4-112.6)	0.932	0.9317
IL_10	18.1 (3.4-34.6)	19.4 (7.8-37.4)	14.7 (2.2-25.7)	14.0 (11.0-42.8)	0.490	0.5884
IL_12P70	34.4 (13.2-74.5)	43.5 (21.9-87.7)	24.2 (14.6-39.6)	26.7 (23.1-97.0)	0.256	0.4551
IL_13	2.2 (1.0-4.2)	2.8 (1.1-5.6)	1.2 (0.0-3.6)	1.5 (0.0-5.3)	0.450	0.5842
IL_15	2.4 (0.3-7.9)	1.3 (0.3-10.7)	4.4 (0.0-4.8)	7.2 (0.0-19.7)	0.710	0.777
IL_17A	18.1 (10.4-35.8)	23.0 (15.2-35.1)	14.5 (8.0-19.1)	24.7 (21.3-32.7)	0.372	0.5514
IL_1B	32.3 (10.0-139.7)	137.4 (29.7-432.0)	61.3 (22.1-67.9)	45.8 (13.8-47.0)	***<.001**	****0.0046**
IL_1RA	7334.1 (3234.9-11417.5)	11371.3 (7025.9-17298.1)	5928.4 (2743.3-12737.9)	9444.2 (2853.0-17699.0)	***<.001**	****0.0046**
IL_2	2.7 (1.3-4.7)	3.8 (1.7-6.8)	0.9 (0.7-5.7)	3.1 (0.6-7.9)	0.378	0.5514
IL_4	0.7 (0.5-1.2)	0.7 (0.5-1.5)	0.4 (0.3-1.5)	0.7 (0.4-1.6)	0.851	0.8693
IL_5	0.4 (0.1-1.4)	0.8 (0.3-2.0)	0.3 (0.0-8.3)	1.1 (0.3-4.0)	0.105	0.2108
IL_6	5.0 (2.0-19.1)	12.2 (3.3-29.6)	11.4 (1.2-45.4)	16.5 (4.3-29.8)	**0.041**	0.0985
IL_7	2.4 (1.0-4.9)	2.8 (1.0-5.6)	1.4 (0.8-3.2)	3.3 (1.2-5.6)	0.462	0.5842
IL_8	341.2 (73.5-954.9)	732.6 (365.5-3217.5)	320.0 (1.3-712.6)	612.3 (125.2-935.9)	***<.001**	****0.0031**
IL_9	4.9 (2.6-8.9)	6.2 (3.7-11.0)	3.4 (0.7-5.1)	4.9 (3.5-16.3)	0.159	0.2934
IP_10	131.1 (33.2-781.6)	1302.8 (161.8-3817.7)	26.4 (21.8-138.9)	2939.4 (58.6-10640.2)	***<.001**	****0.0002**
MCP_1	13.6 (5.1-40.8)	17.3 (8.4-47.2)	10.4 (2.9-13.7)	14.7 (10.0-23.5)	0.481	0.5884
MIP_1A	1.9 (0.9-2.9)	2.4 (1.1-3.3)	1.0 (0.4-3.1)	1.1 (1.1-3.3)	0.390	0.5514
MIP_1B	10.3 (3.9-28.4)	23.6 (8.7-59.4)	5.1 (2.4-37.4)	28.6 (10.3-71.9)	***0.003**	****0.0151**
PDGF_BB	8.6 (4.6-17.1)	14.7 (7.0-29.1)	6.2 (2.6-19.0)	11.4 (9.9-13.4)	0.088	0.1919

Table 4.6 Continued

RANTES	7.7 (3.1-18.2)	19.4 (8.6-49.8)	6.2 (1.2-7.2)	21.1 (13.8-28.7)	__*0.001__	__**0.0071__
TNF_A	9.6 (5.1-19.9)	13.1 (4.8-30.2)	4.6 (4.6-24.8)	6.8 (6.2-23.2)	0.709	0.777
VEGF	298.3 (125.1-677.1)	605.8 (285.9-1126.8)	263.1 (197.4-314.9)	289.3 (252.6-460.9)	__*0.004__	__**0.0151__
CTACK	17.2 (10.1-25.9)	26.4 (14.9-36.1)	11.5 (0.4-18.3)	19.4 (17.6-36.4)	__*0.002__	__**0.0095__
GRO_A	267.4 (43.3-1615.8)	1083.9 (277.6-5014.9)	46.7 (1.7-485.9)	2051.1 (44.9-37751.1)	__*<.001__	__**0.0052__
HGF	142.9 (51.9-436.9)	231.6 (127.6-763.7)	251.4 (14.1-343.0)	123.9 (10.8-343.9)	**0.047**	0.1064
IFN_A2	21.7 (16.0-28.9)	23.0 (17.6-32.2)	18.8 (1.3-24.6)	18.0 (15.3-41.6)	0.568	0.6651
IL_12P40	317.4 (245.7-426.2)	331.2 (266.8-540.3)	465.4 (0.2-494.0)	566.1 (467.3-577.8)	0.136	0.2607
IL_16	37.6 (20.9-72.6)	56.8 (39.9-87.2)	40.1 (1.0-54.8)	46.3 (39.9-78.0)	**0.032**	0.0815
IL_18	102.7 (35.1-333.9)	137.3 (71.6-462.5)	117.5 (2.8-271.6)	59.5 (24.4-847.7)	0.313	0.5189
IL_1A	128.9 (58.5-346.8)	269.6 (97.8-621.3)	57.2 (12.3-128.1)	65.0 (23.7-384.2)	**0.021**	0.0549
IL_2RA	25.6 (16.1-41.2)	40.4 (22.7-56.0)	30.0 (0.1-36.6)	29.5 (25.8-82.3)	__*0.009__	__**0.0257__
IL_3	114.2 (82.0-168.4)	150.2 (82.4-218.7)	120.9 (13.3-188.7)	102.2 (101.5-292.6)	0.272	0.4658
LIF	16.0 (10.8-27.4)	20.3 (11.4-35.4)	17.3 (0.1-34.2)	10.7 (9.1-43.8)	0.423	0.5804
M_CSF	74.6 (39.4-149.3)	116.4 (58.3-212.2)	25.2 (12.5-125.7)	40.9 (33.4-85.2)	__*0.012__	__**0.0336__
MCP_3	17.4 (8.5-28.5)	20.3 (11.2-29.3)	9.7 (0.2-25.7)	26.2 (19.6-33.7)	0.391	0.5514
MIF	3415.9 (460.0-8207.7)	6646.4 (3034.4-12862.1)	1068.4 (37.7-2498.0)	3723.9 (517.6-506541.2)	__*0.006__	__**0.020__
MIG	442.1 (110.6-1734.9)	2820.0 (1052.1-8028.0)	151.0 (3.3-153.8)	2838.7 (515.7-8099.8)	__*<.001__	__**<0.001__
SCF	6.3 (2.7-15.1)	16.5 (9.3-26.0)	5.5 (0.0-9.6)	13.9 (6.2-43.7)	__*<.001__	__**0.0002__
SCGF_B	67.8 (1.6-304.2)	257.0 (59.7-1082.6)	204.8 (1.6-271.6)	389.2 (62.8-398.3)	__*0.005__	__**0.0167__
SDF_1A	90.2 (54.2-141.7)	130.7 (81.0-170.1)	49.5 (5.4-108.0)	160.1 (130.1-168.0)	__*0.005__	__**0.0167__
TNF_B	4.2 (2.3-6.7)	4.6 (1.8-7.6)	2.8 (0.0-6.8)	3.4 (2.0-12.4)	0.842	0.8693
TRAIL	23.4 (5.0-72.2)	32.5 (18.8-101.1)	35.8 (0.0-108.5)	19.5 (16.5-97.1)	0.103	0.2108
B_NGF	0.9 (0.4-2.4)	1.4 (0.5-2.5)	1.2 (0.0-1.3)	0.4 (0.3-7.3)	0.355	0.5514

Kruskal Wallis tests was performed for FGS-HIV, FGS-HIV+, FGS+HIV- & FGS+HIV+ study groups at study exit. Samples were not adjusted for STIs. Significant p values and those withstanding false discovery rate adjustment are indicated in bold text. * Deontes Bonferroni correction to adjust for multiple comparisons was also applied to prevent a Type 1 error and circumvents over estimating statistical significance. ** Denotes a p value that remained significant after false discovery rate adjustment.

Women who were FGS-HIV+ had significantly higher levels of IL-1β (median 137.4 pg/ml, IQR 29.7- 432.0 pg/ml) [figure 4.4 A] than FGS-HIV- women (median 32.3 pg/ml, IQR 10.0-139.7 pg/ml; p<0.0001, FDR adjusted - p=0.0046). Median concentrations of MIF were significantly higher in FGS-HIV+ (median 6646.4 pg/ml, IQR 3034.4-12862.1 pg/ml) women compared to FGS-HIV- women (median 3415.9 pg/ml, IQR 460.-8207.7 pg/ml; p=0.0014, FDR adjusted - p=0.020). The FGS-HIV+ women had significantly higher median cytokine levels of MIF (median 6646.4 pg/ml, IQR 3034.4-12862.1 pg/ml) compared to FGS+HIV- women (median 1068.4

pg/ml, IQR 37.7-2498.0 pg/ml; p=0.034, FDR adjusted - p=0.020). Median cytokine levels of IL-1A were significantly higher in FGS-HIV+ (median 269.6 pg/ml, IQR 97.8-621.3 pg/ml) women when compared to FGS-HIV- (median 128.9 pg/ml, IQR 58.5-346.8 pg/ml; p=0.0088) and FGS+HIV- women (median 57.2 pg/ml, IQR 12.3-128.1 pg/ml; p=0.0499). Women in the FGS-HIV+ group had significantly higher median cytokine levels of IL-6 (median 12.2 pg/ml, IQR 3.3-29.6 pg/ml) when compared to FGS-HIV- women (median 5.0 pg/ml, IQR 2.0-19.1 pg/ml; p=0.0069). Th2 cytokine levels of IL-1RA [figure 4.4 (C)] were significantly higher in FGS-HIV+ (median 11371.3 pg/ml, IQR 7025.9-17298.1 pg/ml) women compared to FGS-HIV- (median 7334.1 pg/ml, IQR 3234.9-11417.5 pg/ml) women (* p<0.0001, FDR adjusted - p<0.0046). Median cytokine levels of IL-2RA [figure 4.5] were significantly lower in FGS-HIV- (median 25.6 pg/ml, IQR 16.1-41.2 pg/ml) women when compared to FGS-HIV+ (median 40.4 pg/ml, IQR 22.7-56.0 pg/ml) women (* p=0.0012, FDR adjusted - p=0.0257).

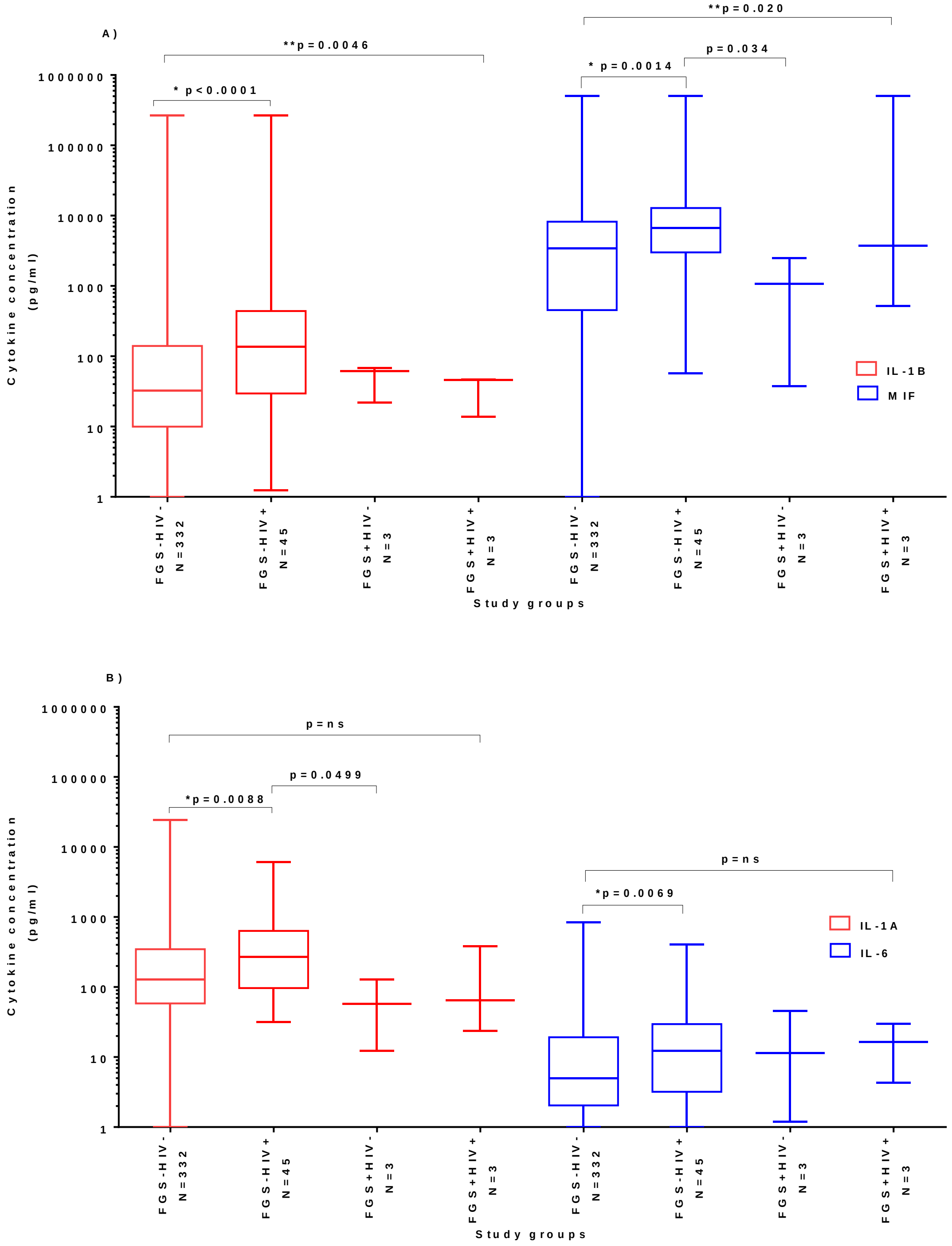


Figure 4.4: Concentrations of Th1 cytokines (IL-1β, MIF, IL-1A & IL-6) investigated between the 4 study groups (FGS-HIV-, FGS+HIV-, FGS-HIV+ & FGS+HIV+) at study exit. Kruskal Wallis tests were performed amongst the four groups. Non-parametric two tailed Mann Whitney tests were performed to test for differences between the FGS- and FGS+ groups. * Denotes Bonferroni correction to adjust for

multiple comparisons was also applied to prevent a Type 1 error and circumvents over estimating statistical significance (p<0.0125). ** Denotes a p value that remained significant after false discovery rate adjustment as depicted in Table 4.6

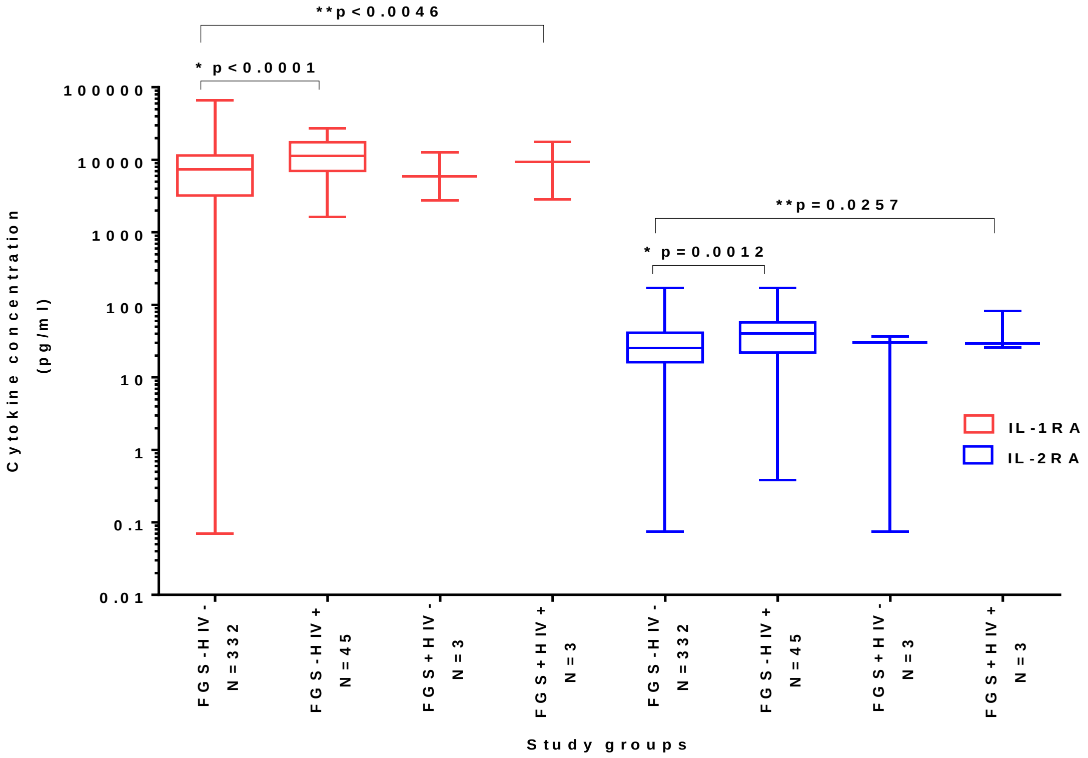


Figure 4.5: T-helper 2 (IL-1RA & IL-2RA) cytokines investigated between the 4 study groups (FGS-HIV-, FGS+HIV-, FGS-HIV+ & FGS+HIV+) at study exit. Kruskal Wallis tests were performed amongst the four groups. Non-parametric two tailed Mann Whitney tests were performed to test for differences between the FGS- and FGS+ groups. * Denotes Bonferroni correction to adjust for multiple comparisons was also applied to prevent a Type 1 error and circumvents over estimating statistical significance (p<0.0125). ** Denotes a p value that remained significant after false discovery rate adjustment as depicted in Table 4.6

In addition, growth factors (G-CSF, VEGF, HGF, MCSF, CSF & SCGF-β) were also investigated at study exit amongst FGS-HIV-, FGS+HIV-, FGS-HIV+ & FGS+HIV+ groups in detail and represented in figure 4.6 & 4.7. Median levels of G-CSF (D) were significantly higher in FGS-HIV+ (median 525.5 pg/ml, IQR 117.2-1813.2 pg/ml) women compared to FGS-HIV- (median 126.2 pg/ml, IQR 38.8-560.3 pg/ml) women (* p=0.0003, FDR adjusted - p=0.0151). Women in FGS-HIV+ study group had significantly higher levels of VEGF (figure 4.6) compared to FGS-HIV- women (* p=0.0002, FDR adjusted - p=0.0151).

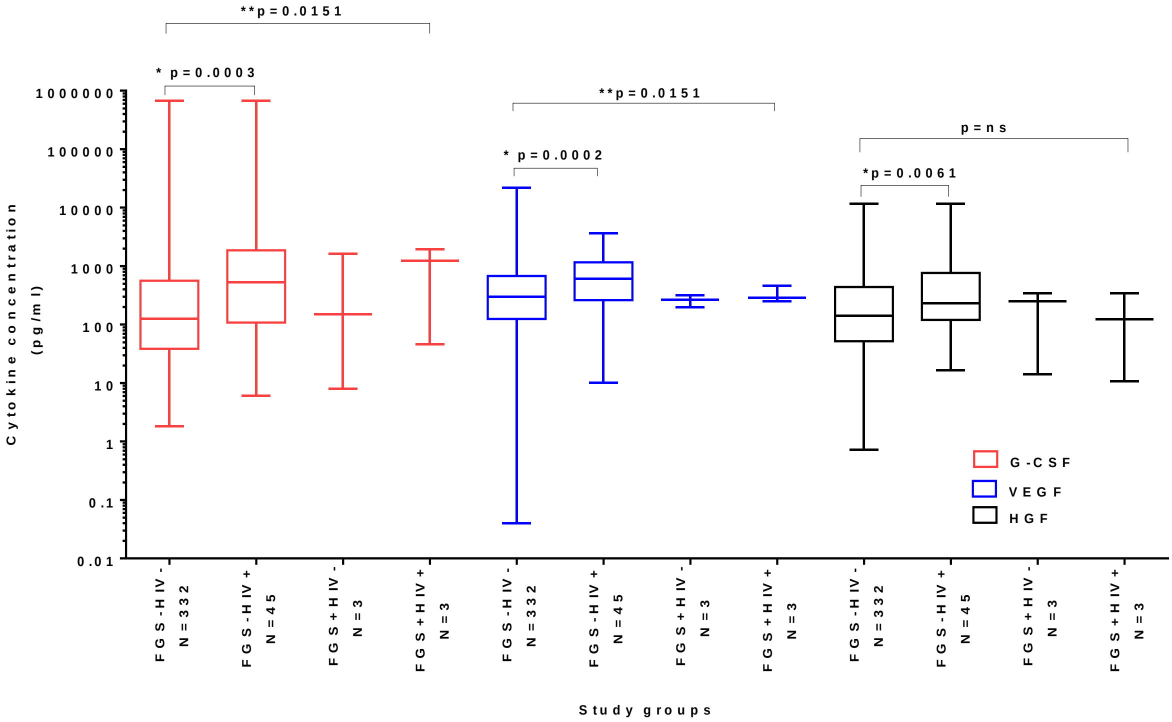


Figure 4.6: Median cytokine levels of growth factors G-CSF, VEGF & HGF were compared in all 4 study groups (FGS-HIV-, FGS+HIV-, FGS-HIV+ & FGS+HIV+) at study exit. Kruskal Wallis test was performed amongst the 4 study groups. Non-parametric two tailed Mann Whitney tests were performed to test for differences between the FGS- and FGS+ groups. *Denotes Bonferroni correction to adjust for multiple comparisons was also applied to prevent a Type 1 error and circumvents over estimating statistical significance (p<0.0125). ** denotes a p value that remained significant after false discovery rate adjustment as depicted in Table 4.6.

Median levels of HGF were also significantly higher in FGS-HIV+ women (median 231.6 pg/ml, IQR 127.6-763.7 pg/ml) compared to FGS-HIV- women (median 142.9 pg/ml, IQR 51.9-436.9 pg/ml) (p=0.0061). Women in FGS-HIV- group had significantly lower levels of M-CSF (figure 4.7) compared to FGS-HIV+ women (* p=0.0041, FDR adjusted - p=0.0336). Growth factor SCGF-β levels (figure 4.7) were significantly higher in FGS-HIV+ women compared to FGS-HIV- women (*p=0.0005, FDR adjusted - p=0.0167). Women in the FGS-HIV+ study group had significantly higher levels of SCF (figure 4.7) compared to FGS-HIV- women (* p<0.0001, FDR adjusted - p=0.0002) and FGS+HIV- women (* p=0.034, FDR adjusted - p=0.0002).

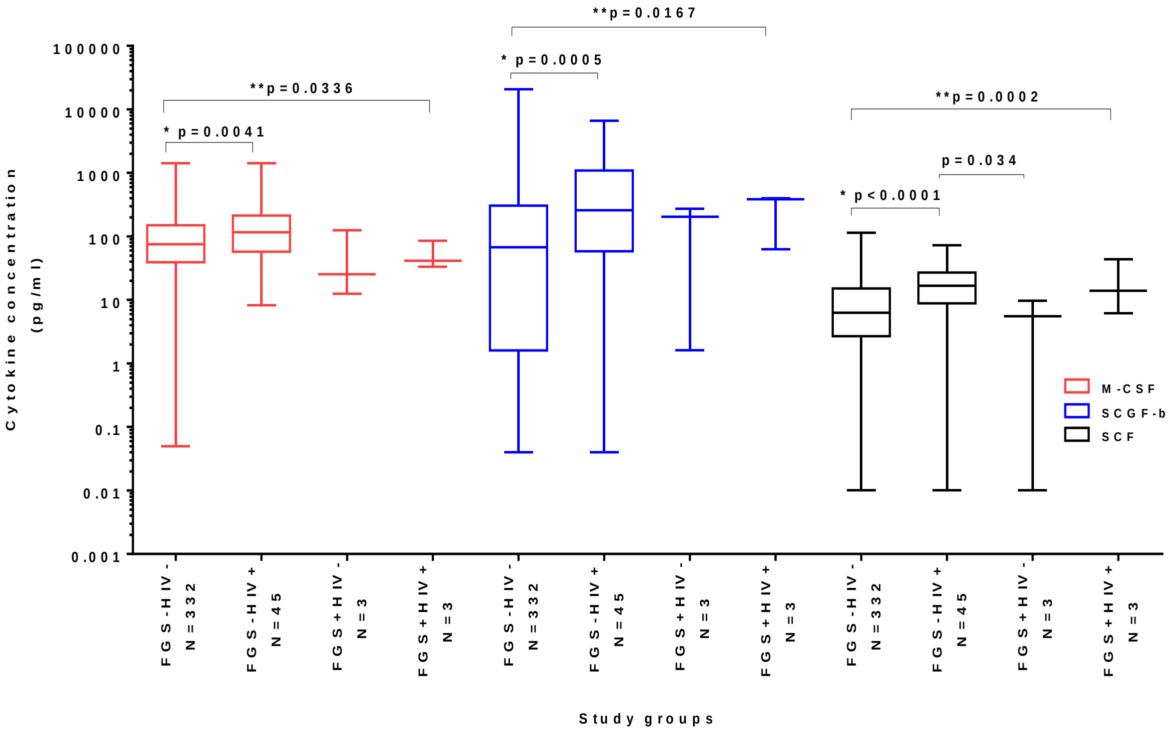


Figure 4.7: Median cytokine levels of growth factors MCSF, SCF & SCGF-β were compared in all 4 study groups (FGS-HIV-, FGS+HIV-, FGS-HIV+ & FGS+HIV+) at study exit. Kruskal Wallis test was performed amongst the 4 study groups. Non-parametric two tailed Mann Whitney tests were performed to test for differences between the FGS- and FGS+ groups. * Denotes Bonferroni correction to adjust for multiple comparisons was also applied to prevent a Type 1 error and circumvents over

estimating statistical significance(p<0.0125). ** Denotes a p value that remained significant after false discovery rate adjustment as depicted in Table 4.6.

Chemokines (IL-8, IP-10, MIP-1β, RANTES, CTACK, GRO-A, IL-16, MIG & SDF-1A) were investigated at study exit amongst FGS-HIV-, FGS+HIV-, FGS-HIV+ & FGS+HIV+ study groups in detail and represented in figure 4.8 & 4.9. Median cytokine levels of IL-8 (figure 4.8) were significantly higher in FGS-HIV+ women (median 732.6 pg/ml, IQR 365.5-3217.5 pg/ml) compared to FGS-HIV- women (median 341.2 pg/ml, IQR 73.5-954.9 pg/ml) (* p<0.0001, FDR adjusted - p=0.0031). Women in FGS-HIV+ study group showed significantly higher levels of IP-10 (figure 4.8) compared to women in the FGS-HIV- study group (* p<0.0001, FDR adjusted - p=0.0002) and women in the FGS+HIV- study group (* p=0.0131, FDR adjusted - p=0.0002). Median cytokine levels of MIP-1β (figure 4.8) were significantly higher in FGS-HIV+ women (median 23.6 pg/ml, IQR 8.7-59.4 pg/ml), compared to FGS-HIV- women(median 10.3 pg/ml, IQR 3.9-28.4 pg/ml) (* p=0.0005, FDR adjusted - p=0.0151).

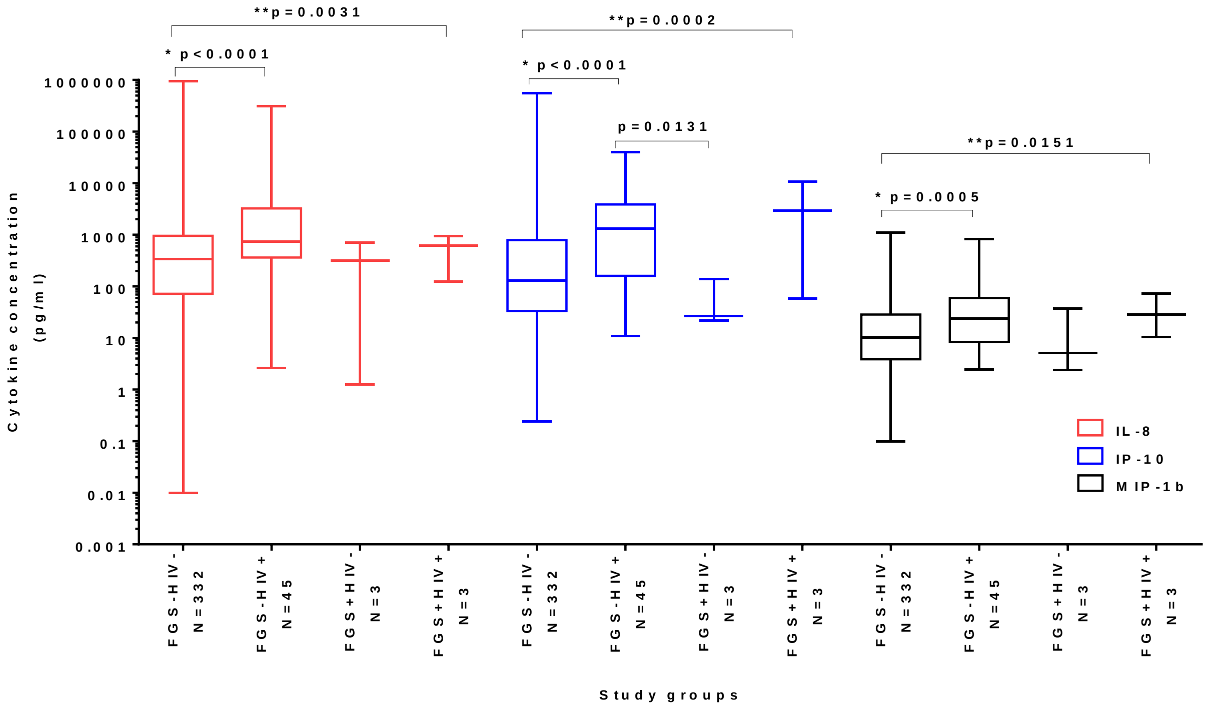


Figure 4.8: Median levels of chemokines IL-8, IP-10 & MIP-1β were investigated and compared between all 4 study groups (FGS-HIV-, FGS+HIV-, FGS-HIV+ & FGS+HIV+) at study exit. Kruskal Wallis test was performed amongst the 4 study

groups. Non-parametric two tailed Mann Whitney tests were performed to test for differences between the FGS- and FGS+ groups. *Denotes Bonferroni correction to adjust for multiple comparisons was also applied to prevent a Type 1 error and circumvents over estimating statistical significance. (p<0.0125). ** denotes a p value that remained significant after false discovery rate adjustment as depicted in Table 4.6.

Women in the FGS-HIV+ study groups had significantly higher levels of RANTES (figure 4.9), compared to FGS-HIV women (* p=0.0003, FDR adjusted - p=0.0071) and FGS+HIV- women (* p=0.0343, FDR adjusted - p=0.0071). Median cytokine levels of CTACK (figure 4.9) were significantly higher in FGS-HIV+ women (median 26.4 pg/ml, IQR 14.9-36.1 pg/ml), compared to FGS-HIV- women(median 17.2 pg/ml, IQR 10.1-25.9 pg/ml) (* p=0.0004, FDR adjusted -p=0.0095) and FGS+HIV- women (* p=0.043, FDR adjusted -p=0.0095). Women in the FGS-HIV+ study group had significantly higher median cytokine levels of GRO-A (figure 4.9), compared to women in the FGS-HIV- study group (* p=0.0001, FDR p=0.0052) and FGS+HIV- women (* p=0.0431, FDR adjusted - p=0.0052).

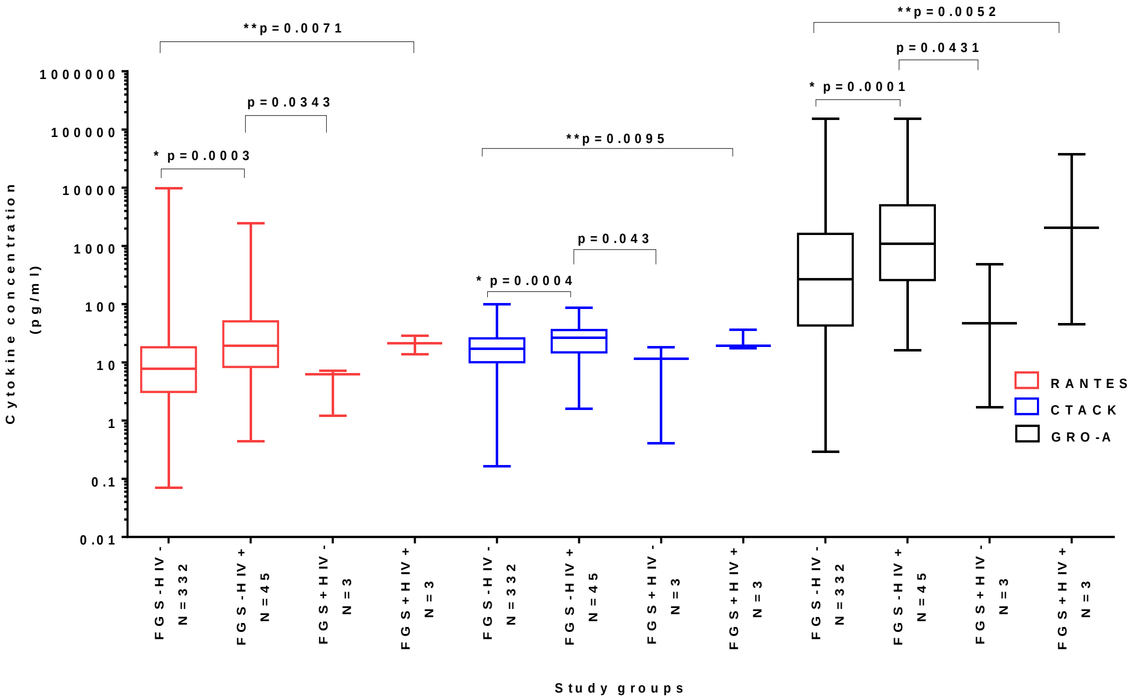


Figure 4.9: Median levels of chemokines RANTES, CTACK & GRO-A were investigated and compared between all 4 study groups (FGS-HIV-, FGS+HIV-, FGS-

HIV+ & FGS+HIV+) at study exit. Kruskal Wallis test was performed amongst the 4 study groups. Non-parametric two tailed Mann Whitney tests were performed to test for differences between the FGS- and FGS+ groups. *Denotes Bonferroni correction to adjust for multiple comparisons was also applied to prevent a Type 1 error and circumvents over estimating statistical significance ($p<0.0125$). ** denotes a p value that remained significant after false discovery rate adjustment as depicted in Table 4.6.

Median cytokine levels of IL-16 (figure 4.10) were significantly higher in FGS-HIV+ women ($p=0.0048$- median 56.8 pg/ml, IQR 39.9-87.2 pg/ml) compared to FGS-HIV- women (median 37.6 pg/ml, IQR 20.9-72.6 pg/ml). Women in FGS-HIV+ study groups had significantly higher levels of MIG (median 2820 pg/ml, IQR 1052.1-8028.0 pg/ml) (figure 4.10)when compared to FGS-HIV- (median 442.1 pg/ml, IQR 110.6-1734.9 pg/ml) women (* $p<0.0001$, FDR $p<0.001$) and FGS+HIV- (median 151 pg/ml, IQR 3.3-153.8 pg/ml) women (* $p=0.0018$, FDR adjusted - $p<0.001$). Women in FGS-HIV+ study groups had significantly higher levels of SDF-1A (figure 4.10) when compared to FGS-HIV- women (* $p=0.0038$, FDR adjusted - $p=0.0167$) and FGS+HIV- women (* $p=0.0428$, FDR adjusted - $p=0.0167$).

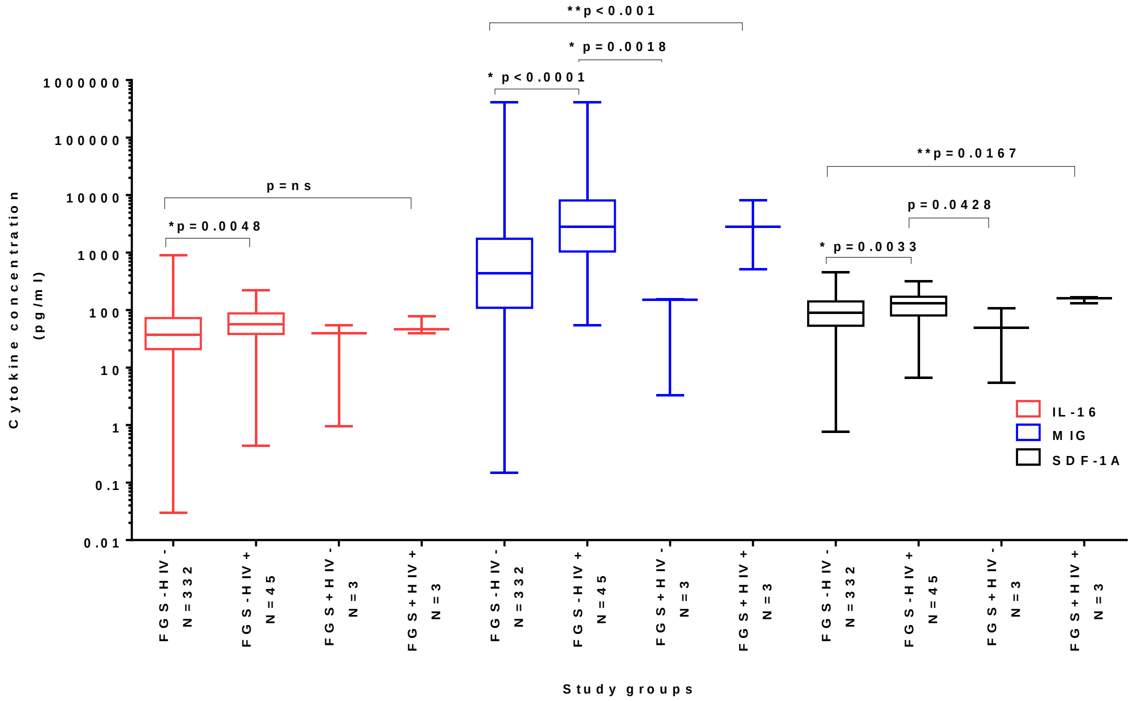


Figure 4.10: Median levels of chemokines IL-16, MIG & SDF-1A were investigated and compared between all 4 study groups (FGS-HIV-, FGS+HIV-, FGS-HIV+ & FGS+HIV+) at study exit. Kruskal Wallis test was performed amongst the 4 study groups. Non-parametric two tailed Mann Whitney tests were performed to test for differences between the FGS- and FGS+ groups. * Denotes Bonferroni correction to adjust for multiple comparisons was also applied to prevent a Type 1 error and circumvents over estimating statistical significance ($p<0.0125$). ** Denotes a p value that remained significant after false discovery rate adjustment as depicted in Table 4.6.

In addition to significant comparisons represented in the above tables 4.5 & 4.6, the following cytokines IFN-γ, IP-10, IL-8, TNF-α, IL-4, IL-5, IL-9 and IL-10 were investigated at study exit between FGS- and FGS + study groups [Figure 4.11 (A-F)] in further detail, due to their associations with helminth infections (Adachi et al., 2014). The Th1 & Th2 cytokines were investigated without HIV as a variable, as represented in figure 4.11 (A-C). FGS+ women had higher levels of IL-6 – [figure 4.11 (A)] compared to FGS- women (p=0.386), however the difference was not significant. Median cytokine levels of MIF – [figure 4.11(A)] showed that FGS+ (median 1783.1 pg/ml, IQR 37.7-506541.2 pg/ml) women had lower levels compared to FGS- (median 3819 pg/ml, IQR 460.0-12862.1 pg/ml) women (p=0.485), with no significant difference observed. No further significant differences were observed in the analyses as shown in figures 4.11 (A-C).

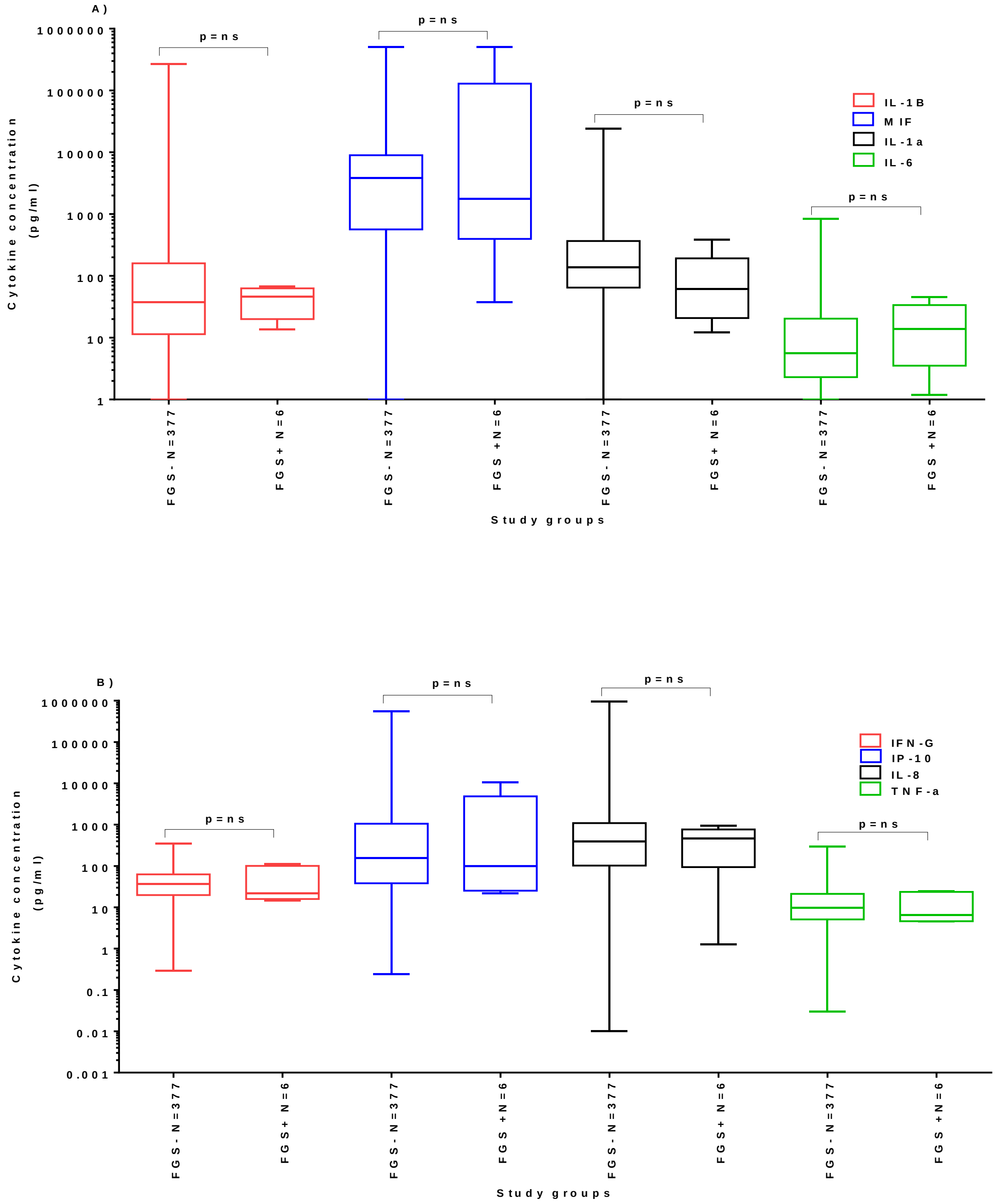
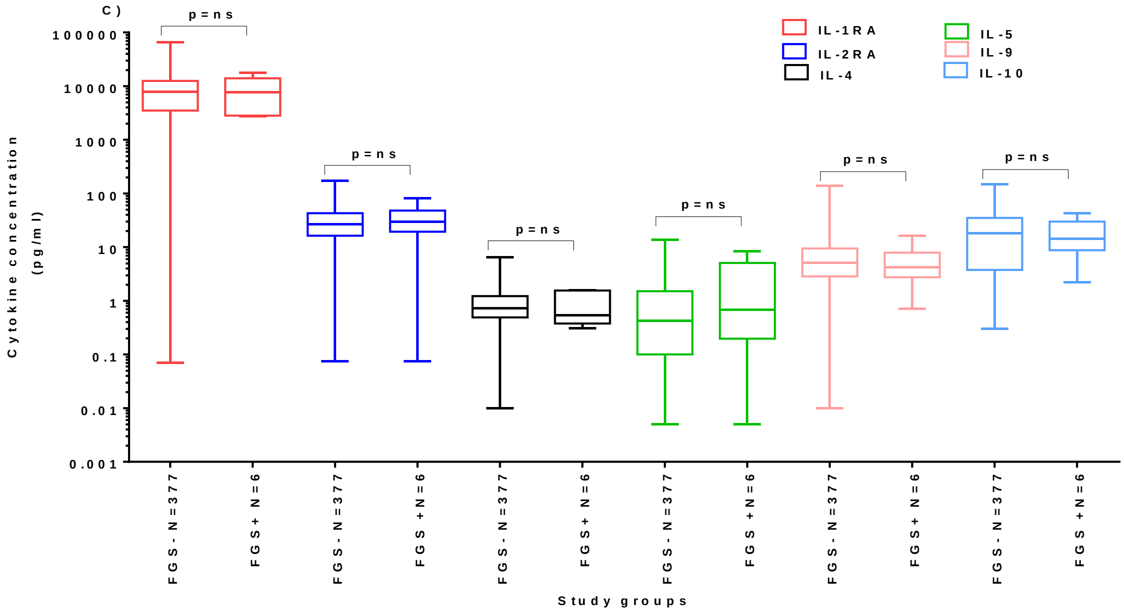


Figure 4.11 (A-B): Median levels of Th1 (IL-1β, MIF, IL-6, IL-1α) cytokines (A) and IFN-γ, IP-10, IL-8 & TNF-α (B) in FGS- women were compared to FGS+ women at

study exit. Non-parametric two tailed Mann Whitney tests were performed between the FGS- and FGS+ groups.



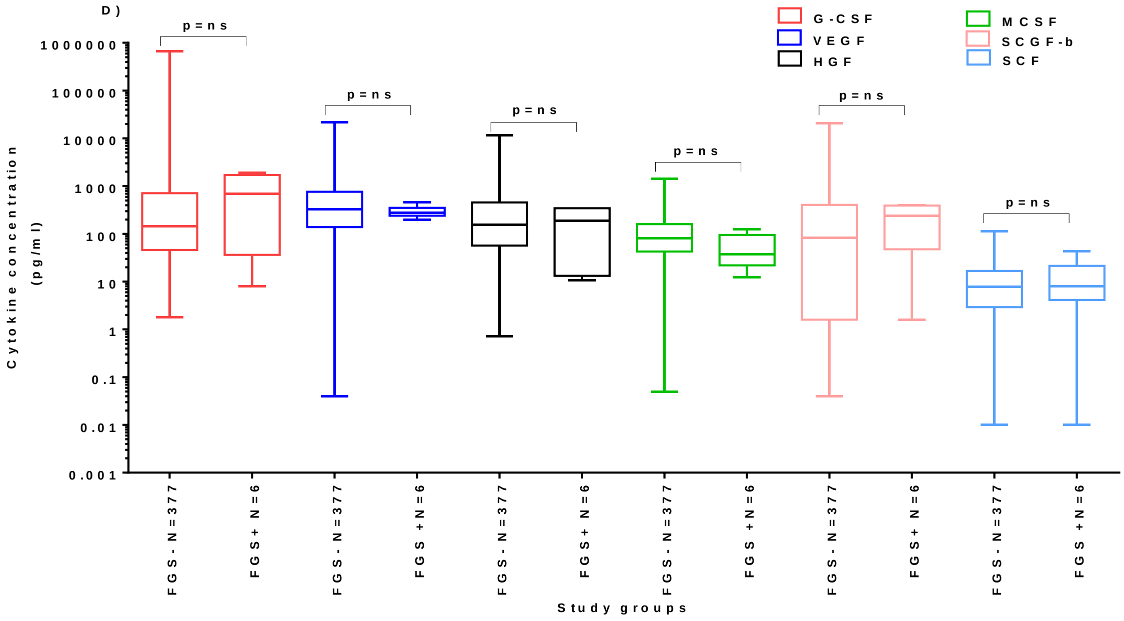
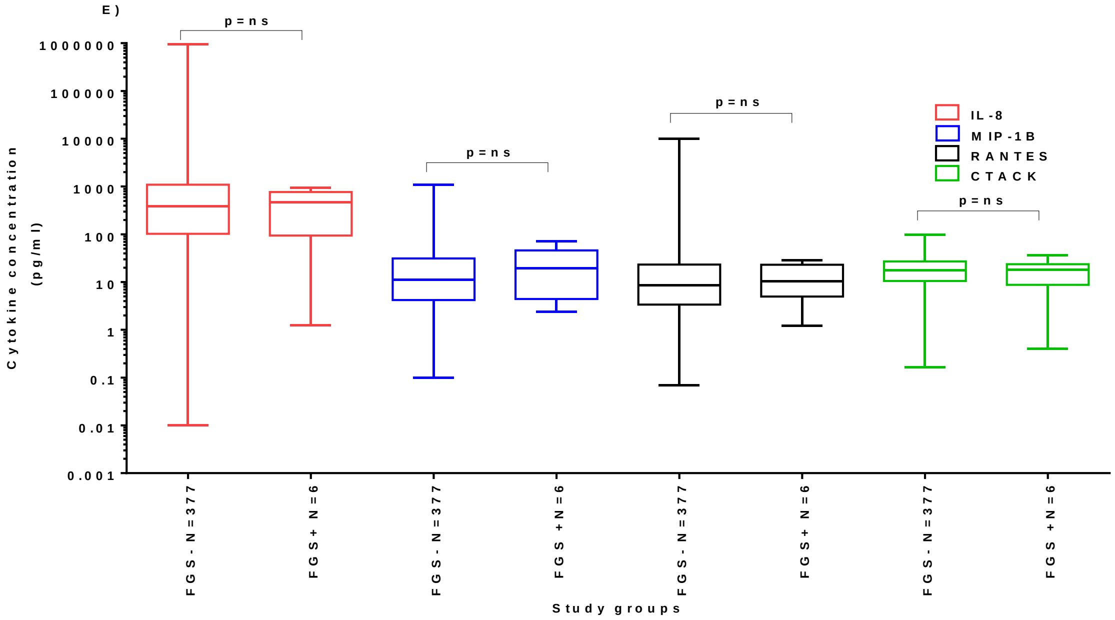


Figure 4.11 (C-D): Median levels of Th2 (IL-1-RA, IL-2RA, IL-4, IL-5, IL-9 & IL-10) cytokines (C) and growth factors G-CSF, VEGF, HGF, CSF, MCSF & SCGF-β

(D) in FGS- women were compared to FGS+ women at study exit. Non-parametric two tailed Mann Whitney tests were performed between the FGS- and FGS+ groups.

Growth factors & chemokines shown in figure 4.11 (D-F) were also investigated in FGS- and FGS+ study groups without HIV as a variable. Women that were FGS+ had higher median levels of G-CSF (median 690.23 pg/ml, IQR 8.0-1928.1) [figure 4.11 (D)] compared to FGS- women (median 145.44 pg/ml, IQR 38.8-1813.2 pg/ml) (p=0.63), with no significant difference observed. Median cytokine levels of SCGF-β was also higher in FGS+ (median 238.18 pg/ml, IQR 1.6-398.3 pg/ml) women compared to FGS- (median 84.4 pg/ml, IQR 1.6-1082.6 pg/ml) women (p=0.5), however this trend observed was not a significant difference. No significant differences were observed between FGS- and FGS+ study groups in the below figures 4.11 (D-F)



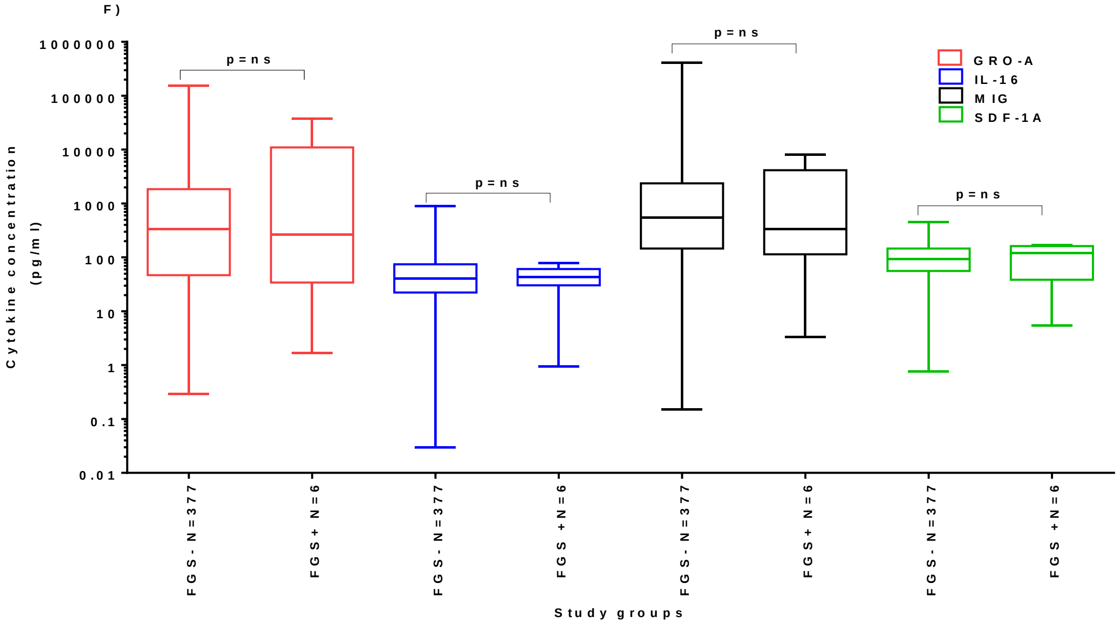


Figure 4.11 (E-F): Median cytokine levels of chemokines IL-8, MIP-1β, RANTES, CTACK, GRO-A, IL-16, MIG & SDF-1A were investigated in FGS- and FGS+ study groups at study exit. No significant differences were observed between study groups. Statistical analysis was performed using the Mann Whitney tests between study groups.

In this study, of the 383 women, 38 were positive for the *S mansoni* IgGs similar levels of IgG were found between the women that had HIV seroconverted compared to the HIV negative women. Only 9 of the 383 women had any evidence of FGS in their genital tracts. At study entry, of the 48 soluble proteins which included cytokines, chemokines and growth factors, only MCP-3, a chemokine was significantly higher in women in the presence of FGS compared to women without FGS. Furthermore, at study exit, in FGS+HIV+ compared to FGS+HIV- women, no differences were found in their genital tract cytokine profiles, which could be due to the low sample sizes for each group. However when the overall HIV-infection status was taken into account at study exit and in the absence of FGS, increased cytokine profiles, were predominant with HIV infection. These data indicate that HIV was primarily driving immune activation, which was evident in the female genital tract. The HIV-infected group displayed a mixture of consistently higher levels of Th1, Th2 responses, chemokines & growth factors compared with the HIV negative group. These data further point to the complexity of immune responses that prevail in the face of helminth infections that may pose additional risk for HIV acquistion and further impacts public health.

Chapter 5: Discussion

The prevalence of *Schistosoma* parasites worldwide and particularly in sub-Saharan Africa and southern Africa, together with the disease and pathogenesis including genital schistosomiasis following parastic infection, places a significant burden on public health. In addition the high incidence and prevalence of HIV-infection in sub-Saharan and southern Africa compounds the disease burden in these afflicted populations, and further highlights the public health challenges faced in these regions. The interplay between these two diseases- schistosomiasis and HIV- remains poorly understood.

The focus of this study was to investigate the relationship between FGS and risk for HIV-acquisition. FGS is a manifestation of the *Schistosoma* parasite that is often overlooked, and largely underdiagnosed particularly in early childhood and young adulthood [reviewed in (Bustinduy et al., 2014)]. The presence of FGS which is associated with increased immune responses in the genital tract may predispose women to HIV infection, and one of the mechanisms for increased HIV infection risk is through of lesions in the genital tract (Kleppa et al., 2014). Lesions in the mucosa surrounding the schistosomal eggs *in situ* create a breach in the genital tissue which may also be a crucial site for HIV-entry [reviewed in (Kjetland et al., 2012)] and may likely also facilitate the transmission of HIV-1 to their sexual partners [reviewed in (Mbabazi et al., 2011)].

Female genital schistosomiasis PCR positive samples are usually associated with the presence of *S. haematobium* ova in the tissue of the female genital tract (Kjetland et al., 2009), which is usually confirmed by colposcopy. It has been found that not all women who test positive for schistosome DNA in the pellet of CVL samples have FGS and not all women with clinical evidence of FGS through colposcopy test positive for schistosome DNA in their lavage pellets (Kjetland et al., 2009). In clinical practice it was reported that cervix, the fallopian tubes and the vagina commonly show *S. haematobium* ova (Arean, 1956, Badawy, 1962, Friedberg et al., 1991). Autopsy specimens of the genital tract also revealed ova in the ovaries and the uterus as well (Al-Adnani and Saleh, 1982, Friedberg et al., 1991, Gelfand and Ross, 1953, Swai et al., 2006). These findings may indicate that FGS may be grossly underdiagnosed and reported in clinical practice if all areas of the female genital tract are not thoroughly examined.

Apart from colposcopic examination, which remains the gold standard for the diagnosis of FGS, PCR in vaginal lavage samples of women is a suitable alternative (Kjetland et al., 2009). Female genital schistosomiasis PCR however may be quite limiting due to calcified eggs in the genital tract which would contain degraded DNA that is unable to amplify, resulting in many false negatives. The need for an internal positive control is warranted in order to distinguish a PCR negative result from PCR inhibition. Detection of FGS using PCR can be a useful tool in diagnostics and could supplement gynaecological examinations (Kjetland et al., 2009), and act as a further confirmatory tool.

In this study, real-time PCR screening yielded a relatively low prevalence of FGS (2.3%) in women from both the urban and rural sites (Ethikwini and Vulindlela). These data may underestimate true prevalence of female genital tract schistosomiasis, as some of the *Schistosoma* eggs may calcify *in situ* (Cheever et al., 1975) and therefore give false negative DNA results. Additionally, prior studies have shown that within 2-6 months of praziquantel treatment, resolution of these lesions occurred (Kjetland et al., 2008, Silva et al., 2005), however, long after puberty, the effects of schistosomiasis may still be seen (King and Dangerfield-Cha, 2008, Kjetland et al., 2008).

This data suggests that FGS is not common in both rural and urban CAPRISA 004 settings. More women from rural KwaZulu-Natal had FGS than the urban setting. However, due to the small sample size of women who tested positive for FGS DNA, the ability to reliably determine the differences in the prevalence rates of FGS in the rural and urban settings are limited and will need to be confirmed in a larger study. In addition to a small number of women who tested positive for FGS DNA, no women had FGS DNA detected at both study entry and study exit and this limitation could be due to the PCR only being able to detect ~67% of Schistosome DNA in the genital tract specimens (Kjetland et al., 2009). Another limitation of this study was the lack of data regarding the places where the participants resided during their childhoods and place of origin before living at the respective sites, as this may provide further insight into specific areas where young women and children may be particularly vulnerable to schistosomal infections.

This risk that FGS poses to acquiring HIV-infection remains a gap and is not fully understood. The data from this study shows that in women with FGS, 44% had acquired HIV infection by study exit and were over four times more likely to be HIV-infected than the FGS- group. This data suggests that the presence of FGS in these women may have predisposed them to HIV infection. A possible mechanism for increased risk for HIV acquisition with co-existing FGS is that there is increased immune responses resulting in inflammation and granuloma formation *in situ* where the eggs are situated, which can lead to extensive tissue damage including scarring and fibrosis (Cheever et al., 2000, Kameh et al., 2004), including calcification which ultimately leads to compromised mucosal tissue integrity. Other studies have also suggested that FGS may facilitate the transmission of HIV (Helling-Giese et al., 1996, Kameh et al., 2004, Kjetland et al., 2005, Poggensee et al., 1998, Ross et al., 2002).

The presence of schistosome DNA, due to egg excretion by *S. haematobium* is mostly found in younger women and in some cases is undetectable in older women as the eggs tend to calcify [(Kjetland et al., 2009), reviewed in (Kjetland et al., 2012)]. As a result, these calcified lesions are less likely to elicit strong immune responses. Many FGS PCR positives may not have been detected due to the calcification of the ova in the genital tissue, indicating non-viable or dead ova. The calcified ova which is foreign antigen, however, can still stimulate the immune system through the induction and influx of immune cells, and this can the lead to oedema and blood vessel proliferation (Jourdan et al., 2011a, Jourdan et al., 2011b, Kjetland et al., 2008, Mbabazi et al., 2011, Silva et al., 2005).

Immune reactions to *S. haematobium* eggs occur at the different phases (Jenkins et al., 2005). The accompanying immune reactions vary during the different phases which could then impact treatment success using praziquantel, the current standard treatment for *Schistosoma* infections, and this could have implications on for treatment success and disease resolution (Rujeni et al., 2012). However, exposure to this parasite generally produces a strong antibody response. Seroprevalence rates of schistsomiasis, ranging as high as between 71 to 100% from Limpopo to KZN, have been previously recorded (Moodley et al., 2003) indicating that these areas are hotspots and highly endemic for this particular parasite. The relatively low seroprevalence of schistosoma, of approximately 5% in our cohort study, may indicate that these areas may not be hotspots. The data from this study may be confounded by the fact that demographic data pertaining to where the participants were born and lived, prior to being in the Vulindlela and eThekwini sites were lacking and data regarding previous praziquantel treatment was not collected. Another factor that may have confounded seroprevalence rates in this and other studies was the possibility of cross reactivity to other nematode species (Mkhize-Kwitshana et al., 2011). This may undermine the accuracy of epidemiological studies looking at prevalence of this parasite based on ELISA antibody testing.

The seroprevalence does not give an indication of how acute or chronic the *Schistosoma* infection is, but merely gives a signal of exposure to the parasite (Bentz et al., 2003). Pathogen specific antibodies can be detected by ELISA up to 8 years post-infection (Murray et al., 2013). Further methods of diagnosis, apart from symptoms associated with the parasite, include stool and urinal analysis for confirmation of the *Schistosoma* eggs. The prevalence data suggests that *S. mansoni* exposure or infection was not common in these women. Additionally, in the women that tested positive for the *Schistosoma* antibody, a potential for antibody cross-reactivity with other nematode species may also influence the data on seroprevalence of *Schistosoma* (Mkhize-Kwitshana et al., 2011). Systemic IgG levels that were specific for *Schistosoma* spp may therefore be a combination of exposure to schistosomiasis itself, or other helminth infections making ELISA or antibody based testing less robust, and reliable.

Seven of the 9 women (78%) who were FGS PCR positive, also had detectable levels of *S. mansoni* specific IgG. This correspondence between the two detection methods used in this study may suggest that these women were very likely exposed to the *Schistosoma* species and indicates that two tests may be needed to confirm the presence of *Schistosoma* infection in the genital tract in the absence of a colposcopy of the genital tract.

From the 21 women who had detectable levels of IgG at study entry, only 2 remained antibody positive throughout the study with persistently detectable levels of *S. mansoni* specific IgG at study exit. This data suggests that 90% of the women that had been positive for *S. mansoni* specific IgG in their blood at study entry had undetectable levels of *S. mansoni* specific IgG by study exit. The decreased detection of sero positivity may be combination of the limitation of the assay, and the stringent optical density of 1.0 to detect the presence of the schistsoma specific IgG and the degradation of the IgG sample over time. Additionally, antigen cross-reactivity between parasites could possibly be the reason for such a trend to have occurred (Mkhize-Kwitshana et al., 2011). At study exit, a total of 19 women had detectable levels of S. mansoni specific IgG, however 17/19 (89%) of these women were antibody negative at study entry. These data may suggest that these 17 women had been exposed to schistosomiasis after their study entry visit, or that there was some degree of cross reactivity of IgGs to other nematodes, helminth proteins, worm species or environmental allergens (Santiago Hda et al., 2015).

Schistosomiasis is thought to impair immune responses to other diseases and viruses including HIV (Chen et al., 2012). However, in this study women who became HIV infected later in the study had higher titres of *S. mansoni* specific IgG compared to HIV uninfected women with detectable levels of *S. mansoni* specific IgG, although these titres were not significantly different between the groups. HIV infection itself induces an autoimmune like condition (Weyand and Goronzy, 1992, Zandman-Goddard and Shoenfeld, 2002) in the HIV-infected individuals, so, it may be highly likely that during HIV-infection, there may be a concommitant increase in antibody titres in people co-infected with parasites which leads to an overall higher antibody response. The study sample size however, precluded the ability to test whether there was a significant difference in the titres of the antibodies specific for the *Schistosoma* species between the groups. A larger sample size in each study group would therefore be more robust in assessing whether there are any significant differences in *S. mansoni* IgG titres in HIV positive women compared to HIV negative women.

Schistosomiasis, along with other helminth infections are known to induce a Th2 anti-inflammatory response (Adachi et al., 2014, Arnon, 1990, Coutinho et al., 2007, Kameh et al., 2004). Presence of both Th1 and Th2 cytokines occurs in the early phases of infection, with the Th2 or anti-inflammatory cytokine response dominating after oviposition (Adachi et al., 2014).

Screening for these biomarkers of genital inflammation in CVL could provide us further understanding into the profiles of cytokines induced due to FGS infection. Cytokines such as IL-4, IL-5, IL-9 and IL-13 are generally associated with *Schistosoma* infections (Silveira-Lemos et al., 2013). A previous study also screened for IFN-γ, IP-10, IL-8, TNF-α, IL-4, IL-5, IL-9 and IL-10, and confirmed that both the pro-and anti-inflammatory cytokines are associated with *Schistosoma* infections (Adachi et al., 2014, Wynn, 2015).

This study found that healthy women who were FGS- showed to have higher cytokine levels of basic FGF compared to women that were FGS+ at study entry. Basic fibroblastic growth factor (FGF) is known for its role in angiogenesis by being activated upon wound healing of normal epithelial tissue (Montesano et al., 1986). Women who are FGS+ are most likely to have smooth epithelial tissue damage [reviewed in (Kjetland et al., 2012)]. The trend in this study does not correlate with those of literature as women who are FGS+ would be expected to have higher cytokine levels of basic FGF than women who are FGS-.

Smooth epithelial tissue damage caused by inflammation can lead to the recruitment of immune cells to the site of infection (Helming, 2011). Monocyte chemotactic protein-3 (MCP-3) is a purified and cloned chemokine that is responsible for activating monocytes, basophils, eosinophils and T lymphocytes (Xu et al., 1995). The production of MCP in response to schistosome eggs has been observed as well as its involvement in Th2 immune responses (Chensue et al., 1995). This study showed that women that were FGS+ at study entry had higher levels of MCP-3 compared to FGS- women. Vaginal epithelial tissue damage caused by FGS may have possibly contributed to the activation of monocytes, eosinophils, T lymphocytes and basophils leading to increased MCP-3 levels in these women.

During helminth infection, Th1 responses are usually observed first before Th2 responses dominate (Smith et al., 2012). Th1 cytokines are generally known to enhance killing of intra-cellular parasites by inducing pro-inflammatory immune responses (Berger, 2000). IFN-γ is a Th1 cytokine that is usually produced by NK cells, and is one of the first innate immune responses to a foreign antigen (Bao et al., 2014). Increased levels of IFN-γ in the genital tract may indicate a presence of *Schistosoma* eggs in the genital tract. This may then also account for the increase in

IFN-γ production leading to the inflammatory signature in the genital tract, which attracts various immune cells to the site of infection. IFN-γ attracts and increases cytotoxic activity of both T cells and macrophages [reviewed in (Arango Duque and Descoteaux, 2014)], promoting genital inflammation. Pro-inflammatory cytokines such as IFN-γ, IP-10, IL-8 and TNF-α were further interrogated at study entry in this study between FGS+ and FGS- study groups, however no significant differences were found between the study groups. A larger study group number for FGS infected individuals could help suggest that Th1 cytokines maybe higher in FGS infected individuals.

Helminth infections are also known to modulate Th2 immune responses (Adachi et al., 2014). In early phases of infection, Th1 immune responses are present whilst Th2 immune responses are known to have a significant increase over the Th1 responses after oviposition (Adachi et al., 2014). Infection with parasites such as the *Schistosoma* species usually result in the production of Th2 cytokines IL-4, IL-5, IL-9 and IL-13 [reviewed in (Wynn, 2015)] which are generally classed as anti-inflammatory cytokines that play a role in regulating inflammatory cytokines.

In this study, IL-4 was found to be similar irrespective of the presence of FGS. IL-4 has been known to stimulate production of IgE which plays a role in protection against re-infection (Medhat et al., 1998). However, measurement of IgE was not performed in this study. IL-9, which has been known for its pleiotropic activity *in vivo*, leads to Th2 cytokine dominating responses (Fallon et al., 2000). In this study however, the levels of IL-9 were similar in all of the study sub-groups. Usually an increase in IL-5 production leads to eosinophilia (Huang et al., 2015), as this is the chemoattractant for eosinophils homing to the location of parasites, their larvae or eggs. It is not conclusive that the women with FGS in this study had elevated levels of eosinophils as this test was not performed during the routine follow-up visits, and this is a limitation of the study. Eosinophilia usually leads to scarring and formation of micro-lesions of the vaginal mucosae through enzyme-directed degradation of the parasite infected or affected tissue (Ramarokoto et al., 2014). Genital scarring and lesions in the vaginal mucosae may facilitate the movement of HIV across the mucosal barrier [reviewed in (Shattock and Moore, 2003)]. Levels of IL-10 are usually expected to be elevated in schistosomal infections (Turner et al., 2013), as this regulatory Th2 cytokine underlies both defence to helminth infections as well as atopic diseases like allergic conditions, asthma etc (Grant et al., 2011). In this study, further investigation of anti-inflammatory cytokines (IL-4, IL-5, IL-9, IL-10 and IL-13) between FGS + and FGS- study groups at study entry showed that no significant differences were observed. The limited sample size for the FGS+ group limited the ability to detect meaningful differences between the groups and draw firmer conclusions for the roles of anti-inflammatory cytokines in the presence of FGS.

Genital inflammation has been identified as a correlate of risk for HIV-acquisition (Masson et al., 2015). Masson et al., (2015) showed that women with genital inflammation, defined as having 5 of 9 key pro-inflammatory cytokines (MIP-1α, MIP-1β, IP-10, IL-8, MCP-1, IL-1α, IL-1β, IL-6, and TNF-α) elevated in the genital tract, had >3 fold increased HIV risk compared to women who did not have genital inflammation. Similarly, in this study we found that women that tested positive for Schistosome DNA in the genital tract samples, had a 4-fold increased risk for HIV acquisition than women who tested negative for schistosome DNA. Together, these studies highlight that both genital tract inflammation and the presence of parasitic DNA in the female genital tract could increase the risk for HIV infection. In addition, in this study, women that were FGS-HIV+ also had higher levels of pro-inflammatory cytokines when compared to FGS-HIV- women. A study showed that in individuals infected with HIV, the immune system is affected in many ways leading to both increases and decreases in cytokine level production [reviewed in (Breen, 2002)]. Pro-inflammatory cytokines act as mediators of immunity in individuals infected with HIV (Connolly et al., 2005). This study showed that FGS-HIV+ study participants had higher levels of pro-inflammatory cytokines (IL-1β, MIF, IL-1α and IL-6) compared to FGS-HIV- women. This trend suggests that HIV in itself has an inflammatory effect leading to the increased production of these Th1 immune responses in study participants. Schistosomiasis has been hypothesized to increase HIV susceptibility through chronic immune modulation (Secor et al., 2003). Both pro-inflammatory & anti-inflammatory cytokines were similar between FGS+HIV+ and FGS-HIV+ study groups, with no significant differences observed at study exit. The data suggests that, female genital schistosomiasis with HIV co-infection modulates the effect of Th1 & Th2 immune responses.

IL-1Ra has been shown to be produced during HIV infection (Zavala et al., 1995). This Th2 cytokine is important in balancing and controlling the inflammatory process of HIV infection (Zavala et al., 1995). This study showed that FGS-HIV+ women had significantly higher levels of IL-1Ra & IL-2Ra when compared to FGS-HIV- women at study exit. This observation suggests that HIV infection may be responsible for the significant production of these Th2 associated cytokines that may counteract the inflammatory actions of HIV infection. Anti-inflammatory cytokine IL-10 also has the ability to act as a B-cell stimulatory factor which in some conditions can suppress HIV replication [reviewed in (Breen, 2002)], however cytokine levels of IL-10 were not significantly different amongst any of the study groups at study exit.

Growth factors involved in cellular growth, healing and proliferation, can also act as signalling molecules between cells (Vander Heiden et al., 2001). Cell proliferation is a necessary to replenish cells that have been lost through cell death (Gutierrez, 2005). Proliferation of cells has also been observed in HIV (Ribeiro et al., 2002). In this study, concentrations of growth factors (G-CSF, VEGF, HGF, MCSF, CSF and SCGF) were significantly higher in FGS-HIV+ women compared to FGS-HIV- women at study exit. This data suggests that HIV may have contributed to the higher levels of growth factors in HIV infected individuals which may be indicative of global dysregulation of soluble growth factors in HIV infection. No significant differences of growth factors were observed in in the small groups of FGS+HIV+ women compared to FGS+HIV- women, indicating that the presence of FGS alone is unlikely modulate these factors.

Chemokines are cytokines or signalling proteins that have the ability to induce the recruitment of nearby responsive cells to the site of infection through chemotaxis (Turner et al., 2014). Release of chemokines are observed in early phases of HIV infection [reviewed in (Suresh and Wanchu, 2006). IL-8 is known to play a key role in recruiting neutrophils to the site of infection (Craig et al., 2009). In this study, FGS-HIV+ women had significantly higher levels of chemokines (IL-8, IP-10, MIP-1β, RANTES, CTACK, GRO-A, IL-16, MIG and SDF-1A) when compared to FGS-HIV- women at study exit. This observation suggests that based on FGS-HIV+ women having significantly higher levels of IL-8 and other respective chemokines mentioned above compared to FGS-HIV- women, that HIV infected patients could also have higher levels of neutrophils, however in the absence of the neutrophil counts and data in this cohort, the overall high immune activation may be attributed to the presence of HIV infection itself. Chemokines usually dictate the intensity of inflammatory response against egg antigen in schistoma infected patients through recruitment of various immune cells (Qiu et al., 2001). No significant differences of chemokine levels were observed in FGS+HIV+ women when compared to FGS-HIV+ women.

Cytokines that showed significant differences between study groups in addition to cytokines that are usually associated with FGS infection (IL-1β, MIF, IL-6, IL-1α, IFN-γ, IP-10, IL-8, TNF-α, IL-1-RA, IL-2RA, IL-4, IL-5, IL-9 and IL-10) were also compared between FGS+ and FGS- women at study exit, without HIV as a variable. However, no significant differences in either the Th1, Th2, chemokines or growth factors were observed at study exit. Th2-dominant systemic responses are expected to be elevated in individuals burdened with helminth infection (Adachi et al., 2014, Grant et al., 2011, Turner et al., 2013). Owing to compartment differences, the foci of infection – the signature of cytokines in the genital tracts for the women who tested FGS+ may be different compared to signature of cytokines in the systemic in response to *Schistosoma* spp infection and warrants further study.

Both pro and anti-inflammatory cytokines may have played a crucial role in driving various immune responses caused by *Schistosoma* infections both systemically and locally (FGS) in the genital tract. However, due to the limitation that the sampling time was different to the time women were infected with schistsoma, likely impacts on our ability to accurately detect the cytokine milieu associated with acute infection with this helminth both locally and systemiocally. The mixture of Th1 and Th2-immune responses in the vaginal tract may partly explain the increased likelihood of acquiring HIV in the presence of FGS. The ability to detect more cases of women with FGS by means of real-time PCR in this study may have been limited due to the fact that many true positives could have not been detected in older woman due to calcification of ova. The DNA in calcified ova may have degraded and therefore was unable to amplify in the real-time PCR reactions. Further study using digital PCR could extend the existing limits of real-time PCR and could aid in the detection of parasitic DNA in CVL pellets at low copy numbers. Cross reactivity of IgG responses to other helminth infections could also have impacted on IgG responses in the *S. mansoni* specific IgG ELISAs. Antibody screening with higher specificity needs to be performed in order to eliminate the probability of antibody cross-reactivity between nematode and trematode species.

Further investigation of these biomarkers of genital inflammation in a larger number of individuals may aid in understanding the role of these biomarkers in driving and promoting inflammation and predisposing women to HIV acquisition. In addition, understanding the role of *Schistosoma* and other parasite infections, profiling in parallel, the systemic and local genital compartment immune responses and cytokines, may better inform understanding of the risks that parasitic infections pose for HIV-acquisition in areas of high HIV endemicity such as KwaZulu-Natal.

This study in conclusion has established an in house PCR detection of FGS DNA in South African women. In this study, the low prevalence of both parasitic DNA from CVL pellets and low plasma IgG observed suggests that FGS and in general *Schistosoma* exposure are not common at these study sites in KZN. However, despite the small number of women who tested FGS+ in this study, there was still a significant risk of HIV acquisition indicating that the presence of FGS DNA in the genital tract may indeed be a risk factor for HIV acquisition as previously hypothesized. Other co-factors such as bacterial vaginosis and sexually transmitted diseases are known to be strongly associated with HIV acquisition risk through altering the pro-inflammatory cytokine profile in the genital tract. Despite low prevalence, women with FGS did have some signatures of genital tract inflammation.

Chapter 6: Conclusion

Data from this study underscores the importance of understanding the immunological mechanisms and risk factors for HIV acquisition in regions endemic for parasitic infections such as *Schistosoma spp* and infectious diseases namely HIV. Although the seroprevalence of schistosomiasis and FGS in the two sites, under study, namely Vulindlela and eThekwini in KwaZulu-Natal (KZN) was low, large-scale studies testing for the presence of these parasites in the regions of high HIV prevalence is warranted. Combining both colposcopy, and DNA PCR for the diagnosis of FGS may be a valuable tool especially in high prevalence regions for both diseases in order to better diagnose and further unravel the exact mechanism/s by which for example a parasitic infection in the genital tract may be increasing the risk for a sexually transmitted infection like HIV. Genital tract inflammation on its own or secondary to bacterial vaginosis and other STDs which results in activated CD4 T cells in the genital mucosae are mechanisms that have been previously described, however, there is a paucity of data available, as to the exact mechanism by which parasites can mediate such risk for HIV acquisition. Genital cytokine data from this study showed that the presence of FGS resulted in MCP-3, a monocyte chemoattractant protein, being elevated. The small number of women that were positive for the presence of parasitic DNA in the genital tract, may have undermined the ability to detect differences in levels of genital cytokines that may have been increased in the presence of *Schistosoma* spp. In the women that did seroconvert, a dysregulation was seen in the genital cytokine profile, which may indicate that HIV in itself drives these responses through increased immune activation which are found locally also in the genital tract. This study does corroborate findings in other studies for significant risk of HIV acquisition, with a 4.0 times likelihood of acquiring HIV in the presence of FGS, despite the small number or women with FGS. Future studies aimed at using combination of diagnostic tools and widescale treatment at public health level for schistosomiasis should be evaluated in an effort to minimize the risk for HIV acquisition in women. Taken together, this study provides further insight into the complexity of ensuing immune responses in the genital tract environment in the presence of parasitic involvement leading to genital inflammation underscoring the need to further study FGS in settings where HIV and schistosomiasis converge.

Chapter 7: References

ABDOOL KARIM, Q., ABDOOL KARIM, S. S., FROHLICH, J. A., GROBLER, A. C., BAXTER, C., MANSOOR, L. E., KHARSANY, A. B., SIBEKO, S., MLISANA, K. P., OMAR, Z., GENGIAH, T. N., MAARSCHALK, S., ARULAPPAN, N., MLOTSHWA, M., MORRIS, L., TAYLOR, D. & GROUP, C. T. 2010a. Effectiveness and safety of tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women. *Science,* 329**,** 1168-74.

ABDOOL KARIM, Q., SIBEKO, S. & BAXTER, C. 2010b. Preventing HIV infection in women: a global health imperative. *Clin Infect Dis,* 50 Suppl 3**,** S122-9.

ADACHI, K., NAKAMURA, R., OSADA, Y., SENBA, M., TAMADA, K. & HAMANO, S. 2014. Involvement of IL-18 in the expansion of unique hepatic T cells with unconventional cytokine profiles during Schistosoma mansoni infection. *PLoS One,* 9**,** e96042.

AL-ADNANI, M. S. & SALEH, K. M. 1982. Extraurinary schistosomiasis in Southern Iraq. *Histopathology,* 6**,** 747-52.

ALI, S. F., RASHID, N., IMANAKA, T. & AKHTAR, M. 2011. Family B DNA polymerase from a hyperthermophilic archaeon Pyrobaculum calidifontis: cloning, characterization and PCR application. *J Biosci Bioeng,* 112**,** 118-23.

ALTFELD, M., ALLEN, T. M., YU, X. G., JOHNSTON, M. N., AGRAWAL, D., KORBER, B. T., MONTEFIORI, D. C., O'CONNOR, D. H., DAVIS, B. T., LEE, P. K., MAIER, E. L., HARLOW, J., GOULDER, P. J., BRANDER, C., ROSENBERG, E. S. & WALKER, B. D. 2002. HIV-1 superinfection despite broad CD8+ T-cell responses containing replication of the primary virus. *Nature,* 420**,** 434-9.

ANAHTAR, M. N., BYRNE, E. H., DOHERTY, K. E., BOWMAN, B. A., YAMAMOTO, H. S., SOUMILLON, M., PADAVATTAN, N., ISMAIL, N., MOODLEY, A., SABATINI, M. E., GHEBREMICHAEL, M. S., NUSBAUM, C., HUTTENHOWER, C., VIRGIN, H. W., NDUNG'U, T., DONG, K. L., WALKER, B. D., FICHOROVA, R. N. & KWON, D. S. 2015. Cervicovaginal bacteria are a major modulator of host inflammatory responses in the female genital tract. *Immunity,* 42**,** 965-76.

ARANGO DUQUE, G. & DESCOTEAUX, A. 2014. Macrophage cytokines: involvement in immunity and infectious diseases. *Front Immunol,* 5**,** 491.

AREAN, V. M. 1956. Manson's schistosomiasis of the female genital tract. *Am J Obstet Gynecol,* 72**,** 1038-53.

ARNON, R. 1990. Life span of parasite in schistosomiasis patients. *Isr J Med Sci,* 26**,** 404-5.

ATA, A. A., EL-RAZIKY, S. H., EL-HAWEY, A. M. & RAFLA, H. 1970. A clinicopathological study of schistosomal colonic polyposis and their pathogenesis. *J Egypt Med Assoc,* 53**,** 762-72.

BADAWY, A. H. 1962. Schistosomiasis of the cervix. *Br Med J,* 1**,** 369-72.

BAO, Y., LIU, X., HAN, C., XU, S., XIE, B., ZHANG, Q., GU, Y., HOU, J., QIAN, L., QIAN, C., HAN, H. & CAO, X. 2014. Identification of IFN-gamma-producing innate B cells. *Cell Res,* 24**,** 161-76.

BASHIR, M. E., ANDERSEN, P., FUSS, I. J., SHI, H. N. & NAGLER-ANDERSON, C. 2002. An enteric helminth infection protects against an allergic response to dietary antigen. *J Immunol,* 169**,** 3284-92.

BEBELL, L. M., PASSMORE, J. A., WILLIAMSON, C., MLISANA, K., IRIOGBE, I., VAN LOGGERENBERG, F., KARIM, Q. A. & KARIM, S. A. 2008. Relationship between levels of inflammatory cytokines in the genital tract and CD4+ cell counts in women with acute HIV-1 infection. *J Infect Dis,* 198**,** 710-4.

BENTWICH, Z., KALINKOVICH, A. & WEISMAN, Z. 1995. Immune activation is a dominant factor in the pathogenesis of African AIDS. *Immunol Today,* 16**,** 187-91.

BENTZ, B. G., EALEY, K. A., MORROW, J., CLAYPOOL, P. L. & SALIKI, J. T. 2003. Seroprevalence of antibodies to Sarcocystis neurona in equids residing in Oklahoma. *J Vet Diagn Invest,* 15**,** 597-600.

BERGER, A. 2000. Th1 and Th2 responses: what are they? *BMJ,* 321**,** 424.

BREEN, E. C. 2002. Pro- and anti-inflammatory cytokines in human immunodeficiency virus infection and acquired immunodeficiency syndrome. *Pharmacol Ther,* 95**,** 295-304.

BULLA, R., DE SETA, F., RADILLO, O., AGOSTINIS, C., DURIGUTTO, P., PELLIS, V., DE SANTO, D., CROVELLA, S. & TEDESCO, F. 2010. Mannose-binding lectin is produced by vaginal epithelial cells and its level in the vaginal fluid is influenced by progesterone. *Mol Immunol,* 48**,** 281-6.

BUSTINDUY, A., KING, C., SCOTT, J., APPLETON, S., SOUSA-FIGUEIREDO, J. C., BETSON, M. & STOTHARD, J. R. 2014. HIV and schistosomiasis co-infection in African children. *Lancet Infect Dis,* 14**,** 640-9.

CARIAS, A. M., MCCOOMBE, S., MCRAVEN, M., ANDERSON, M., GALLOWAY, N., VANDERGRIFT, N., FOUGHT, A. J., LURAIN, J., DUPLANTIS, M., VEAZEY, R. S. & HOPE, T. J. 2013. Defining the interaction of HIV-1 with the mucosal barriers of the female reproductive tract. *J Virol,* 87**,** 11388-400.

CDC. 2014. *Transmission Risk- HIV and the Law- Policies and Programs* [Online]. Available: http://www.cdc.gov/hiv/policies/law/risk.html. .

CHEEVER, A. W., HOFFMANN, K. F. & WYNN, T. A. 2000. Immunopathology of schistosomiasis mansoni in mice and men. *Immunol Today,* 21**,** 465-6.

CHEEVER, A. W., YOUNG, S. W. & SHEHATA, A. 1975. Calcification of Schistosoma haematobium eggs: relation of radiologically demonstrable calcification to eggs in tissues and passage of eggs in urine. *Trans R Soc Trop Med Hyg,* 69**,** 410-4.

CHEN, L., LIU, W. Q., LEI, J. H., GUAN, F., LI, M. J., SONG, W. J., LI, Y. L. & WANG, T. 2012. Chronic Schistosoma japonicum infection reduces immune response to vaccine against hepatitis B in mice. *PLoS One,* 7**,** e51512.

CHENSUE, S. W., WARMINGTON, K. S., LUKACS, N. W., LINCOLN, P. M., BURDICK, M. D., STRIETER, R. M. & KUNKEL, S. L. 1995. Monocyte chemotactic protein expression during schistosome egg granuloma formation. Sequence of production, localization, contribution, and regulation. *Am J Pathol,* 146**,** 130-8.

CONNOLLY, N. C., RIDDLER, S. A. & RINALDO, C. R. 2005. Proinflammatory cytokines in HIV disease-a review and rationale for new therapeutic approaches. *AIDS Rev,* 7**,** 168-80.

COOKE, G. S., LALVANI, A., GLEESON, F. V. & CONLON, C. P. 1999. Acute pulmonary schistosomiasis in travelers returning from Lake Malawi, sub-Saharan Africa. *Clin Infect Dis,* 29**,** 836-9.

COUTINHO, H. M., ACOSTA, L. P., WU, H. W., MCGARVEY, S. T., SU, L., LANGDON, G. C., JIZ, M. A., JARILLA, B., OLVEDA, R. M., FRIEDMAN, J. F. & KURTIS, J. D. 2007. Th2 cytokines are associated with persistent hepatic fibrosis in human Schistosoma japonicum infection. *J Infect Dis,* 195**,** 288-95.

CRAIG, A., MAI, J., CAI, S. & JEYASEELAN, S. 2009. Neutrophil recruitment to the lungs during bacterial pneumonia. *Infect Immun,* 77**,** 568-75.

DOWNS, J. A., MGUTA, C., KAATANO, G. M., MITCHELL, K. B., BANG, H., SIMPLICE, H., KALLUVYA, S. E., CHANGALUCHA, J. M., JOHNSON, W. D., JR. & FITZGERALD, D. W. 2011. Urogenital schistosomiasis in women of reproductive age in Tanzania's Lake Victoria region. *Am J Trop Med Hyg,* 84**,** 364-9.

FALLON, P. G., SMITH, P., RICHARDSON, E. J., JONES, F. J., FAULKNER, H. C., VAN SNICK, J., RENAULD, J. C., GRENCIS, R. K. & DUNNE, D. W. 2000. Expression of interleukin-9 leads to Th2 cytokine-dominated responses and fatal enteropathy in mice with chronic Schistosoma mansoni infections. *Infect Immun,* 68**,** 6005-11.

FELDMEIER, H., POGGENSEE, G. & KRANTZ, I. 1998. Puberty and Age-intensity Profiles in Schistosome Infections: Another Hypothesis. *Parasitol Today,* 14**,** 435.

FRIEDBERG, D., BERRY, A. V., SCHNEIDER, J. & FRIPP, P. J. 1991. Schistosomiasis of the female genital tract. *S Afr Med J,* 80 Suppl**,** 2-15.

GELFAND, M. & ROSS, W. F. 1953. II. The distribution of schistosome ova in the genito-urinary tract in subjects of bilharziasis. *Trans R Soc Trop Med Hyg,* 47**,** 218-20.

GRANT, A. V., ARAUJO, M. I., PONTE, E. V., OLIVEIRA, R. R., CRUZ, A. A., BARNES, K. C. & BEATY, T. H. 2011. Polymorphisms in IL10 are associated with total Immunoglobulin E levels and Schistosoma mansoni infection intensity in a Brazilian population. *Genes Immun,* 12**,** 46-50.

GRAVITT, P. E., MARKS, M., KOSEK, M., HUANG, C., CABRERA, L., OLORTEGUI, M. P., MEDRANO, A. M., TRIGOSO, D. R., QURESHI, S., BARDALES, G. S., MANRIQUE-HINOJOSA, J., CARDENAS, A. Z., LARRAONDO, M. A., COK, J., QEADAN, F., SIRACUSA, M. & GILMAN, R. H. 2016. Soil-Transmitted Helminth Infections Are Associated With an Increase in Human Papillomavirus Prevalence and a T-Helper Type 2 Cytokine Signature in Cervical Fluids. *J Infect Dis,* 213**,** 723-30.

GUTIERREZ, C. 2005. Coupling cell proliferation and development in plants. *Nat Cell Biol,* 7**,** 535-41.

HARVIE, M., JORDAN, T. W. & LA FLAMME, A. C. 2007. Differential liver protein expression during schistosomiasis. *Infect Immun,* 75**,** 736-44.

HELLING-GIESE, G., SJAASTAD, A., POGGENSEE, G., KJETLAND, E. F., RICHTER, J., CHITSULO, L., KUMWENDA, N., RACZ, P., ROALD, B., GUNDERSEN, S. G., KRANTZ, I. & FELDMEIER, H. 1996. Female genital schistosomiasis (FGS): relationship between gynecological and histopathological findings. *Acta Trop,* 62**,** 257-67.

HELMING, L. 2011. Inflammation: cell recruitment versus local proliferation. *Curr Biol,* 21**,** R548-50.

HORSNELL, W. G. & BROMBACHER, F. 2008. Key roles for specific and nonspecific antibody in intestinal nematode expulsion. *Cell Host Microbe,* 4**,** 303-5.

HORSNELL, W. G. & BROMBACHER, F. 2010. Genes associated with alternatively activated macrophages discretely regulate helminth infection and pathogenesis in experimental mouse models. *Immunobiology,* 215**,** 704-8.

HUANG, L., GEBRESELASSIE, N. G., GAGLIARDO, L. F., RUYECHAN, M. C., LUBER, K. L., LEE, N. A., LEE, J. J. & APPLETON, J. A. 2015. Eosinophils mediate protective immunity against secondary nematode infection. *J Immunol,* 194**,** 283-90.

IIJIMA, N., LINEHAN, M. M., ZAMORA, M., BUTKUS, D., DUNN, R., KEHRY, M. R., LAUFER, T. M. & IWASAKI, A. 2008. Dendritic cells and B cells maximize mucosal Th1 memory response to herpes simplex virus. *J Exp Med,* 205**,** 3041-52.

IWASAKI, A. 2010. Antiviral immune responses in the genital tract: clues for vaccines. *Nat Rev Immunol,* 10**,** 699-711.

IWASAKI, A. & MEDZHITOV, R. 2004. Toll-like receptor control of the adaptive immune responses. *Nat Immunol,* 5**,** 987-95.

JENKINS, S. J., HEWITSON, J. P., JENKINS, G. R. & MOUNTFORD, A. P. 2005. Modulation of the host's immune response by schistosome larvae. *Parasite Immunol,* 27**,** 385-93.

JOHNSON, C. & APPLETON, C. 2005. Urban schistosomiasis transmission in Pietermaritzburg, South Africa. *Southern African Journal of Epidemiology and Infection,* 20**,** 103-107.

JOURDAN, P. M., HOLMEN, S. D., GUNDERSEN, S. G., ROALD, B. & KJETLAND, E. F. 2011a. HIV target cells in Schistosoma haematobium-infected female genital mucosa. *Am J Trop Med Hyg,* 85**,** 1060-4.

JOURDAN, P. M., ROALD, B., POGGENSEE, G., GUNDERSEN, S. G. & KJETLAND, E. F. 2011b. Increased vascularity in cervicovaginal mucosa with Schistosoma haematobium infection. *PLoS Negl Trop Dis,* 5**,** e1170.

KAMEH, D., SMITH, A., BROCK, M. S., NDUBISI, B. & MASOOD, S. 2004. Female genital schistosomiasis: case report and review of the literature. *South Med J,* 97**,** 525-7.

KEELE, B. F., GIORGI, E. E., SALAZAR-GONZALEZ, J. F., DECKER, J. M., PHAM, K. T., SALAZAR, M. G., SUN, C., GRAYSON, T., WANG, S., LI, H., WEI, X., JIANG, C., KIRCHHERR, J. L., GAO, F., ANDERSON, J. A., PING, L. H., SWANSTROM, R., TOMARAS, G. D., BLATTNER, W. A., GOEPFERT, P. A., KILBY, J. M., SAAG, M. S., DELWART, E. L., BUSCH, M. P., COHEN, M. S., MONTEFIORI, D. C., HAYNES, B. F., GASCHEN, B., ATHREYA, G. S., LEE, H. Y., WOOD, N., SEOIGHE, C., PERELSON, A. S., BHATTACHARYA, T., KORBER, B. T., HAHN, B. H. & SHAW, G. M. 2008. Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection. *Proc Natl Acad Sci U S A,* 105**,** 7552-7.

KING, C. H. & DANGERFIELD-CHA, M. 2008. The unacknowledged impact of chronic schistosomiasis. *Chronic Illn,* 4**,** 65-79.

KJETLAND, E. F., HOVE, R. J., GOMO, E., MIDZI, N., GWANZURA, L., MASON, P., FRIIS, H., VERWEIJ, J. J., GUNDERSEN, S. G., NDHLOVU, P. D., MDULUZA, T. & VAN LIESHOUT, L. 2009. Schistosomiasis PCR in vaginal lavage as an indicator of genital Schistosoma haematobium infection in rural Zimbabwean women. *Am J Trop Med Hyg,* 81**,** 1050-5.

KJETLAND, E. F., LEUTSCHER, P. D. & NDHLOVU, P. D. 2012. A review of female genital schistosomiasis. *Trends Parasitol,* 28**,** 58-65.

KJETLAND, E. F., NDHLOVU, P. D., GOMO, E., MDULUZA, T., MIDZI, N., GWANZURA, L., MASON, P. R., SANDVIK, L., FRIIS, H. & GUNDERSEN, S. G. 2006. Association between genital schistosomiasis and HIV in rural Zimbabwean women. *AIDS,* 20**,** 593-600.

KJETLAND, E. F., NDHLOVU, P. D., KUREWA, E. N., MIDZI, N., GOMO, E., MDULUZA, T., FRIIS, H. & GUNDERSEN, S. G. 2008. Prevention of gynecologic contact bleeding and genital sandy patches by childhood anti-schistosomal treatment. *Am J Trop Med Hyg,* 79**,** 79-83.

KJETLAND, E. F., NDHLOVU, P. D., MDULUZA, T., GOMO, E., GWANZURA, L., MASON, P. R., KUREWA, E. N., MIDZI, N., FRIIS, H. & GUNDERSEN, S. G. 2005. Simple clinical manifestations of genital Schistosoma haematobium infection in rural Zimbabwean women. *Am J Trop Med Hyg,* 72**,** 311-9.

KLEPPA, E., RAMSURAN, V., ZULU, S., KARLSEN, G. H., BERE, A., PASSMORE, J. A., NDHLOVU, P., LILLEBO, K., HOLMEN, S. D., ONSRUD, M., GUNDERSEN, S. G., TAYLOR, M., KJETLAND, E. F. & NDUNG'U, T. 2014. Effect of female genital schistosomiasis and anti-schistosomal treatment on monocytes, CD4+ T-cells and CCR5 expression in the female genital tract. *PLoS One,* 9**,** e98593.

KROIDL, I., SAATHOF, E., MAGANGA, L., MAKUNDE, W. H., HOERAUF, A., GELDMACHER, C., CLOWES, P., MABOKO, L. & HOELSCHER, M. 2016. Effect of Wuchereria bancrofti infection on HIV incidence in southwest Tanzania: a prospective cohort study. *The Lancet.*

LAMONT, R. F., SOBEL, J. D., AKINS, R. A., HASSAN, S. S., CHAIWORAPONGSA, T., KUSANOVIC, J. P. & ROMERO, R. 2011. The vaginal microbiome: new information about genital tract flora using molecular based techniques. *BJOG,* 118**,** 533-49.

LEE, H. K., ZAMORA, M., LINEHAN, M. M., IIJIMA, N., GONZALEZ, D., HABERMAN, A. & IWASAKI, A. 2009. Differential roles of migratory and resident DCs in T cell priming after mucosal or skin HSV-1 infection. *J Exp Med,* 206**,** 359-70.

LUSTIGMAN, S., PRICHARD, R. K., GAZZINELLI, A., GRANT, W. N., BOATIN, B. A., MCCARTHY, J. S. & BASANEZ, M. G. 2012. A research agenda for helminth diseases of humans: the problem of helminthiases. *PLoS Negl Trop Dis,* 6**,** e1582.

MAGAISA, K., M, T., EF, K. & PJ, N. 2015. A review of the control of schistosomiasis in South Africa. *S Afr J Sci. 2015,* 111(11/12), Art**,** 2014-0427.

MAGGI, E., MAZZETTI, M., RAVINA, A., ANNUNZIATO, F., DE CARLI, M., PICCINNI, M. P., MANETTI, R., CARBONARI, M., PESCE, A. M., DEL PRETE, G. & ET AL. 1994. Ability of HIV to promote a TH1 to TH0 shift and to replicate preferentially in TH2 and TH0 cells. *Science,* 265**,** 244-8.

MAIZELS, R. M., BUNDY, D. A., SELKIRK, M. E., SMITH, D. F. & ANDERSON, R. M. 1993. Immunological modulation and evasion by helminth parasites in human populations. *Nature,* 365**,** 797-805.

MASOOD, R., LUNARDI-ISKANDAR, Y., MOUDGIL, T., ZHANG, Y., LAW, R. E., HUANG, C. L., PURI, R. K., LEVINE, A. M. & GILL, P. S. 1994. IL-10 inhibits HIV-1 replication and is induced by tat. *Biochem Biophys Res Commun,* 202**,** 374-83.

MASSON, L., PASSMORE, J. A., LIEBENBERG, L. J., WERNER, L., BAXTER, C., ARNOLD, K. B., WILLIAMSON, C., LITTLE, F., MANSOOR, L. E., NARANBHAI, V., LAUFFENBURGER, D. A., RONACHER, K., WALZL, G., GARRETT, N. J., WILLIAMS, B. L., COUTO-RODRIGUEZ, M., HORNIG, M., LIPKIN, W. I., GROBLER, A., ABDOOL KARIM, Q. & ABDOOL KARIM, S. S. 2015. Genital Inflammation and the Risk of HIV Acquisition in Women. *Clin Infect Dis,* 61**,** 260-9.

MBABAZI, P. S., ANDAN, O., FITZGERALD, D. W., CHITSULO, L., ENGELS, D. & DOWNS, J. A. 2011. Examining the relationship between urogenital schistosomiasis and HIV infection. *PLoS Negl Trop Dis,* 5**,** e1396.

MCELROY, M. D., ELREFAEI, M., JONES, N., SSALI, F., MUGYENYI, P., BARUGAHARE, B. & CAO, H. 2005. Coinfection with Schistosoma mansoni is associated with decreased HIV-specific cytolysis and increased IL-10 production. *J Immunol,* 174**,** 5119-23.

MEDHAT, A., SHEHATA, M., BUCCI, K., MOHAMED, S., DIEF, A. D., BADARY, S., GALAL, H., NAFEH, M. & KING, C. L. 1998. Increased interleukin-4 and interleukin-5 production in response to Schistosoma haematobium adult worm antigens correlates with lack of reinfection after treatment. *J Infect Dis,* 178**,** 512-9.

MILLER, C. J. & SHATTOCK, R. J. 2003. Target cells in vaginal HIV transmission. *Microbes Infect,* 5**,** 59-67.

MILLIGAN, G. N. 1999. Neutrophils aid in protection of the vaginal mucosae of immune mice against challenge with herpes simplex virus type 2. *J Virol,* 73**,** 6380-6.

MKHIZE-KWITSHANA, Z. L., TAYLOR, M., JOOSTE, P., MABASO, M. L. & WALZL, G. 2011. The influence of different helminth infection phenotypes on immune responses against HIV in co-infected adults in South Africa. *BMC Infect Dis,* 11**,** 273.

MLISANA, K., NAICKER, N., WERNER, L., ROBERTS, L., VAN LOGGERENBERG, F., BAXTER, C., PASSMORE, J. A., GROBLER, A. C., STURM, A. W., WILLIAMSON, C., RONACHER, K., WALZL, G. & ABDOOL KARIM, S. S. 2012. Symptomatic vaginal discharge is a poor predictor of sexually transmitted infections and genital tract inflammation in high-risk women in South Africa. *J Infect Dis,* 206**,** 6-14.

MONTESANO, R., VASSALLI, J. D., BAIRD, A., GUILLEMIN, R. & ORCI, L. 1986. Basic fibroblast growth factor induces angiogenesis in vitro. *Proc Natl Acad Sci U S A,* 83**,** 7297-301.

MOODLEY, I., KLEINSCHMIDT, I., SHARP, B., CRAIG, M. & APPLETON, C. 2003. Temperature-suitability maps for schistosomiasis in South Africa. *Ann Trop Med Parasitol,* 97**,** 617-27.

MOSUNJAC, M. B., TADROS, T., BEACH, R. & MAJMUDAR, B. 2003. Cervical schistosomiasis, human papilloma virus (HPV), and human immunodeficiency virus (HIV): a dangerous coexistence or coincidence? *Gynecol Oncol,* 90**,** 211-4.

MURRAY, K. O., GARCIA, M. N., YAN, C. & GORCHAKOV, R. 2013. Persistence of detectable immunoglobulin M antibodies up to 8 years after infection with West Nile virus. *Am J Trop Med Hyg,* 89**,** 996-1000.

MUTENGO, M. M., MWANSA, J. C., MDULUZA, T., SIANONGO, S. & CHIPETA, J. 2014. High Schistosoma mansoni disease burden in a rural district of western Zambia. *Am J Trop Med Hyg,* 91**,** 965-72.

MWINZI, P. N., KARANJA, D. M., COLLEY, D. G., ORAGO, A. S. & SECOR, W. E. 2001. Cellular immune responses of schistosomiasis patients are altered by human immunodeficiency virus type 1 coinfection. *J Infect Dis,* 184**,** 488-96.

NDHLOVU, P. D., MDULUZA, T., KJETLAND, E. F., MIDZI, N., NYANGA, L., GUNDERSEN, S. G., FRIIS, H. & GOMO, E. 2007. Prevalence of urinary schistosomiasis and HIV in females living in a rural community of Zimbabwe: does age matter? *Trans R Soc Trop Med Hyg,* 101**,** 433-8.

NEUTRA, M. R. & KOZLOWSKI, P. A. 2006. Mucosal vaccines: the promise and the challenge. *Nat Rev Immunol,* 6**,** 148-58.

NIEMANN, MARTI, H. P., DUHNSEN, S. H. & G, B. 2010. Pulmonary Schistosomiasis – Imaging Features. *Journal of Radiology Case Reports,* 4**,** 37-43.

NOLAN, J. P. & SKLAR, L. A. 2002. Suspension array technology: evolution of the flat-array paradigm. *Trends Biotechnol,* 20**,** 9-12.

NORSETH, H. M., NDHLOVU, P. D., KLEPPA, E., RANDRIANASOLO, B. S., JOURDAN, P. M., ROALD, B., HOLMEN, S. D., GUNDERSEN, S. G., BAGRATEE, J., ONSRUD, M. & KJETLAND, E. F. 2014. The colposcopic atlas of schistosomiasis in the lower female genital tract based on studies in Malawi,Zimbabwe, Madagascar and South Africa. *PLoS Negl Trop Dis,* 8**,** e3229.

NOUR, N. M. 2010. Schistosomiasis: health effects on women. *Rev Obstet Gynecol,* 3**,** 28-32.

OLDS, G. R. & DASARATHY, S. 2001. Recent Advances in Schistosomiasis. *Curr Infect Dis Rep,* 3**,** 59-67.

PEARCE, E. J., CASPAR, P., GRZYCH, J. M., LEWIS, F. A. & SHER, A. 1991. Downregulation of Th1 cytokine production accompanies induction of Th2 responses by a parasitic helminth, Schistosoma mansoni. *J Exp Med,* 173**,** 159-66.

PEARCE, E. J. & MACDONALD, A. S. 2002. The immunobiology of schistosomiasis. *Nat Rev Immunol,* 2**,** 499-511.

PITCHFORD, R. J. 1965. Differences in the Egg Morphology and Certain Biological Characteristics of Some African and Middle Eastern Schistosomes, Genus Schistosoma, with Terminal-Spined Eggs. *Bull World Health Organ,* 32**,** 105-20.

POGGENSEE, G., KIWELU, I., SARIA, M., RICHTER, J., KRANTZ, I. & FELDMEIER, H. 1998. Schistosomiasis of the lower reproductive tract without egg excretion in urine. *Am J Trop Med Hyg,* 59**,** 782-3.

PUDNEY, J., QUAYLE, A. J. & ANDERSON, D. J. 2005. Immunological microenvironments in the human vagina and cervix: mediators of cellular immunity are concentrated in the cervical transformation zone. *Biol Reprod,* 73**,** 1253-63.

QIU, B., FRAIT, K. A., REICH, F., KOMUNIECKI, E. & CHENSUE, S. W. 2001. Chemokine expression dynamics in mycobacterial (type-1) and schistosomal (type-2) antigen-elicited pulmonary granuloma formation. *Am J Pathol,* 158**,** 1503-15.

QUINN, T. C. & OVERBAUGH, J. 2005. HIV/AIDS in women: an expanding epidemic. *Science,* 308**,** 1582-3.

RAMAROKOTO, C. E., KILDEMOES, A. O., RANDRIANASOLO, B. S., RAVONIARIMBININA, P., RAVAOALIMALALA, V. E., LEUTSCHER, P., KJETLAND, E. F. & VENNERVALD, B. J. 2014. Eosinophil granule proteins ECP and EPX as markers for a potential early-stage inflammatory lesion in female genital schistosomiasis (FGS). *PLoS Negl Trop Dis*, 8**,** e2974.

RANDRIANASOLO, B. S., JOURDAN, P. M., RAVONIARIMBININA, P., RAMAROKOTO, C. E., RAKOTOMANANA, F., RAVAOALIMALALA, V. E., GUNDERSEN, S. G., FELDMEIER, H., VENNERVALD, B. J., VAN LIESHOUT, L., ROALD, B., LEUTSCHER, P. & KJETLAND, E. F. 2015. Gynecological manifestations, histopathological findings, and schistosoma-specific polymerase chain reaction results among women with Schistosoma haematobium infection: a cross-sectional study in Madagascar. *J Infect Dis,* 212**,** 275-84.

RIBEIRO, R. M., MOHRI, H., HO, D. D. & PERELSON, A. S. 2002. In vivo dynamics of T cell activation, proliferation, and death in HIV-1 infection: why are CD4+ but not CD8+ T cells depleted? *Proc Natl Acad Sci U S A,* 99**,** 15572-7.

ROLLINSON, D. & JOHNSTON, D. 1996. Schistosomiasis: A persistent parasitic disease. *Interdisciplinary Science Reviews,* 21**,** 140-154.

ROSE, W. A., 2ND, MCGOWIN, C. L., SPAGNUOLO, R. A., EAVES-PYLES, T. D., POPOV, V. L. & PYLES, R. B. 2012. Commensal bacteria modulate innate immune responses of vaginal epithelial cell multilayer cultures. *PLoS One,* 7**,** e32728.

ROSS, A. G., BARTLEY, P. B., SLEIGH, A. C., OLDS, G. R., LI, Y., WILLIAMS, G. M. & MCMANUS, D. P. 2002. Schistosomiasis. *N Engl J Med,* 346**,** 1212-20.

RUJENI, N., NAUSCH, N., MIDZI, N., MDULUZA, T., TAYLOR, D. W. & MUTAPI, F. 2012. Schistosoma haematobium infection levels determine the effect of praziquantel treatment on anti-schistosome and anti-mite antibodies. *Parasite Immunol,* 34**,** 330-40.

SAATHOFF, E., OLSEN, A., MAGNUSSEN, P., KVALSVIG, J. D., BECKER, W. & APPLETON, C. C. 2004. Patterns of Schistosoma haematobium infection, impact of praziquantel treatment and re-infection after treatment in a cohort of schoolchildren from rural KwaZulu-Natal/South Africa. *BMC Infect Dis,* 4**,** 40.

SANTIAGO HDA, C., RIBEIRO-GOMES, F. L., BENNURU, S. & NUTMAN, T. B. 2015. Helminth infection alters IgE responses to allergens structurally related to parasite proteins. *J Immunol,* 194**,** 93-100.

SAVINA, A. & AMIGORENA, S. 2007. Phagocytosis and antigen presentation in dendritic cells. *Immunol Rev,* 219**,** 143-56.

SCHABERG, T., RAHN, W., RACZ, P. & LODE, H. 1991. Pulmonary schistosomiasis resembling acute pulmonary tuberculosis. *Eur Respir J,* 4**,** 1023-6.

SECOR, W. E., SHAH, A., MWINZI, P. M., NDENGA, B. A., WATTA, C. O. & KARANJA, D. M. 2003. Increased density of human immunodeficiency virus type 1 coreceptors CCR5 and CXCR4 on the surfaces of CD4(+) T cells and monocytes of patients with Schistosoma mansoni infection. *Infect Immun,* 71**,** 6668-71.

SHATTOCK, R. J. & MOORE, J. P. 2003. Inhibiting sexual transmission of HIV-1 infection. *Nat Rev Microbiol,* 1**,** 25-34.

SHISANA & REHLE 2014. South African National HIV Prevalence, Incedence and Behaviour Survey. *HSRC Press*.

SILVA, I. M., THIENGO, R., CONCEICAO, M. J., REY, L., LENZI, H. L., PEREIRA FILHO, E. & RIBEIRO, P. C. 2005. Therapeutic failure of praziquantel in the treatment of Schistosoma haematobium infection in Brazilians returning from Africa. *Mem Inst Oswaldo Cruz,* 100**,** 445-9.

SILVEIRA-LEMOS, D., FERNANDES COSTA-SILVA, M., CARDOSO DE OLIVEIRA SILVEIRA, A., AZEVEDO BATISTA, M., ALVES OLIVEIRA-FRAGA, L., SOARES SILVEIRA, A. M., BARBOSA ALVAREZ, M. C., MARTINS-FILHO, O. A., GAZZINELLI, G., CORREA-OLIVEIRA, R. & TEIXEIRA-CARVALHO, A. 2013. Cytokine Pattern of T Lymphocytes in Acute Schistosomiasis mansoni Patients following Treated Praziquantel Therapy. *J Parasitol Res,* 2013**,** 909134.

SMITH, K. A., HARCUS, Y., GARBI, N., HAMMERLING, G. J., MACDONALD, A. S. & MAIZELS, R. M. 2012. Type 2 innate immunity in helminth infection is induced redundantly and acts autonomously following CD11c(+) cell depletion. *Infect Immun,* 80**,** 3481-9.

SPURBECK, R. R. & ARVIDSON, C. G. 2011. Lactobacilli at the front line of defense against vaginally acquired infections. *Future Microbiol,* 6**,** 567-82.

STRICKLAND, G. T. 1994. Gastrointestinal manifestations of schistosomiasis. *Gut,* 35**,** 1334-7.

SURESH, P. & WANCHU, A. 2006. Chemokines and chemokine receptors in HIV infection: role in pathogenesis and therapeutics. *J Postgrad Med,* 52**,** 210-7.

SWAI, B., POGGENSEE, G., MTWEVE, S. & KRANTZ, I. 2006. Female genital schistosomiasis as an evidence of a neglected cause for reproductive ill-health: a retrospective histopathological study from Tanzania. *BMC Infect Dis,* 6**,** 134.

TOELLNER, K. M., LUTHER, S. A., SZE, D. M., CHOY, R. K., TAYLOR, D. R., MACLENNAN, I. C. & ACHA-ORBEA, H. 1998. T helper 1 (Th1) and Th2 characteristics start to develop during T cell priming and are associated with an immediate ability to induce immunoglobulin class switching. *J Exp Med,* 187**,** 1193-204.

TURNER, J. D., MEURS, L., DOOL, P., BOURKE, C. D., MBOW, M., DIEYE, T. N., MBOUP, S., POLMAN, K. & MOUNTFORD, A. P. 2013. Schistosome infection is associated with enhanced whole-blood IL-10 secretion in response to cercarial excretory/secretory products. *Parasite Immunol,* 35**,** 147-56.

TURNER, M. D., NEDJAI, B., HURST, T. & PENNINGTON, D. J. 2014. Cytokines and chemokines: At the crossroads of cell signalling and inflammatory disease. *Biochim Biophys Acta,* 1843**,** 2563-2582.

VALORE, E. V., PARK, C. H., IGRETI, S. L. & GANZ, T. 2002. Antimicrobial components of vaginal fluid. *Am J Obstet Gynecol,* 187**,** 561-8.

VANDER HEIDEN, M. G., PLAS, D. R., RATHMELL, J. C., FOX, C. J., HARRIS, M. H. & THOMPSON, C. B. 2001. Growth factors can influence cell growth and survival through effects on glucose metabolism. *Mol Cell Biol,* 21**,** 5899-912.

VENDRAME, C. M., CARVALHO, M. D., YAMAMOTO, C. R., NAKHLE, M. C., CARVALHO, S. A. & CHIEFFI, P. P. 2001. Evaluation of anti-Schistosoma mansoni IgG antibodies in patients with chronic schistosomiasis mansoni before and after specific treatment. *Rev Inst Med Trop Sao Paulo,* 43**,** 153-9.

VERBRUGGE, L. M., RAINEY, J. J., REIMINK, R. L. & BLANKESPOOR, H. D. 2004. Swimmer's itch: incidence and risk factors. *Am J Public Health,* 94**,** 738-41.

WEYAND, C. M. & GORONZY, J. J. 1992. HIV infection and rheumatic diseases--autoimmune mechanisms in immunodeficient hosts. *Z Rheumatol,* 51**,** 55-64.

WHO 2012. Schistosomiasis: Atlas of the global distribution of schistosomiasis.

WHO 2015. Schistosomiasis. *World Health Organization, Fact Sheet,* 115.

WILLEY, J. M., SHERWOOD, L. & WOOLVERTON, C. J. 2008. *Prescott, Harley, and Klein's Microbiology,* McGraw-Hill Higher Education.

WITKIN, S. S., LINHARES, I., GIRALDO, P., JEREMIAS, J. & LEDGER, W. J. 2000. Individual immunity and susceptibility to female genital tract infection. *Am J Obstet Gynecol,* 183**,** 252-6.

WYNN, T. A. 2015. Type 2 cytokines: mechanisms and therapeutic strategies. *Nat Rev Immunol,* 15**,** 271-82.

XU, L. L., MCVICAR, D. W., BEN-BARUCH, A., KUHNS, D. B., JOHNSTON, J., OPPENHEIM, J. J. & WANG, J. M. 1995. Monocyte chemotactic protein-3 (MCP3) interacts with multiple leukocyte receptors: binding and signaling of MCP3 through shared as well as unique receptors on monocytes and neutrophils. *Eur J Immunol,* 25**,** 2612-7.

ZANDMAN-GODDARD, G. & SHOENFELD, Y. 2002. HIV and autoimmunity. *Autoimmun Rev,* 1**,** 329-37.

ZAVALA, F., RIMANIOL, A. C., BOUSSIN, F., DORMONT, D., BACH, J. F. & DESCAMPS-LATSCHA, B. 1995. HIV predominantly induces IL-1 receptor antagonist over IL-1 synthesis in human primary monocytes. *J Immunol,* 155**,** 2784-93.

Chapter 8: Appendix

Annexure 1: Supplementary information

Expedited ethics approval

UNIVERSITY OF
KWAZULU-NATAL
INYUVESI
YAKWAZULU-NATALI

RESEARCH OFFICE
BIOMEDICAL RESEARCH ETHICS ADMINISTRATION
Westville Campus
Govan Mbeki Building
Private Bag X 54001
Durban
4000
KwaZulu-Natal, SOUTH AFRICA
Tel: 27 31 2604769 - Fax: 27 31 260-4609
Email: BREC@ukzn.ac.za
Website: http://research.ukzn.ac.za/ResearchEthics/BiomedicalResearchEthics.aspx

14 March 2016

Mr Sahil Tulsi
2nd Floor DDMRI Building
719 Umbilo Rd
Nelson R Mandela School of Medicine
Moodleyd33@ukzn.ac.za

Dear Mr Tulsi

PROTOCOL: Role of Local or Systemic Schistosoma Infections in Driving Inflammation and HIV risk in women enrolled in the CAPRISA 004 cohort: BE333/13

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved: 14 October 2015
Expiration of Ethical Approval: 13 October 2016

I wish to advise you that your application for Recertification dated 15 February 2016 in relation to the above protocol has been noted and approved by a sub-committee of the Biomedical Research Ethics Committee (BREC) for another approval period. The start and end dates of this period are indicated above.

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

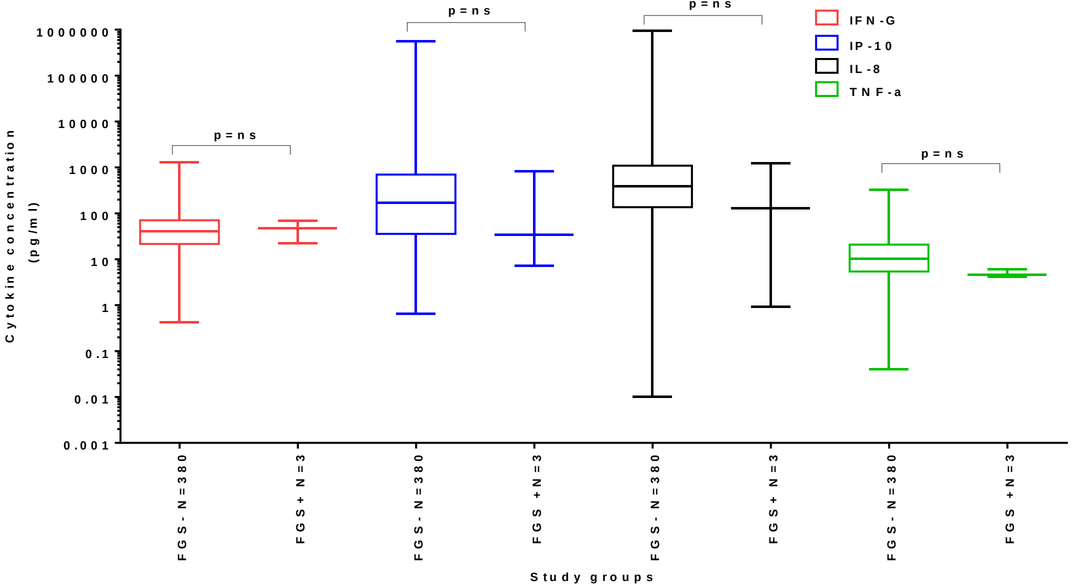
The approval will be ratified by a full Committee at a meeting to be held on **12 April 2016**.

Yours sincerely

Ms A Marimuthu
Senior Administrator: Biomedical Research Ethics

Annexure 2: Supplementary figures & information

8.1



8.2

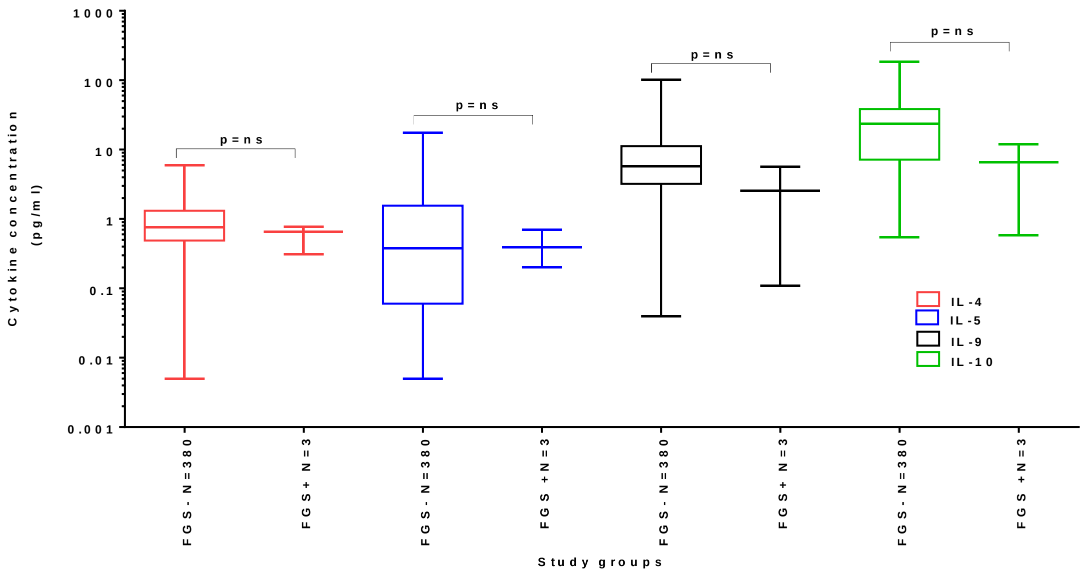


Figure 8.1-8.2: Comparison of Th1 (8.1) and Th2 (8.2) cytokine concentrationss in FGS- women were compared to FGS+ women. Mann Whitney test was performed to test for differences between the FGS- and FGS+ groups.

Table 8.1: Conventional FGS PCR reaction volumes

Master mix reagents	**Master Mix for 1X**	**Master Mix for 12X**
H_2O	17.4µl	208.8µl
Ssp48F (5pmol/µl)	0.25µl	3µl
Ssp124R (5pmol/µl)	0.25µl	3µl
10X PCR Buffer	2.5µl	30µl
Platinum *Taq*	0.1µl	1.2µl
dNTP (10mM)	0.5µl	6µl
$MgCl_2$ (50mM)	1µl	12µl
DNA template	3µl	
Total volume	25µl	

Table 8.2: Cycling conditions of conventional FGS PCR

Temperature (^oC)	**Time**	**# cycles**
94	5 min	
95	30 sec	55
60	30 sec	
72	30 sec	
72	5min	
4	∞	

ELISA solutions and buffers:

ELISA Buffers : Carbonate Coating Buffer

1.6g Na_2CO_3 (15mM)

2.9g $NaHCO_3$ (35mM)

4.2g NaCl (71mM) Make up to 1L with ddH_2O; adjust to pH 9.5 with 1M Citric Acid Filter sterilize and store at 4˚C

Blocking Buffer – 2%

20g Elite Fat Free Powdered milk

1L 1X PBS

Dissolve powder milk in 1L 1X PBS. Store at 4°C.

Dilution Buffer 1% BSA PBS

10g Bovine Serum Albumin (BSA)

1L 1X PBS

Dissolve BSA in 1L 1X PBS. Store at 4°C.

Substrate Buffer (for Alkaline Phosphatase)

0.2g NaN3

97ml Diethanolamine

0.8g MgCl2·6H2O Dissolve all dry compounds in 900ml ddH2O and add the liquified diethanolamine; adjust pH. Add ddH_2O to 1L. Store at 4°C.

20X Wash Buffer

20g KCl

20g $KH_2HPO_4\cdot 2H_2O$

880g NaCl

50ml Tween-20

100ml 10% NaN_3

Dry compounds dissolved in 4500ml ddH_2O, and then added Tween-20 and 10%NaN_3.

Add ddH_2O to 5L.