SUSCEPTIBILITY AND DIAGNOSTIC PROTEOMIC BIOMARKERS FOR URINARY SCHISTOSOMIASIS AND ASSOCIATED BLADDER PATHOLOGIES AMONG ADULTS IN EGGUA, OGUN STATE, NIGERIA

BY

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ABSTRACT

The failure to elicit an adequate immune response to the adult *Schistosoma haematobium* worm, and continuous strong inflammatory responses to the eggs have been the main causes of bladder pathology in chronic schistosomiasis. The identification of bladder pathology-associated biomarkers is necessary to enable early detection of the disease in a non-invasive manner. The aim of this study was to identify candidate-biomarkers for susceptibility and diagnosis of schistosomiasis and schistosomiasis-associated bladder pathologies in adults.

A total of 371 respondents, comprising 130 males and 241 females from Eggua, Ogun State were randomly recruited into a cross sectional study from August 2012 to May 2014. Semi-structured pretested questionnaires were administered to obtain information from consenting respondent. They were screened for *S. haematobium* ova and bladder pathologies by microscopy and ultrasonography, respectively. Host susceptibility to bladder pathologies and schistosomiasis was determined by Polymerase Chain Reaction genotyping of glutathione-S-transferase (*GSTT1* and *GSTM1*) genes, and Interleukin (*IL4* and *IL13*) genes, respectively. Label-free quantification mass spectrometry-based proteomics approach was used to identify protein biomarkers in the urine. Samples were categorised as Schistosomiasis, Bladder Pathology (BP), Pathology and Schistosomiasis (PS). No Pathology and Schistosomiasis (NPS) served as controls. Descriptive statistics, odds ratios (OR) and Chi-square test were used at $\alpha_{0.05}$ to determine association between schistosomiasis and bladder pathologies. False Discovery Rate (FDR) analysis was also used to determine significant biomarkers.

The mean age of respondents was 48.6 ±0.6 years. The prevalence of schistosomiasis was in 42 (11.4 %) males and 66 (17.9 %) females. Majority (74.1%) had light mean intensity of infection (33.3±0.04 eggs/10mL urine). Bladder pathologies included abnormal bladder wall thickness (29.0%), abnormal bladder shape (7.1%), bladder masses (3.1%) and bladder calcification (2.2%). There was a significant association between urinary schistosomiasis and BP. Respondents with *GSTM1* and *GSTT1* polymorphisms expressed elevated risks of BP (OR = 4.3, 95% CI 2.0 - 9.2 and OR =

4.2, 95% CI 1.5 – 12.0, respectively); with the PS having more GST polymorphisms than BP. Polymorphisms in *IL 4-590* and *IL 13-1055* were observed in 24.1% and 9.3% schistosomiasis cases, respectively. The *IL 13-1055* polymorphisms did not indicate susceptibility to schistosomiasis in males (OR 0.7, 95% CI 0.3-2.1) but a slight risk was found in females (OR 1.1, 95% CI 0.7-1.7). A total of 1306 proteins and 8752 unique peptides were observed (FDR = 0.01). Human host (54) and parasite-derived (36) potential biomarkers were found for schistosomiasis and associated pathologies. These included new potential biomarkers in schistosomiasis (Sialidase-1, Growth factor 15, Programmed cell death 1 ligand-2) and PS (Arylsulfatase A and Phosphatidylethanolamine-binding protein 4).

Candidate proteins were identified for the generation of new diagnostic markers for chronic urinary schistosomiasis and its bladder pathologies.

Keywords: *Schistosoma haematobium*, Bladder pathology, Glutathione-S-transferase Polymorphism, Growth factor 15, Arylsulfatase A

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CERTIFICATION

I certify that this work was carried out by Olugbenga Samson ONILE in the Cell Biology and Genetics Unit of Department of Zoology, University of Ibadan, Ibadan, Nigeria.

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DEDICATION

This work is dedicated to:

My family whose support has been overwhelming

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CHAPTER ONE

INTRODUCTION

1.1 Background

Human urinary schistosomiasis (bilharziasis) due to *Schistosoma haematobium* is widespread, ranking second to malaria in terms of socio-economic and public health significance in tropical and sub-tropical areas (Yunusa *et al.*, 2016), with an estimated 732 million persons being vulnerable to infection worldwide in well-known transmission areas (Adenowo *et al.*, 2015). It is the most prevalent of the water-borne diseases, with a great risk on the health of rural populations (Biu *et al.*, 2000). In 2014, more than 61.6 million people were treated globally for schistosomiasis, 67% of those from sub-Saharan Africa only (WHO, 2014; WHO, 2016). An estimated 200,000 deaths per year was recorded due to the infection (USAID 2016, Yunusa *et al.*, 2016). Approximately 120 million individuals in sub-Saharan Africa have schistosomiasis-related symptoms while about 20 million undergo hardship as a result of chronic presentations of the disease (Chitsolu *et al.*, 2000; Adenowo *et al.*, 2015).

Schistosomiasis is endemic in Nigeria (Agbolade and Odaibo, 1996; Nmorsi *et al.*, 2007; Agere *et al.*, 2010; Adenowo *et al.*, 2015; Yunusa *et al.*, 2016). Investigation of *Schistosoma haematobium* infection in Edo State, Nigeria revealed a prevalence of 31.2%, with children having a higher prevalence (41.1%) compared to adults (20.0%) (Nmorsi *et al.*, 2007). *Schistosoma haematobium* infection is reported to be more widespread than *Schistosoma mansoni* infection (Agbolade and Odaibo, 1996). Several investigations have linked the disease to the personal habits and livelihood of individuals who frequently visit fresh water habitats (Agere *et al.*, 2010; Adenowo *et al.*, 2015). Parasitic infections like schistosomiasis are regular and lasting health problems in developing countries that are characterized by continuing health threat and inflammatory challenges to the populations who are exposed to long-term daily

risk of infection (Hotez *et al*., 2008). *Schistosoma haematobium* is a long-lived parasite that is easily acquired, and a direct aetiological link between the parasite and cancer has been suggested (Fritsche *et al.*, 2006; Botelho *et al.,* 2010; European Association of Urology, 2016. Of approximately 110 million cases due to *Schistosoma haematobium* in sub-Saharan Africa, 70 million are associated with hematuria, 18 million with major bladder wall pathology, and 10 million with hydronephrosis leading to kidney damage (Rinaldi *et al*., 2014).

Genetic studies of human susceptibility to *Schistosoma* (blood fluke) infections have previously identified a genetic locus determining infection intensity with *Schistosoma mansoni* in the chromosome 5q31–33 region of the human genome that is known to contain the Th2 immune response cluster, including the genes encoding the IL-4, IL-5, and IL-13 cytokines (Ellis *et al*., 2007). According to Ellis *et al.*, (2007), these cytokines are key players in the inflammatory immune responses and have previously been implicated in human susceptibility to infection with the Asian species, *S. japonicum.* Many immunological studies have demonstrated the role of these cytokines in the immunomodulation of several helminths, including schistosome infections in murine models (Finkelman *et al.*, 1997; Gause *et al*., 2003) and in humans (Jackson *et al*., 2004a; Quinnell *et al*., 2004; Turner *et al*., 2003). Other studies have shown a marked increase in the levels of IL-5 and IL-13 in individuals identified as being resistant to schistosome infection (Al-Sherbiny *et al.*, 2003; Leenstra *et al.*, 2006). Furthermore, two polymorphisms [21055C (rs 1800925) and 2591A (rs 2069743)] in the IL-13 region gene promoter have been shown to be associated with susceptibility to *S. haematobium* infections (Kouriba *et al.*, 2005).

Chronic infection with *Schistosoma haematobium* has been reported as a possible risk factor in the aetiology of bladder cancer (Fritsche *et al.,* 2006; European Association of Urology, 2016). Nmorsi *et al*., (2007) also recorded higher urinary tract pathology conditions with heavy intensity of *S. haematobium* infection. Histopathological study has also associated *S. haematobium* infection with the development of squamous cell carcinoma of the bladder (Mostafa *et al*., 1999). *S. haematobium* is linked with a two to tenfold increase in the risk of bladder squamous cell carcinoma (European Association of Urology, 2016), and is a potential cause of kidney damage hence the

parasite is considered as a group 1 carcinogen (Driguez *et al*., 2016). In fact, in some of the regions where *S. haematobium* is endemic, bladder cancer is the most common cancer in men and the second in women, behind breast cancer; accounting for as much as 30% of all cancer cases (Betelho *et al.*, 2010). Rinaldi *et al.*, (2014), found that a clinical history of urogenital schistosomiasis accounted for 16% of bladder cancer cases in Egypt. One major study in Kenya sought to examine the parasite-disease association by cytopathological studies in a large community in Coastal Province (Hodder *et al*., 2000). Using examination and evaluation of Papanicolaou (Pap)-stained urine sediment cytology slides, the authors were able to show an association between urinary tract hyperplasia and infection with *S. haematobium* (Hodder *et al*., 2000).

Bladder cancer is the most common malignancy of the urinary system in the US (American Cancer Society, 2016). It is the 7th most commonly diagnosed cancer in males worldwide, with a drop to 11th when both genders where considered (European Urology Association, 2016). It is one of the tumours associated with the highest morbidity and mortality. In the U.S.A. estimated new cancer cases and estimated deaths from it in 2016 are 76,960 and 16,390 respectively (American Cancer Society, 2016).

A retrospective review of clinical records of bladder cancer cases in Sokoto, Nigeria between 1999 and 2004 showed a 4.7 fold rise in the number of bladder cancer cases, with squamous cell carcinoma (SCC); composed of 65.1% histologically verified cases and 50% of the SCC showed evidence of chronic urinary schistosomiasis (Mungadi and Malami, 2007). It is a serious global problem, accounting for some 30% of all cancers around the world and almost twice as many cases of bladder cancer occur in men as in women, cigarette smoking being its leading cause (Vineis *et al.*, 1998). It is the second most common urological cancer, clinically characterized by high recurrent rates and poor prognosis once tumors invade the lamina propia (Kausch and Böhle, 2001). Bladder cancer is one of the most expensive malignancies in the Western world (Smith and Guzzo, 2013; Ghafouri-Fard *et al*., 2014). It was estimated in the US in 2001 that the cost of bladder cancer from diagnosis to death was between $96,000 and $187,000 per patient (Botteman *et al.*, 2003). According to Fritsche *et al.*,

(2006), the most common symptom of bladder cancer is intermittent hematuria (80-85% of patients), while other urinary tract symptoms include increased frequency, urgency and dysuria in about 15-20% of patients (Fritsche *et al*., 2006).

Decreases in activity of carcinogen-metabolizing enzyme glutathione-S-transferase (GST) in human bladder cancer tissues have been associated with *S. haematobium* infection (Sheweita *et al.*, 2004). Somali *et al.*, (2003) and Yajie *et al.,* (2016) found that the deficiencies in the *GSTT1* gene confers an increased risk of bladder cancer. Polymorphisms in metabolic enzymes that are involved with *in vivo* detoxification of carcinogens have also been associated with risk of various cancers (Brockmoller *et al.*, 2000). Glutathione S-transferases M1 and T1, involved in the detoxification of polycyclic aromatic hydrocarbons and N-acetyl transferase-2 involved in acetylation of arylamines, are of interest in bladder cancer (Somali *et al.*, 2003; Ying *et al.*, 2016). Analyses have shown that *GSTM1* deficiency and slow acetylation are major determinants of bladder cancer susceptibility (Johns *et al*., 2000a, Johns *et al*., 2000b). The inhibition of GST activity may enhance the effect of many environmental carcinogens such as N-nitrosamines, thereby reducing the capacity of detoxifying many endogenous compounds in the bladder (Johns and Houlston, 2000; Djukic *et al*., 2013 and Yajie *et al.*, 2016).

Cystoscopy and cytology are currently considered the 'gold standards' for the identification and monitoring for recurrence or progression of bladder cancer (Ghafouri-Fard *et al*., 2014). Frequent cystoscopies facilitate the treatment of recurrences at an early stage, thereby potentially slowing the progression of the disease to muscle invasive disease. However, cystoscopy is an invasive, time-consuming and expensive examination that is not well-accepted by patients (Karakiewicz *et al*., 2006; Goodison *et al*., 2013).

Scientists are therefore interested in identifying reliable non-invasive biomarkers that could be utilized in screening, leading to early detection and/or in predicting the progression of superficial tumours to invasive higher-stage lesions with high specificity and sensitivity. A biomarker can be a protein, a fragment of a protein, DNA or RNA. Biomarkers, specifically cancer biomarkers, are bioindicators of the disease

and by detecting them the existence of that specific cancer can be verified (Young-Eun *et al.*, 2010**)**. Advances in omic profiling technologies using biological mixtures such as blood and urine allow the systemic analysis and characterization of alterations in genes, RNA, proteins and metabolites, and offer the possibility of discovering novel biomarkers and pathways activated in disease or associated with disease conditions (Baumgartner *et al.*, 2011, Chien-Lun *et al.*, 2013).

1.2 Statement of the problem

For people living in *S. haematobium*-endemic areas, early disease detection would be of considerable benefit because Schistosoma bladder cancer appears in the prime of life (age 35-50 years). In fact, bladder cancer is likely to be unrecognized, as the overt urinary tract symptoms (intermittent haematuria, dysuria, increased frequency, urgency and pain with micturition) are so commonly associated with urinary schistosomiasis that when the cancer is manifested the patient is not likely to receive adequate diagnosis and may become severely debilitated, with poor disease prognosis. Detection of bladder cancer at the population level is difficult because direct proof requires detailed histopathological study; but invasive examinations are confined to advanced hospitals (Shiff *et al.*, 2006). The detection of tumourigenic biomarkers preferably isolated from urine thus becomes important. Such biomarkers are now an active area of research and development and will provide tools that could be useful to evaluate specific effects of long-term exposure to *S. haematobium* (Konety *et al.*, 2000, Shiff *et al.*, 2006).

Demonstration of schistosome-associated bladder damage by ultrasound examination is valuable and useful; however, it cannot be used to construe a diagnosis of cancer. It is at this point that cancer-specific urine and blood biomarkers may play an important role in focusing on the extent of the problem in people with long-term infections.

1.3 Justification of the study

Considering that treatment of schistosomiasis relies on a single drug, praziquantel, which raises fears of development of resistance, there is a need for acquiring a deeper understanding of the communication between the parasite and the mammalian host with a view to identifying new methods of controlling schistosomiasis,

schistosomiasis-associated bladder cancer and developing potential diagnostic markers and drugs. One potential approach to investigating the developing relationship between the parasite and its host is proteomics and biological fluids are promising targets of choice when looking for diagnostic, prognostic and treatment based biomarkers, due to their easy accessibility. This is owing to the fact that biological fluids-associated tissues are prone to release protein components in the fluids; and the disease-altered state could change either the spectrum or the amount of released proteins. Such proteins can be diseased associated biomarkers and proteomic analysis of the body fluids should reveal a lot of new diagnostic markers.

Candidate biomarkers can be identified from these models, taking into consideration intra-group variations, sample preparation methods, and spectral data acquisition. Proteomics has been used for human studies of disease and it has been revealed to be a valuable approach for distinguishing disease and generating candidate biomarkers of pathological state (Theodorescu *et al.*, 2006). Changes in protein level may be detectable in biofluids before the appearance of clinical symptoms, making them potentially useful early detection biomarkers (He and Chiu, 2003; Cao et al., 2012). Mass spectrometry analysis of a small number of highly exposed and unexposed subjects has been found to reveal altered expression of several proteins that may be identified as intermediate biomarkers of early effect (Moore *et al.*, 2006). Also, the potential of the urinary proteome as a non-invasive means to identifying biomarkers of carcinogen exposure and metabolism to toxic chemicals have been demonstrated by Moore *et al.*, (2006). Several schistosome oriented proteomics studies have focused on the parasites (Sotillo *et al*., 2015; Driguez *et al*., 2016), with little information on the changes that emanate in host proteins during active schistosomiasis (Shiff *et al.*, 2006), hence this study will bridge this gap.

1.4 Aim of the Study

The aim of the present study therefore is to identify biomarkers for the susceptibility and diagnosis of schistosomiasis and schistosomiasis-associated bladder cancer from adults in rural population in South-west Nigeria where *S. haematobium* is prevalent.

1.5 Specific Objectives

i. To determine the prevalence of schistosomiasis and schistosomiasis-associated bladder pathology in Eggua.

ii. To determine the relationship between *S. haematobium* infection and structural bladder pathology

iii. To determine glutathione-S-transferase (*GSTT1* and *GSTM1*) gene polymorphisms among study volunteers with bladder pathology.

iv. To determine Interleukin (*IL-4* and *IL-13*) gene polymorphisms among volunteers with schistosomiasis.

v. To identify biomarkers for schistosomiasis and schistosomiasis-associated bladder pathologies in Nigeria.

CHAPTER TWO

2 LITERATURE REVIEW

2.1 Biology of the Schistosome

Schistosomiasis is a disease caused by infection with schistosome parasites (Ayanda and Opeyemi, 2009). The parasites are blood-dwelling fluke worms (i.e., they are intravascular obligates that reside in the mesenteric veins and affect many organs including the central nervous system (Annapurna and Uday, 2013). They belong to the Kingdom: Animalia, Phylum: Plathyhelminthes, Class: Trematoda, Order: Digenea, Family: Schistosomatidae, Genus: Schistosoma, and have six species that commonly infect humans, namely *Schistosoma haematobium*, *S. mansoni*, *S. japonicum*, *S. intercalatum*, *S. guineensis* and *S. mekongi* (Webster *et al.*, 2006). They differ in their final location in the human host, intermediate (snail) host in their life cycle, number, shape, size of the eggs produced and the pathology they induce (WHO, 2016). Schistosomes feed on blood particles through anaerobic glycolysis (Rumnajek, 1987; Sanaa and Manal, 2014). There is an alternation of generations among all sexual Digeneans, as the asexual reproduction occurs in the intermediate host and sexual reproduction occurs in the definitive (mammalian) host (IARC, 2011).

Of the family Schistosomatidae, 13 members are unusual in four ways when comparing with the ~2700 Digenean genera: they do have two hosts rather than three; they are dioecious (with male and female reproductive organs in separate individuals); they infect their hosts by directly penetrating the body surface, but not through oral consumption; and they parasitize the intravascular niche (Cribb *et al.*, 2001, Annapurna and Uday, 2013). The body of the male schistosomes forms a gynaecophoric channel (groove), in which it holds the longer and thinner female. As permanently embraced couples, the schistosomes live within the perivesical (*S. haematobium*) or mesenteric (other species) venous plexus. The adult worms are about 1–2 cm long (Table 1), having a cylindrical body that features two terminal suckers, a

complex tegument, a blind digestive tract, and reproductive organs. The parasite lives for long periods, with no evidence of immune-mediated clearance of adult worms, and it has evolved to use host factors for developmental signaling (Agnew *et al.*, 1993)

Table 1: Physical features of *Schistosoma* spp at different stages in the life cycle in man

***Schistosoma* spp**	***S. mansoni***	***S. japonicum***	***S. haematobium***
Adult Male			
Length (mm)	6-12	12 – 20	10 – 14
Breath (mm)	2.00	0.50 – 0.55	0.75 – 1.00
No of testes	4 – 13	6 – 9	4 – 5
Adult Female			
Length (mm)	7 – 17	16 – 28	16 – 20
Breath (mm)	1.00	0.30	0.25
No of eggs in uterus	Usually 1	50 or more	10 – 100
Schistosomulum (All species – length x breath)			
Size during penetration (mm) – approximately 0.10-0.12 x 0.030			
Size in the lung (mm)- approximately 0.12-0.18 x 0.029 – 0.037			
Size on arrival in the liver (mm)- approximately 0.16 – 0.20 x 0.023 -0.040			

Source: Annapurna and Uday, 2013

2.2 Life cycle of *Schistosoma haematobium*

As part of the life cycle of *S. haematobium* (Figure 1) is the female worm which produces hundreds of eggs per day throughout her life (Yassir *et al*., 2017). The eggs (144x58 μm, with a characteristic terminal spine) penetrate through the bladder wall where they are excreted with urine. Each ovum contains a ciliated larva (miracidium), which secretes proteolytic enzymes that help the eggs migrate into the lumen of the bladder. About half of the eggs produced do not reach the vesical lumen, and are carried away with the bloodstream, and/or trapped in the tissues.

A granulomatous inflammatory response, which is the main cause of pathology in the human host, is provoked by the retained eggs (Wilson *et al.*, 2007). The viable excreted eggs hatch if they come into contact with water, and release the relatively short-lived miracidium that emerges to infect a suitable intermediate molluscan host. The miracidium are able to locate a suitable freshwater snail host (i.e. *Bulinus* spp. for *S. haematobium*) within 48 hours of their viability and this is achievable with the use of external stimuli such as light and snail-derived chemicals (Yassir *et al*., 2017). Asexual reproduction takes place in the snail, where several generations of multiplying larvae (sporocysts) are developed. These sporocysts ultimately produce large numbers of infective larvae with a typical bifurcated tail (free-swimming cercariae). These cercariae leave the snail at a rate of thousands per day after a period of weeks.

Shedding of cercariae can continue for months; one snail infected by one miracidium can shed thousands of cercariae every day for months (Gryseels *et al*., 2006). The cercariae survive for up to 72 hours and use water turbulence and skin-derived chemicals to locate the human host. They attach to and penetrate the human skin within 3–5 minutes. On finding a host, the cercariae penetrate the skin of human and other mammalian host that act as reservoirs for infection. Percutaneous penetration of the cercariae can provoke a temporary urticarial rash that can manifest within hours and persist for days as maculopapular lesions. In temperate zones, a similar “swimmers itch” is also frequently seen with avian trematode cercariae (Bouree and Caumes, 2004). The presentation of delayed-onset dermatitis, manifest as urticarial or angioedema, which can occur within 48 hours and the skin lesions are often pruritic.

Because of the temporary association with water exposure, the diagnosis is usually suspected clinically. The cercariae lose their bifurcated tail, and the young parasites (schistosomulae) migrate with the bloodstream via the lungs to the liver, where they mature into adult worms in the portal vein and mate. The paired worms migrate against the bloodstream to the perivesicular veins, where in 4–7 weeks after infection they start producing eggs throughout their adult life. The lifespan of an adult worm averages 3–5 years, but can be as long as 30 years with the reproduction potential of one schistosome pair estimated to be up to 600 billion schistosomes (Wilkins, 1987; Gryseels *et al.*, 2006). An infected person probably harbours an average of hundreds (range, 10s–1000s) of worms (Gryseels and De Vlas, 1996).

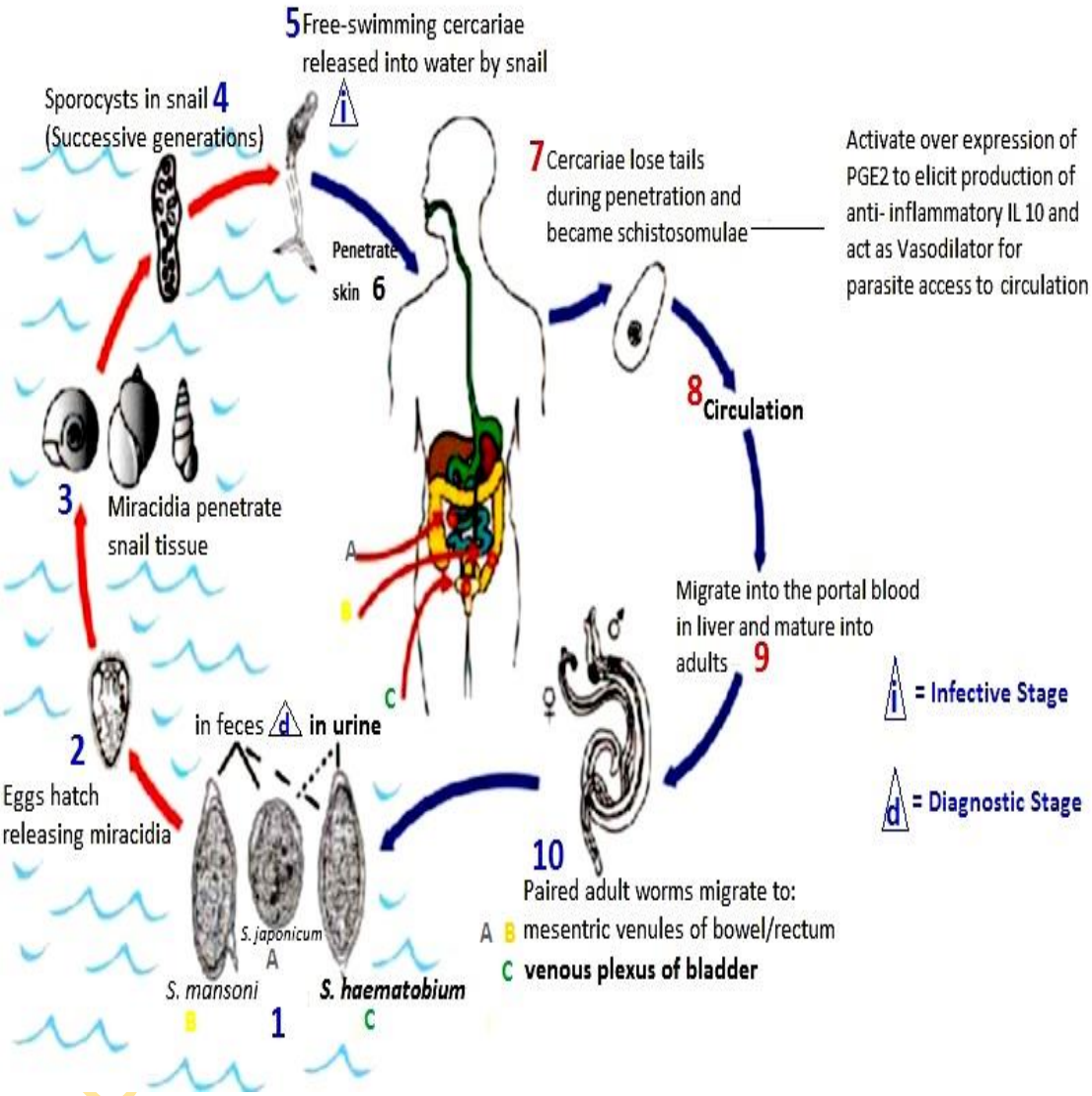

Figure 1: The life cycle of schistosomes showing intermediate and vector host

Source: (Modified from Sanaa and Manal, 2014)

2.3 Epidemiology of infection

2.3.1 Prevalence and geographic distribution

Human schistosomiasis is endemic in large areas of the (sub) tropics. Schistosomiasis currently affects some 76 countries and is found in 48 African countries (WHO, 1999; Ayanda and Opeyemi, 2009), while more than 1 billion people are at risk of infection, and not less than 700 million persons are globally estimated to be infected with the disease (Badawi, 1996; Chitsulo *et al*., 2000; Gibodat, 2000; IARC, 2011 and Yassir *et al.*, 2017), of which 120 million are symptomatic and 20 million have the severe disease (Ayanda and Opeyemi, 2009). Estimates suggest that about 93% of all schistosomiasis cases are in sub-Saharan Africa (Chitsulo *et al*., 2000; Ayanda and Opeyemi, 2009; IARC, 2011; Adenowo *et al.*, 2015). Nigeria is one of the most severely affected countries in Africa (Agbolade and Odaibo, 1996; Pukuma and Musa, 2007; Ayanda and Opeyemi, 2009; Agere *et al*., 2010; Adenowo *et al.*, 2015). It is estimated that 101.28 million people are at risk of infection while about 29 million are infected with *Schistosoma haematobium*, *Schistosoma mansoni* and *Schistosoma intercalatum* in Nigeria (Chitsulo *et al.*, 2000; Ayanda and Opeyemi, 2009; Adenowo *et al.*, 2015), closely followed by the United Republic of Tanzania (19 million), in prevalence of infection; Ghana, and the Democratic Republic of Congo (15 million) make up the top five countries in Africa with schistosomiasis (Figure 2.1).

However, underestimation of the true prevalence of schistosomiasis is suspected; it has been proposed that the prevalence of schistosomal-related diseases may be more than 400–600 million globally (Adenowo *et al.*, 2015). About 95% of the schistosomiasis cases are due to *S. mansoni* and *S. haematobium* infections. *Schistosoma. haematobium* is endemic in 53 countries, in the Middle East and most of the African continent (Chitsulo *et al.*, 2000). The disease is common in the Niger basin and is found in every country within the West African sub-region (Brown and Wright, 1985, Ayanda and Opeyemi, 2009). Largely, Schistosomiasis is found in rural areas (Yunusa *et al.*, 2016), although urban infection is on the increase in many countries (Mott *et al.*, 1990). For few decades, artificial reservoirs and irrigation systems, as well as population growth and migration, have contributed to the spread of infection in

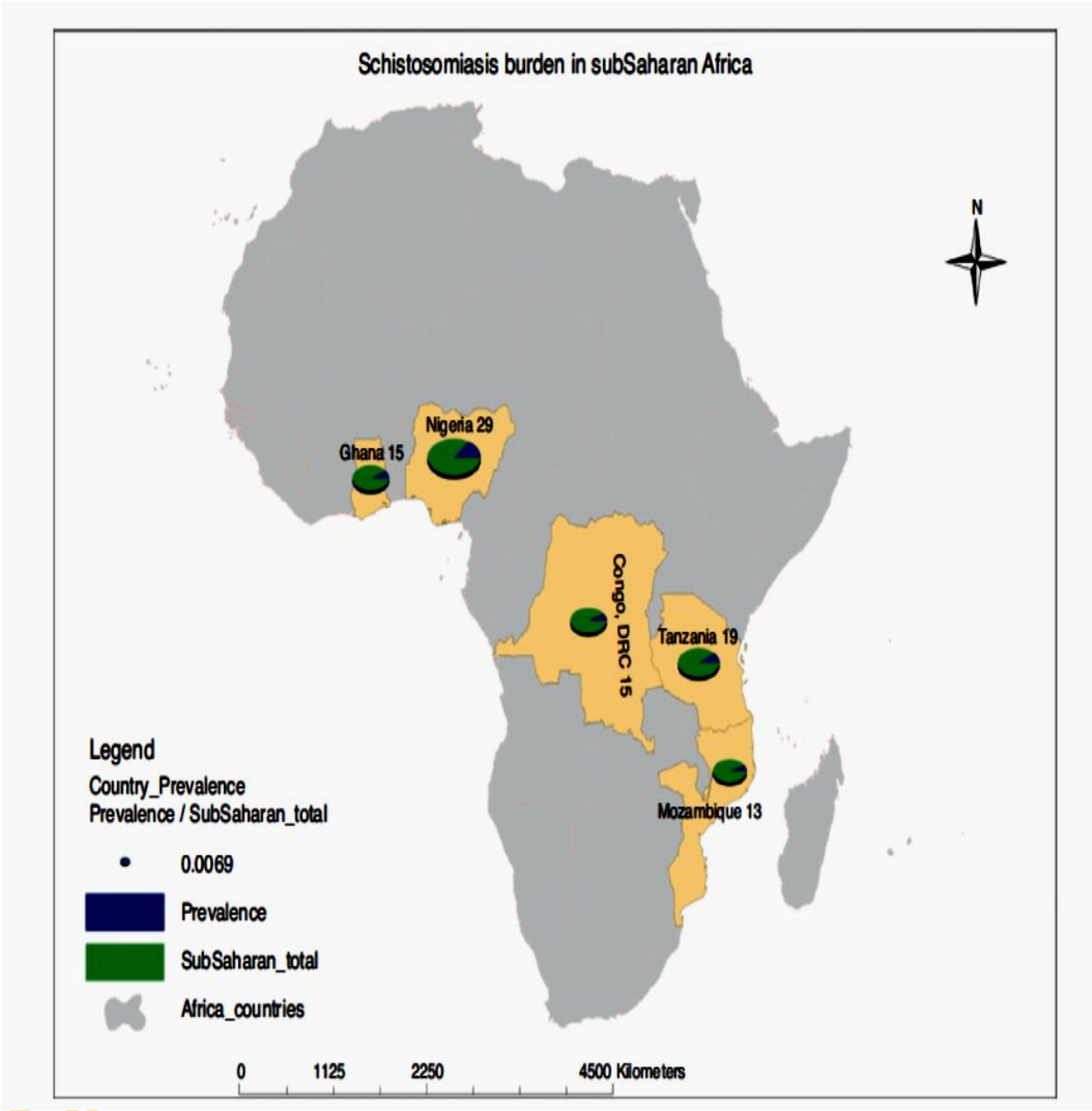

Figure 2.1: Estimated schistosomiasis burden in sub-Saharan African countries shown in yellow.

Source: Adenowo *et al.*, 2015.

addition to the known natural streams, ponds and lakes which are typical sources of infection (Gryseels *et al.*, 2006; McManus and Loukas, 2008).

The distribution of schistosomiasis can be very vital within countries, regions and villages, depending on variations in snail populations and human–water contact behaviour (Gryseels and Nkulikyinka, 1988; Brooker, 2007). Also, the distribution of schistosomiasis can be highly irregular among individuals. Prevalence and intensities of infection generally show a typical convex-shaped curve with a peak at the ages of 10–19 years (Ogbe, 1995; Egwuyenga *et al.*, 1994; Ejima and Odaibo, 2010), and a decrease in adults (Pukuma and Musa, 2007; Agere *et al*., 2010; IARC, 2011). Sex-related patterns of infection vary in relation to behavioural, professional, cultural, and religious factors (Jordan and Webbe, 1993). A meta-analysis of estimated disease burden showed that morbidity and mortality attributed to schistosomiasis has increased with DALYs (disability-adjusted life years), to about ~20% increase in the past 20 years (Murray *et al*., 2013; Driguez *et al*., 2016)

2.3.2 Transmission and risk factors for Schistosoma infection

Transmission of infection is well encouraged in less developed countries, mostly among poor and illiterate/less educated residents who cannot access proper health care or undertake infection preventive measures. Such endemic communities are used to indiscriminate urination and defecation in water frequently used for both domestic and agrarian purposes which include bathing, washing, farming and fishing. Contamination of fresh water with excreta containing schistosome eggs, presence of intermediate hosts and human contact with cercariae containing water are responsible for continual transmission of infection (Jordan & Webbe, 1993; Okoli and Iwuala, 2004; Annapurna and Uday 2013; Sanaa and Manal, 2014, WHO, 2016).

Transmission can take place in almost any type of aquatic habitat ranging from large lakes and rivers to small seasonal ponds and streams. Thus the main risk factor of infection is contact with contaminated water. Another host-related and environmental factors that may influence the risk of acquiring and/or influencing the distribution, prevalence, intensity of infection, morbidity and mortality of schistosomiasis are genetic factors (Quinnell, 2003), behaviour, household clustering (Bethony *et al.*,

2001), climate, immune response of the host, and concomitant infections like hepatitis (IARC, 2011).

Schistosome transmission is seasonal, primarily due to the variation in temperature (Liu *et al.*, 2013). The prevalence and intensity of infection are directly related to the patterns of variation with age. The main risk age groups are 10-20 years children which is accompanied by a decline in adults, specific occupational groups (fishermen, irrigation workers, farmers), and women and other groups using infected water for domestic purposes (WHO Expert Committee, 2002). Difference in the peak age-related prevalence of the disease is due to the gradual development of immunity and changes in the extent of water exposure (Barbosa, 2006). Adults who also migrate to endemic areas are as susceptible to infection as young children (Hotez *et al.*, 2007).

2.3.3 Latency of *S. haematobium* infection with respect to development of bladder cancer

Little is known about the latency between the onset of infection and the appearance of cancer, or about the steps that might lead to cancer.

Infection with *S. haematobium* is not synonymous with clinical disease, and many infections are asymptomatic. Of those infected, a small proportion develops serious chronic disease, after varying durations of exposure and infection (Homeida *et al.*, 1988; Vennervald and Dunne, 2004). Mostafa *et al.* (1999) noted that the incidence of bilharzial bladder cancer in various African countries peaks between the ages of 40–49 years, while infection with *S. haematobium* begins in childhood (as early as 6 months of age), and peaks usually in the second decade of life (between the ages of 5–15 years). This would imply a latency period of 20–30 years.

2.4 Schistosome modulation of the host's immune response

Molecules of the host parasite that are in contact with the host are called host-parasite interface, and these include proteins secreted by the parasite and those on the surface of the parasite (Hernandez-Gonzalez *et al.*, 2010; De la Torre *et al.*, 2011). This interface involves a response to the parasite antigens by the immune system of the host, and the manipulation of the host immune response by the parasite for its survival

inside the host through secretion of proteins and enzymes (De la Torre *et al.*, 2011; van der Ree and Mutapi, 2015).

The modulation of the immune system due to schistosomiasis could be effected through various encounters with the transmission stages, either by the cercaria, the adult and the egg; or during penetration through the skin, migration through the circulation, the incubation of the adult schistosomes, production of eggs and excretion of the eggs (Hewitson *et al*., 2009). Most immune responses related pathologies are widely observed in chronic schistosomiasis. In the early stages of pathogenesis, immunological responses are modulated by the schistosome excretory/secretory (ES) products while late stage immune response modulation is influenced by the soluble egg antigens (SEA). *Schistosoma* ES products are released or secreted from epithelial surfaces of the gut and/or tegument as well as other specialized ES organs throughout almost all life stages of the parasite. Factors present in the host fluid such as blood cells, phagocytic cells, hormones and complement proteins might be responsible for the production and secretion of these products (Hewitson *et al.*, 2009).

Soluble egg antigens of *S. mansoni* have been shown in proteomic studies to induce expression of Jagged-1(a cell surface ligand for notch receptors that may influence haematopoietic cell fate decision) in macrophages and stimulates a mild Th2 type immune response; however it was not enough to generate a Th2 type immune response by itself (van der Ree and Mutapi, 2015). Due to the complexity in collection and harvesting of ES products from host tissue and the inability to mimic in vivo environment in an in vitro environment, studies on the immune modulation by ES products is a daunting challenge for researchers. In the adult worms, ES products are mostly secreted by the excretory cells and co‐localized to the tegumental and sub-tegumental region along with the gut epithelium (Hewitson *et al.*, 2009; Liu *et al.*, 2009). Six of these ES products have been suggested as potential vaccine targets (Paramyosin, glutathione S‐transferase, IrV‐5, Triose phosphate isomerise, Sm23 and Sm14) (Bergquist and Colley, 1998). The ES comprises of important components of all the intra-mammalian stages including the egg (Driguez *et al.*, 2016) (Figure 2.2).

2.4.1 Immune response against cercariae and schistosomula

Protective immunity to helminths develops slowly, and the effector mechanisms for eliminating parasites in humans are not well described; however, animal models have defined a set of Th2-dependent pathways that mediate protection (McSorley and Maizels, 2012). The entry of the schistosomula through the skin elicits an inflammatory response due to infiltration of polymorphonuclear and mononuclear cells that is followed by the localized production of pro‐inflammatory cytokines IL‐1b, IL‐12, TNF‐a, MIP‐1a and IL‐6 (Ramaswamy *et al.*, 2000; Angeli *et al.*, 2001; Hogg *et al.*, (2003)a; Hogg *et al*.,

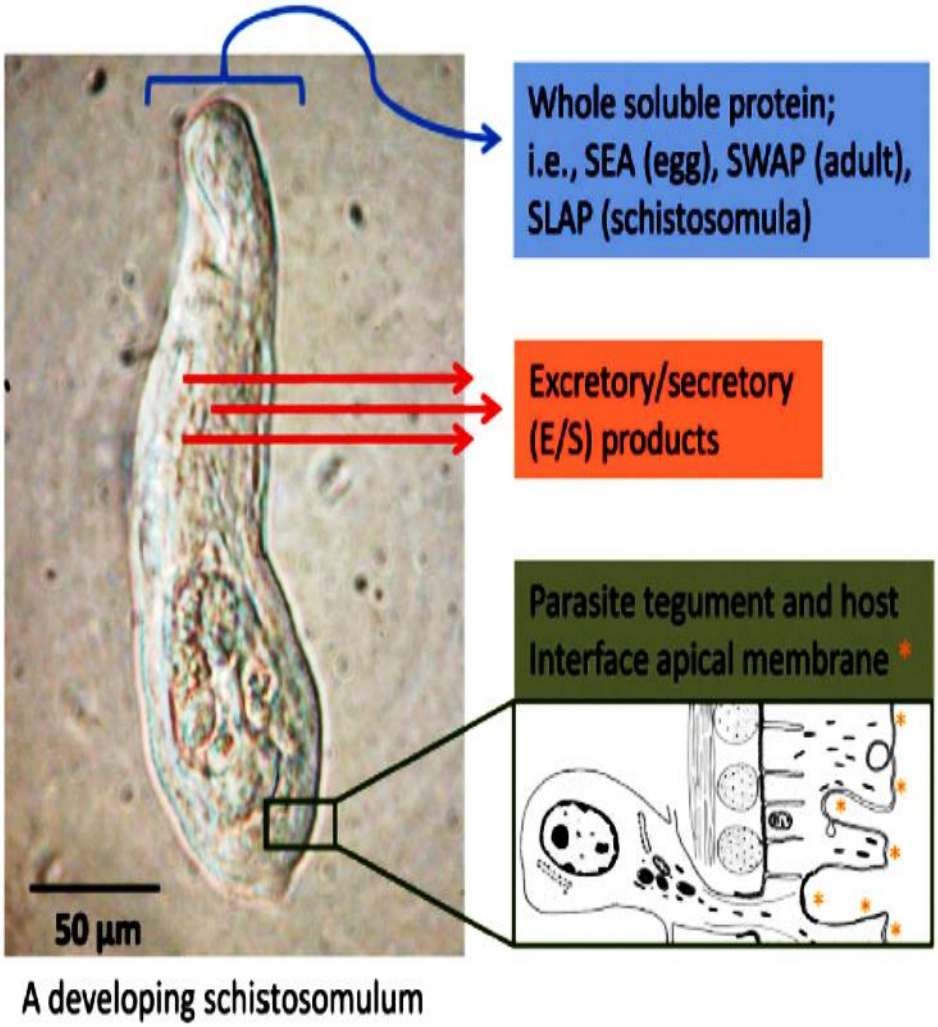

Figure 2.2: Sources of parasite materials for proteomics studies from a developing schistosomulum showing some key compartment and sources of previously studied schistosome proteins.

SWAP (Soluble Worm Antigen Preparation); SLAP (Soluble Lung Antigen Preparation); SEA (Soluble Egg Antigen); ES products (Excretory and Secretory products).

Source: Driguez *et al.*, 2016

(2003)b). The invasion and consequent infection by the schistosomes leads to a predominantly Th2 immune response. One of the main immunomodulatory cytokine induced following exposure to cercariae is the anti-inflammatory IL-10 (Kumar and Ramaswamy, 1999; Ramaswamy *et al.*, 2000; He *et al.*, 2002). Cytokine analysis showed a rapid increase in the levels of IL-10 within a few hours of the parasite entry into the skin, along with significant reduction in the levels of IL-1a and IL-1b and increased levels of IL-1ra (Ramaswamy *et al.*, 1995; Kumar and Ramaswamy, 1999).

Keratinocytes have been suggested as the main source of IL-10 in the skin along with dendritic cells (DC), macrophages and B1 lymphocytes (Ramaswamy *et al.*, 2000). Cercariae ES products stimulate the production of inhibitory molecules which include prostaglandins such as prostaglandin E2 (PGE2) and parasite-derived prostaglandin D2 (PGD2) in all *Schistosoma* spp and IL-1ra (IL-1 receptor antagonist) in *S. mansoni* and *S. haematobium* (He *et al.*, 2002). The production of the prostaglandins (PGE2) due to Schistosomula invasion leads to an increased production of IL-10 in the skin and acts as a potent vasodilator which might facilitate the easy passage of the parasite into the circulation (Salafsky and Fusco, 1987; Ramaswamy *et al.*, 2000). PGE2 aids the production of IL-10 through a cyclooxygenase 2-dependent pathway (Harizi *et al.*, 2002).

Parasite-derived PGD2 which is the ES component of the schistosomula, has been reported to inhibit migration of epidermal Langerhans cells to the site of invasion (Angeli *et al.*, 2001). Physiologically, Langerhans cells are found anchored to neighboring keratinocytes and when the skin is penetrated by parasites, both keratinocytes and Langerhans cells produce pro-inflammatory cytokines such as TNF-a and IL-1b. The expression of these cytokines, in turn, leads to the diminished expression of E-cadherin and stimulates actin-dependent movements of the Langerhans cells. However, during a schistosoma infection, the migration of Langerhans cells is inhibited due to the parasite-induced production of PGE2 by the host cells and parasite-derived PGD2 that both lead to an increased production of IL-10. IL-10 impedes migration of Langerhans cells by downregulating the production of IL-1b and TNF-a by epidermal cells (Wang *et al.*, 1999). Thus, the

purpose of the schistosome‐induced IL‐10 production is to create anti-inflammatory cytokine environments which can downregulate the host immune response against the invading parasite (Ramaswamy *et al.*, 2000; Hoffmann *et al.*, 1999). The interruption of the migration of antigen presenting cells from site of exposure to the draining lymphoid tissue is another strategy adopted by the parasites to modulate the host's immune response.

The schistosomula also adopts additional strategies to evade the host immune response. The ES products from the schistosomula can induce in vitro mast cell degranulation, and hence, lead to production of IL‐4, release of histamine and 5‐hydroxytryptamine in an IgE‐independent manner (Machado *et al.*, 1996). One of the components of the ES products, termed *S. mansoni* apoptosis factor (SMAF), has been shown to induce apoptosis specifically in the CD4+ lymphocyte population via a Fas protein and Fas ligand interaction. The CD4+ apoptosis allows the schistosomula to escape detection by the host immune system (Chen *et al.*, 2002). Once the schistosomula evaded the immune response, it gains entry into the portal veins and remains in the circulatory system. Within 1–3 weeks, it turns into a sexually active adult that adheres to the inner lining of the veins. The male and female adult schistosomes form a pair and can adhere to their chosen vein lining, escaping the host's immune response for decades.

2.4.2 Immune responses triggered by schistosome eggs

The worms themselves have been identified to be poor inducers of a Th2 response (Holland *et al*, 2000), it is known that schistosome eggs or soluble antigens that are derived from the eggs induce an intense Th2 response without the need for additional adjuvant (Pearce *et al*., 1991; Vella *et al*., 1992). The onset of egg production by the adult schistosomes is associated with the skewing of the CD4 response toward the Th2 polarization, characterized by production of IL‐4, IL‐5 and IL‐13 (Annapurna and Uday, 2013). IL‐4 is one of the key cytokines that play a role in the regulation of the development of the Th2 response. IL‐4 is produced in small amounts by naive CD4 cells. This IL‐4 in turn acts in an autocrine manner to induce GATA3 expression, and establish the Th2 phenotype. The resultant IL‐4/IL‐4R/Stat6 signaling pathway plays

an important role in stabilizing and expanding the Th2 cell populations (Ho *et al.*, 2009).

In mouse models in which the egg antigens were injected, rapid induction of strong Th2 responses were observed (MacDonald *et al.,* 2001). Dentritic Cells (DCs), as the most potent antigen presenting cells and the sentinels of cell-mediated adaptive immunity, are known to play a central role in initiation and polarization of T-cell responses. *S. mansoni* eggs preparations have been shown to prime Th2 cells through the functional modulation of DCs (MacDonald *et al.*, 2001; de Jong *et al.*, 2002; Everts *et al.*, 2009; Zaccone *et al.*, 2011). Some studies have shown that carbohydrates on egg antigens are integral to this process (Okano *et al.*, 1999; Williams *et al.*, 2001) and, specifically, that a polylactosamine sugar (lacto-*N*-fucopentaose III) acts as a Th2 adjuvant (Okano *et al.*, 2001).The emerging role of carbohydrates as important factors in inducing immune response during schistosomiasis opens up the possibility that innate pattern-recognition receptors that identify carbohydrates might have a crucial role in the induction of a Th2 response (Pearce and MacDonald, 2002). Some other studies also reported Omega-1 and *S. mansoni* glycoprotein w-1 as inducers of Th2 responses.

Several cytokines other than IL-4 have been implicated in Th2 development. However, these cytokines have been identified to be of little importance for the expression of this type of immune response during schistosomiasis. IL-13, which is closely related to IL-4, seems to be crucial for granuloma formation and fibrosis, rather than Th2 development *per se* (Fallon *et al.*, 2000). IL-6 can direct the development of IL-4-producing T cells. IL-6 does not have a main role during the development of Th2 responses to schistosome eggs *in vivo* (La Flamme *et al.*, 1999), though it might be involved at some level in the regulation of IFN-γ and IL-12 production (La Flamme *et al.*, 2000). One direct correlation of Th2 polarization is the presence of M2 macrophages in the granuloma, which undergo alternative activation by IL-4 and IL-13; this in turn is important for the immune response to parasites as opposed to the classical macrophage activation induced by IFN-γ, which triggers a pro-inflammatory response that is required to kill intracellular pathogens (Gordon and Martinez, 2010).

IL-1R-related molecule T1/ST2 have also been implicated in the induction of a Th2 response (Pearce and Macdonald, 2002). T1/ST2 expression has been implicated in Th2 cytokine production *ex vivo* after the intravenous injection of *S. mansoni* eggs (Townsend *et al.*, 2000). The expression of T1/ST2 seems to be enhanced on CD4+ T cells that are isolated from schistosome egg induced lung granulomas or from the livers of infected mice (Pearce and Macdonald, 2002).

2.4.3 Contribution of various immune cells to the immunopathology of schistosomiasis

Chronic morbidity during schistosomiasis develops as a result of schistosome eggs that lodge in the liver, bladder, gut or other organs causing extensive tissue damage. An immunocompetent host mounts a vast immunological rebuttal to parasite eggs with the development of a vigorous collagen-rich granulomatous response around the eggs (Wilson *et al.*, 2007).

Although granulomatous inflammation is principally triggered by CD4+ cells, cytotoxic CD8+ T lymphocytes, B cells, alternatively activated M2 macrophages, eosinophils and mast cells are also engaged in the development and maintenance of granuloma. Tissue eosinophil infiltration is aided by IL‑5 and IL‑13 in the granuloma (Sher *et al.*, 1990; Sher *et al.*, 1990b; Reiman *et al.*, 2006). However, transgenic mice deficient in eosinophils and infected with *S. mansoni* show no apparent defect in parasite load, granuloma formation and fibrosis (Swartz *et al.*, 2006).These infected mice, with no eosinophil detected in bone marrow and granuloma and high IL‑5 serum levels, were comparable to their wild type counterparts in terms of granuloma number, size, or fibrosis. The role of eosinophils as well as mast cells in *S. mansoni* induced immunopathology remains unclear. T‑cell deficient mice show impaired granuloma formation leading to mortality due to infection within 4–6 weeks (Doenhoff *et al.*, 1981). Without CD4+ T cells, the granuloma has preponderance of neutrophils rather than eosinophils, with extensive damage to liver. In the immunocompetent mice, the liver has normal functions, suggesting that granuloma formation may be helpful to the human host in order to sequester the eggs whose secretion can induce hepatotoxicity (Doenhoff *et al.*, 1981; Dunne *et al.*, 1991). CD4+ T cells have also been shown to be important for egg excretion in mice and humans

(Doenhoff *et al*., 1981; Karanja *et al*., 1997). Regulatory T cells (Tregs) with CD4+CD25+Fox3+ phenotype have been shown to suppress IL‑4 in the murine chronic stage that is reflected in the reduction in the size of granuloma (Turner *et al.*, 2011). Thus, Tregs may play a role in limiting the pathogenesis in the chronic stage of the disease.

2.5 Susceptibility and resistant factors for schistosomiasis

There is an obvious pattern of age-dependent intensity of infection where individuals below the age of puberty are heavily infected by parasites, and those in older age brackets are generally less heavily infected (Pearce and MacDonald, 2002). Identification of specific genes that are associated with infection susceptibility provided evidence that immune-related and especially Th2-related genes, tended to associate with helminth infection (Russell *et al.,* 2015). Mice inability to make TH2 responses make them acutely sensitive to infection with schistosomes and highly susceptible to intestinal helminth infections (Finkelman *et al.,* 2001). Immune-epidemiological data have shown that the intensity of infection is influenced by a major gene called the *SM1* gene that is mapped to a region of chromosome 5 in the 5q31–q33 that codes for proteins that are associated with regulation of the Th2 response such as cytokines like IL-3, IL-4, IL-5, IL-9, and IL-13 and IgE (Marquet *et al.*, 1996; Dessein *et al.*, 2001; Gatlin *et al.*, 2009).

Polymorphisms in these cytokines that lead to an increase or decrease in cytokine levels could influence the antibody isotypes and cellular interactions that in turn may contribute to resistance or susceptibility of individuals to reinfection with schistosomiasis (Gatlin *et al.,* 2009). Kouriba, *et al*., (2005) reported that IL-13 21055C (rs 1800925) and 2591A (rs 2069743) were associated with the upper 10% infection levels in individuals infected with *S. haematobium* (susceptibility). There have been varied reports on effect of IL-4 -590 C/T (rs 2243250) polymorphism on IgE levels, one of which showed that infants with a IL-4 -590 C allele had a higher risk of elevated IgE in their cord blood (Wen *et al.,* 2006). However, it was also reported that total IgE levels were significantly elevated in children with severe malaria carrying the -590T allele (Verra *et al.,* 2004, Gatlin *et al*., 2009). IL-4 is also well known for its important role in IgE class switching (Bacharier and Geha, 2000).

Gatlin *et al.*, (2009) in an univariate analysis reported a significant correlates between resistance to reinfection with *S. mansoni* and the heterozygous (C/T) IL-13 -1055 genotype, any T allele in the IFN-c +874 genotype, and the heterozygous (C/T) in the IL-4 -590 genotype.

Analysis of *S. haematobium* infection in Mali revealed that in chromosomal region 5q31-q33, polymorphisms in the *IL13* gene promoter at −1055 and −591 were associated with the infection rate: alleles −1055C and −591A were preferentially transmitted to children with the 10% highest infection rate, whereas −1055T associated with the lowest infection levels (Kouriba *et al.*, 2005). Another study in Mali revealed an association between a single-nucleotide polymorphism in the STAT6 gene at 12q13.3 and intensity of infection by *S. haematobium*; this polymorphism had an additive effect with IL13 −1055 (He *et al*., 2008).

Comparison of immune responses of susceptible individuals and those who are resistant to reinfection has shown that there is a correlation between immunoglobulin-E responses to worm (not egg) antigens and immunity, which implicates IgE in the protective effector mechanism (Pearce and MacDonald, 2002). Drug treatment of affected populations followed by careful assessment of reinfection status has shown that children usually become heavily reinfected, whereas older individuals might become reinfected, but remain less heavily infected than they were before treatment. So, in endemic areas, older individuals are resistant to reinfection.

Mice that are infected with *S. mansoni* are unable to clear the primary infection, but nevertheless are partially resistant to superinfection. However, the use of mice for studies of resistance to reinfection has been questioned on two points. First, resistance in mice might, in large part, be due to the development of portosystemic vascular shunts (Pearce and MacDonald, 2002). In these animals, immature parasites of a secondary infection might find it difficult to localize to the portal vasculature and, instead, will be carried by the blood flow, through varices, to non-permissive areas of the vasculature. This resistance is, therefore, more anatomical than immunological, and it is related to pathological changes that are more prevalent in infected mice than in infected humans (Dunne and Pearce, 1999). Moreso, the cellular distribution of the

high-affinity receptor for IgE (FcεR1) on mouse cells differs from that on human cells. As IgE dependent eosinophil-mediated ADCC is a possible effector mechanism of protective immunity in humans, the lack of FcεR1 on mouse eosinophils is of particular concern when attempting to model human immunity using the mouse (Dombrowicz *et al.*, 2001).

2.6 The relation of *Schistosomia hematobium* to bladder cancer

Schistosomiasis was first linked to urinary bladder cancer in Egypt in the year 1911 (Fergusson, 1911). The incidence of urinary bladder cancer in the Middle East and Africa is greater in areas with high rather than low *S. haematobium* prevalence. There are several factors that may contribute to the oncological potential of schistosomia infection. The ova deposited in the bladder provoke an intense inflammatory reaction, associated with the production of oxygen-derived free radicals, which may induce genetic mutations or promote the production of carcinogenic compounds (such as N-nitrosamines and polycyclic aromatic hydrocarbons) (Rossin *et al.*, 1994; Zaghloul, 2012), leading to malignant transformation. It is known that schistosomiasis is often accompanied by chronic bacterial super-infection, which may in itself predispose to squamous cell (SC) neoplasia (Shokeir *et al.*, 2004). Bacteria that usually accompany schistosomiasis can promote the formation of N-nitrosamines. International Agency for Research on Cancer (IARC) found that the intensity of infection is determined by urinary egg counts and compounded by smoking, and the combination was strongly considered (IARC, 1994; IARC, 2011). Positive association between bladder cancer and SH infection was detected, with odd ratios ranging from 2 to 14 (IARC, 1994; IARC, 2011).

2.6.1 Pathology of schistosomiasis and schistosoma-associated bladder cancer (SA-BC)

The pathological findings in schistosomiasis are mainly due to inflammatory and immunological responses to egg deposition. Granulomatous areas form around the eggs induce an exudative cellular response consisting of lymphocytes, polymorphonuclear leukocytes and eosinophil. The peri-oval granulomas, fibrosis and muscular hypertrophy are detected histologically. In the urinary bladder, masses of large granulomatous inflammatory polyps containing eggs are found at urinary bladder

walls. Polyps may ulcerate and slough, producing hematuria. Bladder ulcers, sandy patches, irregularly thickened or atrophic bladder mucosa, fibrosis and granulomas containing calcified or disintegrated eggs were also seen (Smith and Christie, 1986; Zaghloul, 2012). The response to egg deposition could lead to calcification of the urinary bladder, infection, stone formation and mucosal proliferation (Zaghloul, 2012).

2.6.2 Carcinogenesis of chronic schistosoma infection

Efforts have been made to study the specific genes involved in the induction of SA-BC. Cell exposed to *S. haematobium* cell total antigen (worm extract) was found to divide faster than those not exposed to the antigen and died much less, probably due to the increased level of bcl2 (Botello *et al*., 2009). Murine urothelium exposed to *S. haematobium* total antigen showed dysplasia, low grade intra-urothelial neoplasm, non-invasive malignant flat lesions in 70% of the tested mice. Bladder carcinoma harbors gene mutations that constitutively activate the receptors tyrosine kinase Ras pathway (Wu, 2005). Botelho *et al.*, (2010) suggested that the parasite extract has carcinogenic ability possibly through oncogenic mutation of Kras gene.

Among the most common genetic changes in bladder cancer is the loss of heterozygosity (LOH) on chromosomes 9p and 9q, which is found regardless of tumor grade and stage (Jacobs *et al*., 2010; Mc Conkey *et al.*, 2010). No line of demarcation between schistosomiasis-associated and non-schistosomiasis-associated bladder cancer detected in terms of LOH of microsatellite markers on chromosome 9. This suggests that data obtained from SA-BC can be extrapolated to bladder cancer induced by other etiologic mechanism (Abdel Wahab *et al*., 2005). Bladder cancer is a very heterogeneous disease cytogenetically, which suggests that the pathogenesis of the disease may not be consistent for every case. The overexpression of the BCL-2 gene in SA-BC patients was found to be up-regulated in squamous but not transitional cell cancers.

Therefore, this BCL-2 overexpression is consistent with the predominance of SCC in SA-BC. Mutations of TP53 were detected in 73% of tumors, BCL-2 expression in 32% and abnormalities of both TP53 and BCL-2 in 13% (Chaudhary *et al*., 1997). Cyclooxygenase-2 is also overexpressed in SA-BC (Zaghloul, 2012). H-RAS, deletion

of p16 and p15, increased epidermal growth factor receptor, c-erb-2 and tumor necrosis factor-alpha are additional mutation reported. These changes increase tumorigenicity by decreasing cell apoptosis and/or creating immunosuppression. Prostaglandin products of cyclooxygenase-2 cause tumor progression and eventual metastasis by down-regulating adhesion molecules, increasing the degradation of extracellular matrix and increasing angiogenesis (El-sheikh *et al*., 2001).

2.6.3 Schistosoma-associated bladder cancer (SA-BC)

The association between **s**chistosoma-associated bladder cancer and *S. haematobium* was initially established through case-controlled studies and through the close correlation of the incidence of bladder cancer with the prevalence of *S. haematobium* within different geographic areas (Zaghloul, 2012). The association was based on the frequent association of tumors with the presence of parasitic eggs and egg-induced granulomatous pathology involving bladder tissues. However, there is yet, no clearly defined cellular mechanisms linking *S. haematobium* infestation with bladder cancer formation.

Schistosoma-associated bladder cancer was known by characteristic pathology (i.e. squamous cell carcinoma, transitional cell carcinoma, or adenocarcinoma, rather than predominantly transitional) and cellular and molecular biology that may differ from non-schistosoma- associated bladder cancer (NSA-BC). The cytogenetic and molecular genetic abnormalities were scarcely studied in SA-BC. Some compared DNA copy number changes in **s**chistosoma-associated bladder cancer and NSA-BC (Kallioniemi *et al*., 1992; Muscheck *et al.,* 2000; Fadl-Elmula *et al*., 2002). Muscheck *et al.* (2000), demonstrated deletion similarities in Schistosoma-associated transitional cell carcinoma (SA-TCC) and Schistosoma-associated squamous cell carcinoma (SA-SCC), compared to what has been previously reported by Kallioniemi *et al*., (1992) on SNA-TCC and Tsutsumi *et al.,* (1998) on SNA-SCC. Armengol *et al.,* (2007) in pools of tissue arising from patients having similar pathological subtypes revealed recurrent primary changes that prevail in each subtype, The pooled specimens of SA-BC tumors showed no schistosomiasis specific changes, compared with pools of NSA tumors. The comparison between SA-TCC and SNATCC and that between SA-SCC and SNA-SCC were similar. DNA copy number profiles of urinary bladder SA adenocarcinoma

revealed similarities to those of SA-TCC and SA-SCC (Vauhkonen *et al*., 2007). Detailed individual gene analysis revealed a set of genes with the same copy number changes in all bladder carcinomas, including both SA and SNA tumors. There were no major cytogenetic differences among different urinary bladder epithelial tumors, regardless of the suspected predisposed carcinogen (Armengol *et al.*, 2007).

Abnormalities in chromosomes 1, 3, 5, 7, 9 and 17 are the most frequently involved chromosomes in urothelial bladder cancer (Heim and Mitelman, 1995). Aly *et a*l. (2012) used Fluorescence in situ hybridization (FISH) technique to show changes in SA-BC. This had previously been detected using Comparative genomic hybridization CGH technique in SA-BC together with aberrations in chromosomes 3, 4, 5, 6 and 11 (Lopez-Beltran and Chen, 2006; Hogland, 2012). It was shown that the most commonly found chromosomal deletion in all stages in SA and SNA-BC involves deletions in chromosome 9 (Jacobs *et al.*, 2010; Mc Conkey *et al*., 2010; Aly *et al.*, 2012), resulting in the loss of their gene encoding proteins that activate the Rb and P53 tumor suppressor pathways. Furthermore, chromosome 9 harbors the TSC1 tumor suppressor that down-regulates the antiapoptotic Akt/mTOR pathway (Abraham *et al.*, 2007). Therefore, deletions on one chromosome may have a crucial influence on the initial steps in tumor development. Furthermore, these mutations may overactivate the fibroblast growth factor receptor 3 protein, which likely directs bladder cells to grow and divide abnormally leading to the formation of bladder tumor (Aly *et al.*, 2012). This suggested that cytogenetic profiles of chemical and schistosoma-induced cancer are largely similar (Fadl-Elmula *et al.*, 2002; Armengol *et al.* 2007). The decreased intensity of schistosomiasis in Egypt led to a changing pattern of the clinicoepidemiologic features of SA-BC. The reported clinicoepidemiologic differences between SA-BC and SNA-BC are now continuously minimizing and the features of SA-BC are slowly approaching those of SNA-BC as reported by Zaghloul *et al*. (2008). If these changes continue, SA-BC is expected to become identical in features to that of western countries SNA-BC (Zaghloul *et al*., 2008; Lopez-Beltran and Chen, 2006; Salem and Mahfouz, 2012).

2.6.4 Age and gender distribution among schistosoma-associated bladder cancer population

In schistosome-free countries throughout the world, the peak incidence of bladder cancer is in the sixth or seventh decade of life (La Vecchia *et al*., 1991) and is maximal between the ages of 65 and 75 years (Burnham, 1989); only 12% of bladder cancer cases occur in people younger than 50 years (Payne, 1959). By contrast, in Egypt, Sudan, Iraq, Zambia, Malawi, and Zimbabwe, the mean age of the highest incidence of bilharzial bladder cancer is between 40 and 49 years (Al-Adnami, 1983; Malik *et al.*, 1975; El-Bolkainy *et al.*, 1981; Lucas 1982; Elem and Purohit, 1983; Ibrahim, 1986), which clearly contrasts with the findings for nonschistosomal areas. The ratio of bladder cancer incidence (males to females) in countries with endemic infection was reported to be 5:1 (El-shebai, 1978) but may vary within the range of 4:1 to 5.9:1 (Ibrahim, 1986). The relatively higher gender ratio in the countries with endemic infection (c.f. 3:1 in countries of nonendemicity) has been suggested to be due to agricultural activities done by men who come in contact with infected waters during agricultural activities (Aboul-Nasr *et al.*, 1986). Hosni and Soheir, (2012) have reported that mean patient age increased from 41 ± 11.2 years to 52 ± 8.6 years, and the male to female ratio changed from 5.6:1 to 4.2:1.

2.7 Bladder cancer studies as a window into SABC pathophysiology

Urinary bladder cancer occurs frequently, with the highest incidence in developed countries. It is typically diagnosed in older patients (55 years of age or older) (Ploeg *et al.*, 2009). Up to 95% of the patients develop urothelial cell carcinoma. The remaining types include adenocarcinoma, squamous cell carcinoma and other rare histological types (Ploeg *et al.*, 2010). Urothelial cell carcinoma also can include such histological variants, which can influence prognosis (Amin, 2009). About 75% of patients are diagnosed with non-muscle-invasive bladder cancer (NMIBC), containing the clinical stages Ta, T1 and CIS (carcinoma *in situ*). The Ta stage is characterized by the presence of noninvasive papillary lesions of low and high grade (with the latter occulting most frequently). T1 tumors invade into the subepithelial connective tissue but not into the muscle layer. T1 tumors harbor a higher risk of progression to muscle-invasive disease, and even come into consideration for cystectomy. Carcinoma *in situ*

is a flat high-grade lesion and has a higher progression rate to muscle invasive bladder cancer (MIBC) (Babjuk *et al.*, 2011). The treatment of NMIBC involves the removal of the tumor by transurethral resection (TUR). Surgical removal is usually followed by a course of intravesical installations of chemotherapy or immunotherapy, which reduce the risk of recurrence.

However, the impact on progression and cancer-specific survival remains uncertain (Van den Bosch and Alfred, 2011).The remaining 25% of patients are diagnosed with MIBC (T2 stage or higher). Due to the high rate of progression to metastatic disease, MIBC, if still confined to the bladder, is mostly treated by radical cystectomy (Sawhney *et al.*, 2006), but systemic chemotherapy, and radical radiotherapy are also widely used in some countries (Merseburger and Kuczyk, 2007). The main risk factor for developing bladder cancer is tobacco smoking, accounting for an estimated 50% of cases among men and 35% of cases among women (Murta-Nascimento *et al.*, 2007). Smoking cessation reduces the risk of developing bladder cancer immediately. However, due to the fact that many carcinogens are present in tobacco smoke, which alter gene expression and damage the DNA, the increased risk is still present even after 25 years (Volanis *et al.*, 2010). Bladder cancer risk is also correlated with various occupational (mainly aromatic amines) and environmental exposures (e.g., arsene in drinking water). Other risk factors include exposure to ionizing radiation, chronic inflammation or schistosomiasis (Kiriluk *et al.*, 2012).

Urinary bladder cancer (UBC) is a common malignancy of the urinary tract, with 180,500 estimated new cases each year and 38,200 deaths in the European Union (Burger *et al.*, 2013). There are 76,960 (54,950 males and 18,010 females) estimated new cases and 16,390 (11, 820 males and 4, 570 females) estimated deaths in United States (Siegel *et al.*, 2012; American Cancer Society, 2016; Figure 2.3). Despite many years of research and the identification of several genes involved in bladder cancer pathogenesis, the large part of its heritability remains unknown (Kiemeney *et al.*, 2009). In the past, many hypothesis-driven candidate gene studies were performed. However the majority of results were not reproduced, with the exception of *GSTM1* and *NAT2* genes (Chung *et al.*, 2010).

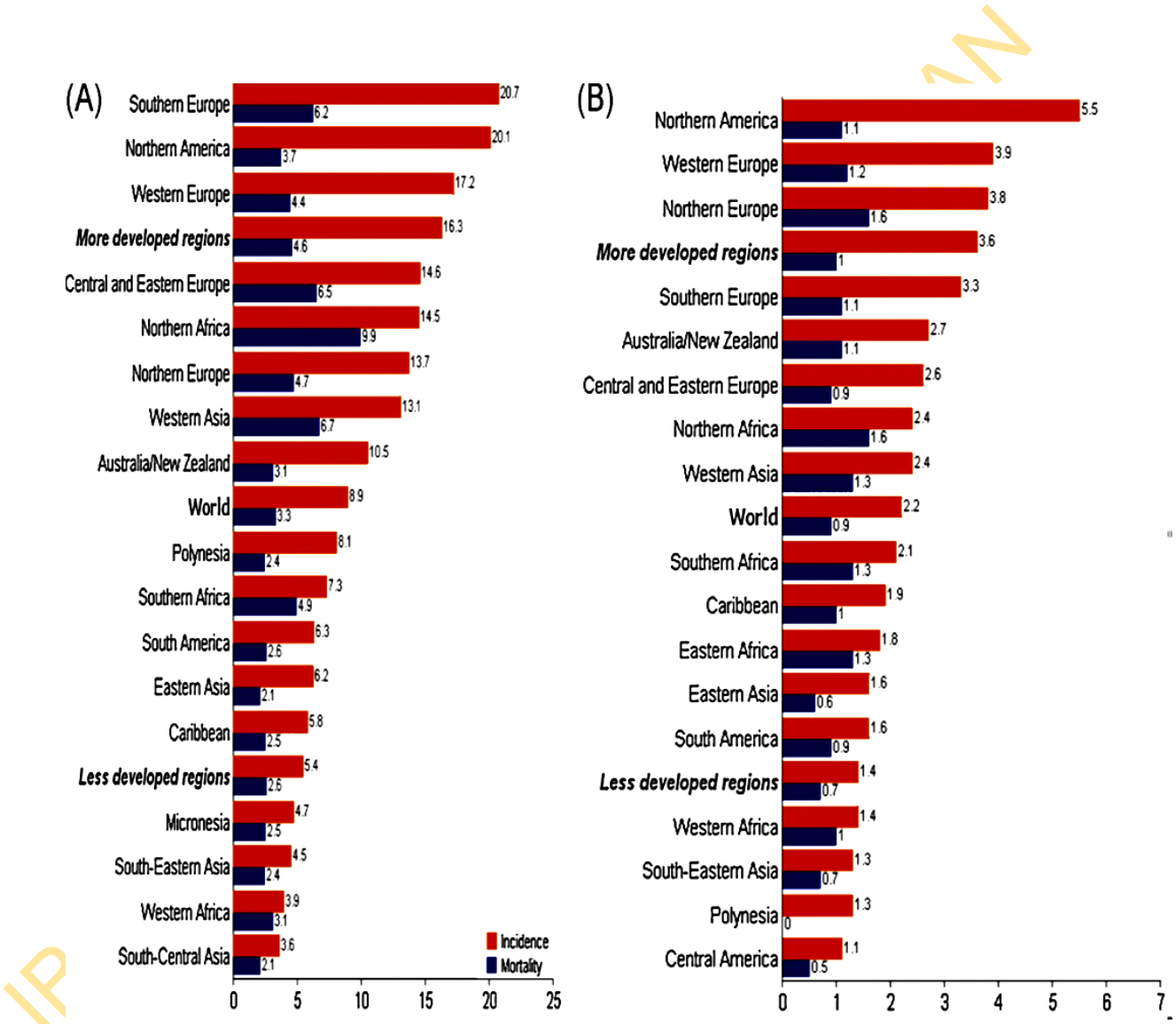

Figure 2.3. Global estimated age-standardized incidence and mortality rates from urothelial bladder cancer per 100 000 in (A) men and (B) women. (**Source:** Burger *et al.*, 2013)

2.8 Genetic susceptibility to bladder cancer risk

2.8.1 Candidate gene studies

Since the establishment of the major environmental risk factors of bladder cancer, basic understanding of the biology of carcinogen action and host defense, major efforts have been made to study candidate genetic variations in cellular signaling pathways. Genes hypothesized to be involved in the carcinogenesis processes, including metabolism of carcinogens, DNA repair, cell cycle checkpoints, apoptosis and inflammatory response have also been considdered (Wu *et al*., 2008; Grotenhuis *et al.*, 2010).

Two genotypes, *N*-acetyltransferase 2 (*NAT2*) slow acetylator and glutathione *S*-transferase µ1 (*GSTM1*)-null genotypes, have been replicated and conferred relatively strong associations with bladder cancer risk (Moore *et al.*, 2004). In addition, pooled analyses and meta-analyses showed weak associations between several DNA repair gene SNPs and bladder cancer risk (Stern *et al.*, 2009).

2.8.2 GSTM1 and GSTT1-null genotype on 1p13.3

Glutathione *S*-transferases are a major family of phase II enzymes that function in detoxifying environmental carcinogens. GSTM1 detoxifies a number of carcinogens including polycyclic aromatic hydrocarbons (PAHs) such as benzopyrene. A meta-analysis of 28 studies (5072 cases and 6466 controls) showed that *GSTM1*-null genotypes conferred a 50% increased bladder cancer risk, which was further confirmed in a recent larger study of 7552 cases and 9688 controls (Gu and Wu, 2011**)**. Interestingly, this association was strongest in never smokers and became progressively weaker in former and current smokers. This observation suggests that *GSTM1*-null genotype lowers the risk of bladder cancer through mechanisms that are not specific to the detoxification of tobacco carcinogens. In never smokers, GSTM1 may protect cells from oxidative damage through metabolism of reactive oxygen species and loss of *GSTM1* allele would lose the protection and lead to increased bladder cancer risk. In current smokers, the heavy tobacco carcinogen exposure may overwhelm the genetic effect of *GSTM1* genotype.

Univariate conditional logistic regression analyses of individuals carrying either the *GSTM1* or *GSTT1* polymorphic variant (null) genotypes had revealed slightly elevated risks of bladder cancer when compared to those carrying the corresponding active allele(s). Also bladder cancer risks association was greater with combinations of the inactive variants *GSTM1* and *GSTT1* polymorphisms than having either one alone (Moore *et al*., 2004).

2.8.3 *N*-acetyltransferases (NAT) 2 slow acetylator

N-acetyltransferases (NATs) are Phase II metabolism enzymes that catalyze the acetylation of aromatic and heterocyclic amine carcinogens and therapeutic drugs. There are two major isoforms of NAT in human cells: NAT1 and NAT2, both are polymorphic in human cells and can categorize the human population into NAT1 (or NAT2) rapid, intermediate and slow acetylator phenotypes (Vatsis *et al.*, 1995; Hein, 2002). NATs may either activate or inactivate carcinogens depending on the specific type of acetylation that occurs on the substrate: *N*-acetylation is typically a detoxifying reaction, whereas *O*-acetylation usually activates (Hein, 2002). For cancers in which *N*-acetylation is a prominent detoxification mechanism such as aromatic amine-related bladder cancer, NAT2 slow acetylator phenotype confers increased risk because it has a decreased capacity to detoxify aromatic amines by *N*-acetylation (Hein, 2002). In humans, NAT2 slow acetylator phenotype can be represented by combinations of several SNPs (Hein, 2002).The association of *NAT2* slow acetylator genotype with increased bladder cancer risk has been compellingly demonstrated by large case–control studies and meta-analyses (Garcia-Closas *et al.*, 2005; Gu *et al*., 2005). In the meta-analysis of 22 studies (5091 cases and 6501 controls), *NAT2* slow acetylator genotype conferred a 40% increased bladder cancer risk (Gu *et al*., 2005; McGrath *et al*., 2006). In addition, there was a significant interaction between *NAT2* genotype and smoking (p for interaction = 0.009). These observations were further confirmed in a publication of even larger sample size of 10,519 cases and 13,218 controls (Rothman *et al.*, 2010). A SNP (rs1495741), located at the 3′-end of *NAT2* on chromosome 8p22, was found to tag NAT2 acetylation phenotype. The G allele was associated with a reduced bladder cancer risk. The AA genotype, which tags the slow acetylator as compared with the GG and AG genotypes that tag rapid and intermediate acetylator,

conferred a significantly increased bladder cancer risk. Moreover, the association was only evident in smokers, but not in never smokers, confirming a significant genotype–smoking interaction (Gu and Wu, 2011). The association between bladder cancer risk and *NAT1* acetylator genotypes has been inconsistent, mostly with null results (Garcia-Closas *et al.*, 2005; Gu *et al*., 2005; Agundez, 2008).

2.8.4 DNA repair gene SNPs

Stern *et al*. (2009), performed meta-analyses and pooled analyses on polymorphism in DNA repair genes in association to bladder cancer risk, these include 5282 cases and 5954 controls of non-Hispanic white origin. Weak but consistent associations were observed for *ERCC2* D312N (rs1799793), *NBN* E185Q (rs1805794) and *XPC* A499V (rs2228000). The association with *NBN* E185Q was only evident in ever smokers (p for interaction = 0.002). (Gu and Wu, 2011)

2.8.5 Genome Wide Association Studies (GWAS) on susceptibility to bladder cancer

While NAT2 slow acetylation and GSTM1-null genotypes exhibited similar associations among noninvasive and invasive Urothelial Bladder Cancer (UBC), Kiemeney *et al*., (2010) reported data from a large genome-wide association study demonstrating a sequence variant on 4p16.3 not only associated with UBC but also located close to the well-established oncogene fibroblast growth factor receptor 3 (FGFR3), which is often mutated in low-grade, noninvasive urothelial bladder cancers. In addition, the frequency of this sequence variant is higher in urothelial bladder cancers carrying an activating FGFR3 mutation, demonstrating a link between germline variants, somatic mutations of FGFR3, and risk of Urothelial bladder cancer (Guey *et al*., 2010; Kiemeney *et al.*, 2010). Three large genome-wide association studies demonstrated eight common sequence variants associated with Urothelial bladder cancer located at 8q24.21, 3q28, 8q24.3, 4p16.3, 22q13.1, 19q12, 2q37.1, and 5p15.33 (e.g., missense variant rs2294008 in the prostate stem cell antigen gene (PSCA) and T allele of rs798766 on 4p16.3 (Guey *et al*., 2010; Kiemeney *et al.*, 2010; Wu *et al.*, 2009), which were all replicated extensively (Rothman *et al.*, 2010). Data from these studies were recently reported, suggesting genetic predisposition in relation to the solute carrier family 14 (urea transporter) gene (SLC14A) that is associated with

renal urine concentration, and thus with variations in contact of carcinogens with urothelial surfaces (HR: 1.17; 95% CI, 1.11–1.22) (Rafnar *et al.*, 2011).

Genetic disposition has been suggested to affect the individual susceptibility to extrinsic carcinogens, mainly tobacco smoke. N-acetyl transferase enzymes (NAT1, NAT2) are involved in bioactivation and detoxification of such carcinogens; a slow NAT2 acetylator genotype was found to be a significant risk factor for urothelial bladder cancers pronouncedly in smokers (HR: 1.31; 95% CI, 1.01–1.70) (Gu *et al.*, 2005). Increasing evidence suggests a significant influence of genetic predisposition on incidence, especially via the impact on susceptibility of other risk factors.

2.9 Biomarkers already developed for bladder cancer

2.9.1 Biomarkers overview

The National Institutes of Health's (NIH's) National Cancer Institute (NCI), describes biomarkers in its dictionary of cancer terms as a"biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease. A biomarker may be used to see how well the body responds to a treatment for a disease or condition. Biomarkers are also called molecular marker and signature molecules." (Alok and Mukesh, 2010)

2.9.2 Protein markers for bladder cancer

Many soluble protein markers in voided urine have been explored for bladder cancer diagnosis and screening. These markers include blood group antigens, tumour associated antigens, proliferating antigens, oncogenes, peptide growth factors and their receptors, cell adhesion molecules, tumour angiogenesis and angiogenesis inhibitors, and cell cycle regulator proteins (Fei *et al.*, 2014). Several of these markers, including bladder tumour antigen (BTA-stat, BTA-TRAK), nuclear matrix protein-22 (Bladder Check and Bladder Cancer Test) and fibrinogen degradation products (ACCU-DX), have been approved by the FDA for clinical use.

A previous study aimed at establishment of a complete two-dimensional database of proteins from the urine of patients with bladder cancer, has listed 339 proteins expressed in urine samples of bladder cancer patients with potential application as

prognostic tumour markers (Ghafouri-Fard *et al.*, 2014). Because proteins are the main executor bio-molecules in cells, protein biomarkers are more important than DNA or RNA biomarkers (Ravichandran *et al*., 2004; Srivastava *et al*., 2005). Protein molecules influence the molecular pathways in normal and transformed cells. Therefore, proteomic markers are closer and more relevant to the disease state initiation and progression. The only FDA-approved biomarkers currently available for clinical use are protein molecules. Protein-based signatures are derived from the techniques of classical two-dimensional (2-D) fluorescence difference gel electrophoresis (DIGE); polycarylamide gel electrophoresis (PAGE); and high throughput platforms, such as Mass Spectroscopy (MS), Matrix Associated Laser Absorption Desorption Ionization Time of Flight (MALDI-TOF), Surface Enhanced Laser Absorption Desorption Ionization Time of Flight (SELDITOF), and reverse phase microarray (Verma *et al*., 2003; Ravichandran *et al*., 2004; Everly *et al*., 2004; Paweletz *et al*., 2001; Zhou *et al*., 2002; Cheng-Han *et al*., 2013). Quantum dots and nanoparticles are recent additions to the technologies available to assess the potential of protein molecules as cancer biomarkers (Seydel, 2003). Quantitative proteomics has been utilized to discover cancer biomarkers in different organ sites, with techniques such as Stable Isotope Labeling with Amino Acids in Cell culture (SILAC) for prostate cancer (Everly *et al*., 2004); iTRAQ, Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS), Capillary electrophoresis-mass spectrometry (CE-MS) for bladder cancer (Theodorescu *et al.*, 2006; Chien-Lun *et al.*, 2013; Goodison *et al.*, 2013).

The diagnostic utility of various proteins that have previously been associated with bladder disease status has been tested. The majority of studies have tested single biomarkers in diverse cohorts, or combined novel and current tests in combination (Kelly *et al*., 2012), but increasingly multiplex combinations are being evaluated. A study by Abogunrin *et al.*, (2012), evaluated 23 previously reported protein biomarkers for bladder disease in urine from a cohort of 80 patients with bladder cancer and 77 controls. Univariate analysis revealed that nine biomarkers were significantly differentially expressed with respect to cancer burden, some of which include Bladder Tumor Antigen (BTA), Nuclear Matrix Protein (NMP22), Epidermal

Growth Factor (EGF), Thrombomodulin (TM). Multivariate algorithms that combined demographic information (age and smoking history) with molecular data significantly improved performance compared to demographic information alone. A combination of NMP22, BTA, serum CEA, EGF, and thrombomodulin enabled sensitivities of up to 91 %, and specificities of up to 80 % (Abogunrin *et al.,* 2012).

Based on the fact that cancer often involves inflammatory processes, Margel *et al.* (2011) monitored a panel of immune modulators in urine to investigate biomarker potential. The panel included 15 heat shock proteins and cytokines, monitored by commercial ELISA assays. The combined urinary concentrations of HSP60 and IL-13 significantly improved the performance over any single factor. The discovery of novel protein biomarker panels has surged recently due to advances in high-throughput proteomic technologies (Goodison *et al.,* 2013, Adeola *et al.,* 2015). The appropriate use of these approaches has the potential to provide highly efficient biomarkers for bladder cancer detection and monitoring. Protein-based biomarkers have several advantages over nucleic acid targets. Only proteomic profiling enables the evaluation of global changes in gene expression that result from both transcriptional, translation, and post-translation modifications.

Although genomics may be more amenable to comprehensive surveys, phenotypic changes can only manifest themselves through altered protein expression, so the identification of protein factors involved I n bladder disease can best inform us of tumour biology. Beyond their diagnostic and prognostic value, protein biomarkers provide potential therapeutic targets and represent markers of disease progression, treatment response, and other clinical utilities.

Proteome profiling studies have tended to identify factors that can classify tumours or predict patient outcome or disease recurrence, but biomarkers identified in solid tissue may be subsequently translated into a serum or urinary test, and some have shown promise as urinary diagnostic markers. Examples from proteomic analyses include psoriasin for squamous cell carcinoma (Celis *et al*., 1996), Tumor-Associated Calcium Signal Transducer 2 (TACSTD2) (Chen *et al*., 2011), and cystatin B (Feldman *et al*., 2009), and panels for serum-based diagnosis (Schwamborn *et al*., 2009), but direct

proteomic analysis of the urine is more likely to reveal promising non-invasive diagnostic biomarkers.

Early urinary profiling studies used gel-based technologies to define the urinary proteome and to begin to identify proteins associated with bladder cancer (Rasmussen *et al*., 1996; Irmak *et al*., 2005; Saito *et al*., 2005; Orenes-Pin˜ero *et al*., 2007). However, advances in MS technology have been rapidly applied to the profiling of bladder tissues, serum, and urine. Vlahou *et al*. (2001) used SELDI-TOF to compare the proteomic profiles of urine samples from healthy controls and patients with transitional cell carcinoma of the bladder. Multiple protein changes were reproducibly detected in the cancer group, including five potential novel biomarkers and several protein clusters. One of the biomarkers, alpha-defensin, was subsequently shown to be present in bladder tumor cells. The combination of the biomarkers and protein clusters significantly improved the accuracy of patient classification. In a separate cross-validation study by the same authors (Holterman *et al*., 2006), alpha-defensin monitoring was used to detect Bladder cancer with better sensitivity and specificity than commercial tests. Theodorescu *et al*. (2006) used capillary electrophoresis (CE)–mass spectrometry to identify urinary biomarkers for bladder cancer in a set composed of 46 patients with urothelial carcinoma and 33 healthy volunteers. These were further refined using CE-MS spectra of another cohort of urine samples from healthy volunteers and patients with malignant and nonmalignant genitourinary diseases. Using this two-step approach, a diagnostic biomarker signature of 22 urinary peptides was established. In a validation study, this signature enabled the correct classification of all urothelial carcinoma patients in a test set containing 31 urothelial carcinoma patients and 138 nonmalignant genitourinary disease patients (Theodorescu *et al.,* 2006).

Another study used an isobaric tag for relative and absolute quantitation (iTRAQ) technique to discover proteins that were differentially expressed between pooled urine samples from bladder cancer patients and non-tumor controls. This strategy identified 55 candidate biomarker proteins (Goodison *et al.,* 2013). Conventional techniques confirmed that the level of apolipoprotein A-I (APOA1) was significantly elevated in urine samples from bladder cancer patients. Using a commercial ELISA assay,

APOA1 was confirmed to have high diagnostic potential in more sample set (Chen *et al*., 2010; Urquidi *et al.*, 2013). Using a glycoprotein enrichment strategy to profile urine samples from 100 subjects (54 with cancer), Yang *et al*., (2011), identified a panel of glycoproteins associated with Bladder cancer. The most discriminatory protein in that study was alpha-1-antitrypsin (A1AT), also known as SERPINA1. In an independent validation cohort of 70 subjects, A1AT measurement by ELISA had a sensitivity of 74% and a specificity of 80% (Yang *et al*., 2011). Through integration of proteomic and genomic urine sample profiling data, several authors, identified panels of promising biomarkers for inclusion in diagnostic urinalysis assays (Feng *et al*., 2009; Yang *et al*., 2011; Rosser *et al*., 2009; Urquidi *et al*., (2012)d). Combinations of 2–3 biomarkers were analyzed by ELISA in a series of studies (Urquidi *et al*., (2012)a; Urquidi *et al.*, (2012)b; Urquidi *et al*., (2012)c; Goodison *et al.*, 2012), and multivariate analysis identified an eight-protein biomarker panel that achieved 92% sensitivity and 97% specificity in cohorts of 64 patients with bladder cancer and 63 controls. The performance was far better than current urinalysis tests in the same cohort (Goodison *et al.*, 2012).

Bladder cancer biomarkers discovered by proteomic profiling have also been derived from serum samples. Discriminating protein patterns in serum using magnetic bead-based separation followed by MALDI–TOF MS have been search for by Schwamborn *et al.*, (2009). Multidimensional analyses of serum samples from 105 patients with Bladder cancer, 98 healthy controls, and 45 prostate cancer patients generated algorithms capable of distinguishing between cancer patients and healthy individuals. The best algorithm achieved 96% sensitivity and 86% specificity (Schwamborn *et al.*, 2009). The studies described above show the power of MS-based urinary analysis for the discovery of biomarkers. The latest proteomic technological developments, such as arrays for phosphoproteins, glycoproteins, or phospholipoproteins, can reduce the sample complexity that plagues the proteomic analysis of biological fluids. As these techniques are applied to bladder cancer samples, there will likely be further advances in urinary biomarker discovery.

2.10 Other bladder cancer biomarkers

2.10.1 DNA markers

The cancer genomics era is developing rapidly, fueled by the emergence of many advanced technologies, including array Comparative Genomic Hybridization (CGH), DNA micro-array, next-generation sequencing, etc. The completion of the cancer genomic landscape not only helps our understanding of the mechanistic basis underpinning particular disease subtypes but also provides opportunities for discovery of new biomarkers for diagnosis, prognosis, and prediction of response (Fei *et al*., 2014). Furthermore, such work facilitates the identification of novel therapeutic targets. Complex chromosomal changes as well as certain mutations have been found to correlate with different stages of bladder cancer (Fei *et al*., 2014). For instance, loss of chromosome 9 is a very distinctive change commonly seen in Ta/T1 and less frequently in muscle invasive bladder cancers (MIBC) (Ghafouri-Fard *et al*., 2014). Since loss of chromosome 9 is recurrently seen as the single abnormality, it has been considered to be an early event in bladder cancer progression (Lindgren *et al.*, 2006). Cytogenetic loss of chromosome 9 is demonstrated by the frequent loss of heterozygosity (LOH) on this chromosome mostly in the region including CDKN2A. LOH on chromosome 9 is shown to be associated with tumour development rather than initiation (Lindgren *et al*., 2006).

2.10.2 UroVysion FISH Assay

This test is designed to detect aneuploidy for chromosomes 3, 7, 17 and deletion of the chromosome 9p21 locus in urine specimens using Flourescence In situ Hybridization (FISH) (Ho *et al*., 2013). It is FDA approved for analysis of urine specimens from subject suspected of having bladder cancer. It is among the few markers that have achieved extensive clinical use. In comparison to urinary cytology it has more sensitivity but less specificity. It has been shown to be more powerful than cytology in diagnosing stage Ta bladder cancer patients. It is also beneficial for monitoring patients with superficial bladder cancer after treatment with intravesical bacillus Calmette-Guerin (BCG) especially when cytology results are ambivalent (Smith and Guzzo, 2013). Chromosomes 4p, 8p, 9p, 11p, and 17p also often display loss of heterogeneity in patients with bladder cancer

2.10.3 Epigenetic changes

Epigenetic changes noticeably DNA methylation have extensive influenced on gene expression. Recent data have indicated that aberrant DNA methylation happens commonly and early in human carcinogenesis (Hattori and Ushijima, 2016). It has been shown to occur extensively in cancer cells and in the same promoter regions. As a result, analysis of a few loci is satisfactory for diagnosis of cancer and this is the main advantage of these markers for detection of cancer (Chihara *et al.*, 2013). Promoter methylation has been shown to occur commonly in both normal urothelium and CIS samples from patients with urothelial carcinoma. During the progression from normal to invasive urothelial carcinoma promoter methylation is increased at both specific loci and in general. Promoter methylation seems to be a good biomarker for early detection of bladder cancer (Dhawan *et al.,* 2006).

2.10.3.1 High throughput DNA methylation profiling in urine and tissue samples

Both differential hypermethylation and hypomethylation have been seen in tumour tissues compared to normal tissues (Hattori and Ushijima, 2016). The diagnostic accuracy of these markers in urine samples has been high, with 100% sensitivity and specificity. According to preliminary data, diagnostic markers based on differential DNA methylation at specific loci can be applied for non-invasive and reliable detection of bladder cancer (Chihara *et al*., 2013).

2.10.3.2 Detecting DNA methylation of the BCL2, Cyclin-Dependent Kinase Inhibitor 2A CDKN2A and Nidogen 2 (NID2) genes in urine

A highly specific and sensitive nested methylation specific polymerase chain reaction (PCR) assay was developed to detect bladder cancer in small volumes of patient urine (Scher *et al.,* 2012). In a pilot clinical study its sensitivity and specificity to differentiate bladder cancer from other urogenital malignancies and non-malignant conditions have been shown to be more than 80% (Scher *et al*., 2012).

2.10.3.3 Detecting DNA methylation of APC, ARF, CDH1, GSTP1, MGMT, CDKN2A, RARb2, RASSF1A and TIMP3 genes in urine

The promoter methylation pattern in urine has been similar to the primary tumours. In more than two third of patients promoter methylation has been seen in at least one of

these genes (CDKN2A, ADP-ribosylation factor (ARF), O^6-methylguanine DNA methyltransferase (MGMT), and GSTP1), while none of controls have displayed such methylation. A combined two-stage predictor strategy for detection of promoter hypermethylation of these 9 genes has 82% sensitivity and 96% specificity. Therefore, quantitative methylation-specific PCR assay of a small panel of genes can be a powerful non-invasive tool for the detection of bladder cancer (Hoque *et al.*, 2006).

2.10.4 Point mutations in bladder cancer

The point mutation assays are diagnostic tools to identify patients who will benefit from targeted therapies. They are also potential biomarkers for recurrences during disease surveillance.

2.10.4.1 FGFR3, HRAS, KRAS, NRAS and PIK3CA Mutations:

In a study of mutational analysis of oncogenes performed on 257 primary bladder tumours and 184 recurrences from 54 patients, it was suggested that surveillance by mutation analysis for Fibroblast Growth Factor Receptor 3 (FGFR3), Phosphatidylinositol-4, 5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (PIK3CA) and the RAS genes could be a practical follow-up policy for patients suffering from a non-muscle invasive bladder cancer (NMIBC), and grade 1-2 primary tumour. In addition, the mutation assays may be a diagnostic tool to define patients with MIBC in whom therapies targeting FGFR3 or other receptors and downstream targets may be beneficial. However, mutations in the RAS and PIK3CA genes were not predictors for either recurrence-free or disease-specific survival (Kompier *et al.*, 2010).

2.10.4.2 FGFR3 Expression and Mutation Analysis in Cancer Samples

Expression profiling, mutation analysis and LOH analysis have been used to molecularly characterize a large cohort of early-stage bladder cancer. Two types of tumours have been defined by this method. Low-grade tumours are distinguished by FGFR3 activity, either by FGFR3 mutation or by expression, high protein synthesis and low cell-cycle activity. Whereas high grade tumours show less or no dependence of the FGFR3 receptor, low levels of protein synthesis and high cell-cycle gene activity. It has been suggested that FGFR3 receptor is critically involved in low grade/stage bladder cancers (Lindgren *et al.*, 2006).

2.11 Metabolomic Markers

The most recent developments in biological fluid analyses have come in the field of metabolomics. The application of urine-based metabolomics using high-pressure liquid chromatography (HPLC) or nuclear magnetic resonance (NMR) with multivariate analysis can identify specific metabolites or profiles that can aid cancer diagnosis (Hyndman *et al.*, 2011; Yidong *et al*., 2015). To date, only a few studies have investigated differential urine metabolite profiles associated with bladder cancer (Alberice *et al.*, 2013; Pasikanti *et al.*, 2013). Using HPLC, Issaq *et al.*, (2008), profiled urine samples from 41 patients with bladder cancer and 48 healthy controls. Statistical analyses allowed at least 40 of the 41 bladder cancer cases to be predicted correctly, but the specific metabolites identified in that study have not been confirmed elsewhere. In a similar study format, Pasikanti *et al.*, (2010), identified a 15-marker metabolite model that achieved a 100 % detection rate for Bladder cancer in 24 patients. Another study of 58 clinical specimens identified 35 metabolites associated with Bladder cancer. The metabolic signature distinguished both normal and benign bladder from Bladder cancer, and even showed promise in distinguishing tumor stages (Putluri *et al*., 2011). Severaal studies have identified upregulation and downreguation of glucose, lipid, amino acid and nucleotide metabolites as distinguished signatures for bladder cancer (Lin *et al*., 2012; Dettmer *et al*., 2013; Pasikanti *et al*., 2013; Yidong *et al*., 2015). In a study of 67 patients and 25 healthy controls, serum sample profiles from bladder cancer patients suggested perturbed metabolic pathways of aromatic amino acids, glycolysis and the citrate cycle, and lipogenesis (Cao *et al.*, 2012). Changes at the metabolite level may be detectable in biological fluids before the appearance of clinical symptoms, making them potentially useful early detection biomarkers, and metabolic profiling can provide insights into bioprocesses perturbed during tumor development and progression. However, there are major problems with urinary metabolomic profiling. The analytes are small metabolites that are not always filtered by the kidney, so confounding factors such as polypharmacy or even recent dietary intake can create large variations between individuals; thus, such studies require large cohorts and standardization of sample collection and processing.

2.11.1 MicroRNA (miRNA) Markers

miRNAs are 18-24 nucleotide RNA molecules which are endogenous inhibitors of gene function acting by either degradation of RNA or inhibition of translation and they have been proved to be involved in pathogenesis of cancer (Ghafouri-Fard *et al.*, 2014).

A growing body of evidence suggests that microRNAs (miRNAs) contribute to bladder cancer development, progression and metastasis. Genome-wide miRNA expression signatures have been used to rapidly and precisely identify aberrant miRNA expression in bladder cancer. Examination of the differential expression of miRNAs between bladder cancer and normal bladder tissue has led to the elucidation of 11 bladder-cancer-specific miRNA expression signature sets (Yoshino *et al.*, 2013). Among the 11 signature sets, the authors reported 15 miRNAs were down-regulated and 7 miRNAs were up-regulated in bladder cancer that have been isolated in three or more expression studies. They also reported 15 miRNAs associated with bladder cancer diagnosis and prognosis. In a parallel review of the current scientific reports that link differences in miRNA expression with the pathogenesis of bladder cancer (Zabolotneva *et al.*, 2011), the authors created the first comprehensive database of miRNA with biased expression profiles in bladder cancer. They identified in total 95 differentially expressed miRNAs, 48 up-regulated in bladder cancer, 35 down-regulated, and 12 contradictory. The molecular targets of these miRNAs have been shown to be involved in crucial cell mechanisms, such as apoptosis, cell cycle progression and epithelial-mesenchymal transition (EMT). A few of these microRNAs and their target mRNAs have been utilized in the The Cancer Gemone Atlas (TCGA) study to cluster bladder cancer patients. For instance, one cluster shows significantly lower expression of miR-99a and miR-100, and up-regulation of their target gene FGFR3. Similarly, two clusters show lower expression of members of the miR-200 family of miRNAs (which target multiple regulators of EMT) and consistent down-regulation of the epithelial marker E-cadherin.

There have been extensive efforts in the past decade to identify genetic susceptibility loci for bladder cancer. Recent cancer association studies by candidate gene and genome-wide association study (GWAS) approaches identified at least ten low-

penetrance genetic susceptibility loci for bladder cancer (Gu and Wu, 2011). The ten validated genetic loci include NAT2, GSTM1, 8q24.21 (MYC), 3q28 (TP63), 8q24.3 (PSCA), 5p15.33 (CLPTM1L-TERT), 4p16.3 (TACC3-FGFR3), 22q13.1 (APOBEC3A-CBX6), 19q12 (CCNE1) and 2q37.1 (UGT1A). A meta-study of GWAS also identified four more loci that achieved or approached genome-wide statistical significance, but require further studies for confirmation (Figueroa *et al*., 2014). There have been numerous candidate gene studies reporting positive associations between SNPs and bladder cancer recurrence, progression, and survival. Chang *et al*., (2012), summarized them into several carcinogenesis-related processes, including cell cycle and apoptosis (TP53, MDM2, CDKN2A), DNA repair (ERCC6, XPD, XPG, XPF), growth factor signaling (EGFR, TGFBR1), PI3K-AKT (AKT2, PIK3R1, RAPTOR), stem cell signaling (GLI2, SHH, GLI3), inflammation (PPARG, IL-6, NF-kB1), cell adhesion (CDH1) and oxidative stress (HIF1A). However, most of the candidate gene pathway studies were of limited sample size and had not been validated in independent populations.

CHAPTER THREE

3 MATERIALS AND METHODS

3.1 Study Area and Study Design

The study was carried out from August 2012 to May 2014 in Eggua, a rural agrarian community where *S. haematobium* infections have been prevalently reported (Hassan *et al.*, 2012). Eggua lies between latitude 7^{o} 6′4.811″ N and longitude 2^{o} 52′ 43.776″ E in a derived savanna zone. The area is largely dominated by Yoruba speaking people. It consists of settlements at Sagbon, Imoto, Tata, Agbon- Ojodu, and Igan Alade. It shares boundaries with Igbogila, Ilaro, Ijoun and Benin Republic. Two major rivers (Yewa and Iju) flowing through the area serve as the main water source, resulting in high water contact by the inhabitants (Fig. 3.1). These rivers are used for religious, domestic and entertainment activities which enhance the transmission of schistosomiasis. A cross sectional study design was employed for this study (Fig. 3.2). Children were excluded from the study because the main objective was to determine the effect of chronic urinary schistosomiasis on the health of the adults within the community.

A total of 371 participants between the ages of 30 and >60 years were drawn from the village without any selection criteria, because the people were attended to as they conveyned at the community health centre following the initial announcement by the community leader. The sample size was calculated using the formula which was adopted from Naing *et al*., (2006).

3.2 Ethical considerations

Informed consent was obtained from each volunteer under a protocol approved by the Local Government and health officials of the area studied. Ethical approval was also obtained from the University of Ibadan and University College Hospital (UI/UCH) Ethical Committee and Ogun State Ministry of Health.

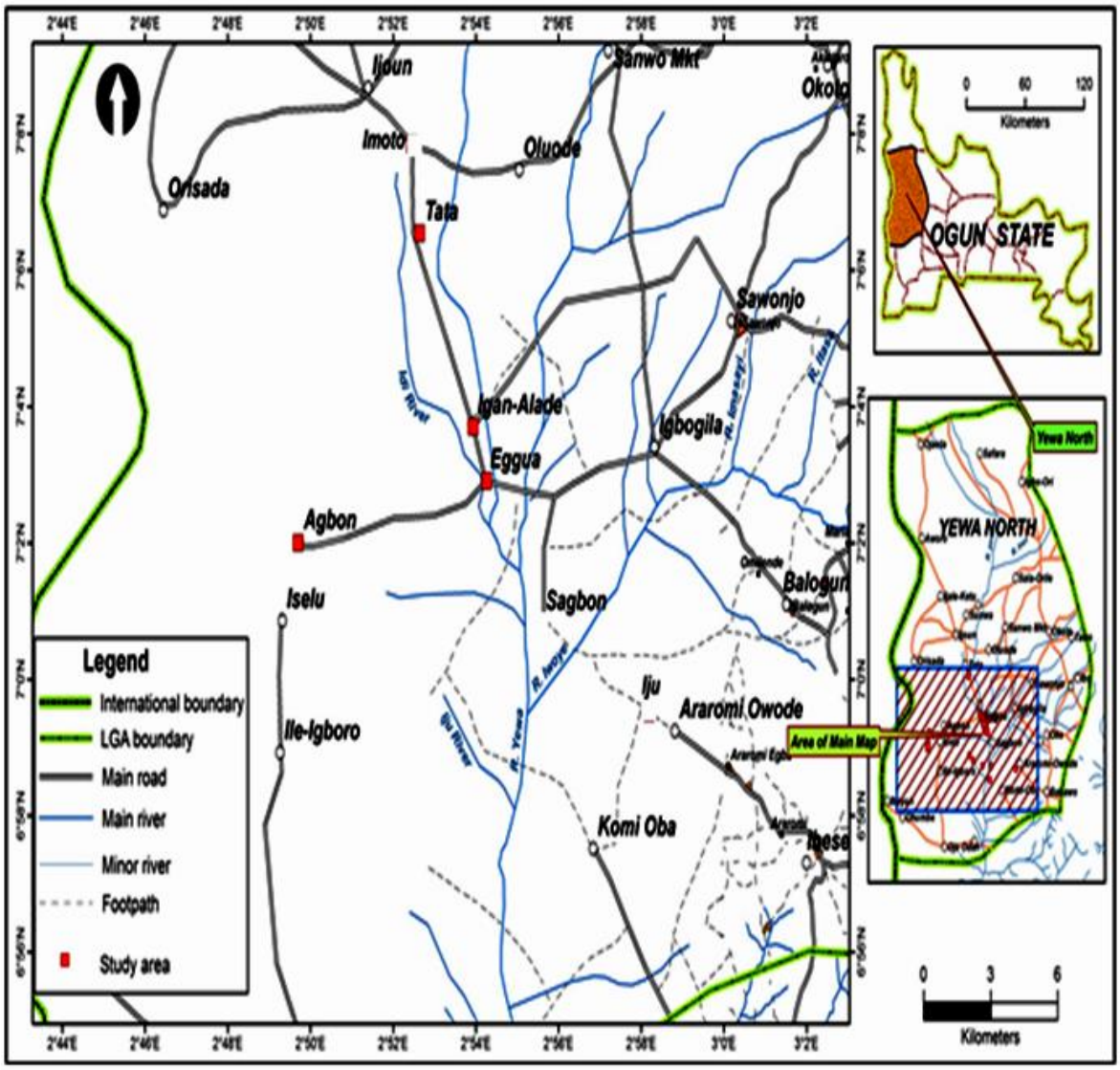

Figure 3.1: Map of Yewa North LGA showing the study areas

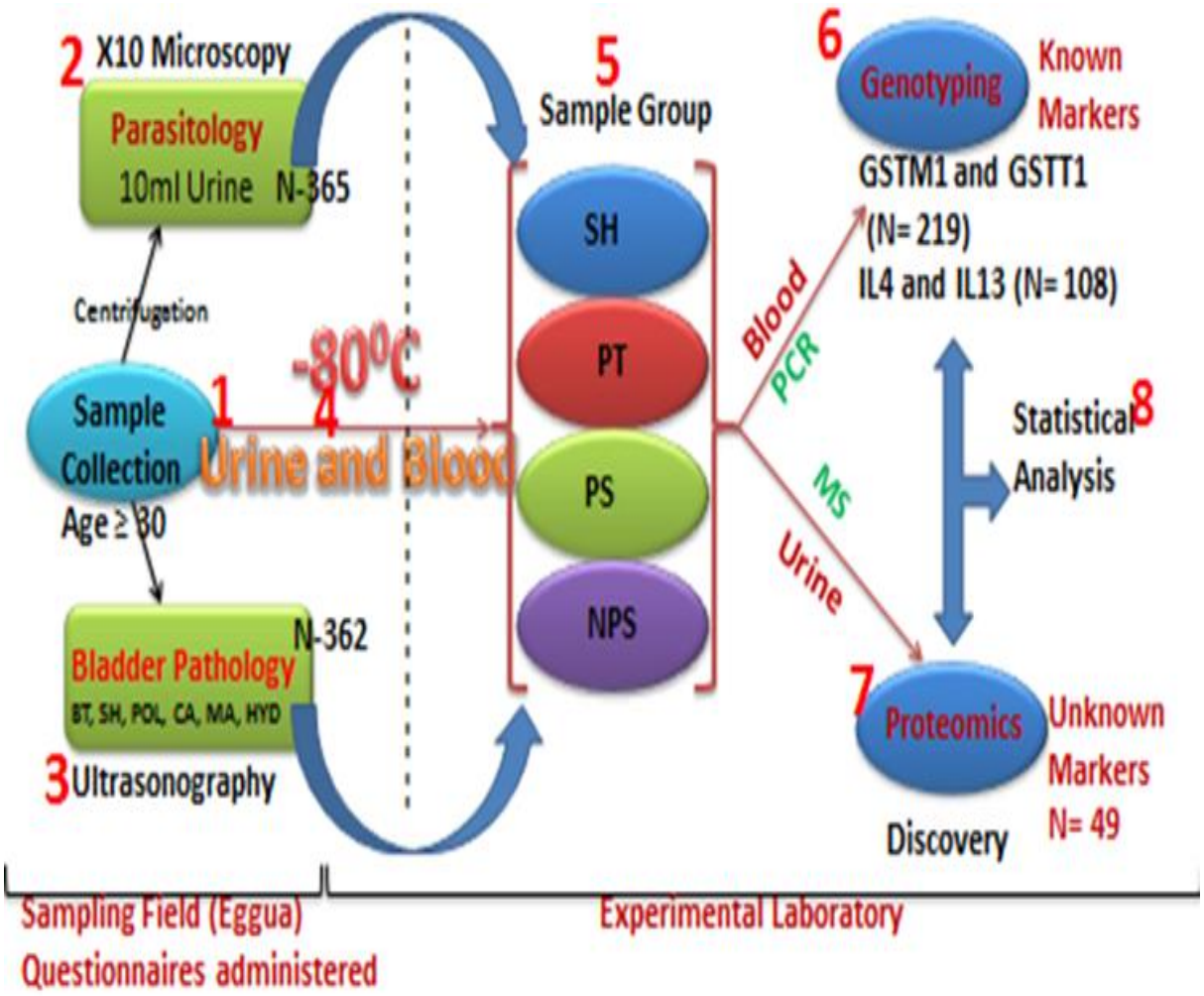

Figure 3.2: Workflow showing design of the field and experimental work.

The study was carried out in two phases (Field sampling and Laboratory experiments). The abbreviations represent different sample groups namely: SH- *S. haematobium* infected groups, PT- Bladder Pathology group, PS- group with combination of pathology and *S. haematobium* infection and NPS- No pathology and schistosomiasis (control group). GSTs- Glutathione-S-transferase, IL- cytokine Interleukine; BT- Bladder wall thickness, SH-Abnormal bladder shape, POL- Polyps, MA-bladder mass, CA-Calculi, HYD- Hydronephrosis in Kidney, PCR- Polymerase Chain Reaction, MS- Mass Specrometry

3.3 Data collection

3.3.1 Questionnaire

A semi-structured questionnaire was used to access some basic epidemiological information on schistosomiasis from the study participants. All participants were interviewed regarding their occupation, whether the occupation involved water contact, the length of time in their current occupation, previous water contact, smoking and alcohol behaviour and length of residence in the village.

3.3.2 Bio-fluid samples

Study volunteers provided blood (2 mL) by venipuncture and urine (for egg count) specimens.

3.4 Parasitology

The urine samples were collected between 10:00am and 2:00pm for maximum egg yield and were processed for parasitological examination and egg count (Nmorsi *et al.,* 2007; Hassan *et al.,* 2012). Participants were given clean wide mouthed universal bottles and shown how to provide urine for the study. The specimens were labelled appropriately, placed in a cold ice packed box and taken to the laboratory for analysis. The urine sedimentation method was achieved by centrifuging 10ml of the urine sample at 5000 rpm for 5 minutes. The supernatant was then discarded and the sediment transferred onto clean grease free glass slide, covered with a cover slip and examined microscopically to identify *Schistosoma haematobium* ova which is characterised by the presence of a terminal spine. The eggs (Figure 3.3) were counted and the intensity of infection classified as light if ≤50 eggs/10 mL of urine and heavy if >50 egg/10 mL urine were present (Nmorsi *et al.,* 2007).

3.5 Ultrasound and Pathology

Approximately one hour after drinking a large volume of water (0.1-1.5 litre depending on their age) that was given to participants to distend the bladder, a blind ultrasound examination was carried out by a radiologist for each participant in the study. The classification of bladder damage was based on the definition of the WHO (WHO, 1999b; WHO, 2000) and Shiff *et al*., (2006). The following abnormalities

were documented: abnormal bladder shape, bladder wall irregularities, bladder masses, presence of polyps, calcification and presence of hydronephrosis. In this study, bladder lesions were considered severely abnormal when four of the above conditions are

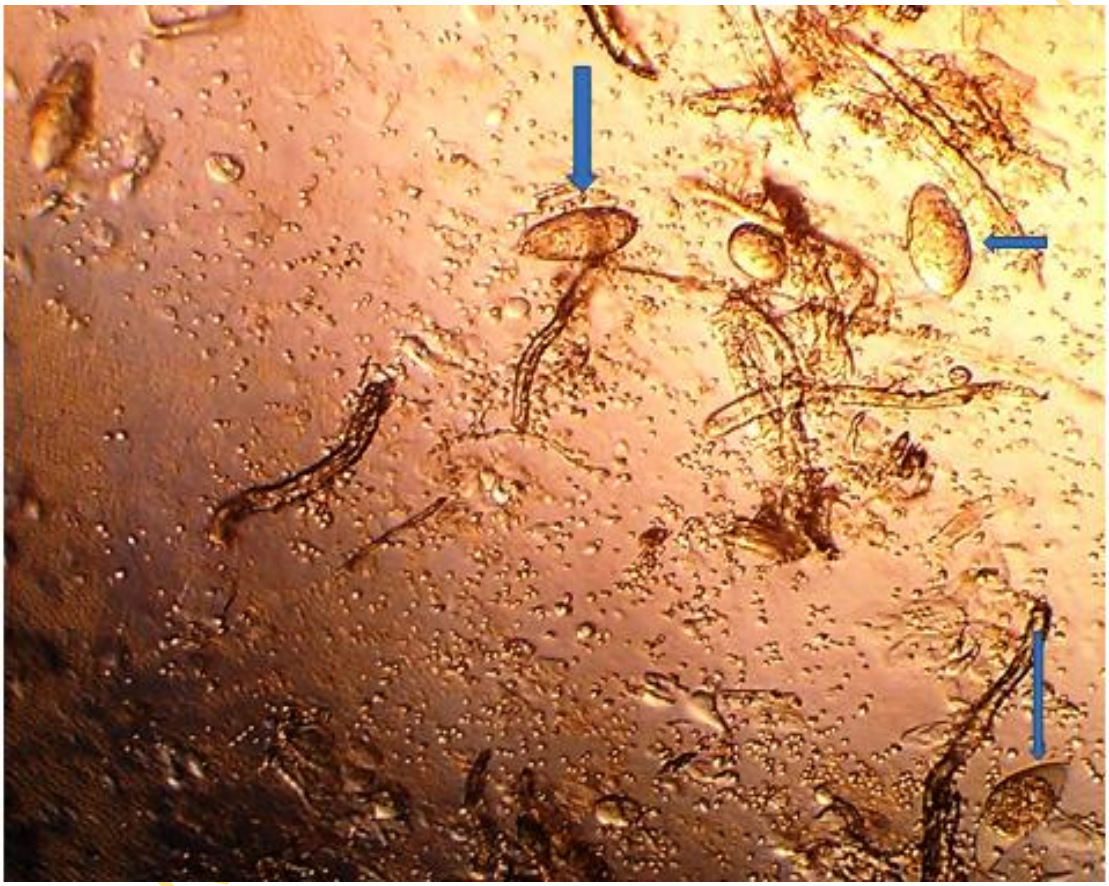

Figure 3.3: Schistosome eggs (blue arrows) as shown by microscopy in the urine of *S. haematobium* infected participant

present (or three with hydronephrosis) in a single individual. Lesions were considered moderate if fewer conditions are seen or negative when no specific lesions are observed (Nmorsi *et al*., 2006). This classification was used throughout this study. Statistical analysis was done using SPSS version 20.0 (P value set at <0.05)

3.6 DNA Extraction and Purification

DNA was purified from the blood samples using Thermos Scientific GeneJET Whole Blood Genomic DNA purification kit (Lithuania), following the manufacturer's instructions. DNA concentration was measured by spectrophotometry. Aliquots (10 µL) of all samples was taken and subsequently adjusted to provide standard stock solutions of 20ng/µL. The *A*280/*A*260 ratio was estimated to provide an indication of the quality of the sample. Only samples that provided a yield >20 ng/µL and *A*280/*A*260 ratio >1.6 and <1.95 were included for genotyping analysis.

3.7 GSTM1 and GSTT1 Genotyping

3.7.1 Genotyping method for GSTM1 Polymorphism

In this study, a total of 219 samples were genotyped for GSTs polymorphism among the bladder pathology group. These included 118 urinary tract pathology and 101 control cases. The control cases were randomly selected from among participants in Eggua, who gave blood sample and had no bladder pathologies. This reaction was used to distinguish between GSTM1-active and GSTM1-null individuals. Two primers were used to amplify for GSTM1 complementary DNA sequences (G1: 5′-CTGCCCTACTTGATTGATGGG- 3′; G2: 5′-CTGGATTGTAGCAGATCATGC -3′) (Brockmoller *et al.*, 2000). The G1 and G2 amplified a 500base pairs (bp) product specific for the GSTM1 gene. The presence of a GSTM1-null polymorphism was concluded from the absence of the specific 500-basepair fragment. 5µL DNA was amplified in a final volume of 25µL amplification reaction of 30 cycles, using the Hot Start Taq 2X Master Mix (M0496L, BioLabs, New England) according to the manufacturer's instructions.

3.7.2 Genotyping for GSTT1 Polymorphism

The 25 µL volume amplification reaction for *GSTT1* gene was done in a duplex PCR assay with a Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers (Forward: 5′- CAA AGC TTG TGC CCA GAC TGT- 3′; Reverse: 5′- CGC CCA ATA CGA CCA AAT CT- 3′) used as an internal control, for determination of GSTT1 and GSTT1-null genotype. A 5 µL DNA template was amplified in a final volume of 25 µL amplification reaction of 30 cycles, using the Hot Start Taq 2X Master Mix (M0496L, BioLabs, New England) according to the manufacturer's instructions. The primers used for GSTT1 include Forward: 5′- TCT GCC GCC CGA AAC CTT- 3′ and Reverse: 5′- ACG TCC TCT TGT CCC CCA TTC- 3′ (Sanyal *et al*., 2003)

3.7.3 Genotyping for *IL 4* and *IL 13* Genes

A total of 108 participants were screened for polymorphisms in interleukin (*IL*) *4* and *IL 13* genes. PCR for IL-13 and IL-4 SNPs was performed using different amplifying methods enhanced optimal amplification (Table 3.1).

PCR for IL-13 -1055 C/T was conducted in a 25 µl reaction containing 100 ng DNA Initial denaturation was performed at 95^{o}C for 3 min followed by 30 cycles of PCR with the following conditions: 95^{o}C for 30 sec, 62^{o}C for 30 sec for annealing, 7^{o}C for 1min, and a final 72^{o}C for 3 min. This was done using the Hot Start Taq 2X Master Mix (M0496L, BioLabs, New England).

PCR for IL-13 -591 A/G was conducted in a 25 µL reaction containing 100 ng DNA and was done using the Hot Start Taq 2X Master Mix (M0496L, BioLabs, New England). Initial denaturation was performed at 94^{o}C for 5 min followed by 30 cycles of PCR with the following conditions: 94^{o}C for 1 min, 61^{o}C for 45 sec for annealing, 72^{o}C for 45 sec, and a final 72^{o}C for 3 min.

PCR for IL-13 +130 G/A was conducted in a 25 µL reaction containing 100 ng DNA and the PCR reaction was done using the Hot Start Taq 2X Master Mix (M0496L, BioLabs, New England). Initial denaturation was performed at 94^{o}C for 5 min followed by 34 cycles of PCR with the following conditions: 94^{o}C for 1 min, 60^{o}C for 45 sec for annealing, 72^{o}C for 45 sec, and a final 72^{o}C for 3 min.

PCR for IL-4 -590 C/T was conducted in a 25µL reaction containing 100 ng DNA, using the Hot Start Taq 2X PCR Master Mix (M0496L, BioLabs, New England). Initial denaturation was performed at 95^{o}C for 5 min followed by 30 cycles of PCR with the following conditions: 94^{o}C for 30 sec, 59^{o}C for 30 sec for annealing, 72^{o}C for 30 sec, and a final 72^{o}C for 3 min.

3.8 Biomarker Discovery

3.8.1 Sample preparation and In solution protein digestion

A total of 49 individual urine samples were placed into four different categories, namely 12 Schistosomiasis cases (SH), 12 bladder pathology cases (BP/PT), 15 combined pathology and schistosomiasis (PS) cases and 10 controls (no pathology and Schistosomiasis- NPS) Table 3.2. Four milliliter (4 mL) of urine was used for methanol-chloroform precipitation followed by in solution tryptic digestion prior to mass spectrometry (MS) analysis. Precipitated protein was resuspended in denaturation buffer (6M urea, 2M thiourea, 10 mM Tris buffer, pH8.0), and then a Bradford assay was carried out to determine protein concentration (Adeola *et al*., 2015). 100µg of protein was further reduced by incubation at room temperature for 1 hour in reduction buffer (1M dithiothreitol (DTT); 50mM ammonium bicarbonate-ABC). An alkylating buffer (550 mM iodoacetamide (IAA); 50 mM ABC) was then added to protein and incubated in the dark at room temperature for an hour. The sample was then diluted with 4 volumes of 50mM ABC and proteolysed overnight for 16 hours at 37^{o}C using Trypsin-Ultra^{TM}, Mass Spectrometry Grade (New England BioLabs) according to the manufacturer's instructions. An equivalent of 10µg of the peptide solution was then transferred to in house prepared stage tips for off-line SPE, desalting, and clean-up of sample as described in Rappsilber *et al.,* (2007); Adeola *et al*., (2015) and the desalted peptides were then dried in a refrigerated speedy vac (SPD 111v-230 Speed VAC Thermo Savant, New York, USA).

Table 3.1: The set of primers used to genotype for *IL 4* and *IL 13* genes in the study

S/N	Primer Name	Primer Sequence	Source
1	IL13 -1055 C/T	Forward 5′-ATGCCTTGTGAGGAGGGTCAC-3′	
		Reverse 5′-CCAGTCTCTGCAGGATCAACC- 3′	
2	IL13 -591 A/T	Forward 5′-CCAGCCTGGCCCAGTTAAGAGTTT-3′	
		Reverse 5′-CTAATTCCTCCTTGGCCCCACT- 3′	Saric *et al.*, 2008
3	IL13 +130 G/A	Forward 5′-TGGCGTTCTACTCACGTGCT-3′	
		Reverse 5′-CAGCACAGGCTGAGGTCTAA- 3′	
4	IL4 -590 C/T	Forward 5′-ACTAGGCCTCACCTGATACG-3′	Gatlin *et al.*, 2009
		Reverse 5′-GTTGTAATGCAGTCCTCCTG-3′	

3.8.2 Ultra-High Performance Liquid Chromatography (HPLC)

Peptide samples were resuspended by diluting the desalted, dried peptides to 200 ng/µL using 2% acetonitrile (ACN) in HPLC grade water containing 0.1% v/v formic acid (FA) before MS analysis. Nanoflow ultra-HPLC was carried out on a Dionex UltiMate R 3500 RSnano UPLC system (Thermo Fisher, San Jose, CA, USA) using an RP precolumn trap (100 µm×2 cm, 5 µm, 100 A° , C-18) and analytic column (70 µm × 20 cm, 5 µm,100 A° , C-18). Gradient chromatography was carried out at 23^0C with a flow rate of 300 nL/min and peptides were eluted with a 6–40% gradient of water–ACN from 0 to 120 min. The binary mobile phase system used was as follows: buffer A contained water and 0.1% FA, while buffer B contained ACN and 0.1% FA. The elution gradient for peptides was 6% B from 10min to 40% B at 60 min, then increasing to 80% B for 10min before returning to 2% B for equilibration.

3.8.3 Mass spectrometry conditions

Discovery proteomic analysis of each sample was carried out on a QExactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher). Analysis of samples introduced from the in-line HPLC system was achieved with the following system settings: Acquisition of mass spectra (MS) was done at a resolution of 70 000 with a maximum injection time of 250 ms or a target automatic gain control value of 3×10^6. High-energy collision dissociation and normalized collision energy set at 27 were used for peptide fragmentation. Continuous tandem mass spectra acquisition resolution was set at 17 500 at a maximum injection time of 120 ms or target value of 2×10^5 ions. Data-dependent automated full scan cycles were performed with automatic switching between MS/MS and MS scans at a scan range of 300–1650 *m/z*. The top ten most abundant precursor ions selected by the quadrupole during the initial MS scan were subjected to fragmentation using in-source high-energy collision dissociation with normalized collision energy at a pressure of 1.2 mTorr and a dynamic exclusion time of 30 s. The abundance threshold for ion selection was 0.001 with charge exclusion of $z = 1$ ions.

Table 3.2: Characteristics of the sample subgroups used for the Mass spectrometry study

Sample Group	Gender	(N=49)	*S. haematobium* infection	Bladder Pathology
	Male	8		
PS	Female	7	Present	Present
	Male	5		
SH	Female	7	Present	Absent
	Male	5		
PT	Female	7	Absent	Present
	Male	4		
NPS	Female	6	Absent	Absent

Legend

SH-Schistosomiasis

PT (BP)-Bladder Pathology

PS-Pathology and Schistosomiasis

NPS-No Pathology and Schistosomiasis (controls)

3.8.4 Mass spectrometry data processing and statistical analysis

All raw MS Xcalibur files acquired were analysed using the default settings of MaxQuant software (version 1.5.3.12) with minor modifications as described in detail elsewhere (Adeola *et al.*, 2015). MaxQuant results for proteins groups, peptides and evidences were retrieved from the combined output folder in text format. The label – free quantification (LFQ) values from MaxQuant results were imported into Perseus software (version 1.5.3.1) for differential expression statistical analysis and visualization. Several normalization techniques were applied which include scatter plot smoothing, global adjustment and logarithmic data transformation. Three separate independent t-tests were carried out, with Bonferroni correction using LFQ data for NPS versus SH, NPS versus PT and NPS versus PS. One way ANOVA was also carried out to statistically validate the differentially expressed potential biomarkers. Venn diagrams were plotted using an online based applicationVENNY[2.1] (http://bioinfogp.cnb.scic.es/tools/venny). Proteins which were determined to be significantly differentially expressed between groups were further subjected to a GO-enrichment analysis using Blast2GO (Conesa *et al.*, 2005; Sotillo *et al.*, 2015) (Figure 3.4).

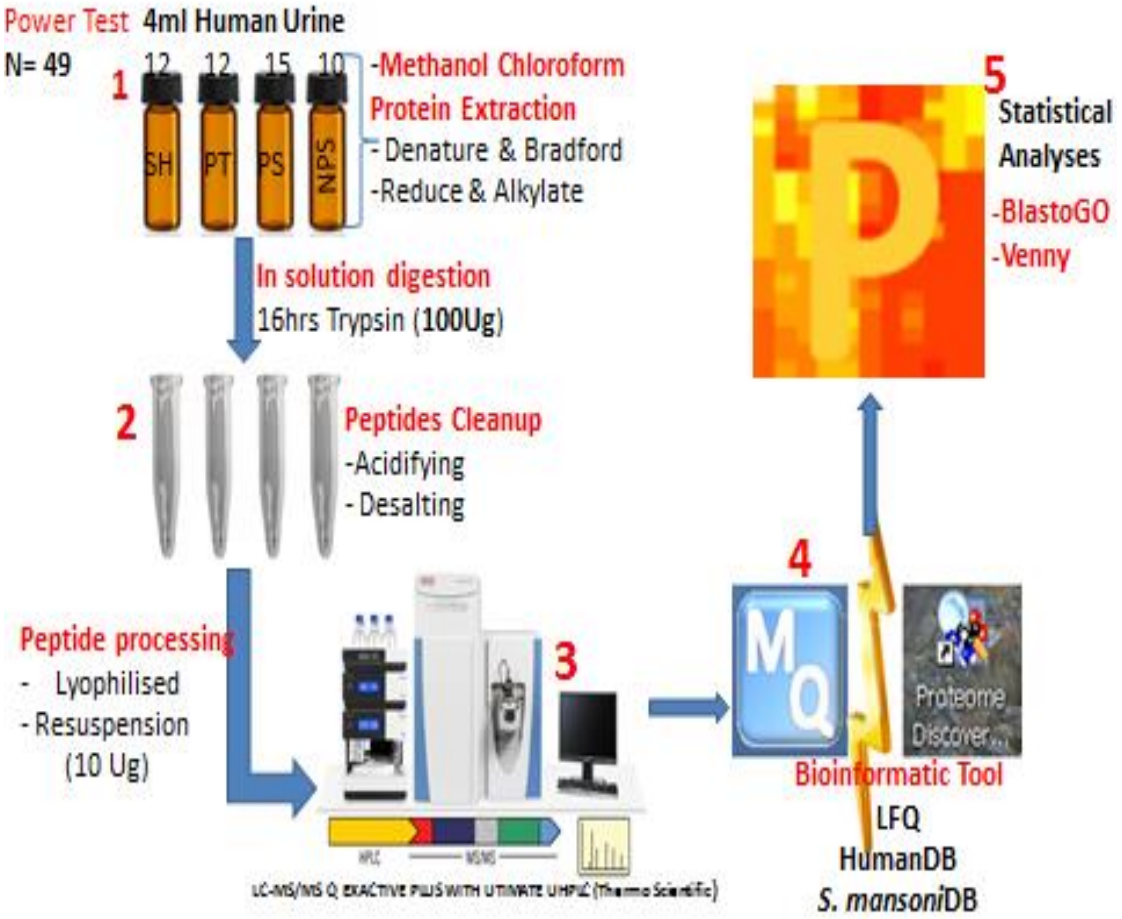

Figure 3.4: Workflow for biomarkers discovery study design.

The study was carried out in two phases (Field sampling and Laboratory experiments). The abbreviations represent different sample groups namely: SH- *S. haematobium* infected groups, PT- Bladder Pathology group, PS- group with combination of pathology and *S. haematobium* infection and NPS- No pathology and schistosomiasis (control group)

CHAPTER FOUR

4 RESULTS

4.1 Prevalence of urinary schistosomiasis and bladder pathology

A total of 371 participants (130 males and 241 females) aged 30-90 years were recruited for the study. While 369 were eventually screened for *S. haematobium* infection and 362 for bladder pathologies, some of the volunteers were excluded from the study using the exclusion criteria (Table 4.1a). The mean age of participants was 48.6 ±0.6 years. The overall prevalence of *S. haematobium* in the sampled population was 29.3% (108/369), 42 (11.4%) in males and 66 (17.9%) in females (Table 4.1a and 4.2). The highest prevalence of infection was observed in participannts over 60 years old 31/108 (28.7%). The majority of those positive for *S. haematobium* had a light intensity of infection 80 (74.1%) with the egg mean intensity of 33.3 ±0.04 eggs/10mL. Eggua community had the highest prevalence of infection 58 (16.0%), while Ibeku 9 (2.5%) had the least prevalence of infection (Table 4.1b). The Yewa river was the main source of water for most (81.3%) of the participants infected with *S. haematobium* (Figure 4.4).

Bladder pathologies were observed in 32.3% (117/362) of sampled population and included abnormal bladder wall thickness which the most common abnormality 29.0% (104/362), abnormal bladder shape 7.1% (26/362), bladder wall irregularities 26/362 (7.1%), bladder masses 11/362 (3.1%), and bladder calcification 8/362 (2.2%) (Table 4.5; Figure 4.1a-4.1c). Among the participants, 63 (57.9%) with bladder pathologies also had existing schistosomiasis, 17/63 (27%) of which were heavy intensity and 46/63 (73.0%) were light intensity. Thus, there was an association between urinary tract pathology and *S. haematobium* infection (χ^2 = 45.451, df= 2, *P*= 0.001). Furthermore, the statistical odd ratios revealed lower risk of bladder pathology among participants without *S. haematobium* (OR= 0.5, 95% CI 0.4-0.7) (Table 4.3).

Among participants with structural bladder pathologies, 47/117 (39.8%) were male and 70/117 (60.2%) females. Mild bladder pathology was more common than severe bladder pathology in this study and was found in 109/117 (93.1%) of the participants. The highest frequency of urinary tract pathology was observed in participants over 60 years old 35 (29.9%) (Table 4.4). There was higher incidence of bladder pathologies among female participants (Table 4.4); bladder calculi and hydronephrosis were also seen only in female participants (Table 4.5). Hydronephrosis (figure 4.2a-c) was present in only four participants with light infection while calcification was identified in only one participant with heavy infection. No bladder polyp was detected. There was no significant relationship between cigarette smoking and bladder pathology in the study and very few 42 (36.2%) participants with bladder pathology admitted to consuming alcohol (Figure 4.3).

4.2 Genetic susceptibility to bladder pathology and schistosomaisis

Amplification of GSTM1 and GSTT1 genes yielded 500bp (figure 4.5a-e) and 400bp (figure 4.6a-c) fragments, respectively among sampled population. The GSTMI amplicons were found in 36 (30.8%) pathology cases and 7 (7.1%) control samples while GSTT1 was found in 20 (17.1%) pathology cases and 4 (4.1%) control samples. Most of the participants with bladder pathologies had GSTM1 81 (69.2%) and GSTT1 97 (82.9) null genotype respectively. These revealed an elevated risk of bladder pathologies for participants carrying either the GSTM1 (OR= 4.3, 95% CI 2.0-9.2) and GSTT1 (OR= 4.2, 95% CI 1.5-12) polymorphic null genotypes when compared to those with the corresponding genotype. However there were wide confidence intervals (Table 4.6).

The risks associated with combined enzyme activities of the GSTM1 and GSTT1 was also examined and the risk with having either one active (OR= 3.4, 95% CI 1.9-6.2) was greater than having both null (OR= 0.6, 95% CI 0.6-0.8). There was no distinctive risk found in pathology intensity with any of the GSTs genotype when the combined variant and active genotype were considered but there was a slight risk with having mild bladder pathology in participants with both inactive variants (both null genotype) as compared to those with either one alone (OR= 1.2, 95% CI 0.7-2.4). The association of schistosomiasis and smoking in development of bladder pathology

among participants was examined within genotypes. It showed *S. haematobium* infected participants had more variant GSTM1 73.1% (OR= 1.7, 95% CI 1.0-3) and GSTT1 85.9% (OR= 1.5, 95% CI 0.7-3.1) null polymorphisms, revealing a slight risk of bladder pathology (Table 4.7). The case was different among smokers with null genotype for the GSTM1 and GSTT1 genotype with higher risk of bladder pathology (OR= 3, 95% CI 1-7, *P*= 0.05; OR= 4, 95% CI 1-11, *P*= 0.006 respectively). When the combination of GSTM1 and GSTT1 genotype was considered for smokers and *S. haematobium* infected participants, bladder pathology risk was only observed in participants with one active genotype both in smokers and participants with schistosomiasis (Table 4.7). There was slight risk of bladder pathology among smokers and *S. haematobium* infected participants with GSTT1 and GSTM1 null polymorphism (Table 4.8). These risks were significant among non-smokers with GSTT1 (OR= 1.6, 95% CI 1.1-2.1, *P*= 0.02) and GSTM1 (OR= 1.8, 95% CI 1.4-2.2, *P*= 0.001) null genotype in the population studied. Participants with or without *S. haematobium* infection having the null GSTT1 and GSTM1 genotype also had a relatively small elevation in risk while some differences in the magnitude of risk associated with *S. haematobium* infection was apparent between polymorphism variants of GSTM1 genotype (OR 7.8, 95%CI 0.9-63.1, *P*= 0.02).

None of the *S. haematobium* infection cases amplified for *IL 13 +130* and *IL 13 -591* gene polymorphisms while genetic polymorphism in *IL 4 -590* and *IL 13 -1055* was found in some of the study infected participants. *IL 4 -590* and *IL 13 -1055* amplified at 200bp and 230bp, respectively (figure 4.7a-d). Only 10 samples from infected participants (9.3%) amplified for *IL 13 -1055* polymorphism when analyzing for genetic susceptibility to schistosomiasis, and this showed no risk of infection in males 3 (7.5%) (OR 0.7, 95% CI 0.3-2.1) and slight risk in females 7 (10.3%) (OR 1.1, 95% CI 0.7-1.7). Among 26 (24.1%) *S. haematobium* infected participants with *IL 4 -590* polymorphisms, slight risk of infection was found both in male 10 (25%) (OR 1.05, 95% CI 0.5-01.8) and female 16 (23.5%) (OR 1.05; 95% CI 0.5-01.8) participants (Table 4.9). Extremely low number of light and heavy infection cases 19 (23.7%) and 7 (25.0%) were found to be polymorphic for *IL 4 -590* genes respectively and this was

also the case in *IL 13 -1055* polymorphism found in 7 (8.8%) and 3 (10.7) cases with light and heavy infection respectively.

4.3 Proteomic biomarkers

Sample size power calculation carried out for the proteomic section of the study showed that 44 individual samples (*N*) were required for a statistical power 0.9 at significance level 0.05. Hence, urine samples from 49 individuals distributed across four groups (Schistosomiasis, Bladder Pathology (BP/PT), Pathology and Schistosomiasis (PS) and No Pathology and Schistosomiasis- NPS (controls)) were analysed to identify potential biomarkers for schistosomiasis and its associated pathologies (Table 3.2). High levels of correlation between the urinary protein components of these sample groups was demonstrated by scatterplots, hierarchical clustering (Heatmap) and principal component analysis (PCA) (Figure 4.15). However, hierarchical clustering of proteins groups identified against "SH, PT and PS" and "NPS" samples showed clear molecular differences between groups. As expected, differences in the proteomic signature were seen between the control group (NPS) and all disease groups and all four disease groups clustered distinctly. The SH and PS group were more closely related to one another compared to the PT samples (Figure 4.15A and B), although few samples of the pathology group clustered proximally to the control group (Figure 4.15B).

In order to assess the technical variability of the analysis, the expression of proteins which are expected to be stably expressed in urine samples regardless of disease state were examined. The expression of uromodulin (UMOD), mannan-binding lectin (MASP) and hemoglobin subunits (HBA1) was found to be dependable as internal standards for normalization of protein quantification within groups, as shown by protein profile plots of LFQ values in the experiments. However, uromodulin and MASP were found to be more abundant in normal healthy (NSP) group compared to the disease group while HBA1 was more abundant in the diseased group. These proteins were therefore suitable for within group normalisation, but not between groups.

A total of 213182 spectra were acquired and used to assign peptides and unique protein group identities, leading to the identification of 1306 protein groups by MaxQuant software. 36 *Schistosoma* proteins were identified in the host urine when the MS output (Figure 4.8, 4.9, 4.10, 4.12) was searched against a combination of human and *Schistosoma* databases (Table 4.11), many of which are membrane proteins (Figure 4.12) and most function as binding proteins (Figure 4.16). These 36 identities were considered to be confident identities due to the relatively small size of the *Schistosoma* database compared to the human database in the combined database. More (124) parasite protein groups were observed when the MS output was searched against the Schistosoma DB only, but only 31 *Schistosoma* proteins were significant by multiple ANOVA using label-fee quantification (LFQ) values. Some *Schistosoma* specific proteins were found in samples from individuals earlier diagnosed and classified as negative for *S. haematobium* infection by microscopy (Figure 4.13B and 4.14).

Venn diagrams were generated using Venny to identify proteins unique to each group. Out of the 36 total *Schistosoma* protein groups confidently identified, 5 (15.6%), 4 (12.6%) and 2 (6.3%) proteins were unique to SH, PS and PT group respectively while only 8 proteins (25%) were found common to all study groups (Figure 4.13B). Heat shock protein 70, elongation factor 1-alpha, camp-response element binding proteins-related, histone H4 and venom allergen-like (VAL) 3 proteins were found to be unique to SH group while tubulin alpha chain, calreticulin autoantigen homolog, heat shock protein HSP 60 and putative adp ATP carrier protein were found only in PS group. Two (2) potential biomarkers unique to the PT group include cytoplasmic dynein light chain and putative actin 1. 13 (36.1%) of the predicted Schistosoma protein were membrane-associated, 8 (22.2%) nuclear based, 4 (11%) cytoplasmic and 3 (8.3%) cytoskeletal and mitochondrial, 1 (2.8%) ribosomal and 3 (8.3%) unknown.

Three independent t-tests with Bonferroni correction performed using LFQ values for "NPS versus SH", "NPS versus PT" and "NPS versus PS" revealed a total of 54 candidate human protein biomarkers for schistosomiasis and bladder pathology. The proteins are distributed into 43, 8 and 7 for "NPS versus SH", "NPS versus PT" and

"NPS versus PS" respectively (Table 4.12, 4.13 and 4.14 respectively). 37 and 2 proteins were unique to SH and PT groups respectively while none were unique to the PS group (Figure 4.13A). A search for possible marker overlap across study groups showed that cathepsin B (P07858) was shared by all disease groups; arylsulfatase A (A0A0C4DFZ2) and phosphatidylethanolamine-binding protein 4 (Q96S96) were shared by PS and SH group; and PT and SH groups were found to have 4 proteins in common namely: transthyretin (P02766), plasma retinol-binding protein (Q5VY30), phosphatidylcholine-sterol acyltransferase (P04180), cartilage intermediate layer protein 2 (K7EPJ4). The majority of the human proteins identified were predicted to be membrane associated and perform "binding" molecular activities (Figure 4.16A-C). Some new human host proteins biomarkers were identified for schistosomiasis in this study and include sialidase-1, programmed cell death 1 ligand 2 (PD-1–PD-L 2), growth/differentiation factor 15 (GDF15)

Table 4.1a: Summary of studied parameters (*S. haematobium* infection, bladder pathology, gender and age range) in Eggua

S. haematobium Infection		Bladder Pathology		Gender		Age Range	
	No (%)		No. (%)		No. (%)		No. (%)
Heavy	28 (7.5)	**Mild**	109 (30.1)	**Male**	130 (35.0)	**35-39**	73 (19.7)
						40-44	78 (21)
Light	80 (21.8)	**Severe**	8 (2.21)	**Fema**	241 (65.0)	**45-49**	56 (15.1)
Total	108 (29.3)		117 (32.3)	-	371	**50-54**	86 (23.2)
						55-59	0 (0)
						60 and Above	78 (21)

Table 4.1b: Prevalence of schistosomiasis among the settlements at Eggua

Participants Residence	Status of *S. haematobium* infection		Total N (%)	Prevalence/ villages (%)
	Positive N (%)	Negative N (%)		
Eggua Central	58 (16.0)	149 (41.2)	207 (57.2)	28.0
Iganalade	10 (2.8)	27 (7.5)	37 (10.2)	27.0
Agbon-Ojodu	31(8.6)	56 (15.5)	87 (24.0)	35.6
Ibeku	9 (2.5)	18 (5.0)	27 (7.5)	33.3
Tata	0(0.0)	4 (1.1)	4 (1.1)	0
Total	108 (29.8)	254 (70.2)	362 (100.0)	

Table 4.2: Prevalence and intensity of schistosomiasis by sex and age group in Eggua, Nigeria

PARAMETERS		*S. haematobium* Infection				
		Status of Infection		Intensity of Infection		
		Positive (%)	Negative (%)	Heavy (%)	Light (%)	Total (%)
AGE		**χ^2 = 9.738, df= 4, P= 0.045**		**χ^2 = 1.717, df= 4, P= 0.788**		
RANGE	35-39	18 (4.9)	54 (14.4)	6 (1.6)	12 (3.3)	18 (4.9)
(Years)	40-44	14 (3.8)	63 (16.9)	4 (1.1)	10 (2.7)	14 (3.8)
	45-49	18 (4.9)	39 (10.4)	3 (0.8)	15 (4.1)	18 (4.9)
	50-54	27 (7.3)	59 (15.5)	6 (1.6)	21 (5.6)	26 (8.2)
	60 and Above	31 (8.4)	46 (12.5)	9 (2.4)	22 (6.0)	31 (8.4)
	Total	108 (29.3)	261 (70.7)	28 (7.5)	80 (21.8)	108 (29.3)
GENDER		**χ^2 = 369.852, df= 6, P= 0.001**		**χ^2 = 0.015, df= 1, P= 0.566**		
	Male	42(11.4)	89 (23.6)	11 (10.2)	31 (28.7)	42 (38.9)
	Female	66 (17.9)	172 (46.1)	17 (15.7)	49 (45.4)	66 (61.1)
	Total	**108 (29.3)**	257 (69.7)	28 (25.9)	80 (74.1)	108 (100)

Table 4.3: Distribution of bladder pathology with intensity of *Schistosoma haematobium* infection.

Bladder Pathology	Status of *S. haematobium* infection $\chi^2 = 45.451$, df=2, P= 0.001			
	Positive (%)	Negative (%)	[b]OR (95% [a]CI)	P (2 Tailed)
Present	63 (57.9)	54 (21.5)	1.00ref	0.001
Absent	45 (42.1)	197 (78.5)	0.5 (0.4-0.7)	
Total	108 (100)	251 (100)		

[a]Confidence Interval, [b]odd ratio

Table 4.4: Distribution of bladder pathology intensity in relation to age range, intensity of infection and gender

Parameters	Pathological Intensity		
AGE RANGE (Years)	**Mild Pathology**	**Severe Pathology**	
χ^2 = 2.047, df= 4, P= 0.727	**(%)**	**(%)**	**Total (%)**
35-39	13 (11.1)	0 (0)	13 (11.0)
40-44	18 (15.4)	1 (0.9)	19 (16.3)
45-49	15 (12.8)	2 (1.7)	17 (14.5)
50-54	30 (25.6)	3 (2.6)	33 (28.2)
60 and Above	33 (28.2)	2 (1.7)	36 (29.9)
Total	109 (93.1)	8 (6.7)	117
***S. haematobium* Intensity**			
χ^2 = 5.826, df= 2, P= 0.054			
Heavy	14 (12.0)	3 (2.6)	17 (14.6)
Light	41 (35.0)	5 (4.3)	46 (39.3)
No infection	54 (46.1)	0 (0)	55 (46.1)
GENDER			
χ^2 = 0.822, df= 1, P= 0.472			
Male	45 (40.9)	2 (25.0)	47 (39.8)
Female	64 (59.1)	6 (75.0)	70 (60.2)
Total	109 (100)	8 (100)	117

Table 4.5: Distribution of bladder pathology among gender and intensity of infection

Pathology	Gender			Intensity of infection		
	Male (%)	Female (%)	Total % within Gender	Heavy (%)	Light (%)	Total % within Infection
Bladder Calculi	0 (0.0)	8 (2.2)	8 (2.2)	1(0.9)	0 (0.0)	1 (0.9)
Hydronephrosis	0 (0.0)	4 (0.3)	4 (0.3)	0(0.0)	4 (3.7)	4 (3.7)
Abnormal Bladder Shape	14 (3.9)	12 (3.3)	26 (7.1)	10 (9.4)	13 (12.3)	23 (21.7)
Bladder Mass	6 (1.7)	5 (1.4)	11 (3.1)	7 (6.6)	3 (2.8)	10 (9.4)
Bladder Wall Thickness	43 (12.0)	61 (17.0)	104 (29.0)	15 (14.2)	37 (34.9)	52 (49.1)
Irregular bladder wall	14 (3.9)	12 (3.3)	26 (7.1)	10 (9.4)	13 (12.3)	23(21.7)
Polyps	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Hydrocalycosis	0 (0.0)	1 (0.3)	1 (0.3)	0 (0.0)	4 (3.7)	4 (3.7)

Table 4.6: Association between *GSTT1* and *GSTM1* polymorphisms and bladder pathology risk among study participants

Genotype	Pathology				Pathology Intensity			
	Cases N (%)	Control N (%)	OR[b] 95%CI[a]	*P* 2 tailed	Mild N (%)	Severe N (%)	OR[b] 95%CI[a]	*P* 2 tailed
GSTM1								
Active	36 (30.8)	7 (7.1)	1.00ref	0.001	34 (30.2)	2 (30.0)	1.00ref	1.00
Null	81 (69.2)	91 (92.9)	4.3 (2.0-9.2)		74 (69.8)	6 (70.0)	1.0 (0.4-2.7)	
GSTT1								
Active	20 (17.1)	4 (4.1)	1.00ref	0.002	18 (16.5)	2 (20.0)	1.00ref	0.63
Null	97 (82.9)	94 (95.9)	4.2 (1.5-12)		91 (83.5)	6 (80.0)	0.8 (0.2-3.1)	
Combined Genotype								
Both Active	7 (6.0)	0 (0)	1.00ref	0.1	7 (6.6)	0 (0)	1.00ref	0.49
Either Active	42 (35.9)	11 (11.2)	3.4 (1.9-6.2)		36 (34.0)	4 (50.0)	0.7 (0.4-1.4)	
Both Null	68 (58.1)	87 (88.8)	0.6 (0.6-0.8)		63 (59.4)	4 (50.0)	1.2 (0.7-2.4)	

[a]Confidence Interval, [b]odd ratio, ref-Reference

Table 4.7: Association between *GSTT1* and *GSTM1* polymorphisms within possible bladder pathology risk factors among study participants

Genotype	Status of *S. haematobium* infection				Cigarette Smokers			
	Positive N (%)	Negative N (%)	OR[b] 95%CI[a]	P 2 tailed	Smokers N (%)	Non-smokers N (%)	OR[b] 95%CI[a]	P 2 tailed
GSTM1								
Active	21 (26.9)	21 (15.4)	1.00ref	0.05	7 (41.2)	36 (18.2)	1.00ref	0.05
Null	57 (73.1)	115 (84.6)	1.7(1.0- 3.0)		10 (58.8)	162(81.8)	3 (1-70)	
GSTT1								
Active	11 (14.1)	13 (9.6)	1.00ref	0.369	6 (35.3)	18 (9.1)	1.00ref	0.006
Null	67 (85.9)	123 (90.4)	1.5 (0.7-3.1)		11 (64.7)	180 (90.9)	4 (1-11)	
Combined Genotype								
Both Active	3 (3.8)	4 (2.9)	1.00ref	0.06	1 (6.2)	6 (3.0)	1.00ref	0.691
Either Active	26(33.3)	26 (19.1)	1.7 (1.1-2.8)		3 (18.8)	50 (25.1)	1.6 (1-3)	
Both Null	49 (62.8)	106 (77.9)	0.8 (0.7-1.0)		12 (75.0)	143 (71.9)	0.8 (0.5-1)	

[a]Confidence Interval, [b]odd ratio, ref- Reference

Table 4.8: Relative risk estimates of bladder pathology associated with smoking and *S. haematobium* infection after stratification by genotype

Risk Factors	*GSTs* Genotype	Pathology Status Cases N (%)	Control N (%)	[b]OR (95% [a]CI)	P value (2 Tailed)
Cigarette Smoking					
GSTT1					
Smokers	Active	6 (40.0)	0 (0)	1.0ref	0.4
	Null	9 (60.0)	2 (100)	1.2 (0.9-1.6)	
Non Smokers	Active	14 (13.7)	4 (4.2)	1.0ref	0.02
	Null	88 (86.3)	92 (95.8)	1.6 (1.1-2.1)	
GSTM1					
Smokers	Active	7 (46.7)	0(0.0)	1.0ref	0.48
	Null	8 (53.3)	2 (100)	1.3 (0.9-1.7)	
Non-smokers	Active	29 (28.4)	7 (7.3)	1.0ref	0.001
	Null	73 (71.6)	89 (92.7)	1.8	
***S. haematobium* Infection**					
GSTT1					
Positive	Active	10 (16.4)	1 (5.9)	1.0ref	0.43
	Null	51 (83.6)	16 (94.1)	1.2 (0.9-1.5)	
Negative	Active	10 (18.2)	3 (3.7)	1.0ref	0.007
	Null	45 (81.8)	78 (96.3)	2.1 (1.4-3.1)	
GSTM1					
Positive	Active	20 (32.8)	1 (5.9)	1.0ref	0.02
	Null	41 (67.2)	16 (94.1)	7.8 (0.9-63.1)	
Negative	Active	15 (27.3)	6 (7.4)	1.0ref	0.002
	Null	40 (72.7)	75 (92.6)	4.6 (1.7-13.0)	

[a]Confidence Interval, [b]odd ratio, ref- Reference

Table 4.9: Estimates of cytokines in susceptibility to schistosomiasis after stratification by gender

Cytokines			GENDER Male N (%)	GENDER Female N (%)	Total N (%)
IL 4					
χ^2 = 0.03, df= 1, *P*= 1.0					
S. haematobium Positive	IL 4 -590 C/T	Present	10 (25.0)	16 (23.5)	26 (24.1)
		Absent	30 (75.0)	52 (76.5)	82 (75.9)
		Total	40 (100.0)	68 (100.0)	108 (100.0)
		OR (95% CI)	**1.05 (0.5-1.8)**	**0.97 (0.7-1.4)**	
IL 13					
χ^2 = 0.23, df= 1, *P*= 0.74					
S. haematobium	IL 13 -1055 C/T	Present	3 (7.5)	7 (10.3)	10 (9.3)
		Absent	37 (92.5)	61 (89.7)	98 (90.7)
		OR (95% CI)	**0.7 (0.3-2.1)**	**1.1 (0.7-1.7)**	

Table 4.10: Estimates of cytokines in susceptibility to schistosomiasis after stratification by gender

Cytokines	Intensity of *S. haematobium* infection		Status of *S. haematobium*
IL 4- 590 C/T GENE	Heavy Infection	Light Infection	(Total)
χ^2 = 0.03, df= 1, *P*= 0.9	N (%)	N (%)	N (%)
Present	7 (25.0)	19 (23.7)	26 (24.1)
Absent	21(75.0)	61(76.3)	82 (75.9)
Total	28 (100.0)	80 (100.0)	108 (100.0)
IL 13 -1055 C/T GENE			
χ^2 = 0.95, df= 1, *P*= 0.7			
Present	3(10.7)	7 (8.8)	10 (9.3)
Absent	25 (89.3)	73 (91.2)	98 (90.7)

Table 4.11: Identified schistosome proteins (36) across all urine sample groups, and their predicted functions.

Protein ID	Identified Schistosome Protein	PEP Scores	Location	Predicted Functions
C4Q4S5	Tubulin alpha chain	1.86E-18	Cytoskeleton	Structural/GTPase activity
C4Q5I7	Calreticulin autoantigen homolog	0.00034	Mitochondria	Binding
C4QBN1	Histone H4	0.002522	Cytosol /Nucleus	Binding
G4LWI2	Heat shock protein HSP60	0.001447	Cytoplasm	Heat Shock protein
G4LYN4	ADP-ribosylation factor, arf	0.000271	Membrane	Transporter
G4M1M0	DNA polymerase	0.000801	Nucleus	Binding and catalytic
G4V6R4	Putative rab9	6.42E-05	Membrane	Binding
G4V8L4	Putative heat shock protein 70	3.61E-43	Cytosol /Nucleus	Binding/Heat Shock protein
G4V910	Putative heat shock protein 70 (Hsp70)	0.001976	Cytosol /Nucleus	Binding/Heat Shock protein
G4V8L4	Putative heat shock protein 70	2.60E-29	Cytosol /Nucleus	Binding/Heat Shock protein
G4VAC9	Putative uncharacterized protein	0.001085	Unknown	Unknown
G4VAD2	Elongation factor 1-alpha	1.72E-36	Cytoplasm	Binding/GTPase activity
G4VAW0	Serine/threonine kinase	4.72E-05	Nucleus	Binding
G4VB75	cytoplasmic dynein light chain	0.000763	Cytoskeleton	Structural/Motor
G4VB79	Voltage-gated potassium channel, KCNQ	0.001114	Membrane	Transmembrane Transporter
G4VDD2	Eukaryotic translation initiation factor 5A	0.000224	Ribosome	Binding
G4VG19	Phosphoglycerate kinase	1.25E-43	Mitochondria	Kinase
G4VG20	Phosphoglycerate kinase	2.68E-07	Mitochondria	Kinase
G4VGA0	Sodium/potassium-transporting ATPase subunit alpha	0.000264	Membrane	Transporter
G4VH98	Putative fimbrin/plastin	0.002	Unknown	Binding
G4VHN3	ATP synthase subunit beta	7.63E-34	Membrane	Transporter/ Bindng
G4VIM7	Camp-response element binding protein-related	0.000471	Nucleus	Binding
G4VKT8	Putative atp synthase alpha subunit vacuolar	0.000308	Membrane	Transporter/ Binding

Table 4.11 (Continuation): Identified schistosome proteins across all urine sample

Protein ID	Identified Schistosome Protein	PEP Scores	Location	Predicted Functions
G4VLJ0	ATP synthase subunit alpha	0.000104	Membrane	Transporter/ Binding
G4VLJ8	Fidgetin like-1	0.000756	Nucleus	Binding and catalytic
G4VLN5	Putative uncharacterized protein	3.70E-70	Unknown	Binding
G4VLW1	Putative actin	6.14E-19	Membrane	Binding
G4VLW2	Putative actin-1	2.35E-33	Membrane	Binding
G4VM26	Putative zinc finger protein	0.000636	Nucleus	Binding
G4VMG4	Venom allergen-like (VAL) 3 protein	0.001866	Membrane	Unknown
G4VMT3	Adapter-related protein complex 3, beta subunit	0.001906	Membrane	Transporter
G4VP51	Putative adp,ATP carrier protein	0.000161	Membrane	Structural/ Transporter
G4VPE8	Putative inorganic pyrophosphatase	6.22E-23	Cytoplasm	Binding
G4VPU8	Putative cytoplasmic dynein intermediate chain 2	9.23E-05	Cytoskeleton	Structural/Motor
G4VQ01	Uncharacterized protein	0.00185	Membrane	Unknown
G4VQ58	Phosphopyruvate hydratase	8.38E-06	Cytoplasm	Binding

groups, and their predicted functions.

*A posterior error probability (PEP) score cutoff of <0.01 was applied to all protein identities in order to ensure confident protein assignments.

Table 4.12: Identified human proteins (43) and predicted functions for target purposes among individuals infected with *Schistosoma haemtobium*

Protein ID	Identified Human Protein	PEP Scores	Location	Predicted Functions
Q99519	Sialidase-1	2.68E+08	Membrane	Binding
A0A075B6I5	Ig lambda chain V-I region NIG-64;Ig lambda chain V-I region BL2	9.46E+08	Membrane Associated	Binding/Immune
Q9BQ51	Programmed cell death 1 ligand 2	4.76E+08	Membrane	Binding/Immune
P09467	Fructose-1,6-bisphosphatase 1	4.59E+08	Cytosol/Nucleus	Binding
Q96L35	Receptor protein-tyrosine kinase;Ephrin type-B receptor 4	4.73E+08	Cytoplasm/Mitochondria	Kinases/Binding
Q08174	Protocadherin-1	6.57E+08	Membrane	Binding
P21796	Voltage-dependent anion-selective channel protein 1	4.55E+08	Membrane/Mitochondria	Transport/Binding
A0A087WXM8	Basal cell adhesion molecule	6.27E+08	Membrane Associated	Binding
A0A087X2B5	Basigin	5.07E+08	Membrane	
Q12794	Hyaluronidase-1	3.94E+08	Lysosome	Other Enzymatic
Q13332	Receptor-type tyrosine-protein phosphatase S;Protein-tyrosine-phosphatase	6.35E+08	Membrane	Other Enzymatic
A6NNI4	Tetraspanin;CD9 antigen	4.59E+08	Membrane	Binding/Immune
Q86UN3	Reticulon-4 receptor-like 2	4.69E+08	Membrane	Binding/Immune
P01701	Ig lambda chain V-I region NEW	8.4E+08	Membrane	Binding/Immune
Q99988	Growth/differentiation factor 15	1.17E+09	Exosome	Binding/Immune
P07858	Cathepsin B;Cathepsin B light chain;Cathepsin B heavy chain	1.19E+09	Membrane	Binding

Table 4.12 continuation: Identified human proteins and predicted functions for target purposes among individuals infected with *Schistosoma haemtobium*

Protein ID	Identified Human Protein	PEP Scores	Location	Predicted Functions
Q15113	Procollagen C-endopeptidase enhancer 1	2.59E+09	Membrane	Binding
P53634	Dipeptidyl peptidase 1;Dipeptidyl peptidase 1 exclusion domain chain	1.43E+09	Endoplasmic Recticulum/Golgi Apparatus	Binding/Enzymatic
Q96NY8	Nectin-4;Processed poliovirus receptor-related protein 4	1.98E+09	Cytoskeleton/Membrane	Binding
Q9HCN6	Platelet glycoprotein VI	1.91E+09	Membrane	Binding
Q9NZH0	G-protein coupled receptor family C group 5 member B	7.23E+08	Membrane/Nucleus	Binding
O00560	Syntenin-1	1.24E+09	Membrane	Binding
Q92520	Protein FAM3C	9.39E+08	Membrane Associated	Immune
Q8NBJ4	Golgi membrane protein 1	8.67E+08	Membrane Associated	
P04180	Phosphatidylcholine-sterol acyltransferase	1.19E+09	Extracellular	Enzymatic
K7EPJ4	Cartilage intermediate layer protein 2;Cartilage intermediate layer protein 2 C1	1.04E+09	Membrane	Unknown
P05109	Protein S100-A8;Protein S100-A8, N-terminally processed	1.19E+10	Membrane /cytosol	Binding
O94919	Endonuclease domain-containing 1 protein	1.24E+10	Membrane	Binding/Enzymatic
P02766	Transthyretin	4.96E+09	Cytoplasm	Binding

Table 4.12 continuation: Identified human proteins (43) and predicted functions for target purposes among individuals infected with *Schistosoma haemtobium*

Protein ID	Identified Human Protein	PEP Scores	Location	Predicted Functions
Q16777	Histone H2A type 2-C	8.63E+09	Nucleus	Binding
A0A087WV17	Osteoclast-associated immunoglobulin-like receptor	5.67E+09	Membrane	Binding/Immune
Q5VY30	Retinol-binding protein 4;Plasma retinol-binding protein(1-182)	2.34E+09	Membrane	Transporter/Immune
H0Y755	Low affinity immunoglobulin gamma Fc region receptor III-A	3.96E+09	Membrane/cytoskeleton	Transporter/Immune
Q8NFZ8	Cell adhesion molecule 4	2.09E+09	Membrane	Binding
A0A087WZR4	Low affinity immunoglobulin gamma Fc region receptor III-B	2.11E+09	Membrane/cytoskeleton	Transporter/Immune
Q9H8L6	Multimerin-2	2.72E+09		
A0A0C4DFZ2	Arylsulfatase A;Arylsulfatase A component B and C	1.16E+09	Membrane/ER	Binding
F6X2W2	Neuronal growth regulator 1	3.44E+09	Membrane	Transporter/Binding
P09603	Macrophage colony-stimulating factor 1	6.79E+08	Membrane/Cytoplasm	Binding/Immune
Q96S96	Phosphatidylethanolamine-binding protein 4	1.9E+08	Cytoplasm/Mitochondria	Binding
P31946	14-3-3 protein beta/alpha	3.77E+08	Membrane	Binding
A0A087X0D5	Pro-cathepsin H	5.06E+08	Cytoplasm/Nucleus	Enzymatic
P09619	Platelet-derived growth factor receptor beta	2.68E+08	Membrane	Binding

Table 4.13: Identified human proteins (8) and predicted functions for target purposes among individuals infected with structural bladder pathology (PT)

Protein ID	Identified Human Protein	PEP Scores	Location	Predicted Functions
A0A0G2JM94	Leukocyte-associated immunoglobulin-like receptor 1	1.1E+10	Membrane	Binding/Immune
P02766	Transthyretin	1.24E+10	Membrane	Binding/Immune
Q5VY30	Retinol-binding protein 4;Plasma retinol-binding protein(1-182);Plasma retinol-binding protein(1-181);Plasma retinol-binding protein(1-179);Plasma retinol-binding protein(1-176)	5.67E+09	Cytoplasm	Binding
A0A0C4DFZ2	Arylsulfatase A;Arylsulfatase A component B;Arylsulfatase A component C	2.72E+09	Nucleus	Enzymatic
P07858	Cathepsin B;Cathepsin B light chain;Cathepsin B heavy chain	1.19E+09	Membrane	Binding
P04180	Phosphatidylcholine-sterol acyltransferase	1.19E+09		
K7EPJ4	Cartilage intermediate layer protein 2;Cartilage intermediate layer protein 2 C1;Cartilage intermediate layer protein 2 C2	1.04E+09	Membrane	Unknown
P98160	Basement membrane-specific heparan sulfate proteoglycan core protein;Endorepellin;LG3 peptide	9.34E+10	Membrane	Binding

*A posterior error probability (PEP) score cutoff of <0.01 was applied to all protein identities in order to ensure confident protein assignments.

Table 4.14`: Identified human proteins (3) and predicted functions for target purposes among individuals with combined structural bladder pathology and Schistosoma infection (PS)

Protein ID	Identified Human Protein	PEP Scores	Location	Predicted Functions
A0A0C4DFZ2	Arylsulfatase A;Arylsulfatase A component B;Arylsulfatase A Component C	2.72E+09	Membrane/ER	Binding
Q96S96	Phosphatidylethanolamine-binding protein 4	6.79E+08	Membrane Associated/ Lysosome	Binding
P07858	Cathepsin B;Cathepsin B light chain;Cathepsin B heavy chain	1.19E+09	Membrane	Binding

*A posterior error probability (PEP) score cutoff of <0.01 was applied to all protein identities in order to ensure confident protein assignments.

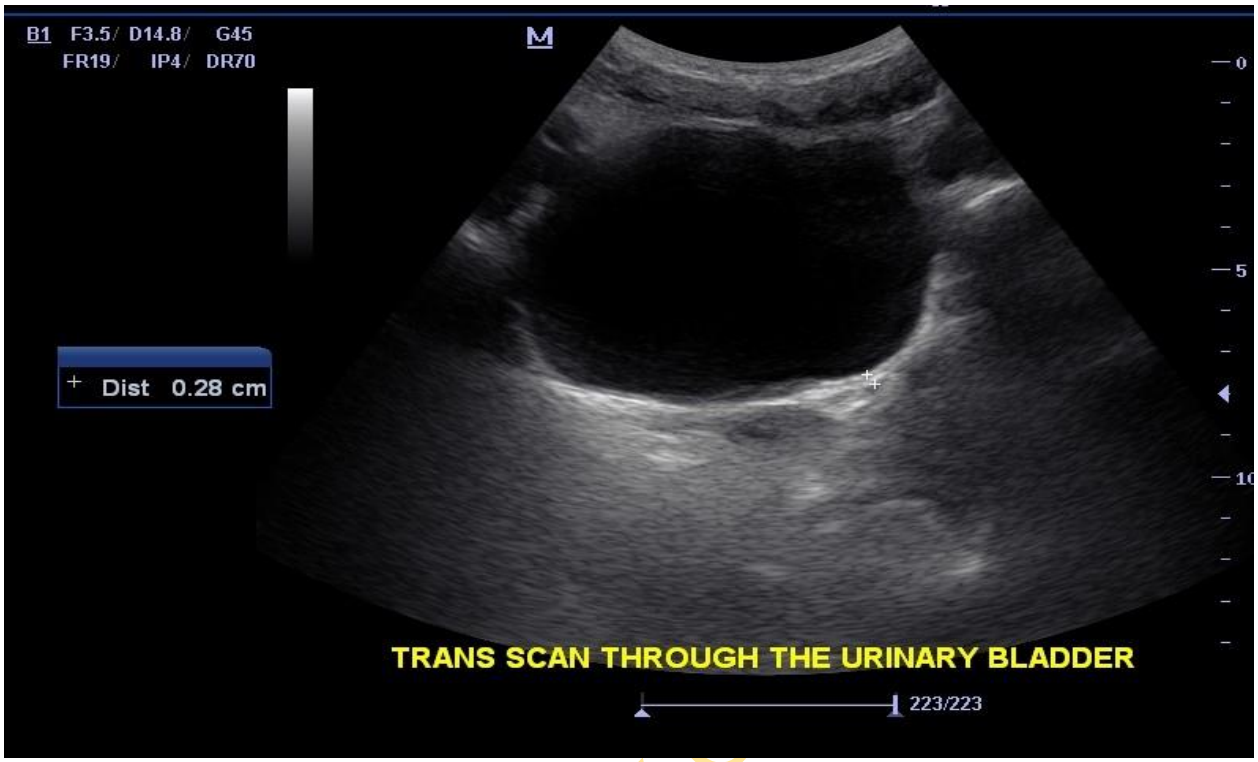

Figure 4.1a: A B mode ultrasound of the bladder showing a fully extended bladder with no pathology

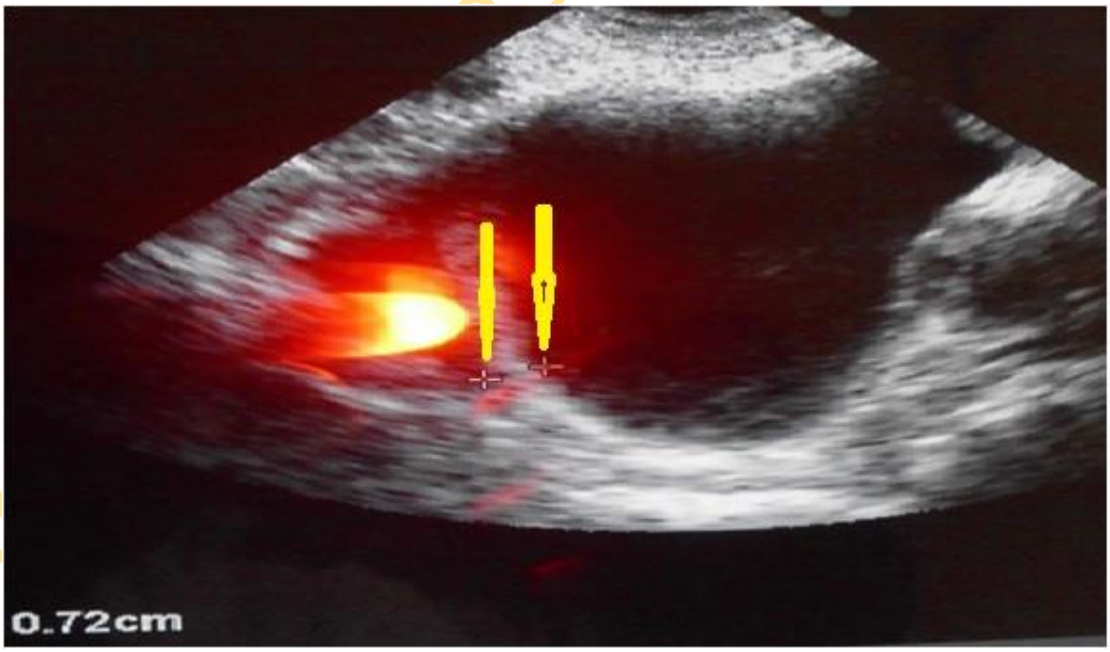

Figure 4.1b: A B mode ultrasound of the bladder showing a thickened bladder wall (arrows)

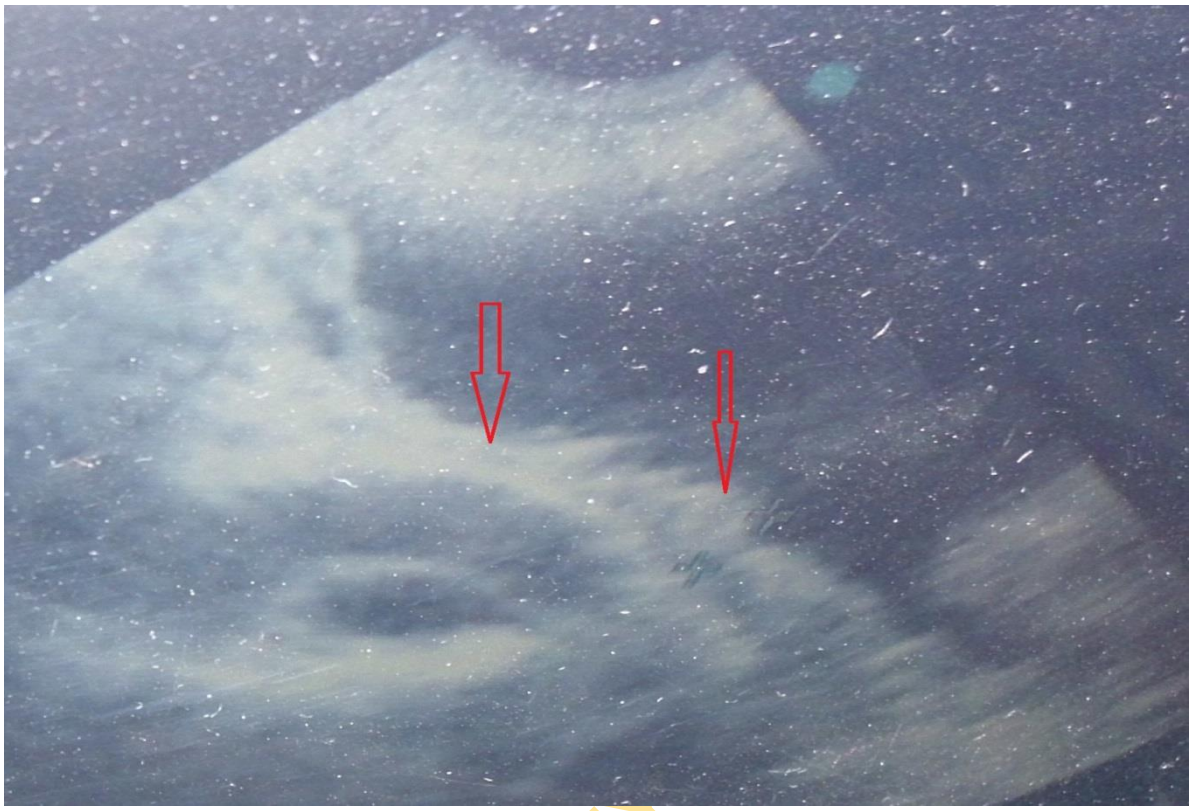

Figure 4.1c: Bladder with masses extending from the wall into the lumen (ignore speckled artefacts)

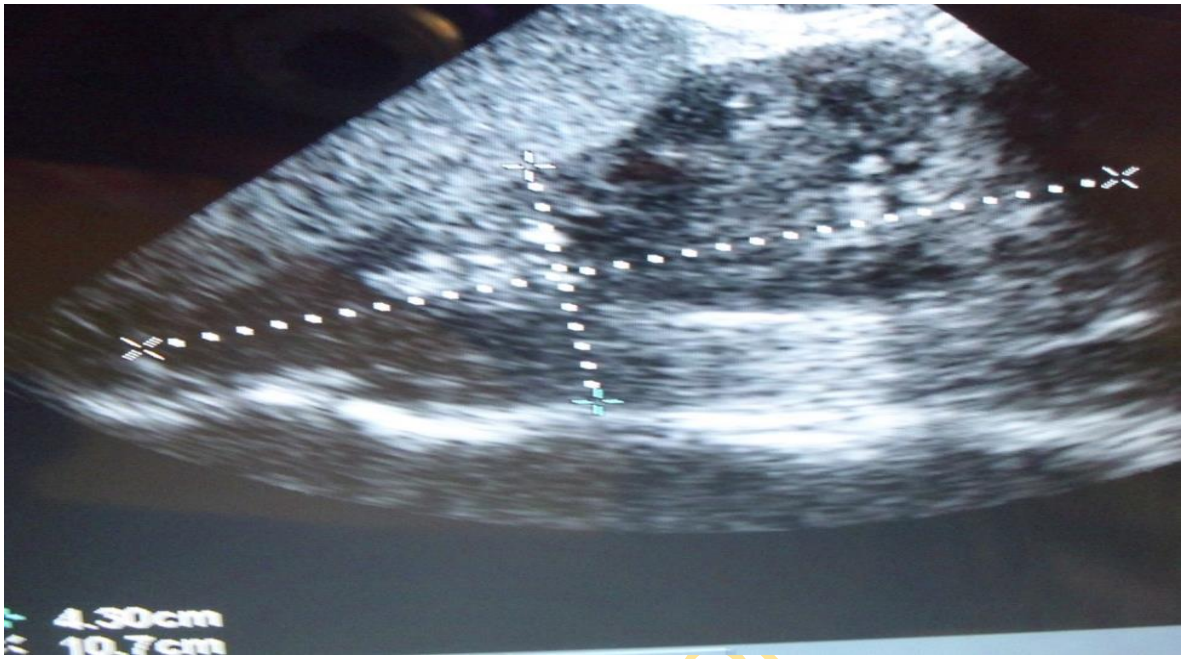

Figure 4.2a: Kidney with no abnormaalities in participants with schistosomiasis in Eggua

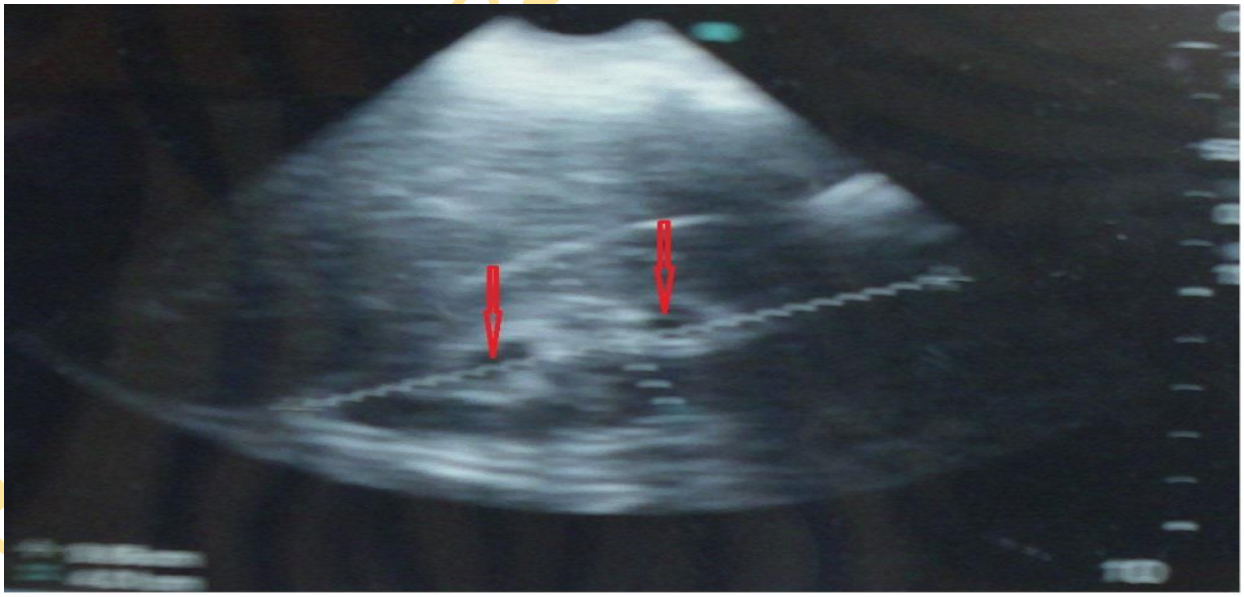

Figure 4.2b: Mild dilation (Arrow) of kidney in participants with schistosomiasis in Eggua

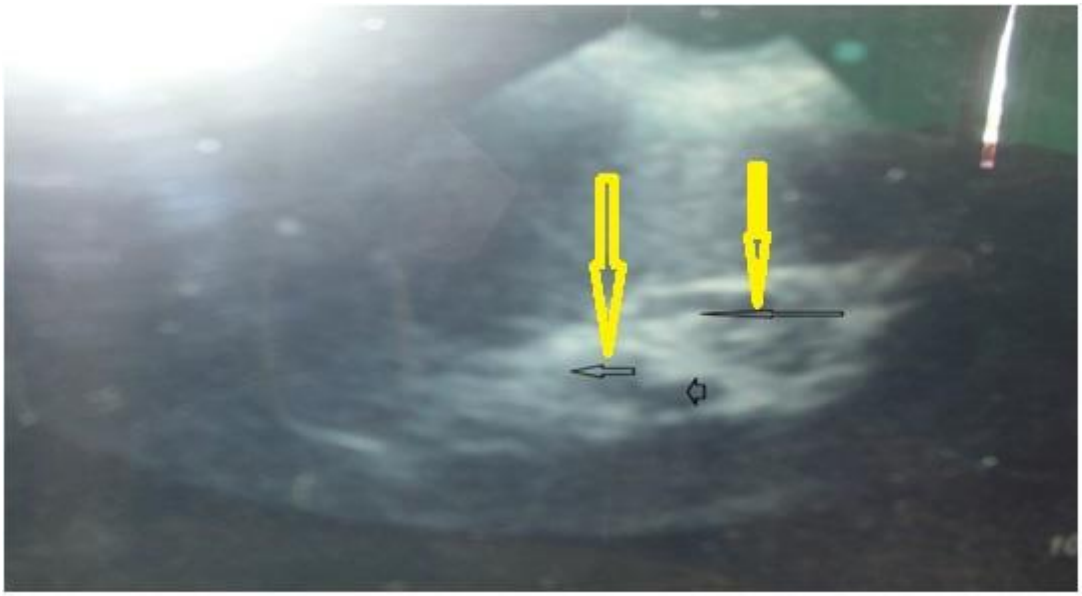

Figure 4.2c: Severe dilation (Arrow) of kidney in participants with schistosomiasis in Eggua

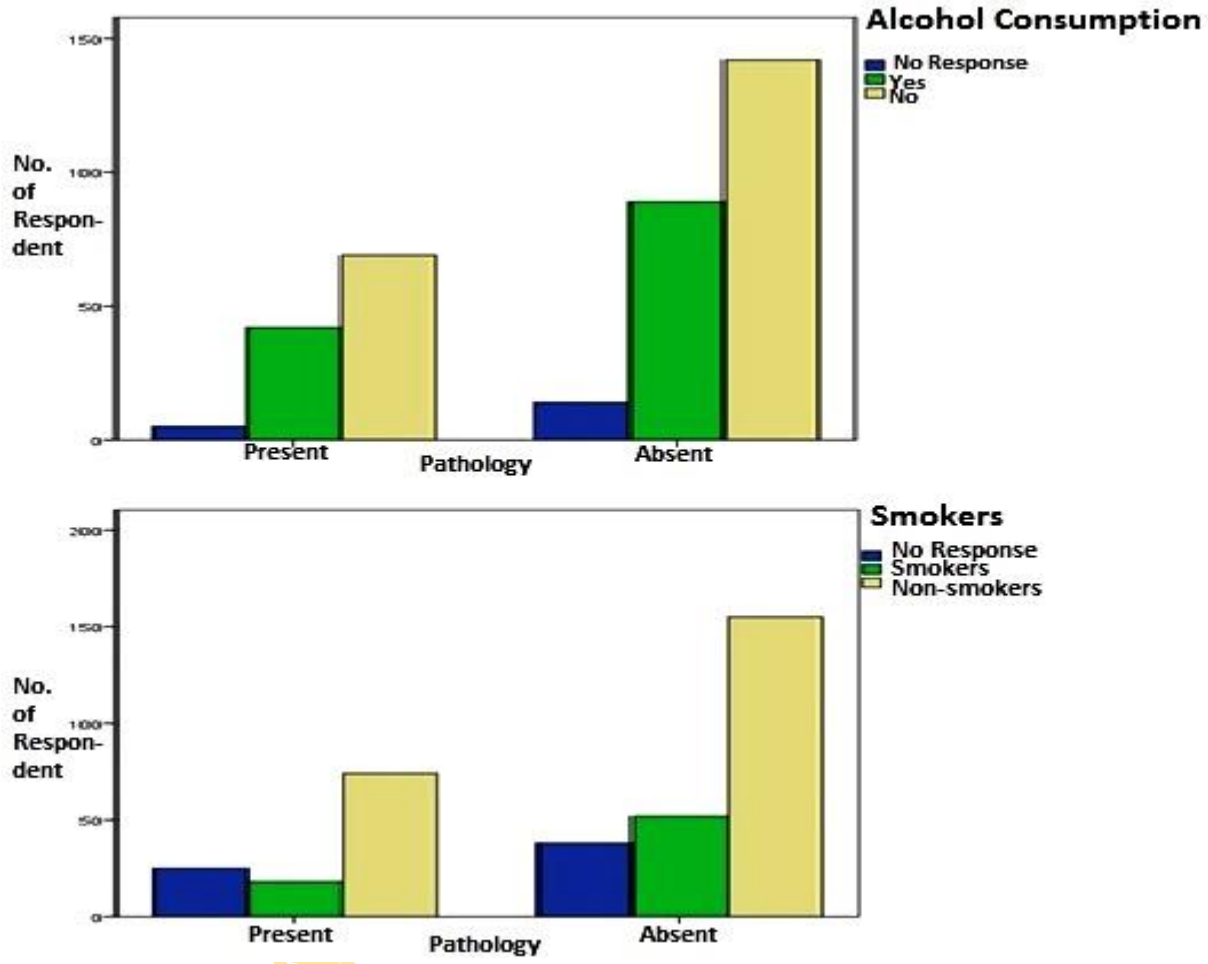

Figure 4.3: Relationship between lifestyle (possible risk factors) and structural bladder pathology

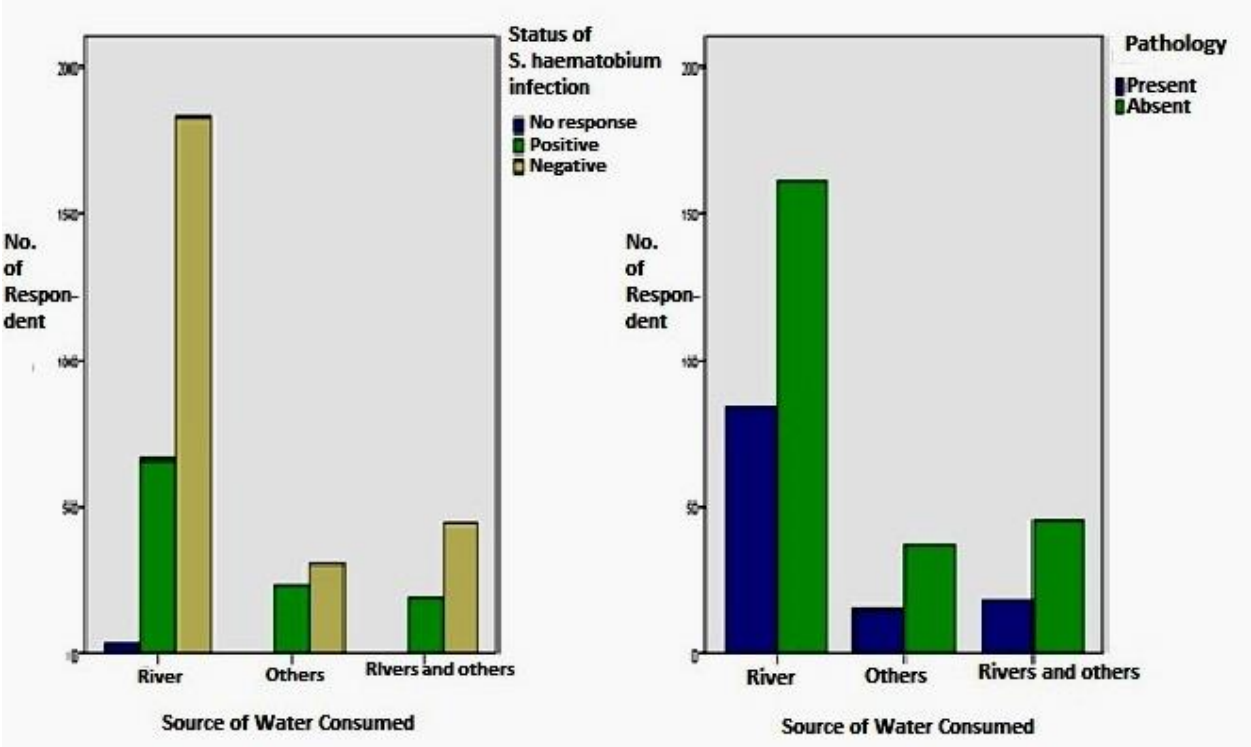

Figure 4.4: Relationship between water contact (possible risk factors) with *S. haematobium* infection and structural bladder pathology

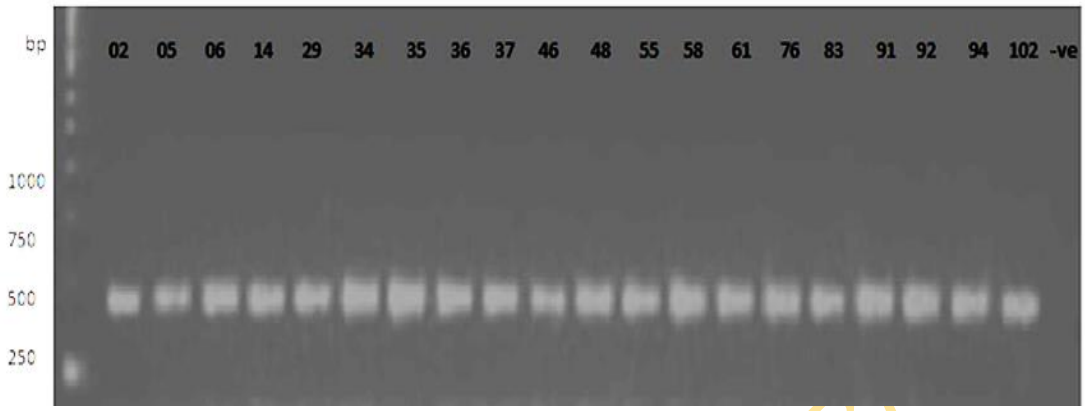

Figure 4.5a: GSTM1 SNP yielded twenty PCR amplicons for bladder pathology cases in Eggua, Nigeria.

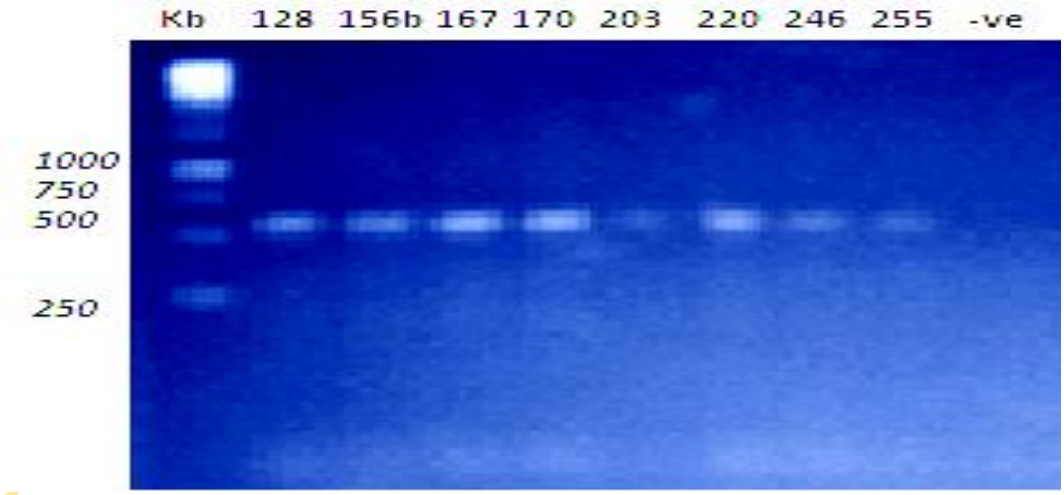

Figure 4.5b: GSTM1 SNP yielded eight PCR amplicons for bladder pathology cases in Eggua, Nigeria.

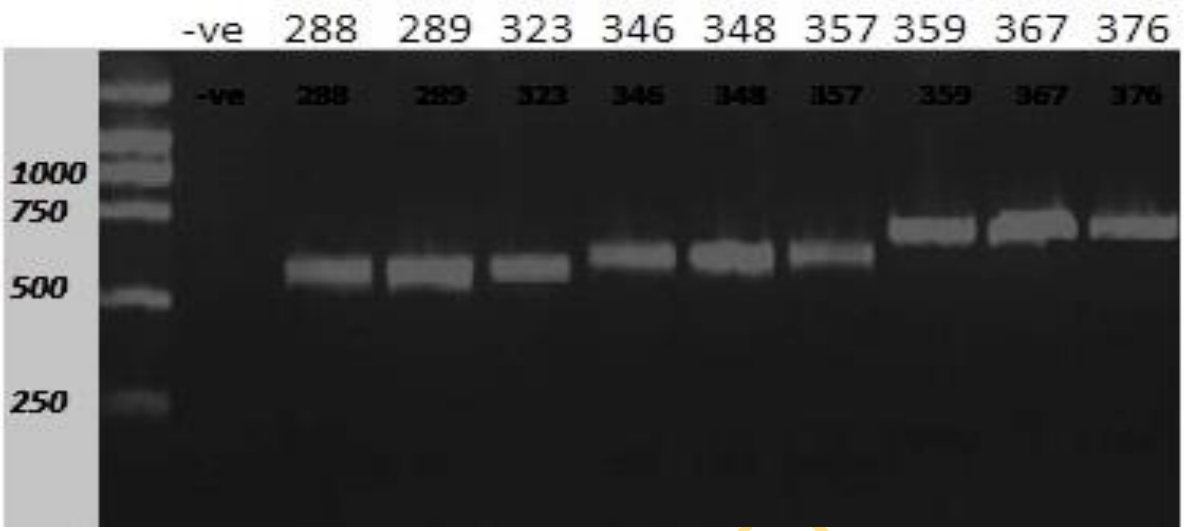

Figure 4.5c: GSTM1 SNP yielded nine PCR amplicons for bladder pathology cases in Eggua, Nigeria.

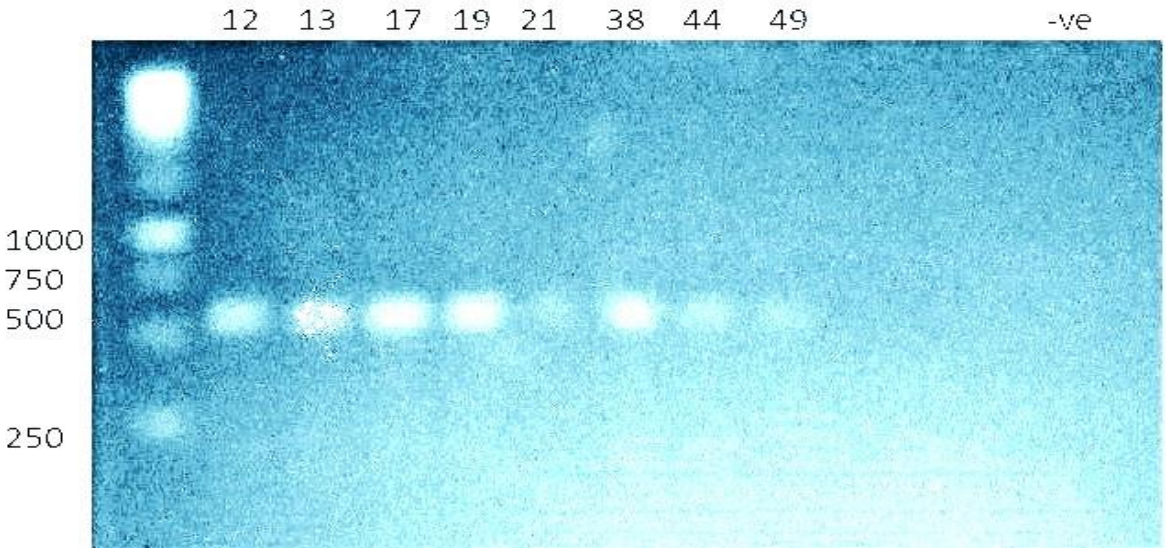

Figure 4.5d: GSTM1 SNP yielded six (12, 17, 19, 38, 44, 49) PCR amplicons for non-bladder pathology cases in Eggua, Nigeria.

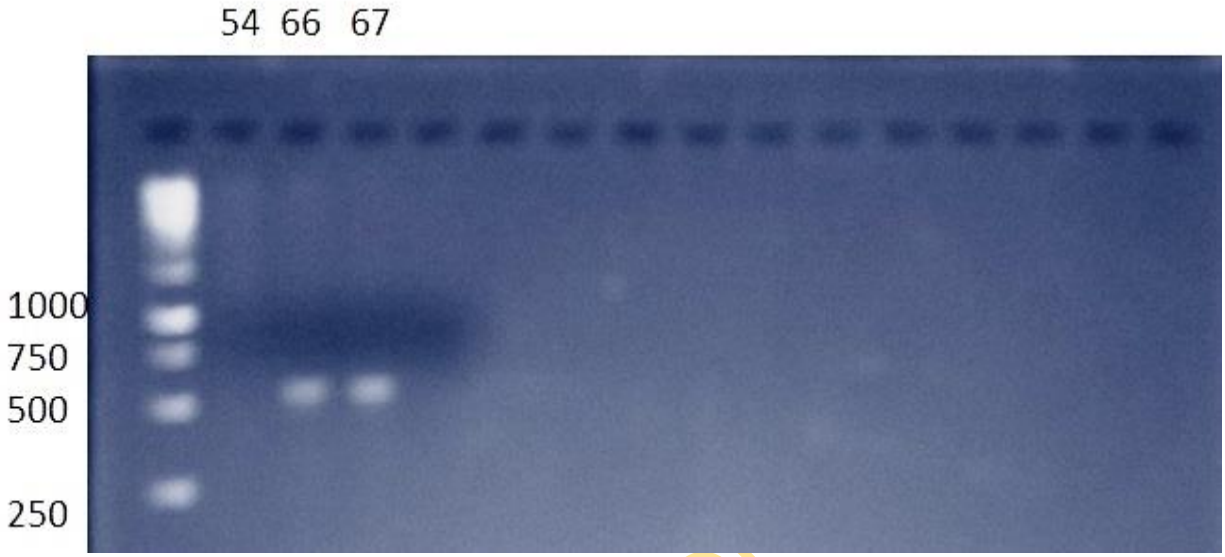

Figure 4.5e: GSTM1 SNP yielded two (66, 67) PCR amplicons for non-bladder pathology cases in Eggua, Nigeria.

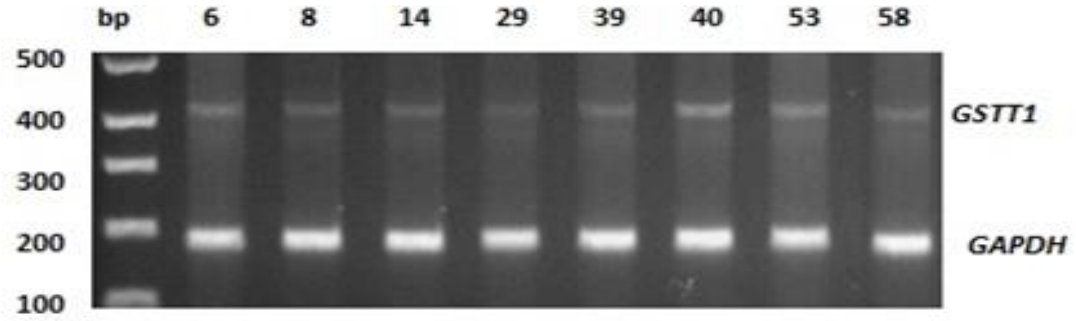

Figure 4.6a: Amplified GSTT1 SNP among bladder pathology (6, 8, 14, 29, 39, 40, 53, 58) and control cases in Eggua, Nigeria.

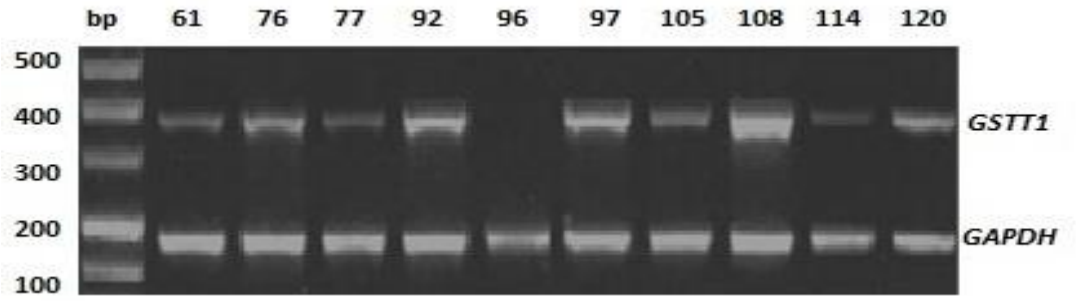

Figure 4.6b: Amplified GSTT1 SNP among bladder pathology (61, 76, 77, 92, 105, 108) and control (96, 114, 120) cases in Eggua, Nigeria

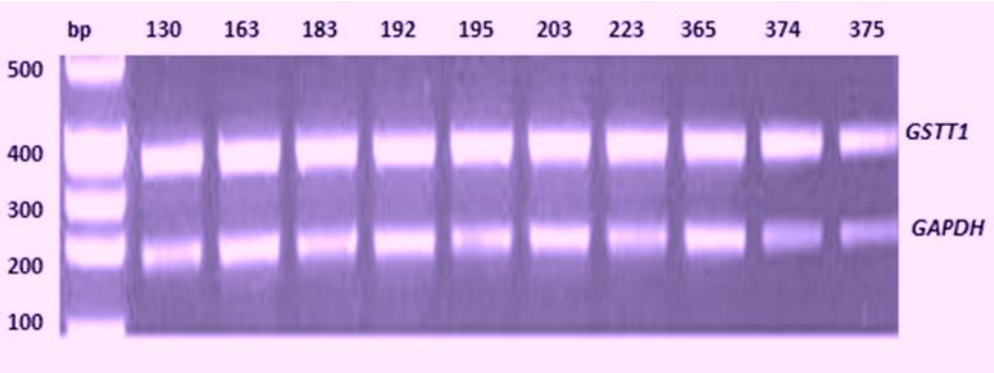

Figure 4.6c: Amplified GSTT1 SNP among bladder pathology (163, 183, 192, 195, 203, 223, 365, 374, 375) and control (130) cases in Eggua, Nigeria

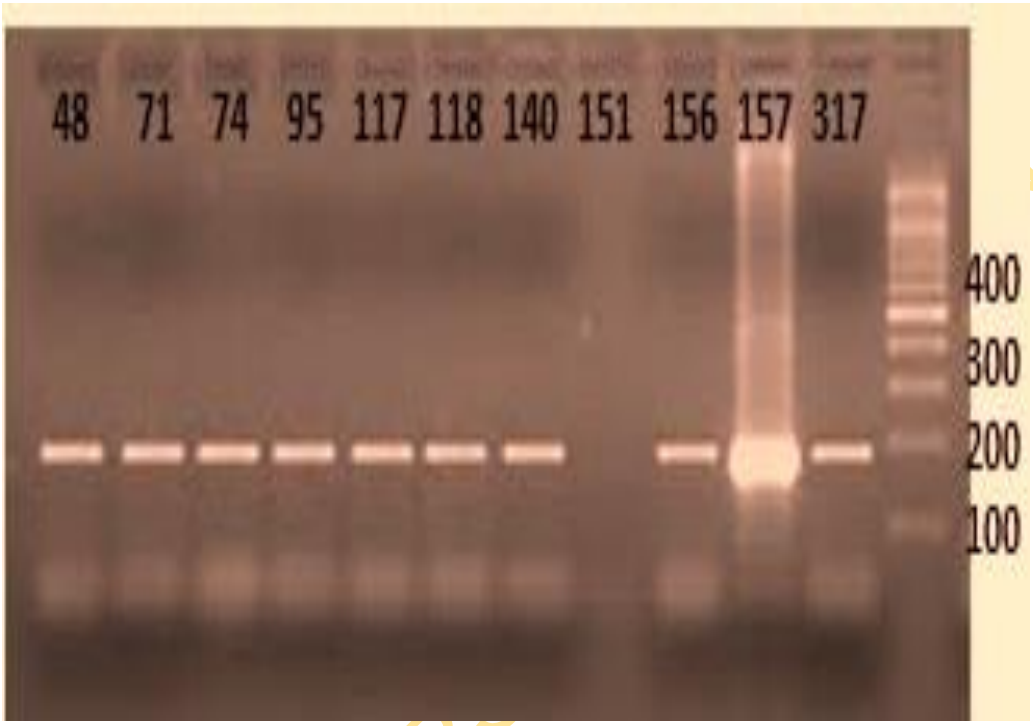

Figure 4.7a: Amplified IL13 -1055 SNP among *Schistosoma haematobium* infected participants in Eggua, Nigeria

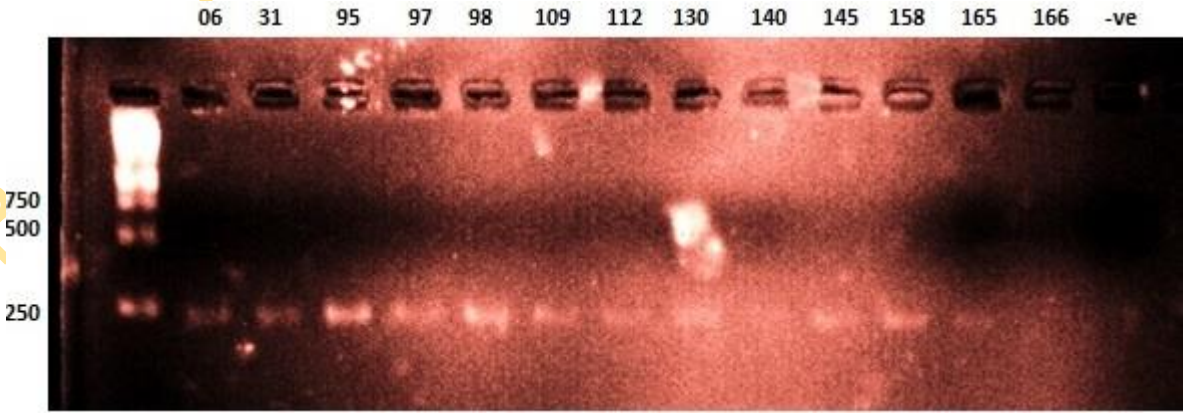

Figure 4.7b: Amplified IL4 -590 SNP (6, 31, 95, 97, 98, 109, 112, 130, 145, 158, 165) among *Schistosoma haematobium* infected participants in Eggua, Nigeria

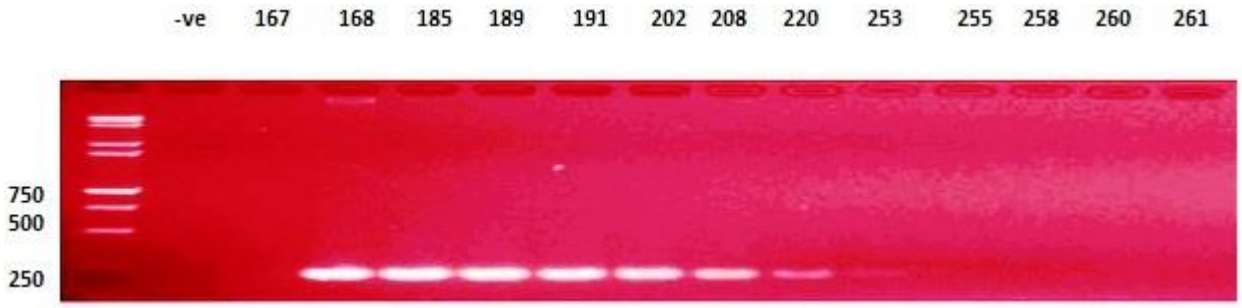

Figure 4.7c: Amplified IL4 -590 SNP among *Schistosoma haematobium* infected participants in Eggua, Nigeria

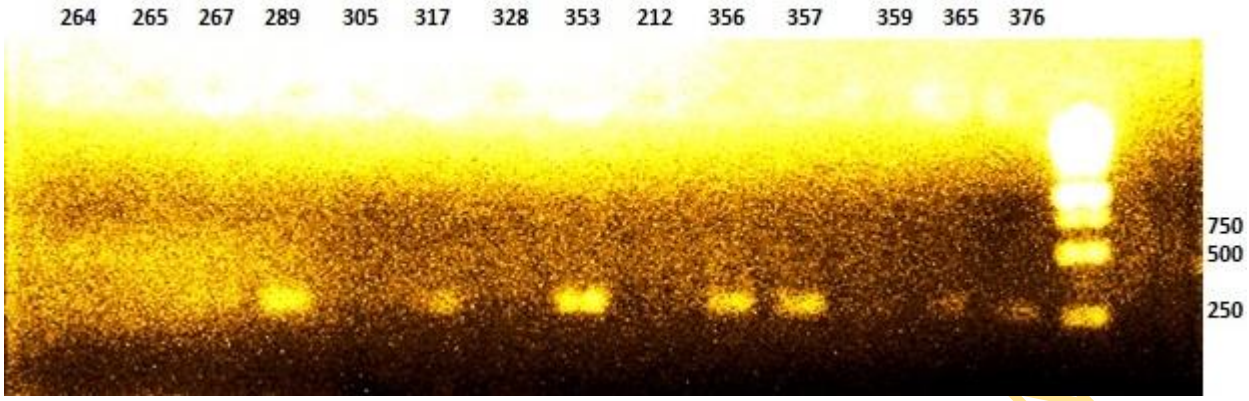

Figure 4.7d: Amplified IL4 -590 SNP (289, 317, 353, 356, 357, 365, 376) among *S. haematobium* infected participants in Eggua, Nigeria

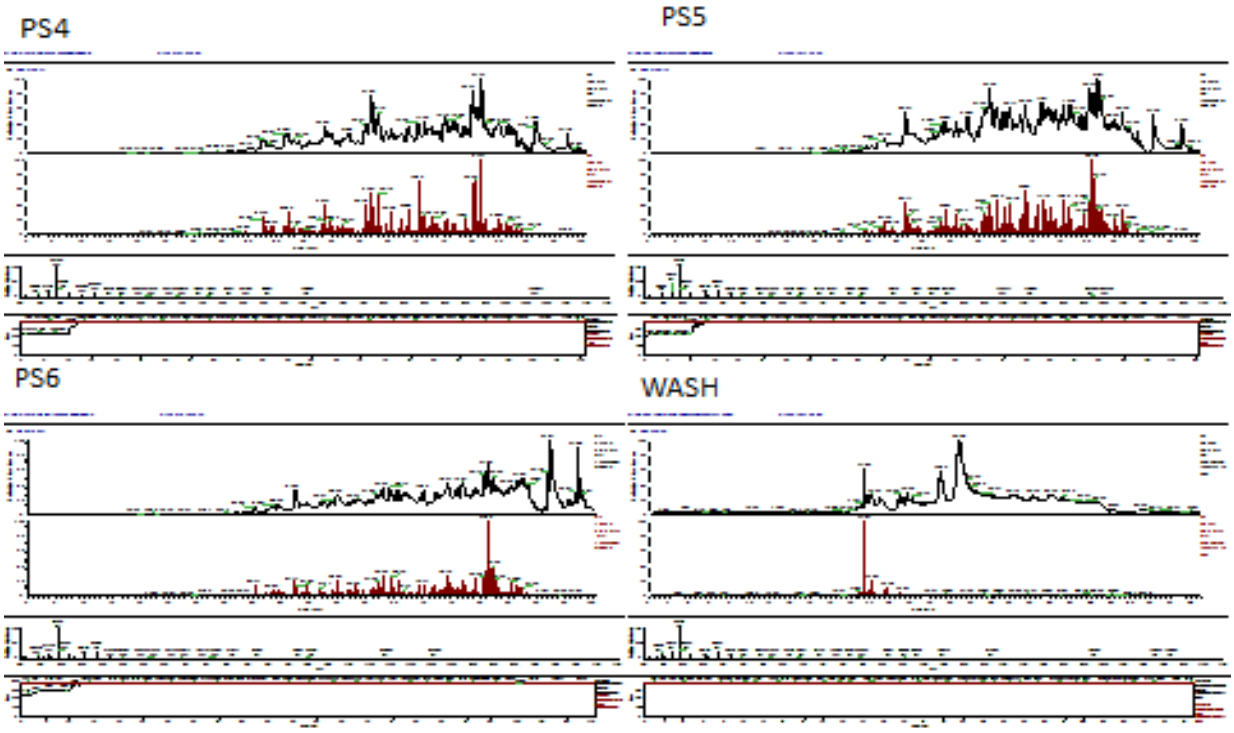

Figure 4.8: Mass Spectrometry-MS/MS spectra output for combined pathology and schistosomiasis (PS) samples and a wash

Legend

Wash- Washing of the HPLC column at regular interval. A wash is done after every three (3) samples analysed by mass spectrometry

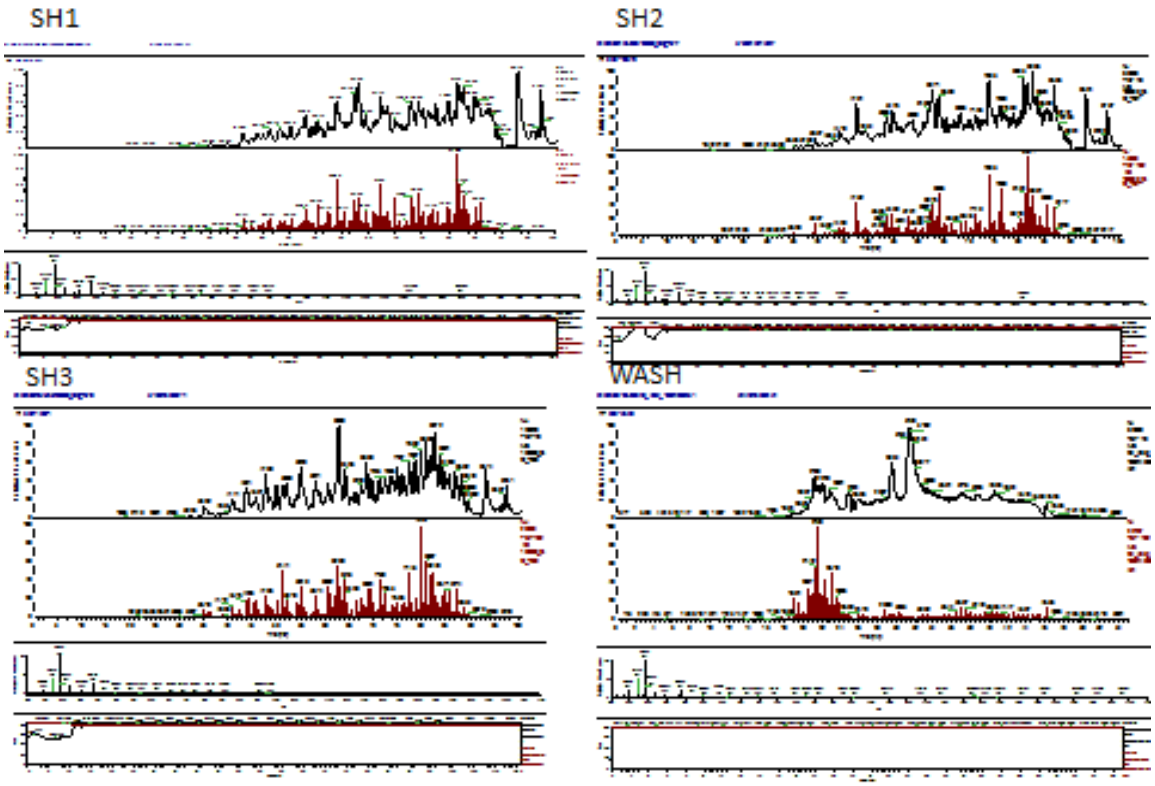

Figure 4.9: Mass Spectrometry-MS/MS spectra output for Schistosomiasis infected (SH) samples and a wash

Legend

Wash- Washing of the HPLC column at regular interval. A wash is done in every three (3) samples analysed by mass spectrometry

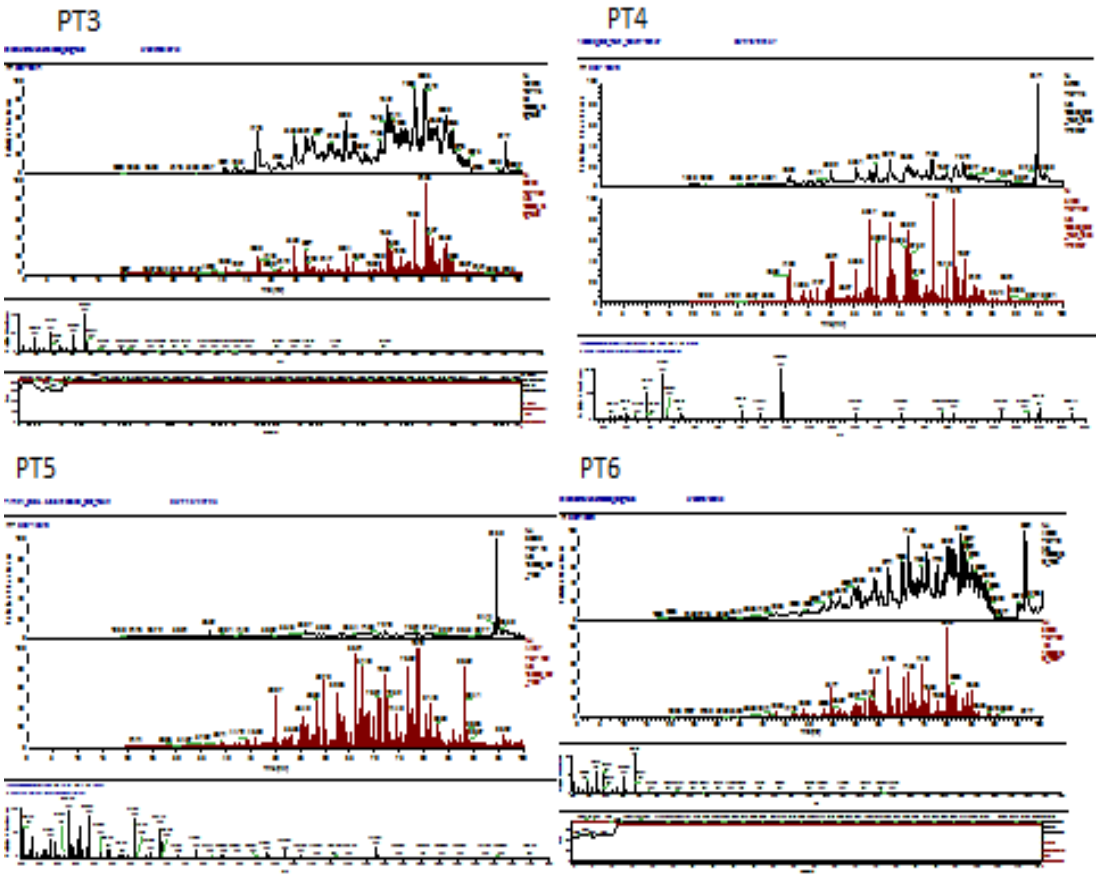

Figure 4.10: Mass Spectrometry-MS/MS spectra output for pathology (PT) samples

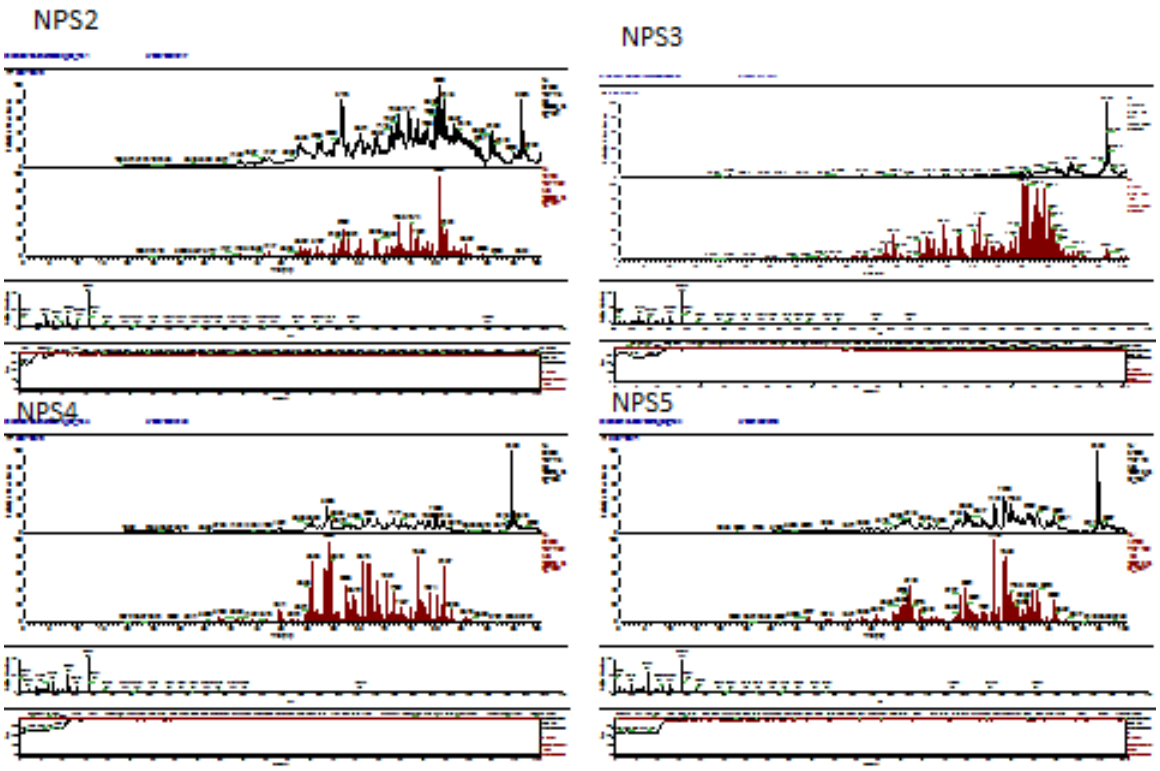

Figure 4.11: Mass Spectrometry-MS/MS spectra output for some samples with No pathology and Schistosomiasis (NPS) – Control

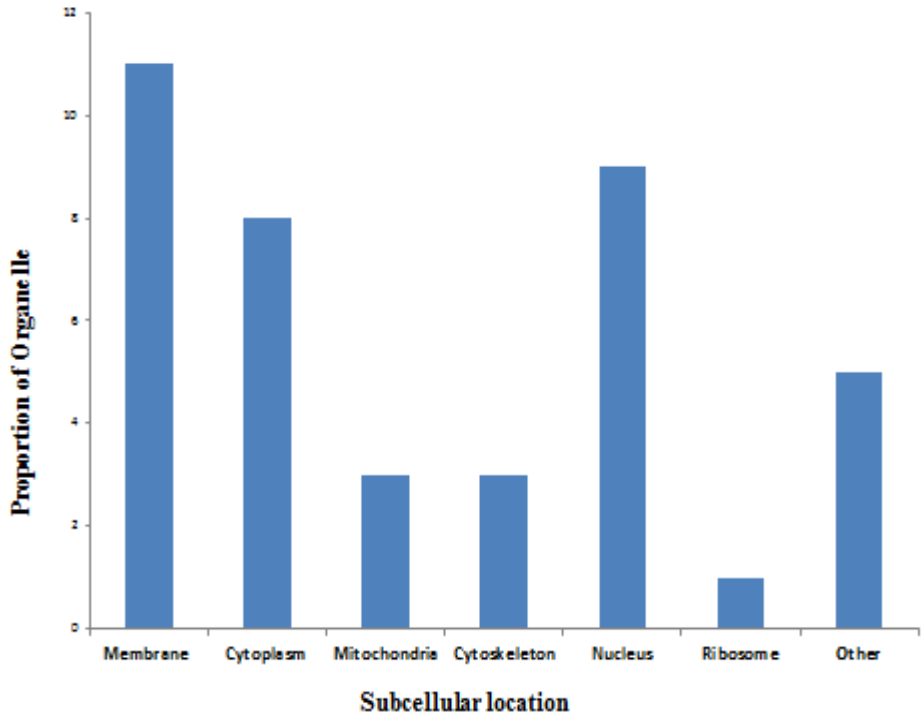

Figure 4.12: Distribution of predicted schistosome proteins to different subcellular location within the parasite

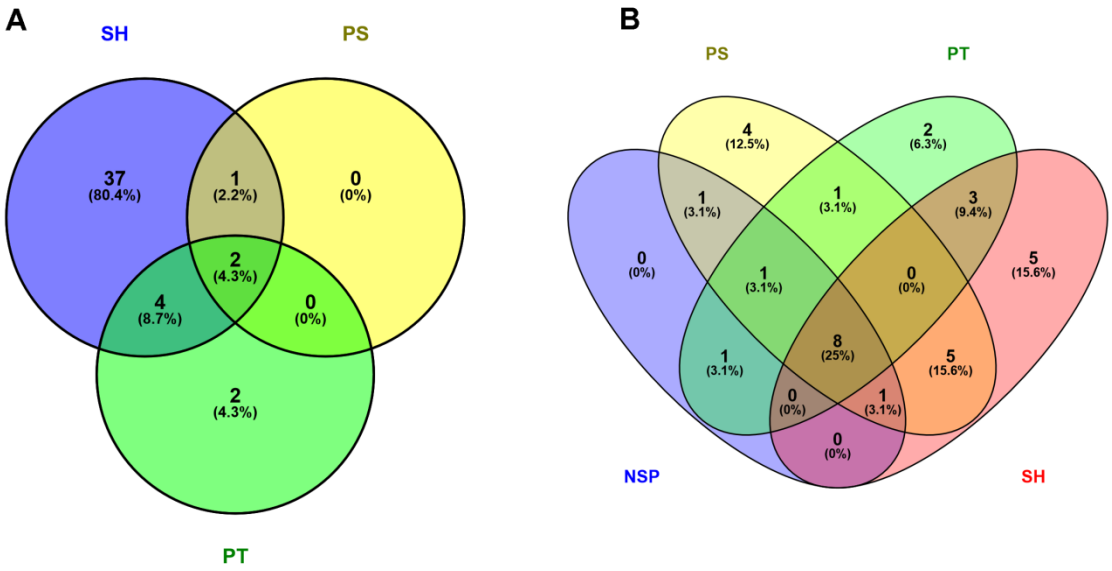

Figure 4.13: Distribution of statistically significant biomarkers originating from (A) humans and (B) schistosomes sample groups

Legend

SH-Schistosomiasis

PT (BP)-Bladder Pathology

PS-Pathology and Schistosomiasis

NPS-No Pathology and Schistosomiasis (controls)

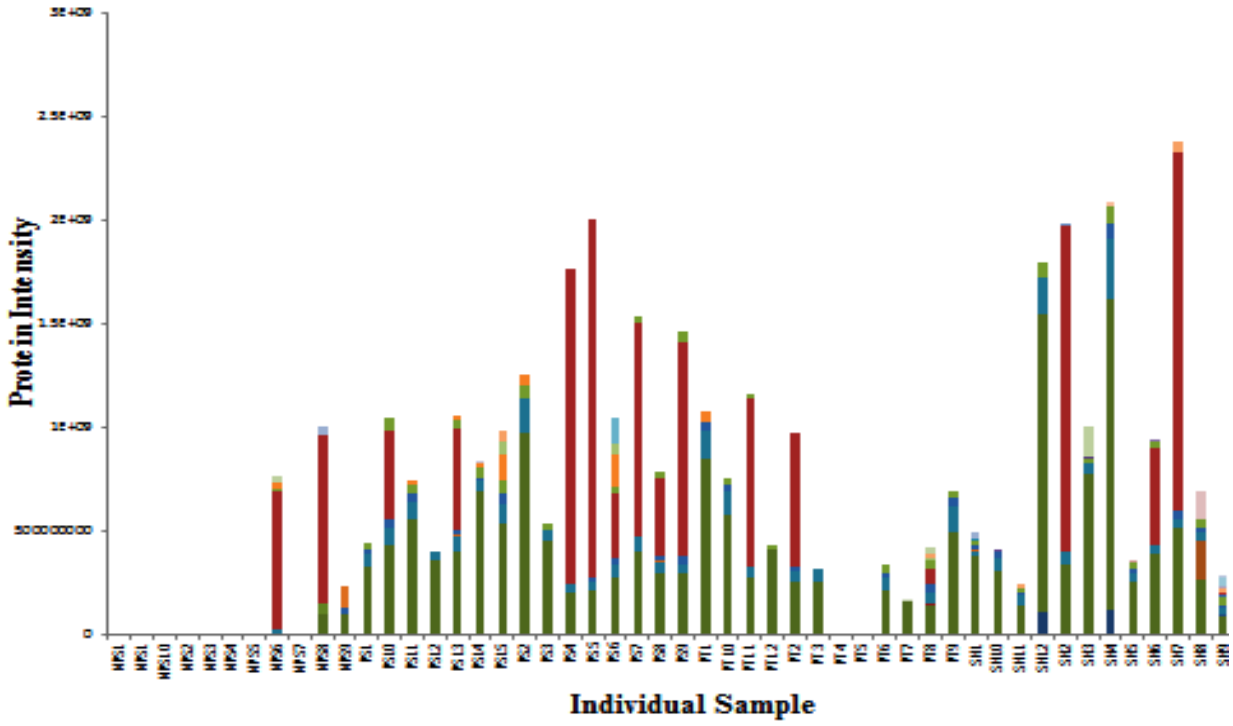

Figure 4.14: Abundance of schistosome proteins and their intensity among individual samples

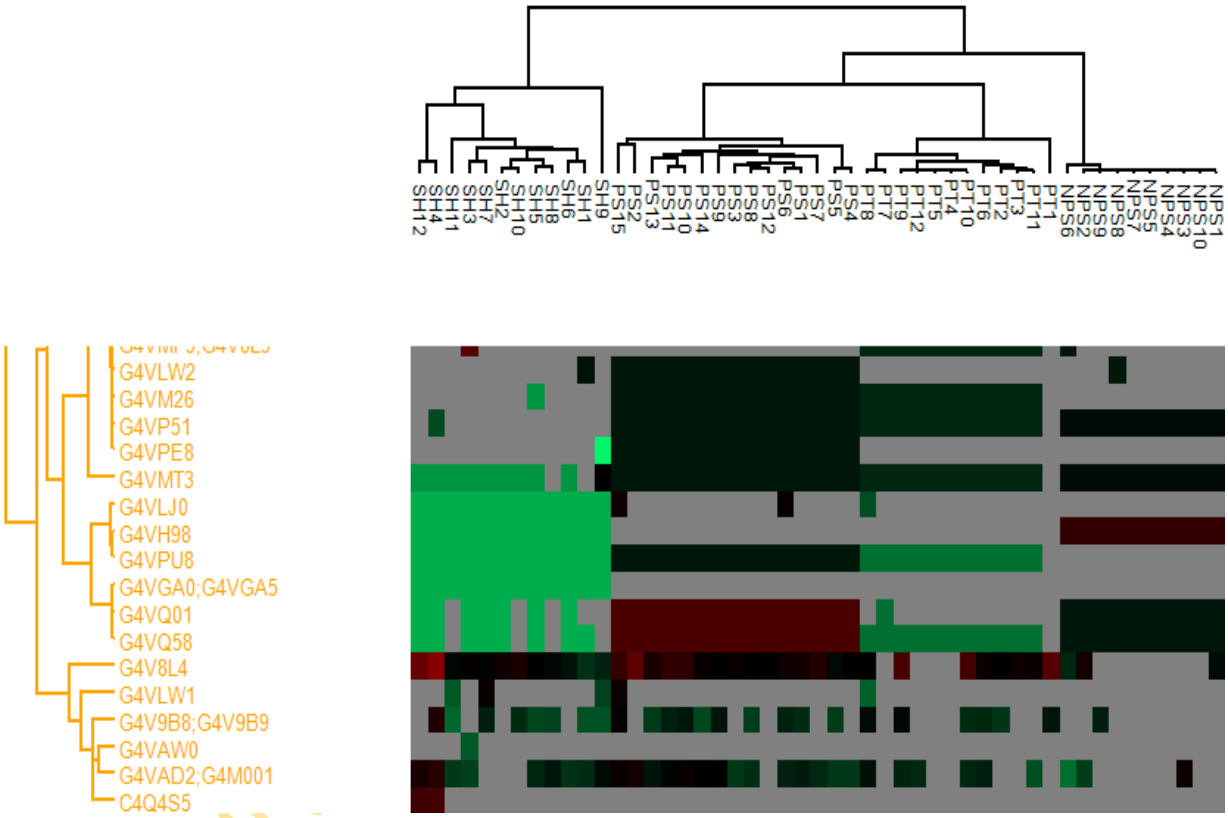

Figure 4.15a: A hierarchical heatmap with distinct clustering of each sample groups (NPS, SH, PS and PT).

Samples in each group clustered together, thereby showing difference in proteome signatures

Legend

SH-Schistosomiasis

PT (BP)-Bladder Pathology

PS-Pathology and Schistosomiasis

NPS-No Pathology and Schistosomiasis (controls)

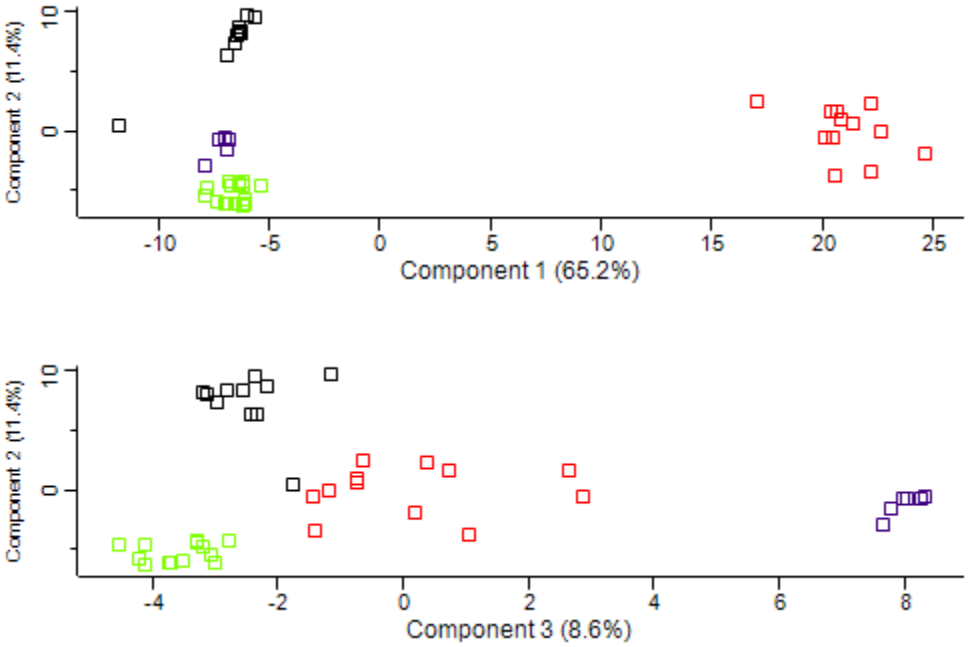

Figure 4.15B: Individual sample analysis of NPS, SH, PS and PT.

Similar clustering with minor overlap was seen in each sample group by multivariate testing using Principal Component Analysis (PCA)

Legend

SH-Schistosomiasis

PT (BP)-Bladder Pathology

PS-Pathology and Schistosomiasis

NPS-No Pathology and Schistosomiasis (controls)

Colour Green represents -PS,

Colour Blue- NPS,

Colour Black-PT and

Colour Red- SH.

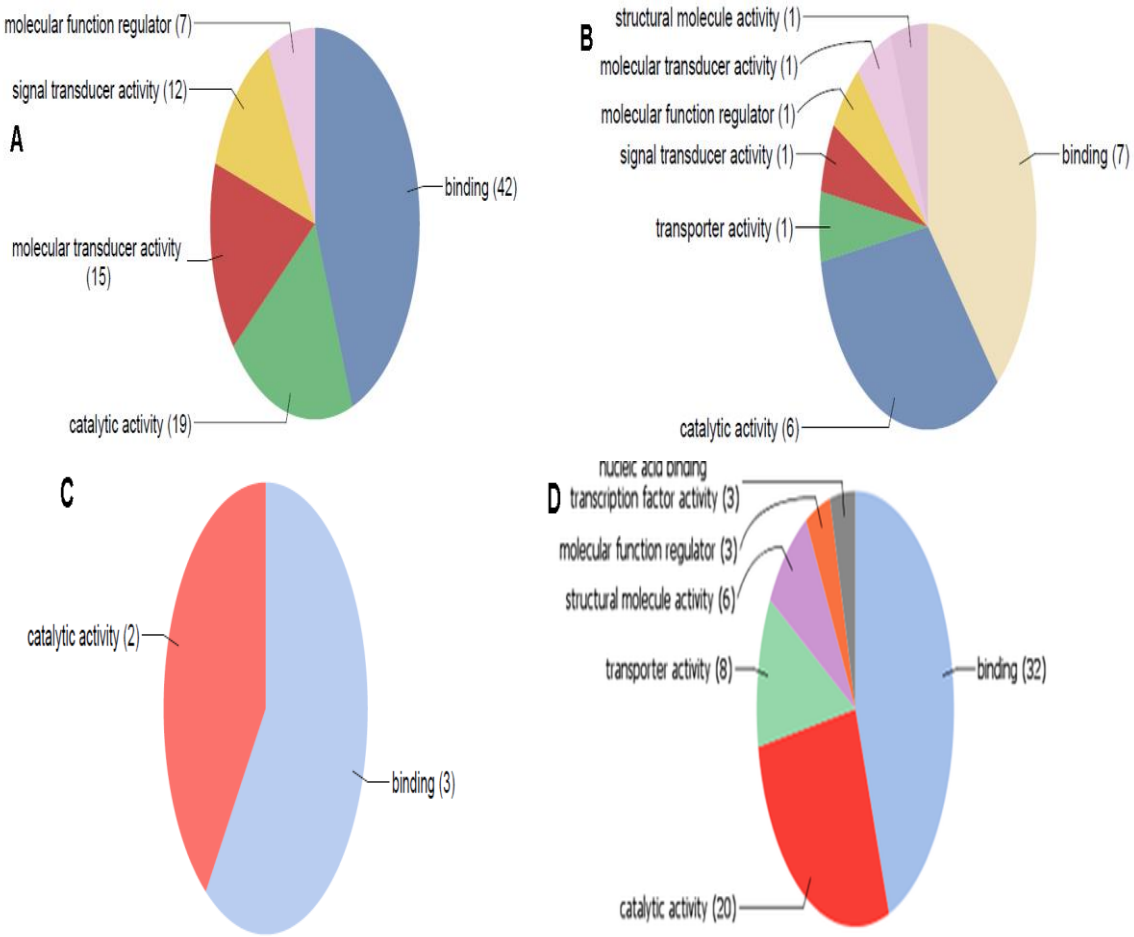

Figure 4.16: Molecular function of the identified human and schistosome proteins as predicted by Blast2GO

Legend

SH-Schistosomiasis

PT (BP)-Bladder Pathology

PS-Pathology and Schistosomiasis

NPS-No Pathology and Schistosomiasis (controls)

A representing SH group, B-PT group, C- group PS and D- Schistosoma proteins

CHAPTER FIVE

5 DISCUSSION

5.1 Infection and Pathology

The overall prevalence rate (29.3%) of adults *S. haematobium* infection recorded in this study was higher than several reported cases in Nigeria (Nmorsi *et al*., 2007; Dawet *et al*., 2012; Ugochukwu *et al*., 2013). This could further explain continuous and longtime exposure to infection and possibly contribute to *S. haematobium* subtle morbidity. Finding higher frequency of infection in adults above 50 years old further strengthens the belief that the population has endured long time and incessant exposure to the infection.

Most (81.3%) of the participants depended solely on the *S. haematobium* contaminated river water, which could account for the higher *S. haematobium* prevalence; and little or no schistosomiasis control (drug) intervention targeted to adults has been recorded in this area.

The higher frequency of light intensity *S. haematobium* infection observed in this study could be explained by some possible level of gradual development of acquired protected immunity by adults in this community due to chronic exposure to schistosomiasis (Barbosa, 2006). Shiff *et al.*, (2006), found that the proportion of egg-positive individuals falls progressively with age and is a feature in populations with lifelong exposure to the parasite. Therefore, chronicity of infections in older people is more likely to be difficult to ascertain using egg count method. Also, according to the WHO Expert Committee (2002), prevalence and intensity of infection have been directly related to the patterns of variation with age with a reported decline in adults, an assertion supported by studies including Nigeria's population (Pukuma and Musa, 2007; Agere *et al*., 2010; IARC, 2011). Pearce and MacDonald (2002) also reported an obvious pattern of age-dependent intensity of infection where those who are below

the age of puberty carry the most parasites, and those in older age groups are generally less heavily infected.

The association between *S. haematobium* infection and the presence of urinary tract abnormalities was similar to previous reports (Warren *et al*., 1979; Serieye *et al.*, 1996; Nmorsi *et al.*, 2007; and Ekwunife *et al.*, 2009). Also, *S. haematobium* infection has been associated with a two to tenfold increase in the risk of bladder squamous cell carcinoma, as well as potential cause of kidney damage (Driguez *et al*., 2016). In fact, in some of the regions where *S. haematobium* is endemic, bladder cancer has been marked as the most common cancer in men and the second in women, just behind breast cancer, and accounts for as much as 30% of all cancer cases (Betelho *et al.,* 2010). A meta-analysis of an estimated disease burden showed that morbidity and mortality attributed to schistosomiasis increases with DALYs (disability-adjusted life years) which had risen to about ~20% increase in the past 20years (Murray *et al.,* 2013; Driguez *et al*., 2016). A retrospective review of clinical records of bladder cancer cases in Sokoto, Nigeria between 1999 and 2004 showed a 4.7 fold rise in the number of bladder cancer cases, with squamous cell carcinoma composed of 65.1% of histologically verified cases and 50% of the squamous cell carcinoma showed evidence of chronic urinary schistosomiasis (Mungadi and Malami, 2007).

The observation of higher frequency of structural bladder pathologies within the age range 50 years and above could suggest a long time exposure to infection before development of cancer. This is supported by Mostafa *et al.* (1999), which reported that the incidence of bilharzial bladder cancer in various African countries peaks between the ages of 40–49 years. The higher frequency of mild bladder pathology observed in this study was also similar to another study in Western Madagascar (Serieye *et al.*, 1996), which observed higher incidence of mild bladder pathology than severe bladder pathology. This could be explained by the low number of participants who claimed to smoke cigarettes and consume alcohol; these conditions may serve as promoting or progression factors either in progression of bladder pathology to cancer or in making the badder pathology more severe. Also, cigarette smoking and alcohol consumption were not significantly associated with urinary tract pathology, thereby suggesting that

these risk factors play a relatively minor role in urinary carcinogenesis in this area which is endemic with *S. haematobium* infection. This is similar to the findings of Hsu *et al.*, (2011), who discounted the possible role of tobacco smoking in urinary carcinogenesis among a population highly exposed to arsenic. More human based study studies are still required to elucidate the role of *S. haematobium* in aetiopathogenesis bladder cancer as the evidence of carcinogenicity in human is less than sufficient despite proven evidence in animal models (Driguez *et al*., 2016).

The presence of hydronephrosis in participants with light infection is however at variance with the report of Nmorsi *et al.*, (2007), although hydrocalycosis (a condition mostly mistaken for hydronephrosis) was observed in some patients with heavy infection, indicating the likely contribution of this infection to kidney pathology. Females (60.2%) had more structural bladder pathology compared to males (39.8%). This may be due to higher water exposure by females; on the other hand, this may be due to higher number of female study participants than an indication of a female predilection to bladder pathology. However, since hydronephrosis and bladder mass or bladder calculi were found together in the female participants, female predilection to bladder pathology may not completely be ruled out. The structural changes to the bladder recorded in this study were in consonance with observations in West Madagascar (Serieye *et al*., 1996) and Nigeria (Nmorsi *et al*., 2007 and Ekwunife *et al.*, 2009) where bladder irregularities and bladder wall thickness were identified as the most common pathologies in individuals infected with *S. haematobium*.

5.2 Genetic Susceptibility

Evidence had shown that imbalance in activation and detoxification by detoxifying enzymes (*GSTs*) due to gene polymorphisms may influence increase in bladder cancer risk due to accumulation of carcinogen metabolites (Mc Grath *et al.*, 2006; Ying *et al*., 2016; Yajie *et al*., 2016 and Jobaida *et al*., 2016). Common polymorphisms occur in almost all members of GSTs (Ying *et al.*, 2016) and several types of allelic variations have been observed (Djukic *et al*., 2013; Matic *et al*., 2016; Yajie *et al.*, 2016) which include *GSTM1* and *GSTT1* class deletion polymorphism (*GSTM1-null* and *GSTT1-null*). The genotypes *GSTM1-null* and *GSTT1-null*, produce no GSTM1 and GSTT1

protein and consequently have complete lack of GSTM1 and GSTT1 enzymatic activity (Matic *et al*., 2016).

In this study, *GSTM1* and *GSTT1* null polymorphisms were shown to significantly increase the risk of structural bladder pathology. This is in agreement with Arnaldo *et al.*, 2000; Aktas *et al.,* 2001; Cengiz *et al.,* 2007; Yajie *et al*., 2016 and Jobaida *et al*., 2016 who also observed a risk of bladder cancer with *GSTM1* and *GSTT1* null polymorphism. Also, Okkels *et al.,* 1996 and Arnaldo *et al.,* (2000) reported that the association of *GSTM1* null genotype with bladder tumour was more apparent in the group with less aggressive tumours. This by implication could further support the presence of *GSTM1* null genotype among the urinary tract pathology cases (an indicator of early stage of possible progression to bladder cancer). Among bladder pathology cases, the distribution of the polymorphisms was relatively similar to the control group and this was also the findings of Arnaldo *et al.*, (2000). It was observed in this study that the absence of *GSTM1* activity was higher in control cases. This is similar but considerably higher than reported in several other studies (Brockmoller *et al.*, 1996; Okkels *et al.,* 1996; Arnaldo *et al.*, 2000 and Jobaida *et al.*, 2016). The role of *GSTT1* null genotype in bladder cancer risk still remains unresolved. Several studies suggested an increased risk (Moore *et al.*, 2004; Marcus *et al.*, 2000; Salagovic *et al.*, 1999; Kempkes *et al.*, 1996; Ying *et al.*, 2016; Yajie *et al.*, 2016 and Jobaida *et al*., 2016), but other suggested no risks or low risks (Mc Grath *et al.,* 2006; Karagas *et al.*, 2005; Lee *et al*., 2005; Matic *et al.*, 2016). This study also found an elevated risk of bladder cancer among *S. haematobiun infected* participants and more in smokers with null *GSTM1* and *GSTT1* null polymorphism, a finding that is similar to those observed by Moore *et al.,* (2004) where elevated risk to bladder cancer was only seen in smokers with the *GSTT1* null polymorphism.

The detection of *IL 4-590 C/T* and *IL13-1055 C/T* polymorphisms among *S. haematobium* infected participants in this study was also similar to other findings in previous studies (Gatlin *et al*., 2008; Kouriba *et al*., 2005; He *et al*., 2008). Gatlin *et al.,* (2008) reported a more resistance to infection among men with the combination of *IL-13 21055* C/T and *IL-4 2590* C/T genotypes when compared with those seen with the sum of the separate effects of *IL-13 21055* C/T and *IL-4 2590* C/T on resistance.

Therefore, to understand the role of this heterozygous cytokine as susceptible or resistance factor, further post treatment follow up study will be required among infected study participants to establish role of these cytokines as done in other similar studies (van der Pouw Kraan *et al*., 1999; Cameron *et al*., 2006; Gatlin *et al*., 2008; Gatlin *et al*., 2009). Individuals with polymorphisms at *IL-13 -1055* and *IL-4 -590* position are more likely to require fewer reinfections and treatments to become resistant to reinfection than individuals who are homozygous at either position (Gatlin *et al.,* 2008). Another study in Mali revealed an association between a single-nucleotide polymorphism in the *STAT6* gene at 12q13.3 and intensity of infection by *S. haematobium*; this polymorphism had an additive effect with *IL13 −1055* (He *et al*., 2008).

Other analyses of *S. haematobium* infection in Mali revealed that in chromosomal region 5q31-q33, polymorphisms in the *IL13* gene promoter at position *−1055* and *−591* were associated with the infection rate: alleles −1055C and −591A were preferentially transmitted to children with the 10% highest infection rate, whereas *−1055T* associated with the lowest infection levels (Kouriba *et al*., 2005). *IL4 −590T* allele has been associated with high IgE production, and thereby increased resistance to infection (Russell *et al.*, 2015).

5.3 Proteomics Biomarkers

Over 2000 proteins in total are estimated to be present in normal human urine (Kalantari *et al.*, 2015), 1823 of these proteins were identified by Marimuthu *et al.* (2011). This observation was not far from the result of this study which recorded 1306 proteins in human urine by MS analysis. Sample clustering analyses by PCA and Heatmap have placed all sample groups into clear-cut strata, with little interference between groups, thereby showing difference in proteome signatures between groups. The recent discovery of high resolution and mass accuracy of mass specrometers has drastically improved the reliability of integrated peaks signals of MS 1 label-free quantification methods (Adeola *et al.,* 2015).

Some of the potential Schistosoma biomarkers identified are likely clear candidates for the generation of new diagnostic markers against schistosomiasis. Majority of these

proteins after a GO (Gene Ontology)-enrichment analysis were assigned binding activity and are involved in several biological and molecular processes. This observation is similar to the report of Sotillo *et al.,* (2015). The parasite markers include 4 heat shock proteins (HSPs) which are known as highly conserved stress-induced proteins found in many trematodes and nematodes (Maizer and Mattei, 1991), and specifically in schistosomes (deWalick *et al.,* 2011). HSP expression in the earliest stages of intra-mammalian schistosomula development has been reported and was suggested to be as a result of thermal changes in the parasite niche or environment i.e. changes between freshwater and the human body (Devaney, 2006; Sotillo *et al.,* 2015).Venom Allergen-like (VAL) protein found in this study has also been reported elsewhere (Sotillo *et al.,* 2015), and have been tried as experimental vaccine against hookworm infections in humans (Diemert *et al.,* 2012). Changes in *VAL* gene and the resultant protein expression denote its functions in different aspect of host-parasite biology, which include snail invasion by miracidium, intra-molluscan sporocyst development and cercarial development and host penetration (Yoshino *et al.,* 2014). VAL protein families are abundant in different helminth species including gastrointestinal nematodes, where they are known for several roles in the infective activities of parasites (Sotillo *et al.*, 2015).

Actin 1 protein as reported in this study could be identified as a possible drug target for treatment of schistosomiasis. Strong association between actin and *S. mansoni* adult worm surface membranes has been confirmed (Hatem and Rashika, 2007; deWalick *et al.,* 2011). Studies have described the role of actin in enhancing the activity of praziquantel (PQZ) treatment of schistosomiasis. It is suggested that PQZ intercalates in the surface membrane lipid bilayers, thereby inducing tegumental changes that leads to antigen exposure, including actin (Linder and Thors 1992; Hatem and Rashika, 2007). Elongation factor 1-alpha, Phosphopyruvate hydrase and Histone-4 were all identified as potential Schistosoma biomarkers in this report and this is similar to deWalick *et al.,* (2011), where these proteins were identified in purified eggshell fragments of *Schistosoma mansoni*. The proteins identified as part of the eggshell protein skeleton are known schistosome antigens and may induce cellular or antibody responses (deWalick *et al.,* 2011). These eggshell markers may be very

useful schistosomiasis diagnostic candidate rather than a vaccine candidate; as such, a vaccine may likely target the eggs and further encourage granuloma formation and pathology rather than enhancing the immune system against the parasite.

The significant parasite proteins as predicted in this study when classified according to their subcellular location were membrane-associated. This was similar to previous reports (deWalick *et al.,* 2011; Sotillo *et al.,* 2015). Also, expression of some membrane associated proteins has been earlier proposed as possible vaccine antigens in different *Schistosoma spp* (Da'dara *et al.,* 2013; Sotillo *et al.,* 2015).

Arylsulfatase A and phosphatidylethanolamine-binding protein 4 were both found in the Schistosomiasis group and Pathology and Schistosomiasis (PS) group. Arylsulfatases A, B, and C (arylsulfo-hydrolases) are a group of hydrolytic enzymes that occur in various tissues and fluids (Mahmoud *et al.,* 2004). An increase in the activities of arylsulfatase B (ASB) has been reported in bladder tumours (Poys and Morgan, 1977). Also, arylsulfatase A (ASA) activity in the livers of schistosome infected mice displayed a non- significant decrease in expression vs the control, while the expression of hepatic ASB was significantly increased in schistosma infected mice in similar study (Mahmoud *et al.,* 2004).

Aminophospholipids, such as phosphatidylserine and phosphatidylethanolamine are described as specific, accessible and stable markers of the luminal surface of tumour blood vessels (Thorpe *et al.,* 2009). There has already been some development of aminophospholipid-targeted diagnostic and therapeutic constructs for use in tumour intervention. Antibody-therapeutic agent conjugates and constructs that bind to aminophospholipids, including methods that specifically deliver therapeutic agents, which include toxins and coagulants, to the stably-expressed aminophospholipids of tumour blood vessels, thereby inducing thrombosis, necrosis and tumour regression, are particularly promising (Thorpe *et al.,* 2009).

One of the 4 proteins shared by bladder pathology and Schistosomiasis samples was Plasma retinol-binding and Transthyretin as observed in this report has also been identified by Yi-Ting *et al.,* (2012), as potential urine biomarkers for bladder cancer. Plasma retinol-binding protein (RBP) is a circulating plasma protein produced in the

liver and adipose tissue that transport active natural metabolites of Vitamin A as retinol around the body (Kotze *et al.*, 2015). Retinoid act pharmacologically to restore differentiation and inhibit growth in some premalignant and malignant cells both in vivo and in vitro (including bladder cancer cases) and also modulate cell proliferation, malignant transformation, apoptosis and immune system (Hameed and El-Metwally, 2008; Kotze *et al.*, 2015). A recent study found that individuals with HIV and *S. mansoni* coinfection significantly have low blood RBP level when compared to participants with HIV and *S. haematobium* coinfection (Kotze *et al.*, 2015).

The programmed cell death (PD) 1 surface receptor binds to two ligands, PD-L1 and PD-L2. Programmed cell death 1-programmed cell death ligand interactions are known to control the induction and maintenance of peripheral T cell tolerance. PD-1 and its ligands have been exploited by a variety of microorganisms to reduce the effect of antimicrobial immunity, thereby facilitating chronic infection (Sharpe *et al.*, 2007). The findings of Alvarez *et al.*, (2010), on the role played by PD-1 during innate immunity against *Mycobacterium tuberculosis* showed that PD-1 signaling might be modulating host innate immunity by inhibiting natural killer (NK) cell responses to the pathogen, contributing to avoidance of immune-mediated pathology caused by excessive host response to the infection. Understanding the functions of PD-1 and its ligands in regulating antimicrobial and self-reactive T cell responses and the possibility of manipulating this pathway may eventually reveal its therapeutic potential in chronic schistosomiasis.

Human Growth/differentiation factor 15 (GDF15) could be another useful diagnostic marker for chronic urinary schistosomiasis. GDF15 is a divergent member of the transforming growth factor β family discovered in a broad range of cells (Corre *et al.*, 2013). Corre *et al.*, (2013) reported that the protein could to be an integrative signal in pathologic conditions and also providing information on severity of a disease. Expression and secretion of GDF15 are heightened in many malignant tissue and cancer cell lines (prostate, pancreatic, oral squamous carcinoma and leukemia) as compared with their normal tissues or cells (Welsh *et al.*, 2001; Koopmann *et al.*, 2004; Zhang *et al.*, 2009; Wang *et al.*, 2014; Park *et al.*, 2010; Zhai *et al.*, 2016).

Human sialidase protein observed among SH group is known for its immunological roles in regulating phagocytosis in macrophages cells (Seyrantepe, 2010). Amith *et al.*, (2010) have reported Neu1 sialidase as a complex with Toll like receptor (TLR)-2, -3 and -4, and it is induced upon ligand binding to either receptor. Activated Neu1 sialidase hydrolyzes sialyl α-2, 3-linked β-galactosyl residues distant from ligand binding to remove steric hindrance to TLR-4 dimerization, MyD88/TLR4 complex recruitment, NFkB activation and pro-inflammatory cell responses (Amith *et al.*, 2010).

5.4 Conclusion

The prevalence of urinary schistosomiasis among adults is relatively high in the study area when compare with other study in Nigeria. There is significant evidence that *S. haematobium* infection is associated with bladder pathology, on ultrasound examination. Individuals with bladder pathologies could have heavy or light intensity of schistosomaisis or have no existing infection at all. However, long term exposure to schistosomiasis is necessary for the development of bladder pathology which eventually may advance to cancer. *GSTM1* and *GSTT1* polymorphisms were associated with elevated risk of bladder pathology with the pathology and schistosomiasis group having more GST polymorphisms than group with only bladder pathogy while slight risk of *S. haematobium* infection were found in *IL 4-590* polymorphisms. Also, *IL 13-1055* polymorphisms did not indicate susceptibility to schistosomiasis in males but a slight risk was found in females. With the use of integrated high throughput technologies we can begin to elucidate how *S. haematobium* and human host systems interact during infection. The comparative proteomics approach undertaken in this study has generated promising hypotheses regarding the mechanisms of pathogenesis that can be tested through manipulation of the host and parasite during infection. These included new potential biomarkers in schistosomiasis (Sialidase-1, Growth factor 15, Programmed cell death 1 ligand-2) and Pathology and Schistosomiasis group (Arylsulfatase A and Phosphatidylethanolamine-binding protein 4). This study demonstrates that urinary proteomics is a viable approach to discovering candidate biomarkers for schistosomiasis and its associated

pathology, but the results presented here require validation in a larger cohort before clinical applications can be considered.

5.5 Contribution to Knowledge

The prevalence of urinary schistosomiasis (29%) and bladder pathology (33%) among adults in Eggua was reported in this study. Also, the association between schistosomiasis and aetiopathogenesis of human urinary bladder for this group of adults in Nigeria was confirmed (χ^2 = 45.451, *P*= 0.001). Genetic susceptibility to bladder pathology was linked to GSTM polymorphisms in adults from Eggua in a moderately significant association and susceptibility to bladder pathology due to urinary schistosomiasis was also provided (OR 4.3, 95% CI 2.0-9.2 and OR 4.2, 95% CI 1.5-12 respectively).

For the first time, data on human host genetic susceptibility factors and biomarkers for urinary schistosomiasis and its associated pathology were provided using comparative genomics and discovery proteomic approach. New human chronic urinary schistosomiasis protein biomarkers identified (Programmed cell death 1 ligand 1, Growth/differentiation factor 15).

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RESEARCH QUESTIONNAIRE ON THE DEVELOPMENT OF BIOMARKERS FOR EARLY DETECTION OF BLADDER CANCER IN ADULTS CHRONICALLY INFECTED WITH *SCHISTOSOMA HAEMATOBIUM* IN NIGERIA

Code;…….. No;……

Instruction: This questionnaire is aimed at generating information from the participants. The data generated will be used for this research only and treated confidentially. Therefore, I implore every participant to respond to the questionnaire appropriately.

SECTION A: BIOSOCIAL DATA

AGE: Below 30-34 ( ), 35-39 ( ), 40-44 ( ), 45-54 ( ), 55- 59 ( ), 60 and above ( )

SEX: Male ( ) Female ( )

STATE OF ORIGIN: ……

EDUCATIONAL QUALIFICATION: Pry School Certificate ( ), Secondary School Certificate ( ), First Degree ( ), Masters Degree ( ), M. Phil or Ph.D ( ), None of the above ( )

OCCUPATION: Farming ( ), Fishing ( ), Artisan ( ), Trading ( ), specify the type of trade……. If none is applicable, specify ……

Religion:

SECTION B: WATER SUPPLY, SOURCES AND USES

Did your occupation require frequent contact with water? (i) Yes (ii) No

If yes, how long have you been with this occupation? 1-5yrs ( ) 6-10yrs ( ) 11-15yrs ( ) 16yrs and Above ( )

What other source of water do you have? [i] River [ii] Pond [iii] Dam [iv]Bore hole [v]Pipe borne water [vi] others, specify ……..

What do you use the water for? [i] Drinking [ii] Laundry [iii] Bathing [iv] Religious purpose [v] others, specify ……

How often do you visit the source of water? [i] Daily [ii] Weekly [iii] Monthly

SECTION C: KNOWLEDGE AND PRACTICES

Have you heard or seen someone passing out blood in urine? [i] Yes [ii] No

Have you at any time seen blood come out withn your urine? [i] Yes [ii] No

Do you have any knowledge on the cause(s) of blood in urine? [i] Yes [ii] No

If yes, what are the causes of blood in urine? [i] Snail [ii] Bad water [iii] Mosquito [iv] Others, specify …….

Do you currently pass out blood in urine or in the last three months? [i] Yes [ii] No

How does the blood come out in the urine? [i] with the urine [ii] last few drops [iii] before urine

Do you frequently experience pain during urination in the last 3months? [i] Yes [ii] No

Have you been diagnosed of schistosomiasis before? [i] Yes [ii] No

If yes, have you at any time been treated? [i] Yes [ii] No

Do you always observe any of these symptoms: high fever ( ), weakness( ), loss of appetite( ), headaches( ), and dizziness( ) (tick as many as possible)

What do you always use to cure the infection (schistosomiasis)? [i] Herbs [ii] injection [iii] Praziquantel [iv] Unknown drug [v] Other drugs……. (tick as many as possible)

Do you know you can still be re-infected even after receiving treatment? [i] Yes [ii] No

Have you been re-infected before even after treatment? [i] Yes [ii] No

If yes, how many times have been treated and re-infected? ……

Do you smoke cigarette? [i] Yes [ii] No, if other specify……

If yes for how long have you been smoking……..

How many cigarettes do you smoke per day? .....

Have any of your family suffer schistosoma infection [i] Yes [ii] No

If yes, mention which of them [i] Brother [ii] Sister [iii] Son [iv] Daughter [v] Wife, any other, specify……

Do you know if they received any treatment [i] Yes [ii] No

If yes, do you know if they suffer any re-infection after treatment [i] Yes [ii] No and how many time have they been re-infected…….

PARTICIPANTS INFORMED CONSENT FORM

IRB Research approval number; ……

This approval will elapse on……

DEVELOPMENT OF BIOMARKERS FOR EARLY DETECTION OF BLADDER CANCER IN ADULTS CHRONICALLY INFECTED WITH *SCHISTOSOMA HAEMATOBIUM* IN NIGERIA.

This study is being conducted by Onile Olugbenga Samson, Cell Biology and Genetics Unit, Department of Zoology, University of Ibadan, under the supervision of Dr. C. I. Anumudu of the Department of Zoology, University of Ibadan.

Purpose of Research:

The purpose of this study is to develop and provide biomarkers for the diagnosis of schistos-associated bladder cancer from adults in rural population in south-west Nigeria considering areas endemic for urinary schistosomiasis. (This is towards the award of a PhD degree)

Procedure of research:

Participants shall be recruited voluntarily into the study. Questionnaire will be self-administered to the participants. Urine and Peripheral blood (5mls) will be collected by venipuncture from the arm and examined for susceptibility to bladder cancer and schistosomiasis infection using PCR and develop a potent biomarker for early detection of schistosomiasis-associated urinary bladder cancer using an NMR spectroscopy. The result obtained from volunteers will be kept confidential.

Duration:

The study will begin as soon as approval is granted by the UCH/UI ethical committee. Collection of sample, laboratory analysis and interpretation of result will be for three years.

Risks:

The level of risks involved in the study is negligible

Benefit:

This study will help develop and provide biomarkers for the early diagnosis of schistos-associated bladder cancer from adults in rural population in south-west Nigeria. Also provide information about the genetic susceptibility to schistos infection and urinary bladder cancer.

Confidentiality:

The questionnaire that will be administered to participants will bear coded numbers with no names recorded. This will be employed in the processing of samples, screening and analysis of result. Personal information will remain confidential and will be not be used in any publication or report in this study. Records will be kept confidential.

Voluntariness:

Participation in this study is entirely voluntary, and therefore volunteers reserve the right to withdraw from the study at any time without prior notice or giving a reason for such decision. Such participant will not be denied any benefit that may arise as a result of the study. Results obtained will be confidential.

Consequences of Participants Withdrawal from Research:

Participants reserve the right to withdraw from the study anytime but it should be noted that some of the information that has been obtained from them before choosing

to withdraw may have been modified. However the researcher promise to make good faith effort to comply with your wishes as much as is practicable.

Statement of person obtaining informed consent:

I have fully explained this research to …….....and have given sufficient information, including the risks and benefits to make an informed decision.

DATE……. SIGNATURE……..

NAME: **ONILE, Olugbenga Samson**

Statement of person giving consent:

I have read the description of the research and have or had it written into the language I understand. I have also talked it over with the doctor to my satisfaction. I understand that my participation is voluntary. I know enough about the purpose, methods, risk and benefits of the research study to judge that I want to take part in it. I understand that I may freely stop being part of this study at any time. I have received a copy of this consent form and additional information sheet to keep for myself.

NAME

……

DATE …… SIGNATURE……...

Contact information:

This research has been approved by the Ethics Committee of the University of Ibadan and the Chairman of this Committee can be contacted at Biode Building, Room T10 2nd Floor, Institute for Advanced Medical Research and Training, College of Medicine, University of Ibadan, Telephone: **08032397993,** E-mail: **uiuchirc@yahoo.com**. In addition, if you have any question about your participation in this research, you can contact the principal investigator, Onile, Olugbenga Samson, Cell Biology and Genetics unit, Department of Zoology, Telephone: **08133109389,** E-mail: **onileg@yahoo.com**. You can also contact the supervisor of the research Dr.

Chiaka I. Anumudu, Department of Zoology, Telephone: **08023590478,** E-mail: **cianumudu@yahoo.com**.

INSTITUTE FOR ADVANCED MEDICAL RESEARCH AND TRAINING (IAMRAT)
College of Medicine, University of Ibadan, Ibadan, Nigeria.

Director: **Prof. Catherine O. Falade,** *MBBS (Ib), M.Sc, FMCP, FWACP*
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UI/UCH EC Registration Number: **NHREC/05/01/2008a**

NOTICE OF FULL APPROVAL AFTER FULL COMMITTEE REVIEW

Re: Development of Biomarkers for Early detection of Bladder Cancer in Adults Chronically Infected with *Schistosoma Haematobium* in South-Western, Nigeria

UI/UCH Ethics Committee assigned number: UI/EC/12/0212

Name of Principal Investigator: **Olugbenga S. Onile**

Address of Principal Investigator: Department of Zoology,
Faculty of Science,
University of Ibadan, Ibadan

Date of receipt of valid application: 16/07/2012

Date of meeting when final determination on ethical approval was made: **N/A**

This is to inform you that the research described in the submitted protocol, the consent forms, and other participant information materials have been reviewed and *given full approval by the UI/UCH Ethics Committee.*

This approval dates from **06/12/2016 to 05/12/2017.** Note that no participant accrual or activity related to this research may be conducted outside of these dates. *All informed consent forms used in this study must carry the* UI/UCH EC *assigned number and duration of* UI/UCH EC *approval of the study.* It is expected that you submit your annual report as well as an annual request for the project renewal to the UI/UCH EC at least four weeks before the expiration of this approval in order to avoid disruption of your research.

The National Code for Health Research Ethics requires you to comply with all institutional guidelines, rules and regulations and with the tenets of the Code including ensuring that all adverse events are reported promptly to the UI/UCH EC. *No changes are permitted in the research without prior approval by the* UI/UCH EC *except in circumstances outlined in the Code. The* UI/UCH EC *reserves the right to conduct compliance visit to your research site without previous notification.*

UI/UCH EC APPROVED COLLEGE OF MEDICINE

Professor Catherine O. Falade
Director, IAMRAT
Chairperson, UI/UCH Ethics Committee
E-mail: uiuchec@gmail.com